Tumor-draining Lymph Nodes of Primary Lung Cancer Patients: A Potent Source of Tumor-specific Killer Cells and Dendritic Cells

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Abstract. Background: The tumor-draining lymph node tissue (TDLT) of lung cancer patients generated killer cells specific to autologous tumor cells when cultured with low dose IL-2. This production of killer cells lasted as long as 2 months after the initiation of the culture (productive phase). Even after this productive phase, TDLT supported the generation of the killer cells when these were co-cultured with peripheral blood lymphocytes (PBL) from the same patients. We tried to analyze the mechanisms of this production of killer cells from TDLT. Materials and Methods: TDLT, tumor tissues as well as PBL were obtained from primary lung cancer patients and cultured in vitro. Cell growth, cell surface markers and specific cytotoxic activity of the lymphocytes were examined. Results: The majority of the cells from TDLT or TDLT+ PBL co-culture (TDL-Pb) were CD3-positive T cells (89-99%) and a 51Cr-releasing assay showed that these cells had a stronger cytotoxic activity against autologous tumor cells than cells from PBL cultured with IL-2. Their activity against allogeneic MHC incompatible target cells was not, however, elevated. Cytotoxic activity against autologous tumor cells was blocked by anti-HLA class I (52.0%), class II (47.9%) and CD8 (46.8%) antibodies, but not by anti-CD56 antibody. The treatment of TDLT with anti-CD8, CD4, CD80 and CD83 all together completely abrogated the ability of TDLT to generate killer cells, with one of these antibodies it did so partially, while treatment with anti-CD56 antibody failed to do so at all. Conclusion: These results collectively suggest that TDLT contains tumor antigen-pulsed DCs as well as precursors of specific killer T cells and gives rise to the generation of killer cells when cultured in a low dose of IL-2.

Tumor-draining lymph node (TDLN) of solid tumors are one critical type of lymphoid organ in immunological responses to tumors and are the first barrier to the spread of the tumor through the lymphatic vessels (1-4). Destruction of this barrier by tumor progression facilitates metastasis to distant organs (1). We have been interested in the role of TDLN in the host-defense mechanisms of lung cancer patients and have previously reported that lymphocytes from regional lymph nodes exhibited significantly higher cytotoxic activity against autologous tumor cells than those from peripheral blood cultured with IL-2 (5). Tumor-draining lymph node tissue (TDLT) is a potent source of specific killer T cells and can continue to generate killer T cells over 4-8 weeks with a low dose of IL-2. After this initial productive phase, the TDLT ceases to support the production of killer T lymphocytes (dormant phase), but resumes its support of specific killer T cell generation when fresh peripheral blood lymphocytes of the same patients are provided in this dormant phase.

In the present study, we analyzed the mechanisms of killer cell production by the TDLN of lung cancer patients and found that the TDLT was a potent source not only of killer cells, but also of mature dendritic cells (DCs).

Material and Methods

Source of regional lymph nodes. The study was approved by the Ethical Board of Chiba Cancer Center, Japan (No. 13-13). Informed consent was obtained from patients both to enroll them in the study and to use lymph nodes, tumor cells and peripheral blood lymphocytes (PBL) before surgery. The surgical
treatment they received was resection of the primary lung cancer and systematic lymph node dissection. Patients who had undergone chemotherapy or radiotherapy before surgery were excluded from the study. One to 2 g of regional lymph nodes was obtained at surgery. Lymph nodes without metastasis were minced with scissors into pieces smaller than 1 mm³ after connective and fatty tissues had been removed aseptically. A lymph node preparation, consisting of a single cell suspension of lymphocytes, monocytes, phagocytes and lymph node tissues, was then suspended in 75 ml medium (KBM400; Kohjin-Bio Tokyo, Japan) containing 175U/ml IL-2 (Proleukin; Chiron B.V., Amsterdam, Netherlands) and put into 75-cm² culture flasks (Iwaki 3110-075 Tokyo, Japan).

Culture of TDLT. To eliminate cells adherent to the plastic surface of each flask, the cell suspension including fine pieces of lymph node tissue was transferred the next day to an empty culture flask. After the transfer, the flask was allowed to stand for 2 min, 50 ml of supernatant was drawn off with an aspirator to remove the dead and floating single cells, and 50 ml of fresh medium containing a low dose of IL-2 was added. This procedure eliminated most of the adherent and single cells, leaving only lymph node tissues. This TDLT was then cultured without changing the medium. Seven to 10 days after the initiation of the culture, when TDLT started shedding lymphocyte colonies, half of the medium was changed, and this was repeated every 2-3 days thereafter. When the cells had grown abundantly, lymphocyte colonies were harvested and transferred to other flasks, leaving only TDLT in the original bottles. Harvested lymphocyte colonies continued to expand for 1-2 weeks until the lymphocyte clusters broke into single cells and ceased proliferating. Fresh medium was then added to the original bottles, and the tissue culture started again. As a result of this procedure, the TDLT continued to release lymphocyte colonies for at least 1-2 months.

Figure 1. Inverted microscope views of TDLT. A) Two days after the initiation of culture (Day 2). Single cells and small clumps of cells consisting of lymphocytes, macrophages, dendritic cells and others were scattered around the lymphoid tissues. B) Day 7. Cell colonies consisting of 40-50 enlarged lymphocytes and DCs were observed among clumps of connective tissue and stromal cells. C) Day 10. Cell colonies consisting of 100-200 lymphocytes. D) Day 14. Large colonies consisting of 1000-10,000 lymphocytes.
Culture of tumor cells. Tumor tissues were removed aseptically from the surgically resected lung, minced with 2 knife blades and transferred into 75-cm² culture flasks. Medium containing 1% collagenase (Sigma Chemical Co., USA) and 0.02% DNase (Sigma) was added, and the tumor tissue was separated by a magnetic stirrer for 6 h at 37°C, in a 5% CO₂ atmosphere. Tissue debris was removed with nylon mesh and the cells were washed 2 times with Hanks Balanced Salt Solution (HBSS; GIBCO, NY, USA). The cell preparation was then resuspended in Cosmedium (Cosmo Bio, Tokyo, Japan) containing 2% fetal calf serum and was cultured.

Stimulation of peripheral blood lymphocytes in IL-2. PBL were collected using a COBE Spectra system (COBE BCT, Inc., Colorado, USA), washed 3 times to remove platelets and cultured in KBM 400 containing 175 IU/ml IL-2 at 2X10⁶ cells/ml for 1-2 weeks.
Cytotoxic test against autologous and allogeneic tumor cells by standard 51Cr-releasing assay. The tumor cell monolayer was rinsed twice with 10 ml of Cosmedium and 3.7 Mbq Na235CrO4 (Daichii Radioisotope Lab., Tokyo, Japan) was added. After 45 min, the cells were rinsed 3 times and treated with 6 ml of trypsin-EDTA (08618: GIBCO, USA) for 5 min at 37 °C in a 5% CO2 atmosphere. The tumor cell suspension was then washed twice with HBSS and resuspended in KBM 400 at 10^5/ml. A serial two-fold dilution from 2X10^6 to 2.5X10^5 effector lymphocytes in a 0.2 ml medium or 0.2 ml 1N HCL was put in 5 ml glass tubes, and 0.2 ml of labelled target cells was added. The resulting target cell-effector cell suspension was incubated for 6 h at 37 °C in a 5% CO2 atmosphere, and then 2 ml of cold medium was added and centrifuged at 900 xg for 10 min. The radioactivity of the supernatant was measured by a gamma-counter (Auto-well Gamma System; ARC-370, ALOKA, Tokyo, Japan). Cytotoxic activity was calculated as follows Cytotoxic activity (%) = 100 X (CPM of test - CPM of medium control)/(CPM of maximum control - CPM of medium control), where medium control was measured in medium alone without effector cells and maximum control was measured in 1N HCl. All tests were performed in triplicate.

HLA typing of tumor cells and lymphocytes. The HLA type of the tumor cells and lymphocytes was determined by DNA-based tissue typing techniques with microsequence-specific primer (SSP) HLA DNA typing trays (One Lambda, Inc., CA, USA). DNA was extracted from the primary culture of tumor cells and lymphocytes and amplified using the polymerase chain reaction (PCR) with microsequence-specific oligonucleotide primers of HLA alleles. After PCR, samples were transferred to 2.5% agarose gel, electrophoresed at 150 volts, and the HLA type was determined on a UV transilluminator.

Inhibition of cytotoxic activity by monoclonal antibodies. Samples of anti-HLA class I, class II, CD4, CD8 and CD56 antibodies (25 μl) were placed in 5 ml glass tubes containing 5X10^5 TDL-Pb effector cells and 1X10^4 51Cr-labelled autologous target cells in 0.4 ml medium. After 6 h of incubation at 37 °C in 5% CO2, 2 ml cold medium was added, and the tubes were centrifuged. The radioactivity of the supernatant was counted by a gamma-counter. Percent inhibition was calculated as follows: Inhibition (%)= 100 X [1-(cytotoxicity with antibody)/(cytotoxicity without antibody)].

Cell surface analysis of cultured lymphocytes. Cell surface analyses were carried out by direct immunofluorescence with a monoclonal antibody. Fluorescein isothiocyanate (FITC)-anti-CD3, CD8, monoclonal antibody and phycoerythrin (PE)-anti-CD4, CD83 and CD14 antibody were purchased from Becton-Dickinson (CA, USA). In each sample, 10,000 cells were analyzed by flow cytometry (FACS Calibur; Becton-Dickinson). Cell clusters released from TDLT were also stained by FITC anti-CD3 and PE anti-CD83 antibody and examined using a confocal laser microscope (Olympus, Tokyo, Japan).

Treatment of TDLT with antibodies. One ml of TDLT suspension equivalent to 100 mg of TDLN was placed in each of a number of 5-ml sterile tubes. Twenty-five μl of CD4, CD8, CD80, CD83 or CD56 monoclonal antibody (Becton-Dickinson) were added and the tubes were incubated at 37 °C in a 5% CO2 atmosphere.

Further, in some tubes, all the antibodies except for the anti-CD56 antibody were added together. Twenty-four h after incubation, TDLT was suspended in KBM-400 with IL-2, transferred to a flask and cultured for 12 days.

Percent cell number was calculated as follows:

Cell number (%) = 100 X (number of cells treated with antibody / number of cells treated without antibody).

Statistical analysis. The results are presented as mean values with associated standard deviation. Student’s t-test was used for the statistical analysis. P values less than 0.05 were considered significant.

Results

Patients. Twenty-seven patients with primary lung cancer were enrolled in the study. There were 16 males and 11 females, 1 stage II, 21 stage III and 5 stage IV, 19 adenocarcinoma, 6 squamous cell carcinoma and 2 other histology cases.

Microscopic view of TDLT cell production. Figure 1 provides an inverted microscope view of TDLT demonstrating an increased colony formation of lymphocytes during tissue culture. Seven days after the initiation of culture, cell colonies consisting of 40-50 enlarged lymphocytes and DCs were observed among clumps of connective tissue. On Day 14, large cell colonies consisting of 1000-10,000 lymphocytes were observed. Microscopic views of the colonies revealed that the lymphocytes and DCs formed cell clusters that came into contact with the dendrites of the DCs. Confocal laser microscopic examination showed that CD83-positive dendritic cells and CD3-positive T cells were the main components of cell clusters (Figure 2).

T cell production by TDLT in low dose IL-2. When TDLT was cultured with a low concentration of IL-2, it started releasing lymphocyte colonies 1 week after the initiation of culture and continued to generate lymphocytes for 1-2 months (productive phase: Figure 3). Figure 3 shows a typical pattern of the cell proliferation of TDLT, PBL and TDLT+PBL (TDL-Pb). When PBL were cultured with IL-2, cell proliferation continued for 2 weeks, and then gradually came to a stop. We obtained a total of 5.8X10^9 lymphocytes 2 weeks after the initiation of culture and continued to generate lymphocytes for 1-2 months (productive phase: Figure 3). Figure 3 shows a typical pattern of the cell proliferation of TDLT, PBL and TDLT+PBL (TDL-Pb).
Figure 3. Pattern of lymphocyte production by TDLT. TDLT co-cultured with peripheral blood lymphocytes (TDL-Pb) and peripheral blood lymphocytes (PBL) in IL-2. When 1.0 g of TDLT (solid line) was cultured with IL-2, TDLT produced 2.4 and 3.1x10^9 lymphocytes on Days 8 and 21 (gray bars) after the initiation of culture, and continued to produce lymphocytes for up to 1 month (productive phase; solid line). When 1x10^9 PBL (dotted line) were cultured with IL-2, 5.8x10^9 lymphocytes (white bar) were obtained on Day 14 but their proliferation gradually slowed and came to a stop within 3 weeks. When 1x10^9 PBL were added to TDLT (fine dotted line) on Day 37, the cells started to proliferate again, and the TDLT then resumed its support of the production and generated a total of 12.2x10^9 lymphocytes (black bar) for 28 days.

Figure 4. Cell surface markers of TDL-Pb. Fourteen days after the second initiation of the culture, the cells were stained with fluorescein isothiocyanate (FITC)-anti-CD3 and phycoerythrin (PE)-anti-CD4 antibody and assayed using flow cytometry. CD8, CD14 and CD83 cell surface markers were also analyzed. The mean percentage of each cell type is indicated under the graph. The cells included CD3-, CD4- and CD8-positive T cells as well as CD83-positive mature DCs.
with 1x10^7 PBL generated 20.8x10^7, 8.5x10^7 and 50.5x10^7 cells, respectively, 1 week after the initiation of the culture (Figure 5). TDL-Pb continued to expand for 2 weeks to produce 1,353x10^6 cells.

Cytotoxicity against autologous and allogeneic tumor cells. The cytotoxic activity of these cells against autologous tumor cells (HLA A24/A24, B52/B52, DRB1 15/DRB1 15) was examined in a 51Cr-releasing assay (Figure 6). TDL-Pb exhibited significantly greater cytotoxic activities than PBL alone (p<0.01). This activity was specific since the cytotoxicity against HLA-incompatible allogeneic target cells (HLA A24/A31, B39/B52, DRB1 08/DRB1 09) did not differ between TDL-Pb and PBL culture.

Blocking of cytotoxicity with monoclonal antibodies. To analyze the cytotoxicity of TDL-Pb, we added monoclonal antibodies to the 51Cr-releasing assay (Figure 6). Cytotoxic activity was blocked with anti-HLA class I (52.6%), class II (47.9%), or CD8 (46.8%) antibody, but not with anti-CD56 antibody.

Mature DCs in TDL-Pb. The ratio of CD83-positive cells (mature DCs) in TDL-Pb and PBL cultured with a low dose of IL-2 was examined in specimens from several lung cancer patients (Figure 8). The mean percentage of CD83-positive cells in TDL-Pb (18.6±6.0%) was significantly higher (p<0.001) than that in PBL culture (7.22 ±5.7%).

Treatment of TDLT with monoclonal antibodies. In order to examine the subpopulations of TDLT responsible for the generation of killer cells, we treated TDLT with several antibodies. Twelve days after the initiation of the culture, the cells were harvested and counted (Figure 9). The cell production from TDLT was not impaired by the treatment using anti-CD56 antibody. However, treatment using anti-CD4, CD8, CD80, or CD83 antibody partially abrogated the cytotoxic activity of TDLT, while treatment with all of these four antibodies together abrogated it completely.

Discussion

The results presented in this paper clearly demonstrate that the tumor-draining lymph nodes of lung cancer patients contain precursors of killer cells and mature dendritic cells. Culture of tumor-draining lymph node tissues with low concentrations of IL-2 continually generated killer cells for 1-2 months. Cytotoxic activity was mediated by killer T cells specific to autologous tumor cells, which were induced by antigen-loaded mature DCs. Furthermore, DCs in lymph node tissues survived longer than lymphocytes and were able to continuously support the production of killer cells when precursors were supplied from other sources.

Immune responses against tumor antigens initially occur in the first TDLN, the sentinel nodes (SN) (6). Effective induction of immune responses can only take place in these secondary lymphoid organs where cell-to-cell interactions are properly guided and cells can meet in an appropriate cytokine-enriched microenvironment (1). At an early stage in the vast majority of cancers, TDLN are the primary sites where the specific recognition of tumor antigens and proper activation of the immune system are initiated (1-4). DCs are the specialized antigen-presenting cells of the immune system. To induce an effective immune response, these cells should not only express high levels of MHC and
co-stimulatory molecules, but should also migrate into the lymph nodes to interact with naive T cells. Mature—but not immature—dendritic cells generated in vitro efficiently migrate into the T cell areas of the lymph nodes of malignant melanoma patients. Immature DCs are strongly adherent, whereas mature DCs remain highly motile (7). Tissue-resident immature DCs take up antigens, and this is followed by a complex maturation and activation process that is characterized by an up-regulation of antigen-presenting MHC molecules and co-stimulatory molecules, as well as a switch in their adhesion and chemokine receptor repertory. These phenotypical changes allow DCs to migrate from peripheral tissue to the lymph nodes, in which they present processed antigens to resting T cells (7).

Accumulating evidence indicates, however, that intra-nodal DC infiltration is suppressed as tumor progression takes place, and that both total paracortical and paracortical subsector areas occupied by DCs are significantly reduced in size (8). The incidence of intra-nodal DC infiltration in patients with submucosal gastric cancer was significantly higher than in patients with tumors that invaded beyond the muscularis propria (9). Melanoma-associated SNs are the most likely sites of early metastases, and their immune functions are strikingly down-modulated. The frequency of paracortical interdigitating dendritic cells (IDC) was reduced in SN, and most IDCs lack the complex dendrites associated with active antigen presentation (8).
mechanisms of this immunosuppression are not clear but may be mediated by immunosuppressive factors released from the tumors (9), regulatory T cells (10,11), IL10 cytokine secretion by tumor infiltrating cells (12), or co-stimulation molecule B7-H1 (13,14) etc.

Liberation of DCs from this immunosuppressive state or non-suppressed lymph nodes presumably makes it possible to obtain from TDLN mature DCs, which are capable of presenting tumor-specific antigens to naive T cells.

In our initial study, we used single-cell preparations obtained by mincing TDLN and filtering the resulting material through a nylon mesh to eliminate the connective tissues of TDLN, but we could not obtain sufficient proliferating cells for the adoptive immunotherapy. When we cultured the connective tissue of TDLN along with lymphocytes, we obtained enough proliferating lymphocyte colonies. It appears that the single cell preparation obtained by the initial preparation of TDLN has a tendency to inhibit cell proliferation. Therefore, we eliminated single cells obtained from the initial preparation of TDLN. Furthermore, since cells adherent to plastic surfaces also inhibited T cell proliferation, we transferred the TDLT to another culture bottle to eliminate adherent cells. Finally, we concluded that the TDLN consist of a mixture of killer cell progenitors and suppressor or regulatory cells that inhibited proliferation of killer T cells as well as of mature DCs. We could not obtain proliferating T cells constantly until we used TDLT after the elimination of the initial single-cell suspension and adherent cells.

Our above procedure may eliminate immunosuppressive factors and liberate DCs from the immunosuppressive state of the tumor-bearing host to produce mature DCs as well as tumor-specific killer T cells. By culturing in vitro, without the continuous stimulation of tumor antigens or immunosuppressive factors released from tumor cells or cytokines, DCs may have been liberated from the suppressive environment inhibiting the killer cell production.

Cells adherent to lymph node stromal cells or to the cell matrix of connective tissues may be responsible for the induction of killer cells and mature DCs. Long-term cultures producing DCs were made possible by a well-developed stromal cell layer obtained from the spleens of
C57BL/6 mice (15). A stromal cell layer containing a mixture of fibroblasts and endothelial cells supports the continuous production of DCs. Cells were shed from foci of dividing cells in contact with the stromal cell matrix (16). Stromal cells as well as a cell matrix of regional lymph nodes may also support the long-term maintenance of DCs. We used neither GM-CSF nor IL-4 but low-dose IL-2 to induce mature DC, but when we examined the culture medium, we detected high levels of Th1 cytokines such as IL-2, GM-CSF and interferon-gamma one week after the initiation of the culture. We could not up-regulate mature DC production by pulsing these lymph nodes with tumor cells or tumor lysate (17-20) (data not shown), probably because the DCs in these lymph nodes had already acquired antigens and gone through the maturation and activation process to present processed antigens to resting T cells.

In the study presented here, we report that we were able to obtain sufficient specific killer T cells from TDLN and mature DCs to allow their use for adoptive immunotherapy of lung cancer patients (21-24). There have been many attempts to use DCs for the treatment of cancer patients. Most of the studies so far use peripheral blood monocytes as a source of DCs. Customizing processes required for ex vivo DC maturation have hindered the broadening of the range of clinical applications of DCs for the treatment of malignant diseases (25). Since these monocytes or DCs are immature, cytokines and tumor antigens or tumor-derived RNA (26) have to be used to induce maturation of the DCs. The DCs in TDLN are mature, but represent a relatively rare cell population and have been difficult to isolate from lymph node tissue (27). Our methods of culturing TDLN are efficient and easy for the preparation of specific T cells and mature DCs and will make possible the opening of a new era of adoptive immunotherapy of cancer patients.

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