Successful Induction of Tumor-specific Cytotoxic T Lymphocytes from Patients with Non-small Cell Lung Cancer Using CD80-transfected Autologous Tumor Cells

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Cytotoxic T lymphocytes (CTL) against human lung cancer cells are difficult to induce by a conventional method using tumor cell stimulation probably due to an insufficiency of tumor antigens (TA) or costimulatory molecules such as CD80. We, therefore, investigated the potential of CD80-transfected tumor cells as stimulators of the in vitro induction of autologous tumor-specific CTL from regional lymph node lymphocytes in patients with lung cancer. Five non-small cell lung cancer cell lines (two adenocarcinomas, 1 squamous cell carcinoma, 1 large cell carcinoma and 1 adenosquamous cell carcinoma) were established from surgical specimens and were successfully transduced with a plasmid constructed with expression vector pBj and human CD80 cDNA, using a lipofection method. CD80-transfected tumor cells (CD80-AT) significantly augmented the proliferation of autologous lymphocytes from all cases as compared with non-transfected tumor cells (AT). AT-stimulated lymphocytes from 4 out of 5 cases did not show any cytotoxicity against AT; however, lymphocytes stimulated with CD80-AT exhibited substantial cytotoxicity against parental AT in all 5 cases tested. AT-stimulated lymphocytes derived from only one out of 5 cases showed major histocompatibility complex (MHC)-class I-restricted cytokine production in response to AT, while the MHC-class I-restricted responses were found in CD80-AT-stimulated lymphocytes from 4 out of 5 cases. These results indicate that CD80 on tumor cells could be a beneficial costimulatory molecule to elicit CTL against lung cancer, and also show that TA recognized by CTL was frequently expressed on lung cancer cells.

Key words: Lung cancer — CTL — CD80 — Regional lymph node — Autologous tumor cells

Several tumor antigens (TA) that are recognized by major histocompatibility complex (MHC) class I-restricted cytotoxic T lymphocytes (CTL) have been identified in human malignant melanoma and renal cell carcinoma (RCC). However, little is known about TA recognized by autologous CTL in other solid tumors because of difficulties in the induction of CTL. Possible major reasons for the failure in the in vitro generation of CTL against lung cancer and other solid tumors include insufficiency of TA or costimulatory molecules on tumor cells. It is now well accepted that T-cells require at least two signals for their full activation. The first signal is delivered through interaction of a T-cell receptor with an MHC-antigen complex. The second is received via interaction of receptors on T-cells (i.e., CD2, LFA-1 or CD28) and costimulatory molecules on antigen-presenting cells (i.e., LFA-3, ICAM-1, CD80 or CD86). The absence of the second signal leads to clonal inactivation or activation-induced cell death of T-cells. In murine models, the costimulation mediated by CD80 plays an important role in the induction of T-cell-mediated antitumor immunity. In human malignancies, a few studies have been performed to assess the effect of CD80 on the induction of tumor-specific CTL from patients with melanoma and cervical carcinoma. We previously reported human lung cancer-specific responses of lymphocytes which were derived from regional lymph nodes, tumor tissues and pleural cavities. However, it remains difficult to induce autologous tumor-specific CTL in patients with lung cancer. The present study was, thus, performed to investigate the potential of CD80 transduction into autologous tumor cells for the induction of tumor-specific CTL.

MATERIALS AND METHODS

Culture medium (CM) CM consisted of RPMI 1640 (GIBCO-BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (GIBCO-BRL), 10 mM
HEPES, 100 units/ml penicillin G and 100 mg/ml streptomycin sulfate.

**Cell lines** Lung cancer cell lines used for the present study were established from resected tumor samples by the enzymatic procedure described previously. All of these cell lines have been maintained for more than a year in monolayer culture by serial passages in CM. Identification of their human leukocyte antigen (HLA) genotypes by polymerase chain reaction was performed by Shionogi Co., Osaka, and the results are shown in Table I. K562 is an erythroleukemia cell line lacking MHC class I expression on the cell surface and is sensitive to natural killer cell cytotoxicity.

**Transfection of CD80 into tumor cells** The lung cancer cell lines described above were transfected with 2 µg of the pBJ/hCD80 plasmid containing human CD80 gene (kindly provided by Dr. M. Azuma, National Children’s Medical Research Center, Dep. Immunol.) using a lipofectin reagent (GIBCO-BRL) according to an instruction manual. These cell lines were selected with G418 (0.4–0.8 mg/ml) for more than 1 month. Successful induction of CD80 in G418-resistant cells was confirmed by a flow cytometry following staining with FITC-conjugated anti-human CD80 (PharMingen, San Diego, CA), and viable cells were expanded to form cell lines.

**Isolation of lymphocytes** Regional lymph nodes from lung cancer patients were obtained at the time of surgery. Each lymph node was divided into two parts for histological diagnosis and for this study. The latter part of each lymph node was squeezed between a pair of glass slides in Hanks’ balanced salt solution (HBSS) and then passed through a gauze filter. The cells were washed twice with HBSS and resuspended in CM.

**Induction of CTL** Lymphocytes obtained from regional lymph nodes were stimulated with solid-phase anti-CD3 monoclonal antibody (mAb) (Ortho Pharmaceutical Corp., Raritan, NJ) for 48 h and expanded in CM containing 50 units/ml recombinant interleukin-2 (rIL-2) (kindly donated by Takeda Chemical Ind., Osaka) in 24-well plates (Nunc, Roskilde, Denmark) for 14 days at 37°C in a 5% CO₂ atmosphere as previously reported. The expanded lymphocytes were aliquoted and cryopreserved. Subsequently, the cryopreserved lymphocytes were rapidly thawed and then stimulated with mitomycin-treated autologous tumor cells (CD80-transfected or parental tumor cells) weekly at a tumor-to-lymphocyte ratio of 1:10 in CM with 50 units/ml rIL-2 for 3 weeks. As a control of tumor stimulations, lymphocytes were also cultured in IL-2 containing CM without tumor stimulation. CTL activity was assessed at the end of the culture.

**mAb** FITC-conjugated anti-HLA-class I and phosphodiethanolamine (PE)-conjugated anti-HLA-DR were purchased from Becton Dickinson, Mountain View, CA. Hybridomas described below were purchased from the American Type Culture Collection (ATCC) (Rockville, MD). The culture supernatants of ATCC HB-145 (IVA12; anti-HLA-DR, DP, DQ mAb), HB-95 (W6/32; anti-HLA A, B, C) and HB-164 (A11.1; anti-HLA A24 and anti-HLA A11) were used as sources for blocking mAb.

**Cytotoxicity assay** Cytotoxicity of CTL was assessed by a standard 31Cr release assay. Briefly, target cells were labeled for 1 h with 50 µCi of 31Cr at 37°C. Labeled cells were then washed twice before plating at 2500 cells/well. Effectors were plated in 96-well round-bottomed plates at an indicated effector to target (E/T) ratio, and then co-incubated with labeled targets for 4 h. Radioactive release was determined in triplicate using a γ-counter. Results are expressed as percent specific lysis, calculated as (experimental release cpm–spontaneous release cpm)/(maximum release cpm–spontaneous release cpm)×100 (%). Spontaneous release was assessed by incubating target cells in medium alone and maximum release was determined in the presence of 2% Triton X.

**Measurement of interferon-γ (IFN-γ) by enzyme-linked immunosorbent assay (ELISA)** The cultured lymphocytes (10⁶/ml) were incubated with the parental autologous tumor cells (10⁶/ml) for 24 h, and the amount of IFN-γ in the culture supernatant was measured using a

### Table I. HLA-class I Types of Lung Cancer Cell Lines Tested

| Histology | Cell line | HLA          |
|-----------|-----------|--------------|
| A         | Class I   | Class II     |
| Adeno     | B203L     | +            | 2402/31012 | 1501/5401 | 0102/0304 |
| B901L     | +         | –            | 0206/2601 | 3901/4006 | 0702/0801 |
| Squamous  | B1203L    | +            | 2402/      | 5201/5401 | 0102/1202 |
| Large     | A904L     | +            | 2402/      | 0702/     | 0702/     |
| Adeq      | A529L     | +            | 2402/      | 5201/     | 1202/     |

*Adeno, adenocarcinoma; Squamous, squamous cell carcinoma; Large, large cell carcinoma; Adeq, adenosquamous cell carcinoma.

b) MHC class I and class II were assessed by flow cytometry. +, positively stained; –, negatively stained.
Human Interferon Gamma ELISA Test Kit (GIBCO-BRL) according to an instruction manual. In the mAb blocking assay, a 1/4-diluted culture supernatant of hybridomas was added to the co-culture of the CTL and parental autologous tumor cells (AT). The hybridoma-derived mAb did not affect detection of IFN-\(\gamma\) in preliminary experiments.

**RESULTS**

**Successful transduction of CD80 into established lung cancer cell lines**  Cell lines from resected tumor samples, obtained at surgery, were successfully established for 8 out of 215 patients (3.7%) with primary lung cancer.\(^{11}\) Five cell lines out of 8 were used in this study because a sufficient number of frozen autologous lymphocytes was available. The five cell lines were B203L, B901L, lung adenocarcinomas, B1203L, a lung squamous cell carcinoma, A904L, a lung large cell carcinoma, and A529L, a lung adenosquamous cell carcinoma. Their HLA genotypes were shown in Table I. In a flow cytometry analysis, MHC class I antigens were expressed on the surfaces of all lung cancer cell lines although allelic loss of genes was found in 2 cell lines (A904L and A529L) when compared with the genotypes in autologous EBV-B cells. MHC class II antigens were only expressed on A529L (Table I). The cell lines used in the present study did not express CD80 on their surfaces (data not shown). The pBj/hCD80 plasmid was transfected into the 5 lung cancer cell lines. Flow cytometry revealed cell surface expression of CD80 molecule in the 5 transfectants, whereas the parental cells did not express CD80 on cell surfaces. As shown in Fig. 1, transfectants from two representative cases (A904L and B203L) expressed CD80.

**Efficient expansion of lymphocytes stimulated with CD80-transfected autologous tumor cells**  Regional lymph node lymphocytes were first expanded in solid-phase anti-CD3 mAb followed by a low dose of rIL-2 (50 units/ml) for 14 days, then these lymphocytes were stimulated with non-modified autologous tumor cells (AT), CD80-transfected AT (CD80-AT), or without any tumor cells (without tumor). Growth curves of lymphocytes stimulated under different conditions are indicated. Open circles, CD80-AT; open squares, without tumor; open triangles, AT.

| Case | Expansion (fold) of lymphocytes after 3 stimulations with Medium | AT | CD80-AT |
|------|-------------------------------------------------------------|----|--------|
| B203 | 1.5                                                        | 0.4| 2.6    |
| B901 | 4.0                                                        | 0.2| 21.0   |
| B1203| 19.1                                                       | 7.7| 39.0   |
| A904 | 2.5                                                        | 0.1| 8.5    |
| A529 | 1.2                                                        | 1.8| 2.4    |

Mean±SD 5.7±7.6 2.1±3.2 14.7±15.5\(^{a}\)

\(^{a}\) Significantly higher than the values of no tumor and AT stimulation with a \(P\) value of less than 0.05 in a paired \(t\) test.

Fig. 1. Surface expression of CD80 on lung cancer cell lines. Lung cancer cell lines were transfected with the pBj/hCD80 expression vector by the lipofection method. Following selection by G418, viable cells were stained with FITC-conjugated anti-human CD80 or control Ig. Representative data from two cell lines are given.

Fig. 2. Efficient expansion of lymphocytes stimulated with CD80-transfected autologous tumor cells. Freshly isolated regional lymph node lymphocytes from patients with lung cancer were first expanded in solid-phase anti-CD3 mAb followed by a low dose of rIL-2 (50 units/ml) for 14 days, then these lymphocytes were stimulated with non-modified autologous tumor cells (AT), CD80-transfected AT (CD80-AT), or without any tumor cells (without tumor). Growth curves of lymphocytes stimulated under different conditions are indicated. Open circles, CD80-AT; open squares, without tumor; open triangles, AT.
the proliferation of lymphocytes during CTL induction using autologous tumor stimulation.

**Cytotoxicity of lymphocytes stimulated with CD80-transfectants against parental AT** The most important purpose of this study was to examine whether AT-specific CTL could be efficiently induced by stimulation with CD80-AT or not. Lymphocytes stimulated with CD80-AT were tested for cytotoxicity against parental AT and K562 in comparison to those stimulated with AT. Cytotoxic activity of lymphocytes (more than 10% cytotoxicity at an E/T ratio of 20/1) without tumor stimulation was not detected in any of the 5 cases. Substantial cytotoxicity against AT was induced in only one (B1203) of the 5 cases by stimulation with AT, whereas anti-AT cytotoxicity was apparent in lymphocytes stimulated with CD80-AT in all 5 cases (Table III). Representative data for A904L are shown in Fig. 3. In the case of B1203, the cytotoxicity generated by CD80-AT was comparable to that generated by AT. Cytotoxic activities against K562 were comparable among cases except B203, in which lymphocytes stimulated with CD80-AT showed a higher value (Table III). These results indicate that CD80-AT stimulation could induce lytic activities against AT even in cases in which AT stimulation could not.

**MHC-class I-restricted IFN-γ production of CTL in response to AT** After stimulation with CD80-AT, lymphocytes exhibited a substantial lytic activity against parental AT, though K562 was also killed by CD80-AT stimulated lymphocytes (Table III, Fig. 3). It seemed that CD80-AT-stimulated lymphocytes consisted of non-specific effectors such as NK cells that lysed K562 and AT-specific CTL. To determine whether CD80-AT-stimulated bulk lymphocytes contained tumor-specific activity, which is expected to be MHC class I-restricted, IFN-γ production of CTL was assessed in response to parental AT. AT-stimulated lymphocytes did not produce a substantial level of IFN-γ in response to AT except for B1203. However, CD80-AT-stimulated lymphocytes produced IFN-γ in response to AT in 4 out of 5 cases (B901, B1203, A904 and A529), and the production was inhibited by the addition of anti-MHC class I mAb (W6/32) but not anti-MHC class II mAb (IVA-12) (Table IV). In the case of B1203, MHC class I-restricted IFN-γ production was observed in lymphocytes without previous stimulation by AT albeit at a lower level as compared with those in the case of AT- or CD80-AT stimulation. Thus, MHC class I-restricted CTL were induced by stimulation with CD80-AT. Overall, stimulations with tumor cells and CD80 molecule were required for successful induction of tumor-specific CTL in patients with lung cancer.

**DISCUSSION**

Recently, lung cancer has become the most frequent cause of death in Japanese males. Among several therapies for lung cancer, surgery is, even now, the only effective means to cure this disease, and the problem of poor prognosis of patients with non-resectable or recurrent disease has not been solved yet. We were therefore interested in immunotherapy with high specificity to tumor cells for patients with lung cancer. For this purpose, establishment of CTL lines or clones is essential for the more precise investigation of immune response and for identification of TA in lung cancer. However, it seems to be difficult to induce tumor-specific CTL derived from patients with lung cancer since only a few investigations of autologous lung cancer-specific CTL have been reported.

Escape mechanisms of tumor cells from antitumor immunity have been ascribed to suppressive factors derived from tumors, immunosuppressive molecules expressed

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**Table III. Enhanced Cytotoxicity of Lymphocytes Stimulated by CD80-transfected Autologous Tumor Cells**

| Case   | % lysis<sup>a</sup> against AT and K562 stimulated with | Medium | AT | CD80-AT |
|--------|----------------------------------------------------------|--------|----|---------|
|        |                                                          | AT<sup>b</sup> | K562 | AT | K562 | AT | K562 |
|        |                                                          | AT<sup>c</sup> | K562 | AT | K562 | AT | K562 |
| B203   | 6                                                        | 12      | 2   | 5 | 23 | 36 |
| B901   | 8                                                        | 67      | 3   | ND<sup>c</sup> | 49 | 81 |
| B1203  | 2                                                        | 14      | 47  | 18 | 45 | 20 |
| A904   | 2                                                        | 10      | 0   | 0 | 45 | 17 |
| A529   | 6                                                        | 6       | 0   | 4 | 13 | 5 |

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<sup>a</sup> E/T ratio=20/1.

<sup>b</sup> Target cells: AT, K562.

<sup>c</sup> Not done.

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**Fig. 3. Cytotoxic activity of lymphocytes stimulated with CD80-transfected autologous tumor cells.** Cytotoxic activities against parental autologous tumor cells and NK-sensitive K562 of lymphocytes stimulated with AT, CD80-AT or without tumor were assessed 7 days after the last tumor stimulation (day 35) by a standard 4 h ⁵¹Cr release assay. Representative data (A904) from 5 cases are shown. Open circles, CD80-AT; open squares, without tumor; open triangles, AT.
on tumor cells\textsuperscript{23, 24} and a lack of costimulators such as CD80.\textsuperscript{25} Among them, we focused on the costimulation delivered through CD80-CD28 interaction. Drastic tumor elimination and T cell-mediated antitumor immune responses \textit{in vivo} were achieved by transfection of CD80 into tumor cells in murine models.\textsuperscript{7, 8} In human malignancies, Yang \textit{et al.}\textsuperscript{9} successfully generated tumor-specific CTL \textit{in vitro} using CD80-transfected melanoma cells from autologous and HLA class I-matched allogeneic lymphocytes. Mogi \textit{et al.}\textsuperscript{26} succeeded in the generation of tumor-specific CTL from lung cancer patients by using CD80-transfected autologous tumor cells with adenovirus vector together with IL-12. In their experiments, primary cultured tumor cells, but not established cell lines, were used for the induction of CTL. Nevertheless, it remains unclear whether tumor-specific CTL can be generated using the CD80 molecule in patients with lung cancer.

Parental tumor cell-stimulated lymphocytes failed to proliferate substantially in 4 of 5 cases (Fig. 2, Table II) although exogenous IL-2 was contained in the culture medium. Because those lymphocytes without tumor stimulation could expand in IL-2-containing CM to a high degree, the poor cell expansion of AT-stimulated lymphocytes might be due to tumor-derived suppressive substances such as those reported in esophageal cancer,\textsuperscript{21} lung cancer\textsuperscript{22} and breast cancer,\textsuperscript{23} but not due to the low expansion potential of lymphocytes from regional lymph nodes. However, if any immuno-suppressive factors were secreted from tumor cells, our results imply that the costimulatory signal via CD80-CD28 can overcome their effects.

Our aim in this study is to investigate the efficacy of CD80-AT stimulation in the induction of AT-specific CTL in lung cancer patients. In 3 cases (B901, A904 and A529), although the conventional method using inactivated AT stimulation could not induce AT-specific CTL, CD80-AT could successfully elicit AT-specific CTL. These findings may imply that tumor antigens recognized by CTL exist in tumor cells derived from many patients with lung cancer. No detectable production of IFN-\(\gamma\) was found in CD80-AT-stimulated lymphocytes from case B203 in response to AT, although a substantial level of cytotoxic activity against AT (23\% cytotoxicity) was detected. If the CTL cytotoxicity is completely parallel to IFN-\(\gamma\) production, this cytolytic activity found in case B203 might be a non-specific activity like natural killer

\begin{table}[h]
\centering
\caption{MHC-class I Restricted IFN-\(\gamma\) Production of Lymphocytes Stimulated with CD80-AT in Response to AT}
\begin{tabular}{cccccccc}
\hline
Case & Responder & Stimulator\textsuperscript{a} & Ab to & IFN-\(\gamma\) production of CTL induced by the stimulation with \\
& & & & medium & AT & B7-AT \\
\hline
B302 & + & – & – & <10 & 11 & <10 \\
& + & + & – & <10 & <10 & <10 \\
& + & + & Class I & <10 & <10 & <10 \\
& + & + & Class II & <10 & <10 & <10 \\
B901 & + & – & – & <10 & ND & <10 \\
& + & + & – & 20 & ND & 123 \\
& + & + & Class I & 19 & ND & 74 \\
& + & + & Class II & 17 & ND & 134 \\
B1203 & + & – & – & <10 & <10 & <10 \\
& + & + & – & 107 & 2070 & 4185 \\
& + & + & Class I & 12 & 140 & 570 \\
& + & + & Class II & 99 & 2250 & 3445 \\
A904 & + & – & – & <10 & <10 & <10 \\
& + & + & – & 10 & <10 & 433 \\
& + & + & Class I & 17 & <10 & 113 \\
& + & + & Class II & 14 & <10 & 465 \\
A529 & + & – & – & <10 & <10 & <10 \\
& + & + & – & <10 & <10 & 26 \\
& + & + & Class I & <10 & <10 & 14 \\
& + & + & Class II & <10 & <10 & 31 \\
\hline
\end{tabular}
\textsuperscript{a} Autologous tumor cells.
\end{table}
(NK) or lymphokine-activated killer (LAK) activity. In the case of B1203, class I-restricted cytokine response was observed not only in AT and CD80-AT-stimulated CTL, but also in lymphocytes receiving no tumor stimulation. A higher production of IFN-γ in response to AT (4185 pg/ml) was found in CD80-AT-stimulated lymphocytes, but even lymphocytes without tumor stimulation exhibited class I-restricted cytokine production at a relatively lower level (107 pg/ml). This might be explained by a stimulatory effect of contaminating tumor cells in freshly isolated lymph node lymphocytes during the first cultivation for 14 days since B1203-tumor cells (B1203L) could have activated autologous lymphocytes without CD80 molecules (Table III). Further investigation is needed to identify costimulatory molecules expressed on B1203L responsible for activating T-cells without the CD80 signal.

In three HLA-A24 positive patients of this study (Table I), preliminary experiments on blocking of cytokine production from CD80-AT-stimulated lymphocytes using anti-HLA-A24 mAb suggested that an HLA-A24 restricted response was found in CTL from case A529, but not those from case B1203 and A904 (data not shown). T-cell cloning from these bulk CTL is now on-going and should give us more precise information about this issue.

Since the identification of the MAGE gene in melanoma,27 knowledge of the human TA recognized by autologous CTL has allowed us to develop vaccine therapies utilizing several antigenic peptides derived from TA. Recently, the effectiveness of vaccine therapy in melanoma patients has been reported from several institutes.28–30 From the viewpoint of antigen-specific immunotherapy for lung cancer, the results of the present study are very significant in terms of finding an effective method to induce AT-specific CTL. This implies a possible antigen-specific immunotherapy for lung cancer patients by adoptive transfer of lymphocytes stimulated with CD80-transfectants or with TA which would be identified by the CTL, as well as by vaccine therapy using the transfectants or TA-derived antigenic peptides.

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