killed only when pulsed with the Ep-CAM peptide were transferred into flasks and were expanded as detailed above.

Enzyme-linked immunospot assay

Enzyme-linked immunospot (ELISPOT) assays were performed as previously described (22). A total of 1×10^3 polyclonal $CD8^+$ T cells were co-cultured with peptide-pulsed T2-A24 cells (5×10^4) in wells of the MultiScreen-HA plates (Millipore) coated with 10 μg/ml of anti-interferon-γ (IFN-γ) monoclonal antibody (R&D Systems). All assays were performed in duplicate. After probing with a polyclonal rabbit anti-IFN-γ antibody (Genzyme, Cambridge, MA), followed by exposure to peroxidase-conjugated goat anti-rabbit IgG (Genzyme), IFN-γ spots were visualized and were counted under a dissecting microscope.

CTL assay

Target cells were labelled with ^{51}Cr as previously described (22). In some experiments, pre-determined amounts of blocking antibodies, W6/32 (anti-HLA class-I), MA2.1 (anti-HLA-A2) and A11.1 (anti-HLA-A24) were added to the wells 30 min before adding effector cells in order to determine the HLA restriction. The plates were incubated for 4 h at 37°C, and the supernatants were counted in a γ-counter. The percentage of specific ^{51}Cr release was calculated as follows: 100 × (experimental release − spontaneous release)/(maximum release −spontaneous release).

Cold target inhibition assays

Cold target inhibition assays were performed as described previously (30). Briefly, T2-A24 cells were incubated with the peptide Ep_{173} or EBV-$LMP2_{419}$ at a concentration of 10 μM for 1 h. After extensive washing, the indicated numbers of peptide-loaded cells were incubated with 2×10^4 cytotoxic effector cells for 1 h, and then 2×10^3 ^{51}Cr-labelled PC9 cells were added to each well. Cytotoxicity was assessed as described above.

Results

Selection of potential HLA-A24-binding peptides within Ep-CAM proteins

The computer program was applied in order to identify potential HLA-A*2402-binding peptides within the amino acid sequence of Ep-CAM and to predict HLA-binding peptides, on the basis of estimation of the half-time dissociation of the HLA/peptide complex. The peptide sequences were reviewed according to peptide motifs for HLA-A24 molecules (31–33), and seven peptides were synthesized (Table 1). MHC stabilization assays were performed in order to test their HLA-A*2402-binding efficiency by using T2-A24 cells. Most peptides increased the HLA-A24 expression on the cells, indicating that they bound and stabilized the HLA complexes on the cell surface, but peptide Ep_{304} gave a low value for the percentage of MFI (Table 1) and was excluded from further studies.

Screening of peptides antigenic for anti-Ep-CAM polyclonal CTL lines by means of ELISPOT assay

In order to identify peptides recognized by Ep-CAM-specific CTLs in the context of HLA-A*2402 molecules, $CD8^+$ T cells of five HLA-$A24^+$ healthy donors were stimulated with autologous DCs pulsed with each of the six peptides. After three rounds of stimulation, T cell lines from four donors produced significant numbers of IFN-γ spots when incubated with T2-A24 cells pulsed with peptide Ep_{173} (Fig. 1). Almost no spots were produced with control peptide ENV_{584} (data not shown). After four-time stimulation, the CTL line established from donor 4 specifically stained with the HLA-A24/Ep_{173} tetramer but not the HLA-A24/ENV_{584} tetramer (37.2 *vs* 0.06% of the total $CD8^+$ T cells, Fig. 2A). The intensity of the tetramer-positive cells was homogeneous and stronger than that of tetramer-negative cells by two- to three-fold on a logarithmic scale.

We established a T-cell clone, designated as C27, from limiting dilution culture of the Ep_{173}-specific polyclonal CTL line of donor 4. The study with tetramers indicated that both polyclonal and monoclonal Ep_{173}-specific $CD8^+$ T cells had high-affinity antigen receptors directed to HLA-A*2402/Ep_{173} complexes (Fig. 2B).

While an Ep_{250}-stimulated CTL line from donor 3 also specifically produced IFN-γ spots when incubated with Ep_{250} (Fig. 1), the establishment of Ep_{250}-specific CTL clones was unsuccessful. We, thus, further evaluated the Ep_{173}-specific CTL clone.

Characterization of the peptide Ep_{173}-specific $CD8^+$ CTL clone

Ep-CAM expression of cancer cell lines was examined by means of RT-PCR, Western blot analysis (Fig. 3) and indirect immunofluorescence (Table 2). The data showed concordance of results obtained with the three approaches. Twelve of 15 (80%) cancer cell lines appeared to express Ep-CAM. When HLA-A24 expression was examined with the help of indirect immunofluorescence by using an HLA-A24 monoclonal antibody, 10 were positive (Table 2).

As shown in Fig. 4(A), the Ep_{173}-specific CTL clone, C27, showed cytotoxicity to T2-A24 cells pulsed with Ep_{173} at a peptide

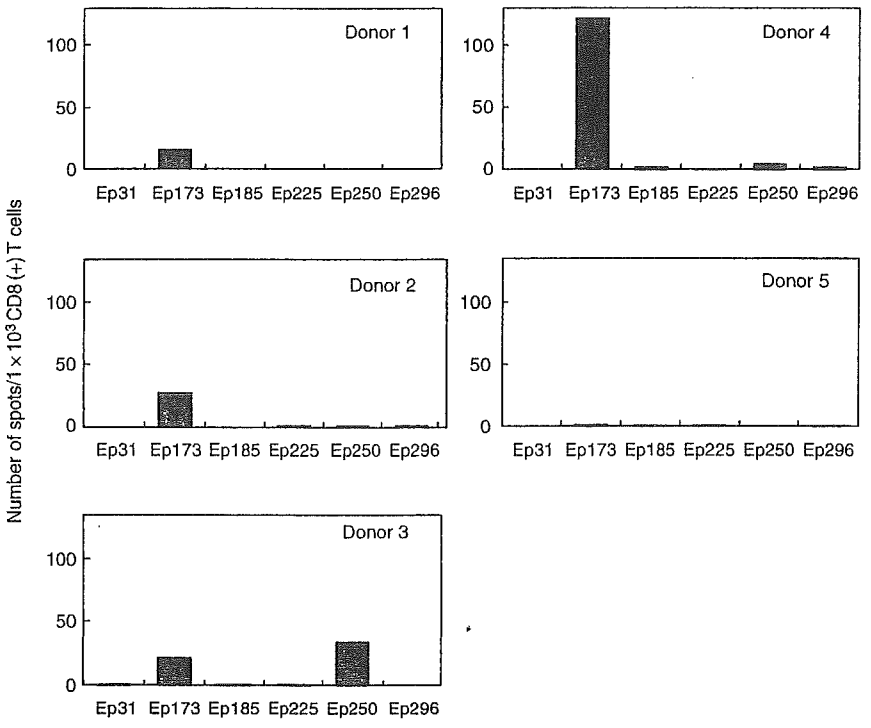

Fig. 1. **Evaluation of CD8⁺ T cell lines by means of enzyme-linked immunospot (ELISPOT) assay.** Aliquots of CD8⁺ T cells (1×10^3) were incubated with T2-A24 cells pulsed with the indicated peptide (10 μM) in wells of the ELISPOT plate. All assays were performed in duplicate.

concentration as low as 100 pM, but not with control peptide EBV-LMP2$_{419}$. Data for cytotoxicity of C27 against various cancer cell lines have been shown in Fig. 5. C27 efficiently lysed lung cancer cell lines PC9, LU99, LC99A and LC-1/sq, an oral squamous cell carcinoma cell line HSC-2 and a gastric cancer cell line MKN45 that expressed both HLA-A24 and Ep-CAM. However, no killing was observed with HLA-A24⁺ Ep-CAM⁻ (11–18, COLO320 DM and A549-A24) or HLA-A24⁻ (either Ep-CAM⁺ or Ep-CAM⁻) cell lines (QG56, A549 and MNK28). After the transfection of the HLA-A*2402 cDNA into HLA-A24⁻ QG56 cells (QG56-A24), they were killed by C27. K562 cells were included in order to assess the degree of NK-like cytotoxicity of C27, which turned out to be negligible.

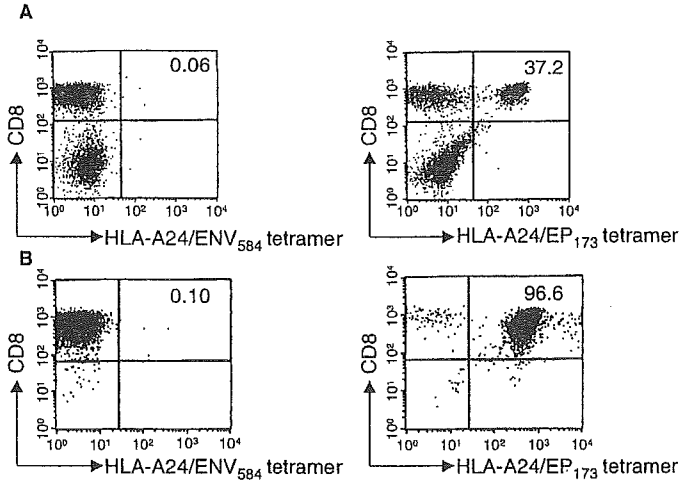

Fig. 2. **Tetramer staining of Ep$_{173}$ peptide-specific CD8⁺ T cells.** Polyclonal CD8⁺ T cells (A) and an Ep$_{173}$-specific cytotoxic T-lymphocyte (CTL) clone – C27 – (B) were stained with HLA-A24 tetramers incorporating Ep$_{173}$ or a control peptide, ENV$_{584}$. The percentages of tetramer-positive cells in CD8⁺ T cells have been shown. HLA, human leucocyte antigen.

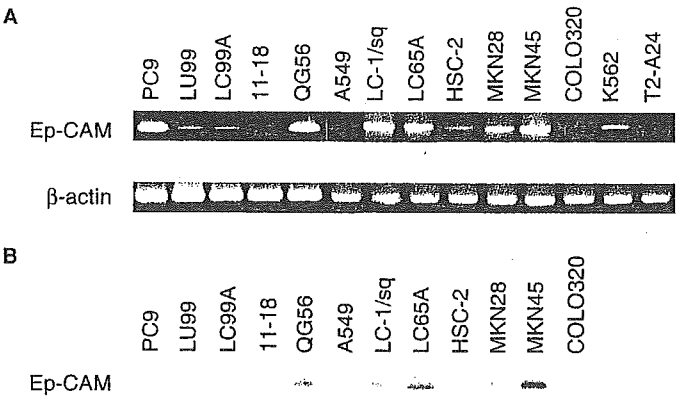

Fig. 3. **Reverse transcription polymerase chain reaction (RT-PCR) and Western blot analysis of epithelial cell adhesion molecule (Ep-CAM) in cancer cell lines.** Semi-quantitative RT-PCR analysis was performed by using specific primers for Ep-CAM and β-actin (A). Western blot analysis of Ep-CAM was performed with a monoclonal antibody specific to Ep-CAM (B).

Characteristics of the cell lines used

Cell line	Origin	Surface expression (MFI[a]) of Ep-CAM	HLA-A24
Lung cancer			
LU99	Giant cell carcinoma	+ (22.55)	+ (87.16)
PC9	Adenocarcinoma	+ (383.67)	+ (43.53)
11–18	Adenocarcinoma	– (4.00)	+ (73.67)
LC99A	Large cell carcinoma	+ (15.32)	+ (96.71)
LC65A	Small cell carcinoma	+ (307.14)	– (3.67)
LC-1/sq	Squamous cell carcinoma	+ (198.94)	+ (70.56)
A549	Adenocarcinoma	– (6.40)	– (3.21)
A549-A24	Adenocarcinoma	Not done	+ (109.73)
QG56	Squamous cell carcinoma	+ (229.13)	– (2.65)
QG56-A24	Squamous cell carcinoma	Not done	+ (84.12)
Gastric cancer			
MKN28	Adenocarcinoma	+ (722.59)	– (7.67)
MNK45	Adenocarcinoma	+ (823.25)	+ (47.99)
Colon cancer			
COLO320DM	Adenocarcinoma	– (3.07)	+ (35.35)
Others			
HSC-2	Oral squamous cell carcinoma	+ (57.15)	+ (34.40)
K562	Chronic myelogeneous leukaemia	+ (41.45)	– (5.23)
T2-A24	B × T hybrid cells	– (3.05)	+ (197.75)
NHBE	Normal bronchial epithelial cells	+ (152.56)	+ (33.52)

[a]Mean fluorescence intensity (MFI) was examined with the help of flow cytometric analysis after cell staining by using anti-Ep-CAM or anti-HLA-A24 monoclonal antibodies and FITC-labelled anti-mouse IgG $F(ab')_2$ fragments.

Table 2

These data demonstrated that Ep_{173}-specific CTLs kill tumour cells expressing both HLA-A24 and Ep-CAM.

We further examined whether C27 might recognize naturally processed peptides presented on the surfaces of tumour cells in the context of HLA-A24. Cytotoxicity of C27 against PC9 cells ($HLA\text{-}A24^+$ $Ep\text{-}CAM^+$ lung cancer cell line) was blocked by monoclonal antibodies specific to HLA-A24 or pan-class-I molecules, but not an anti-HLA-A2 monoclonal antibody (Fig. 4B), confirming the HLA-A24 restriction. Cold target inhibition assays demonstrated that C27-mediated cytotoxicity against PC9 cells was specifically inhibited in the presence of T2-A24 cells pre-pulsed with the cognate but not an irrelevant peptide (Fig. 4C), indicating that C27 recognized peptides that were naturally processed and presented.

Because Ep-CAM is expressed in some sites of normal epithelial cells, there has been concern about potential autoimmune reactions after *in vivo* activation of T cells specific to the molecule. We, therefore, tested an HLA-A*2402-positive, normal human bronchial epithelial cell line, designated as NHBE, with clone C27. As demonstrated in Fig. 6(A), C27 exerted toxicity, especially at higher effector : target ratios (40 : 1 and 20 : 1), which was specifically blocked by T2-A24 cells pre-pulsed with the cognate peptide (Fig. 6B). At lower effector : target ratios (10 : 1 and 5 : 1), however, C27-mediated lysis of NHBE was apparently lower than that of cancer cell lines, such as PC9, LU99, LC99A and HSC-2 (Fig. 5).

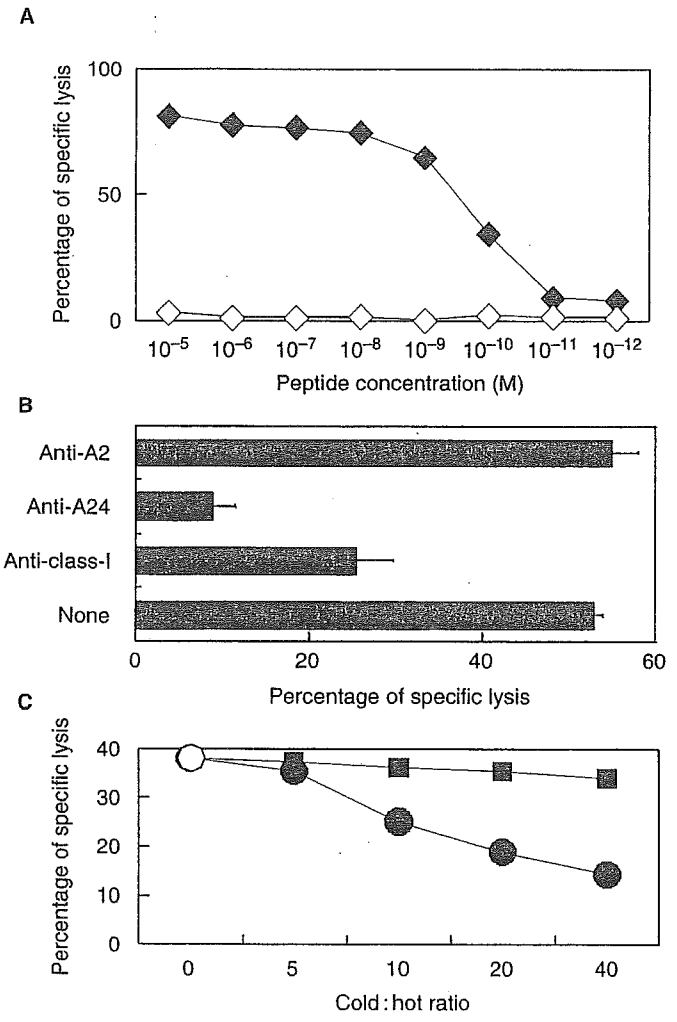

***Fig. 4.* Characterization of an Ep_{173}-specific cytotoxic T-lymphocyte (CTL) clone, C27.** An Ep_{173}-specific CTL clone, C27-mediated target cell (T2-A24) lysis in the presence of Ep_{173} (◆) and control peptide $EBV\text{-}LMP2_{419}$ (◇) has been shown (A). C27-mediated target cell (PC9) lysis was blocked with monoclonal antibodies specific to HLA-A24 and pan-class-I molecules (B). C27-mediated target cell (PC9) lysis was blocked with T2-A24 cells that had been loaded with Ep_{173} (●) but not with a control peptide $EBV\text{-}LMP2_{419}$ (■) (C). The cytotoxic assays were performed at effector to ^{51}Cr-labelled target ratios of 1, 10 and 5, in experiments shown in A, B and C, respectively. HLA, human leucocyte antigen.

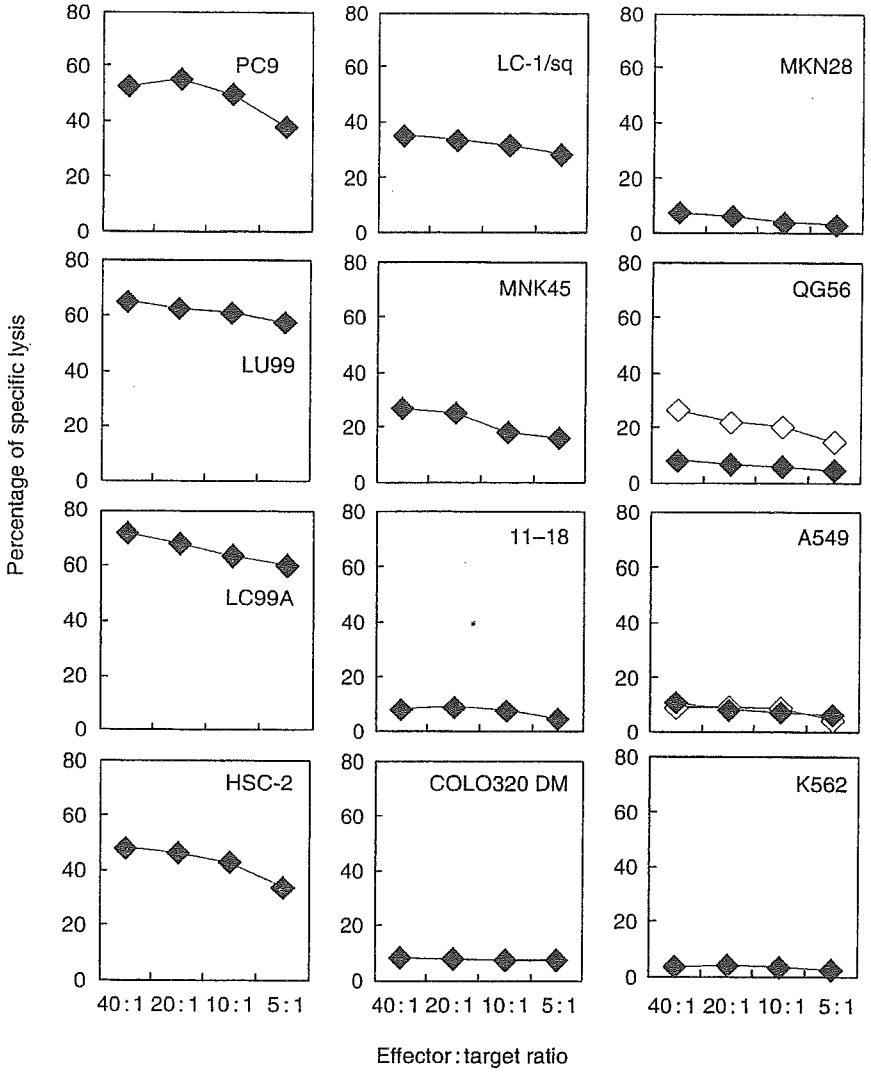

Fig. 5. **Cytotoxicity of the clone C27 against cancer cell lines.** An Ep_{173}-specific cytotoxic T-lymphocyte (CTL) clone, C27-mediated lysis of various cancer cell lines. Cell lines in the left and the centre columns express HLA-A24 on their surface, whereas those in the right do not. All the target cell lines except for 11–18, COLO320DM and A549 express the Ep-CAM. HLA-A*2402 transfectants of QG56 and A549 cells were also used as target cells (◇). K562 was included in order to assess natural killer-like cytotoxicity. HLA, human leucocyte antigen.

Discussion

According to recent findings, overexpression of Ep-CAM, a transmembrane protein mediating Ca^{2+}-independent cell–cell adhesion, is correlated with tumour grade (14, 34, 35), providing a useful marker for diagnosis of micrometastases (36) and a predictor of survival (14). The promoter region that regulates the Ep-CAM transcription has been cloned and it has shown to be negatively regulated by TNF-α (37).

Ep-CAM has become one of the major targets for immunotherapy with monoclonal antibody, because it is expressed in the vast majority of epithelial cell-derived cancers. The administration of Ep-CAM-specific murine monoclonal antibody (17-1A) into Duke's C colorectal cancer patients after surgery, for example, led to the prevention of distant metastasis and to prolonged survival after 7 years of follow-up evaluation (19). Recently, bispecific antibodies against Ep-CAM and CD3 (38, 39) or B7 (40) have been engineered in order to improve cytotoxicity with synergistic effects of antibodies and T lymphocytes. Considering the expression pattern, immunotherapy by using CTL directed against Ep-CAM appears attractive. Indeed, Ep-CAM peptides that elicited cellular immune responses restricted by HLA-A*0201 have recently been identified (4, 20). Of note, a natural T-cell response against Ep-CAM has been observed in colorectal cancer patients (41, 42). The available evidence, thus, suggests the possibility of therapeutic approaches using Ep-CAM as a target of CTLs.

We, in this study, explored immunogenic peptides derived from Ep-CAM that can elicit cellular immune responses against Ep-CAM$^+$ tumour cells in the context of HLA-A24, which is positive in nearly 20% of persons of European descent and more than 60% of Japanese. For that purpose, seven candidate peptides were first predicted from the Ep-CAM protein with a bioinformatic approach. Secondly, MHC

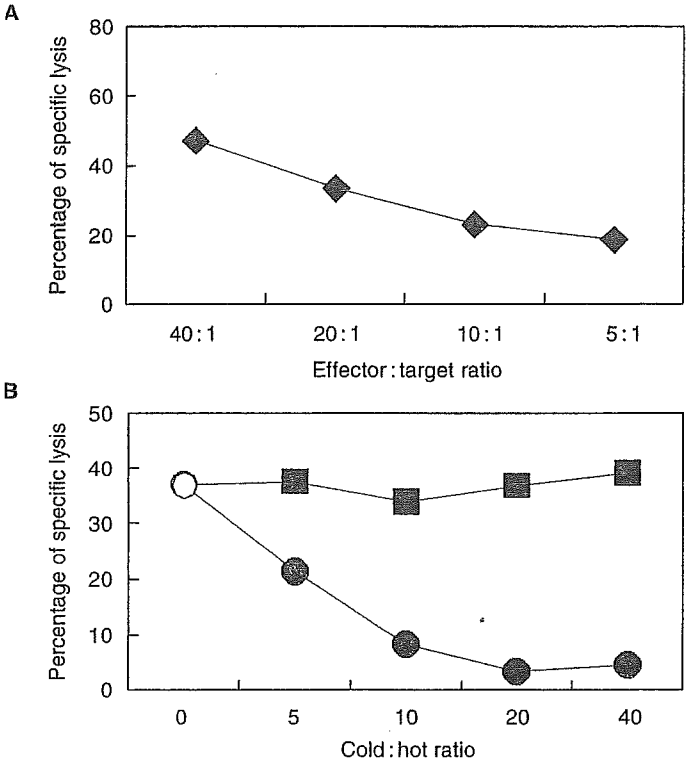

***Fig. 6.* Cytotoxicity of the clone C27 against normal bronchial epithelial cells.** C27-mediated lysis of normal bronchial epithelial cells (NHBE) expressing HLA-A24 and Ep-CAM (A) and C27-mediated NHBE cell lysis were blocked with T2-A24 cells pulsed with Ep_{173} (●) but not with a control peptide EBV-$LMP2_{419}$ (■) (B). The effector to hot target ratio was 20. HLA, human leucocyte antigen.

stabilization assays were used in order to determine the binding affinity of the peptides with HLA-A*2402 molecules, revealing one peptide, Ep_{173} (RYQLDPKFI), to have the highest affinity and Ep_{304} (EMGEMHREL) the lowest. Thirdly, we successfully established Ep_{173}-specific polyclonal CTL lines from four of five healthy donors. A CTL clone, C27, demonstrated fine specificity for Ep_{173}, which was naturally presented on the surfaces of tumour cells, because C27-mediated PC9 cell lysis was blocked by both anti-HLA-A24 monoclonal antibody and Ep_{173}-pulsed cold target cells. An Ep_{250}-specific polyclonal CTL line was generated from donor 3, raising the possibility that Ep_{250} is another CTL epitope presented by HLA-A*2402. We, so far, could not establish CTL clones specific to Ep_{250}, which are required for further evaluation of the peptide.

It is of note that there is no correlation between the level of expression of Ep-CAM and the degree of CTL-mediated lysis (Figs. 3 and 5; Table 2). The level of HLA expression does not seem to explain the issue. We speculate that the Ep-CAM may be more resistant to proteasome degradation in cells with high Ep-CAM expression and low or moderate sensitivity to CTL-mediated lysis (LC-1/sq, MKN45 and QG56). On the contrary, there may be a higher turnover rate of the Ep-CAM in cells with low expression and high sensitivity to CTL-mediated lysis (LU99, LC99A and HSC-2).

Although Ep-CAM is a candidate target for both humoral and cellular immunotherapy, a persistent difficulty arises because of its presentation on normal epithelial tissues. Indeed, the CTL clone, C27, lysed HLA-A24-positive normal bronchial epithelial cells in an epitope-specific fashion, especially at high effector : target ratios (Fig. 6). However, at lower effector : target ratios, such as 10 : 1 and 5 : 1, C27-mediated lysis of NHBE was apparently lower than that of C27-sensitive cancer cell lines (Fig. 5). In addition, C27-mediated lysis of NHBE was more efficiently inhibited by T2-A24 cells pulsed with the cognate peptide (Fig. 6B) than that of the PC9 lung cancer cell line (Fig. 4C), implicating a lower density of HLA-A*2402/Ep_{173} complexes on the surface of NHBE cells.

It has been reported that an HLA-A*0201-restricted Ep-CAM-specific CTL line could not lyse normal bronchial epithelial cells under conditions, whereby epithelial tumour cells were efficiently killed (20). So far, immunization of colorectal carcinoma patients with a recombinant canarypox virus expressing Ep-CAM has been well tolerated and has been shown to induce anti-Ep-CAM CTL responses without causing autoimmune reactions (43). Interestingly, a monoclonal antibody specific to Ep-CAM did not localize to Ep-CAM-positive normal tissues of human Ep-CAM transgenic mice (6), indicating limited *in vivo* accessibility, which might explain any absence of autoimmunity. Another example of vaccination of tissue-specific self-antigen is provided by means of immunotherapy targeting CEA (carcino embryonic antigen). Greiner et al. (44) reported that vaccination of CEA-transgenic mice with a recombinant canarypox virus expressing CEA can generate substantial anti-tumour immunity with little or no autoimmunity. They proposed possible explanations including (i) differential susceptibility of tumour and normal tissues to the immune effector arms and (ii) blockage of the autoreactive T-cell activity by tolerizing antigen-presenting cells or the presence of regulatory T cells in order to terminate the response in the normal epithelia. Further studies are required in order to evaluate potential autoimmune reactions with Ep_{173} immunization. Littermates of human Ep-CAM transgenic mice (6) bred with HLA-A*2402 transgenic mice would provide a suitable model to study this issue.

In conclusion, we present, in this study, a novel HLA-A*2402-restricted epitope, Ep_{173} (RYQLDPKFI), which has the ability to induce $CD8^+$ T cells with high-affinity antigen receptors directed to HLA-A*2402/Ep_{173} complexes. All the data suggest that the epitope-specific CTL responses may play some roles in both anti-cancer and autoimmune reactions. The peptide should prove useful to study anti-Ep-CAM CTL responses among populations possessing HLA-A*2402.

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Int. J. Cancer: **110,** 403–412 (2004)

Publication of the International Union Against Cancer

INTERFERON-γ DIFFERENTIALLY REGULATES SUSCEPTIBILITY OF LUNG CANCER CELLS TO TELOMERASE-SPECIFIC CYTOTOXIC T LYMPHOCYTES

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There is accumulating evidence that peptides derived from the catalytic subunit of human telomerase reverse transcriptase (hTERT) are specifically recognized by $CD8^+$ cytotoxic T lymphocytes. We investigated the cytotoxicity of a human leukocyte antigen (HLA)-A*2402-restricted hTERT-derived peptide 461–469 ($hTERT_{461}$)-specific $CD8^+$ T-cell clone, designated as K3-1, established from a healthy donor by repetitive peptide stimulation. This clone exhibited cytotoxicity against 4 out of 6 HLA-A24-positive lung cancer cell lines with positive telomerase activity but not 4 HLA-A24-negative examples. When the target cells were pretreated with 100 U/ml of interferon (IFN)-γ for 48 hr, the susceptibility to K3-1 increased with PC9 cells but unexpectedly decreased with LU99 cells. However, in both cell lines, the expression of molecules associated with epitope presentation such as HLA-A24, transporters associated with antigen processing, low molecular weight polypeptide 7 and proteasome activator 28 was similarly increased after IFN-γ treatment. Results of CTL assays using acid-extracted peptides indicated that the epitope increased on PC9 cells but not on LU99 cells after IFN-γ treatment. Semi-quantitative reverse transcriptase polymerase chain reaction disclosed that the expression of hTERT was attenuated in LU99 but not in PC9 cells, accounting for the decreased cytotoxicity mediated by K3-1. The attenuation of the hTERT expression and K3-1-mediated cell lysis after IFN-γ treatment was also observed in primary adenocarcinoma cells obtained from pulmonary fluid of a lung cancer patient. Our data underline the utility of peptide $hTERT_{461}$ in immunotherapy for lung cancer, as with other malignancies reported earlier, and suggest that modulation of hTERT expression by IFN-γ needs to be taken into account in therapeutic approach.

Key words: *telomerase; hTERT; immunotherapy; lung cancer; Interferon-γ*

Human chromosomes terminate with 5–15 kilobases of repetitive telomeric DNA $(TTAGGG)_n$,[1] which protect against DNA degradation, end-to-end fusion, rearrangements and chromosome loss.[2] In normal cells, such as cultured skin fibroblasts, telomeric DNA becomes shortened with every round of replication,[3] ultimately leading to replicative senescence. In contrast, with permanently established cell lines from malignant tumors, telomeres are believed to be elongated by a unique ribonucleoprotein enzyme, called telomerase, which adds telomeric sequences *de novo*.[1] Indeed, there is clear evidence that telomerase activity is involved in tumorigenesis.[4,5] Normal tissues display little or no telomerase activity, and activation of the enzyme may therefore play a critical role in cell immortalization.

Human telomerase complexes are composed of telomerase RNA component,[6] telomerase protein 1[7,8] and hTERT.[9] Messenger RNA expression of hTERT is essential for telomerase activation during cellular immortalization and tumor progression,[9] and ectopic expression of the hTERT gene in telomerase-negative cells can induce telomerase activity to levels comparable to those in immortal telomerase-positive cells.[10] The expression of hTERT has been frequently demonstrated in telomerase-positive primary tumors and cancer cell lines but found to be low or undetectable in normal tissues.[9–13] Thus, hTERT could be a candidate universal tumor antigen for immunotherapy and vaccine approaches.

Several studies have been conducted to test the possibility that hTERT could serve as a tumor antigen recognized by specific CTL. Indeed, hTERT peptide-specific CTL have proved cytotoxic to cell lines derived from various malignancies including leukemias,[14,15] osteosarcoma, ovarian carcinoma, non-Hodgkin's lymphoma,[15] multiple myeloma,[15,16] melanoma[15,17] and cancers of breast, colon, lung,[17] prostate[17,18] or kidney.[18] Recent studies revealed that hTERT is expressed in 89%[13] to 93.9%[19] of primary lung cancers.

In our study, we first asked the question whether hTERT-specific CTL recognize and kill lung cancer cells applying an HLA-A*2402-restricted hTERT-derived peptide ($hTERT_{461}$)-specific $CD8^+$ T-cell clone, generated from a healthy donor, and a panel of lung cancer cell lines with positive telomerase activity as targets. The findings confirm and extend previous results, supporting the feasibility of developing CTL-based immunotherapy targeting hTERT in some, if not all, lung cancer patients. In addition, interesting evidence was obtained to demonstrate that IFN-γ treatment of the target cells did not always enhance CTL recognition.

Abbreviations: CD40-B, CD40-activated B; CTL, cytotoxic T lymphocyte; FITC, fluorescein isothiocyanate; hTERT, human telomerase reverse transcriptase; IRF, interferon regulatory factor; HLA, human leukocyte antigen; IL, interleukin; LCL, lymphoblastoid B-cell line; LMP, low molecular weight polypeptide; MAb, monoclonal antibody; MHC, major histocompatibility complex; PBMC, peripheral blood mononuclear cell; PE, phycoerythrin; RT-PCR, reverse transcription polymerase chain reaction; TAP, transporters associated with antigen processing.

Grant sponsor: Ministry of Education, Culture, Sports, Science and Technology of Japan; Grant numbers: 13218152, 12217170; Grant sponsor: Second Team Comprehensive 10-year Strategy for Cancer Control from the Ministry of Health, Labour, and Welfare of Japan; Grant number: 017; Grant sponsor: Japan Society for the Promotion of Science; Grant number: 15590429; Grant sponsor: Nagono Medical Research Grant.

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Received 16 June 2003; Revised 27 August 2003, 22 December 2003; Accepted 7 January 2004

DOI 10.1002/ijc.20139
Published online 1 March 2004 in Wiley InterScience (www.interscience.wiley.com).

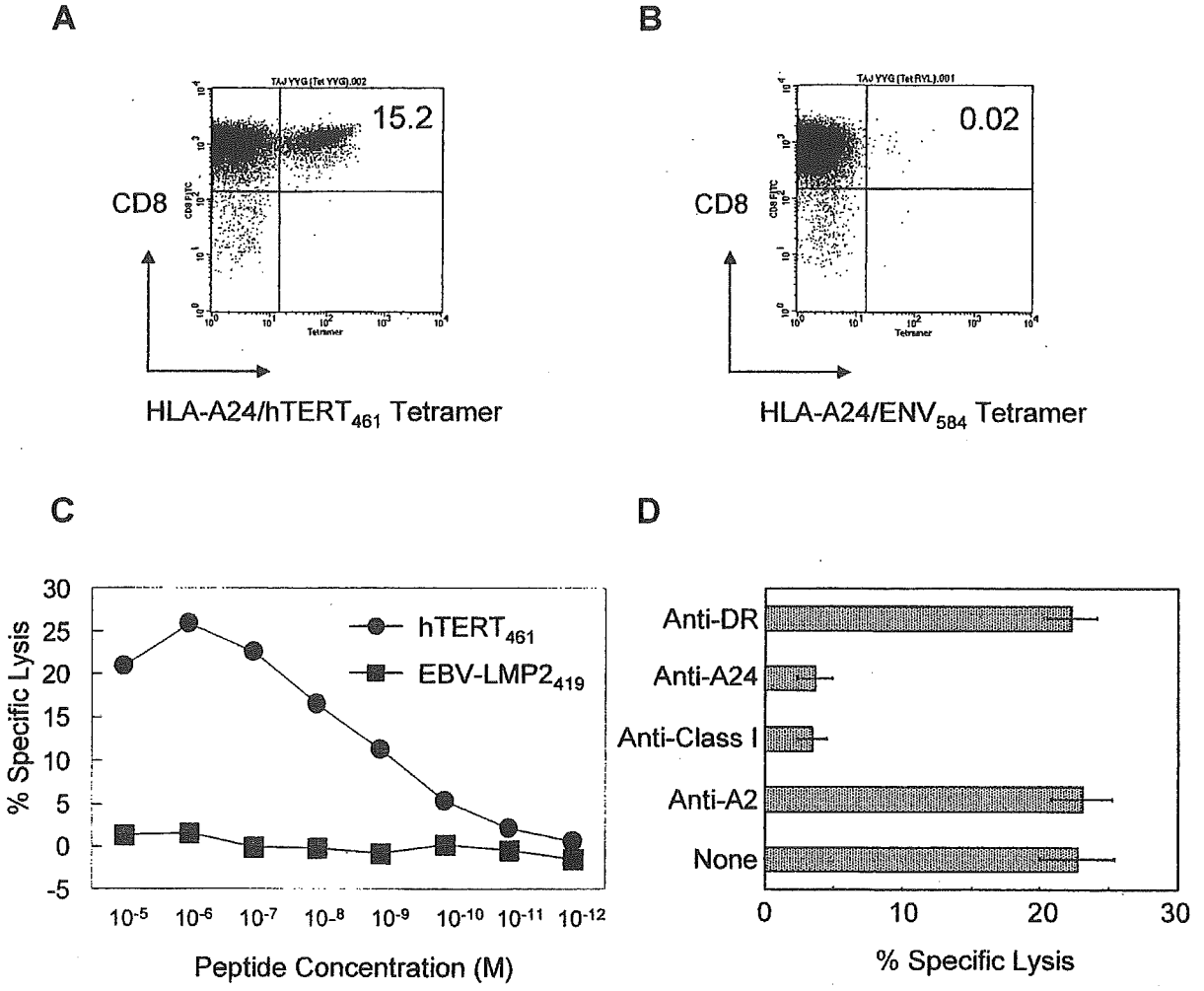

FIGURE 1 – Characterization of polyclonal CTL specific to $hTERT_{461}$. Polyclonal $CD8^+$ T cells after stimulation 4 times were stained with FITC-labeled anti-CD8 antibodies and PE-labeled HLA-A24-tetramers incorporating $hTERT_{461}$ (*a*) or a control peptide, ENV_{584} (*b*), and analyzed by flow cytometry. The percentages of tetramer-positive cells in total $CD8^+$ T cells are shown. (*c*) Results of CTL assays using serial dilutions of $hTERT_{461}$ (closed circle) and an EBV-derived control peptide, $EBV\text{-}LMP2_{419}$ (closed square). Cytotoxicity of polyclonal CTL to T2-A24 cells in the presence of indicated concentrations of each peptide was determined by ^{51}Cr release assays at an effector-target ratio of 1. (*d*) Inhibitory effect of an anti-HLA class I monoclonal antibody or an anti-HLA-A24 monoclonal antibody on cytotoxicity of a CTL clone K3-1 against a HLA-A24-positive cell line, PC9. Chromium-labeled target cells were incubated with either monoclonal antibodies specific to HLA class I, HLA-A24, HLA-A2 or HLA-DR molecules, before addition of K3-1 cells. The cytotoxic assays were done at an effector-target ratio of 10.

MATERIAL AND METHODS

Donors and cell lines

Peripheral blood mononuclear cells (PBMC) were isolated from 4 HLA-A24-positive healthy donors by centrifugation on a Ficoll density gradient. Epstein-Barr virus (EBV)-transformed LCL were established as previously described[20] and cultured in RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal calf serum (FCS) (Life Technologies Limited, Auckland, NZ), 2×10^{-3} M L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml kanamycin and 5×10^{-5} M β-mercaptoethanol (referred to as complete medium). CD40-activated B (CD40-B) cells were generated using NIH-3T3-hCD40 ligand cells (kindly provided by Dr. G. Freeman, Dana-Farber Cancer Institute, Boston, MA) as previously described.[21,22] Pulmonary fluid was obtained from an HLA-A24-positive patient with lung adenocarcinoma for primary culture of the cancer cells. The study design and purpose, which had been approved by the institutional review board of Aichi Cancer Center, were explained fully to all donors. Samples were obtained after informed consent was confirmed.

Human lung cancer cell lines, LC-1/sq and LU99 cells, were purchased from the Japanese Collection of Research Bioresources (Tokyo, Japan) and RIKEN Cell Bank (Tsukuba, Japan), respectively. LC-1/sq cells were maintained in 45% RPMI1640 medium and 45% Ham's F12 (Sigma Chemical Co.) supplemented with 10% FCS, L-glutamine, penicillin, streptomycin and kanamycin. All other lung cancer cell lines (LU99, PC9, 11-18, LC99A, LC65A, LK79, A549, QG56 and RERF-LC-MT) and a chronic megakaryoblastic leukemia cell line, MEG-01, were maintained in the complete medium. K562 cells were maintained in IMDM (Sigma Chemical Co.) supplemented with 10% FCS, L-glutamine, penicillin, streptomycin and kanamycin. HLA-A*2402-trans-

TABLE I - TELOMERASE ACTIVITY AND HLA-A24 EXPRESSION OF TARGET CELL LINES USED IN THIS STUDY

Cells	Tumor origin	Telomerase activity[1]	Surface HLA-A24 expression[2]
Lung cancer cell lines			
PC9	Adenocarcinoma	+	+
11–18	Adenocarcinoma	+	+
LC-1/sq	Squamous cell carcinoma	+	+
LU99	Giant cell carcinoma	+	+
LK79	Small cell carcinoma	+	+
LC99A	Large cell carcinoma	+	+
LC65A	Small cell carcinoma	+	−
RERF-LC-MT	Adenocarcinoma	+	−
A549	Adenocarcinoma	+	−
QG56	Squamous cell carcinoma	+	−
Hematopoietic cell lines[3]			
MEG-01	Leukemia	+	+
K562	Leukemia	+	−
T2-A24	—	Not done	+

[1]Telomerase activity was detected as described in the Material and Methods.–[2] To detect surface expression of HLA-A24 molecules, cells were stained with an anti-HLA-A24 antibody and subsequently with FITC-labeled anti-mouse IgG $F(ab')_2$ fragments and analyzed by flow cytometry.–[3]MEG-01, a control cell line expressing telomerase and HLA-A24 molecules; K562, a representative cell line susceptible to natural killer-like cytotoxicity; T2-A24, a TAP-deficient cell line expressing HLA-A24 molecules.

fected, TAP-negative T2-A24 cells[23] were cultured in complete medium containing 0.8 mg/ml of G418 (Gibco, Grand Island, NY). Pulmonary fluid containing adenocarcinoma cells was diluted with the complete medium and cultured in the presence or absence of IFN-γ for 48 hr. After the incubation, adherent cells were used for RT-PCR analysis and as target cells for hTERT-specific CTL.

A retrovirus encoding HLA-A*2402 was infected into the HLA-A24-negative cell lines, QG56 and A549, as previously described.[24] The infected cells were maintained in complete medium with puromycin at the final concentration of 0.6 (for QG56) or 0.9 (for A549) μg/ml for selection and designated as QG56–A24 and A549–A24, respectively.

Peptides

Two HLA-A24-restricted CTL epitope peptides derived from hTERT,[14] VYAETKHFL (residues 324–332, designated as $hTERT_{324}$) and VYGFVRACL (residues 461–469, designated as $hTERT_{461}$), a human immunodeficiency virus-1 (HIV-1) envelope peptide RYLRDQQLL[25] (residues 584–592, designated as ENV_{584}) and an EBV latent membrane protein 2 peptide TYGPVFMCL[20] (residues 419–427, designated as EBV-$LMP2_{419}$) were synthesized by Toray Research Center (Kamakura, Japan).

Cell staining and flow cytometric analysis

Surface expression of HLA-A24 molecules was examined by indirect immunofluorescence using an HLA-A24 MAb (One Lambda, Inc. Canoga Park, CA) and FITC-labeled anti-mouse IgG $F(ab')_2$ fragments (IMMUNOTECH, Marseilles, France). MHC-tetramers were produced as previously described.[23,26] $CD8^+$ T cell lines were stained with PE-labeled HLA-A*2402-tetramers incorporating $hTERT_{324}$, $hTERT_{461}$ or ENV_{584}. Flow cytometric analysis of the stained cells was performed using a FACSCalibur (Becton Dickinson, San Jose, CA) and the data were analyzed using CellQuest software (Becton Dickinson).

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from cultured cell lines. Gene-specific oligonucleotide primers were synthesized at Proligo (Kyoto, Japan) and used to evaluate the mRNA expression pattern of hTERT,[13] TAP-1, TAP-2[27] and IRF-1.[28] RT-PCR was performed using a thermal cycler (Perkin-Elmer, Wellesley, MA) and the products were analyzed by 1.5% gel electrophoresis and ethidium bromide visualization.

Western blot analysis

Western blot analysis was performed as described previously[29] with slight modifications. Briefly, cells were lysed in lysis buffer (50 mM Tris/HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, 0.5% Triton X-100, 10 μM leupeptin, 2.8 μM pepstatin and 0.85 mM phenylmethanesulfonyl fluoride) for 30 min at 4°C. The post-nuclear supernatant was quantified by absorbance at 280/260 nm for protein concentrations, and aliquots of 130 μg protein were applied to 12% SDS-PAGE. The proteins were blotted onto Immobilon-P membranes (Millipore Corporation, Bedford, MA), blocked with PBS containing 10% low fat dry milk and 0.1% Tween-20 overnight at 4°C and probed with rabbit polyclonal Abs specific to low molecular weight polypeptide 7 (LMP7) and proteasome activator 28 (PA28) α subunits (Affinity, Mamhead, U.K.) followed by peroxidase-conjugated goat anti-rabbit IgG (Zymed, San Francisco, CA). Proteins were visualized using an ECL Western blot detection system (Amersham Biosciences, Buckinghamshire, UK).

Generation of hTERT-specific polyclonal and clonal CTL using peptide-pulsed CD40-B cells as antigen presenting cells

CD40-B cells (2.5×10^5) were pulsed with $hTERT_{324}$ or $hTERT_{461}$ at 1×10^{-5} M for 1 hr and irradiated at 33 Gy. $CD8^+$ T cells (1×10^6) were isolated from donated PBMC with the aid of CD8 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and cocultured with autologous peptide-pulsed CD40-B cells in 2 ml culture medium in the presence of 25 ng/ml IL-7 (Genzyme, Cambridge, MA) and 5 ng/ml IL-12 (R&D Systems, Minneapolis, MN) at 37°C in a 5% CO_2 incubator. On days 7, 14, 21 and 28, $CD8^+$ T cells were restimulated with peptide-pulsed and γ-irradiated CD40-B cells. One day after each restimulation, human recombinant IL-2 (Takeda Chemical Industries, Osaka, Japan) was added to a final concentration of 20 U/ml. If necessary, rapidly growing cells were split into 2 to 3 wells and fed with fresh culture medium containing 20 U/ml of IL-2. Specificity of the T cells was examined with tetramer staining and cytotoxic assays. To establish T-cell clones, limiting dilution of the polyclonal CTL was performed.[23] Briefly, polyclonal $CD8^+$ T cells were seeded at 1 or 3 cells/well in round-bottomed 96-well plates containing the culture medium (0.2 ml) with anti-CD3 MAb (30 ng/ml, Ortho Diagnostics, Raritan, NZ), IL-2 (30 U/ml), γ-irradiated (33 Gy) 1×10^5 PBMC and γ-irradiated (55 Gy) 2×10^4 LCL. After 2 weeks of culture, growing cells positively stained for the HLA-A*2402/$hTERT_{461}$-tetramer were transferred into flasks and expanded as above.

CTL assay

Target cells were labeled with chromium (^{51}Cr) in 100 μl culture medium for 1 h at 37°C. In some experiments, predetermined amounts of blocking antibodies, W6/32 (anti-HLA class I), MA2.1 (anti-HLA-A2), A11.1 (anti-HLA-A24) and HDR-1 (anti-HLA class II) were added to the wells 30 min before adding

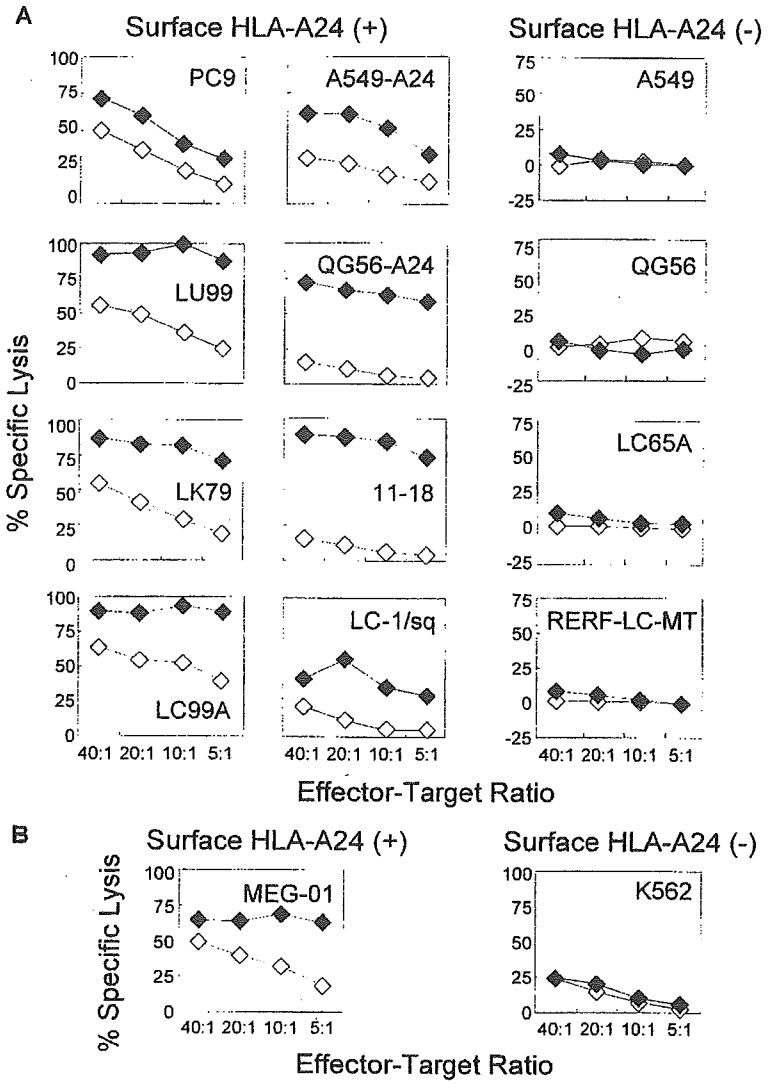

FIGURE 2 – Cytotoxicity of $hTERT_{461}$-specific CTL clone, K3-1 against cancer cell lines. (a) Cytotoxicity of $hTERT_{461}$-specific CTL clone K3-1 for 8 lung cancer cell lines with positive surface HLA-A24 expression and 4 with negative surface HLA-A24 expression as target cells (see Table I). The HLA-A*2402 gene was retrovirally transfected into A549 and QG56 cells, and the resultant transfectants designated as A549-A24 and QG56-A24, respectively. Assays were performed in the presence (closed diamond) or absence (open diamond) of 1×10^{-7} M $hTERT_{461}$ at the indicated effector-target ratios. (*b*) Cytotoxicity of $hTERT_{461}$-specific CTL clone K3-1 against MEG-01, a control hematopoietic cell line expressing telomerase and HLA-A24 molecules, and K562, a representative cell line susceptible to natural killer cytotoxicity, as target cells. Assays were performed in the presence (closed diamond) or absence (open diamond) of 1×10^{-7} M $hTERT_{461}$ at the indicated effector-target ratios.

effector cells to determine the HLA restriction. In others, target cells were treated with 100 U/ml of IFN-γ for 48 hr before chromium labeling. The plates were incubated for 5 hr at 37°C, and the supernatants were counted in a gamma counter. The percentage specific ^{51}Cr release was calculated as follows: 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release).

Extraction of naturally processed peptides from cell lines

Isolation of peptides from cell cultures was performed as previously described[30] with slight modifications. Briefly, confluent PC9 or LU99 cells (either treated or untreated with IFN-γ for 48 hr) in T225 flasks (Costar, Cambridge, MA) were washed 3 times with PBS and incubated with 5 ml of citrate-phosphate buffer (pH 3.3) for 1 min. The buffer containing eluted peptides was harvested and stored at −80°C until use. Peptides were repetitively stripped for 4 consecutive days.

The acid-extracted peptides were filtered and concentrated on SepPak Light C18 Cartridges (Waters Corporation, Milford, MA) according to the manufacturer's instructions. Bound peptides were eluted with 80% acetonitrile and 0.1 % trifluoroacetic acid, concentrated in a Speed-Vac (Savant Instruments, Inc., Hicksville, NY) and pulsed on ^{51}Cr-labeled T2-A24 cells. K3-1-mediated target cell lysis was assessed as described above.

Measurement of telomerase activity

Telomerase activity was measured by the telomeric repeat amplification protocol using Telo TAGGG Telomerase PCR ELISA^{PLUS} (Roche Diagnostics Corporation, Indianapolis, IN) according to the manufacturer's instruction. Samples were considered as telomerase-positive if the difference in absorbance (absorbance of sample − absorbance of heat-treated sample) was more than 2-fold background activity, according to the protocol supplied with the reagents.

RESULTS

Generation of hTERT peptide-specific $CD8^+$ CTLs

To generate hTERT-specific $CD8^+$ T cell lines, $CD8^+$ T cells of 4 HLA-A24-positive healthy donors were stimulated weekly with autologous CD40-B cells pulsed with either of the HLA-A*2402-restricted hTERT-derived peptides, $hTERT_{324}$ or $hTERT_{461}$. After

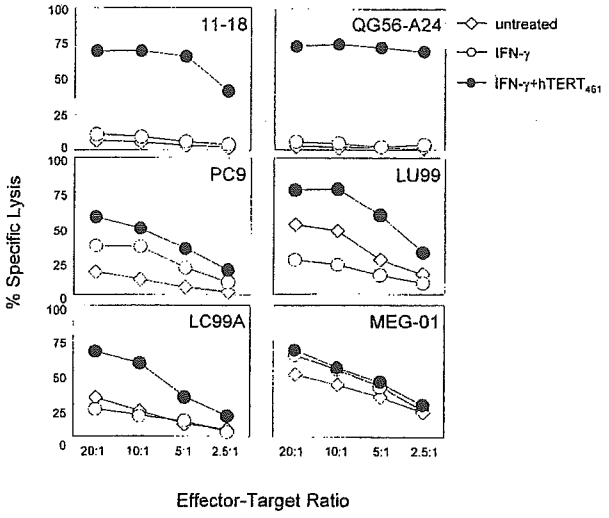

FIGURE 3 – K3-1-mediated lung cancer cell lysis induced by IFN-γ treatment. Cytotoxicity of the $hTERT_{461}$-specific CTL clone K3-1 against HLA-A24-positive lung cancer cells determined with (open circle) or without (open diamond) IFN-γ pre-treatment (100 U/ml for 48 hr). Cytolysis of IFN-γ treated cells was also tested in the presence of 1×10^{-7} M $hTERT_{461}$ (closed circle). Assays were performed at the indicated effector-target ratios.

the fourth stimulation, the T-cell lines were stained with HLA-A24-tetramers incorporating $hTERT_{324}$, $hTERT_{461}$ or ENV_{584}. A T cell line from a donor stimulated with $hTERT_{461}$ was specifically stained with HLA-A24-tetramers incorporating $hTERT_{461}$ but not ENV_{584} (15.2% *vs.* 0.02% in total CD8+ cells, Fig. 1*a*,*b*). This line showed cytotoxicity to T2-A24 cells pulsed with $hTERT_{461}$ dose-dependently but not with control peptide $EBV\text{-}LMP2_{419}$ (Fig. 1*c*). None of the other polyclonal T-cell lines were stained with HLA-A24-tetramers incorporating peptides used for individual stimulation, even after a fifth stimulation (data not shown).

A $CD8^+$ CTL clone was established by limiting dilution of the polyclonal T-cell line and designated as K3-1. The integrity of K3-1 was assessed with the HLA-A24/$hTERT_{461}$-tetramer (data not shown).

Lysis of lung cancer cell lines by the $hTERT_{461}$ peptide-specific CTL clone, K3-1

We next examined K3-1-mediated cytotoxicity against a panel of lung cancer cell lines (Table I). Among the 10 lung cancer cell lines examined, 6 were positive for HLA-A24 expression, and all cell lines featured telomerase activity (Table I). Results for cytotoxicity are summarized in Figure 2*a*, only HLA-A24-positive lung cancer cell lines (PC9, LU99, LK79 and LC99A) being affected. The degree of cell lysis was comparable to that observed for a leukemia cell line, MEG-01 cells (Fig. 2*b*), previously reported to be well recognized by HLA-A24-restricted hTERT-specific CTL.[14] The cytotoxicity of K3-1 against PC9 cells was blocked by an anti-HLA-A24 MAb, but not anti-HLA-A2 or HLA-DR MAbs showing HLA-A24 restriction (Fig. 1*d*). The K3-1-mediated cytotoxicity against PC9 cells was specifically inhibited by the presence of T2-A24 cells pre-pulsed with the cognate but not an irrelevant peptide (data not shown), indicating K3-1 could recognize $hTERT_{461}$ naturally processed and presented on the surfaces of the target cells. As shown in Figure 2*a* (center column), some HLA-A24-positive and HLA-A24-transfected lung cancer cell lines were not effectively lysed by K3-1 despite confirmation of surface expression of HLA-A24 by indirect immunofluorescence and flow cytometry. However, the cytotoxicity against these cell lines was enhanced in the presence of $hTERT_{461}$ in the medium, suggesting insufficient epitope density on these cells.

HLA-A24-negative cell lines, A549, QG56, LC65A and RERF-LC-MT cells were not lysed at all by K3-1 either in the presence or absence of the cognate peptide (Fig. 2*a*, right column). K562 cells were included to assess the degree of NK-like cytotoxicity of K3-1 cells, which turned out to be negligible (Fig. 2*b*).

K3-1-mediated lung cancer cell lysis after IFN-γ treatment

Pretreatment of target cells with IFN-γ is well known to augment epitope processing and presentation.[30] Thus, we asked the question whether IFN-γ treatment augments CTL-mediated cell lysis of the cytolysis-negative cell lines (Fig. 2*a*, center column) by improved epitope processing and presentation.[30] The cytolysis-positive cell lines were also tested. As demonstrated in Figure 3, there was no augmentation of K3-1-mediated lysis in the 11–18 and QG56-A24 cases. Lysis of LC-1/sq and A549-A24 cells was also not augmented by IFN-γ treatment (data not shown). Of note, K3-1-mediated lysis of PC9, LU99, LC99A and MEG-01 cells was differentially affected by IFN-γ pretreatment (Fig. 3). Thus the lysis of PC9 and MEG-01 cells was increased by the treatment, but with LC99A cells, it was unchanged or slightly decreased. Most interestingly, the lysis of LU99 cells was clearly reduced by the IFN-γ treatment.

IFN-γ induces gene expression of components involved in antigen processing and presentation in the lung cancer cells

Unexpectedly, IFN-γ affected K3-1-mediated lysis differently on PC9, LU99, LC99A and MEG-01 cells (Fig. 3). Therefore, we examined whether there is any difference of expression pattern of molecules important for class I antigen presentation. First, HLA-A24 expression was studied and found to be increased after IFN-γ treatment in all the cell lines (Fig. 4*a*). Second, expression of TAP-1 and TAP-2 was studied using semi-quantitative RT-PCR, mRNAs of both being also consistently increased after the treatment (Fig. 4*b*). Third, the expression of the LMP7, 1 of the 3 catalytic subunits of immunoproteasomes, and PA28, a regulator of the immunoproteasome, was studied using Western blotting with specific MAbs. In all the cell lines but QG56-A24, where the expression did not change, both proteins were increased after the treatment (Fig. 4*c*). In summary, we could not detect any difference in expression patterns of these molecules to account for the differential influence of IFN-γ.

Differential susceptibility of lung cancer cell lines to cytotoxicity of the CD8+ CTL clone, K3-1

To disclose the differential susceptibility to K3-1 in more detail, we compared cytotoxicity against 2 lung cancer cell lines, PC9 cells whose lysis was increased by IFN-γ and LU99 cells whose lysis was decreased, in the presence of a wide range of cognate peptide concentrations. After IFN-γ treatment, PC9 cells were efficiently lysed by K3-1 with any concentration of the peptide (Fig. 5*a*). In contrast, PC9 cells without IFN-γ treatment and LU99 cells, irrespective of IFN-γ treatment, demonstrated exogeneous peptide dose-dependent K3-1-mediated cell lysis, which was similar to the results using T2-A24 cells as target cells (compare Fig. 5*a* and Fig. 1*c*). These observations strongly suggest that the epitope density is saturated on the PC9 cells after IFN-γ treatment but not on the PC9 cells without the treatment and LU99 cells either with or without IFN-γ treatment.

In addition, to confirm that the epitope density was increased in PC9 but not LU99 cells after IFN-γ treatment, naturally processed peptides were acid-eluted from the cells, concentrated and tested by K3-1 after pulsing on T2-A24 cells applying ^{51}Cr-release assays. The results demonstrated in Figure 5*b* indicate elevation in the epitope peptides on the surfaces of PC9 but not LU99 cells after the IFN-γ treatment.

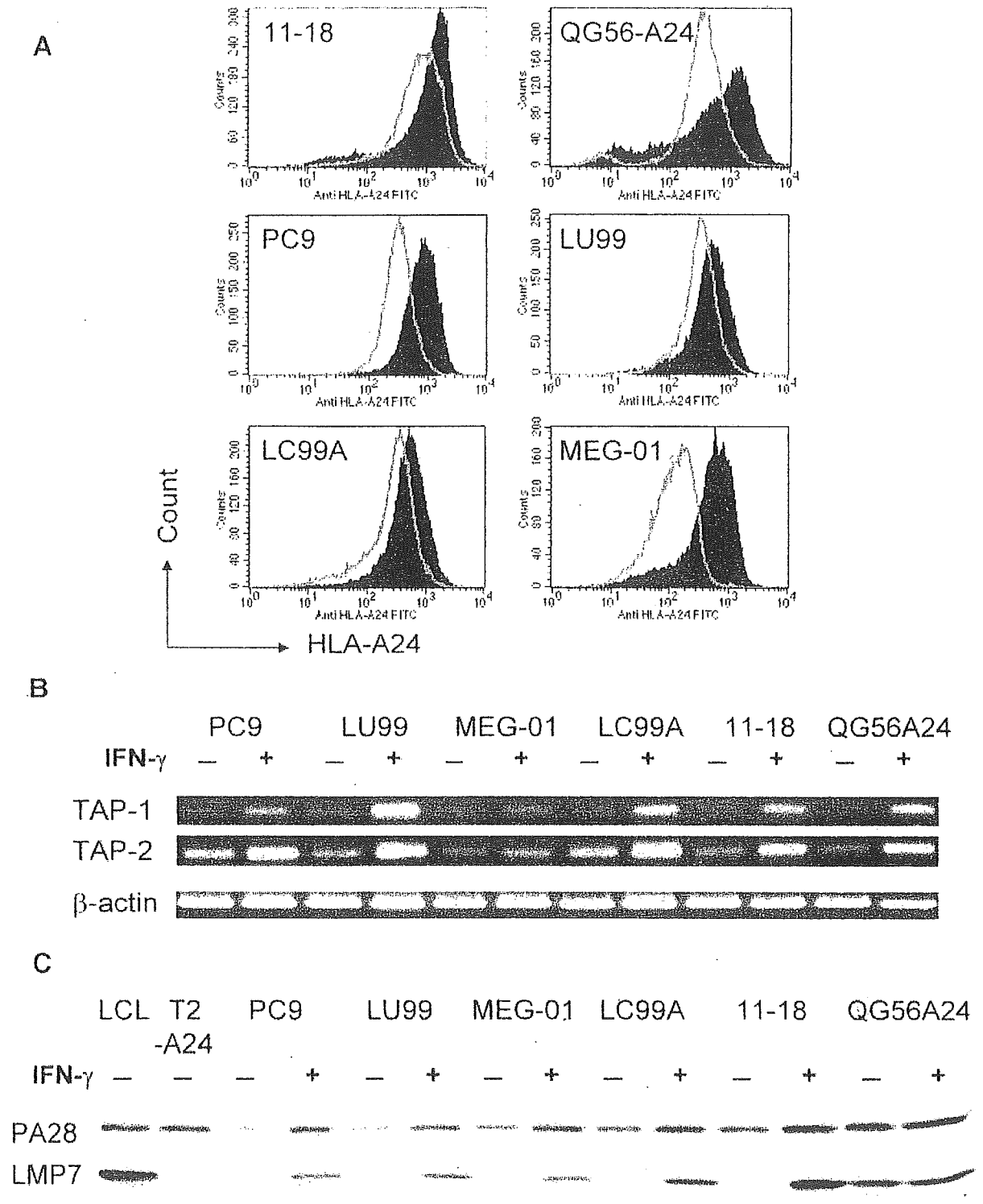

FIGURE 4 – Effects of IFN-γ on the regulation of molecules which play roles in antigen processing and presentation. (*a*) Results for surface HLA-A24 expression with (black shadowed) and without (lined) IFN-γ treatment analyzed by flow cytometry. Surface expression of HLA-A24 molecule was examined by indirect immunofluorescence using an HLA-A24 MAb and FITC-labeled anti-mouse IgG $F(ab')_2$ fragments. (*b*) Results of semi-quantitative RT-PCR analysis of TAP-1, and -2. Primers specific for TAP-1, and -2, as well as β-actin as a control were used for amplification of mRNA from cancer cell lines either treated or untreated with IFN-γ. (*c*) Results of Western blot analysis of PA28 and LMP7 molecules. Samples were obtained before and after treatment of cancer cells with IFN-γ.

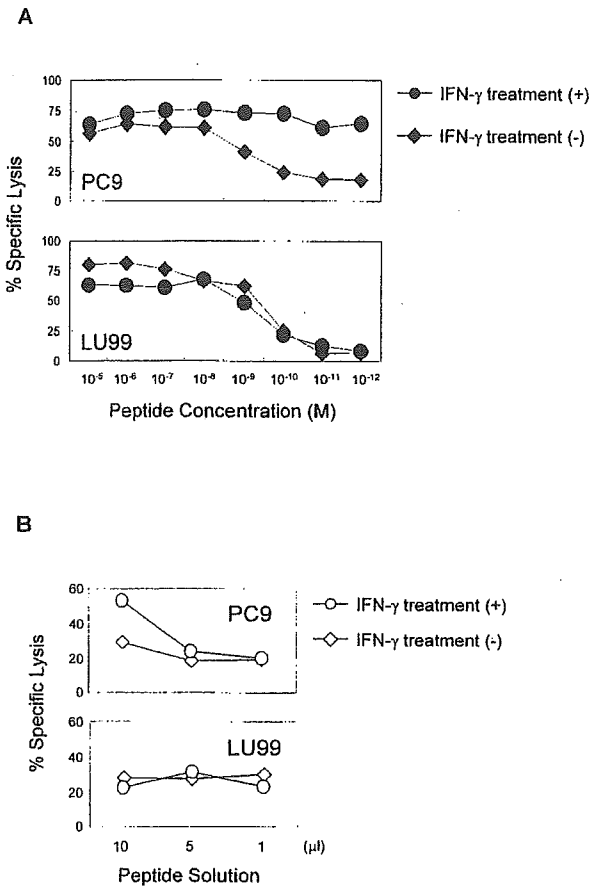

FIGURE 5 – The effects of IFN-γ on susceptibility of PC9 and LU99 cell lines to K3-1. (*a*) Cytotoxicity of the $hTERT_{461}$-specific CTL clone K3-1 against HLA-A24-positive lung cancer cells, PC9 or LU99, either treated (closed circle) or untreated (closed diamond) with 100 U/ml IFN-γ for 48 hr, as determined in ^{51}Cr release assay. The assay was performed in the presence of the indicated concentrations of the peptide $hTERT_{461}$ at an effector-target ratio of 5. (*b*) Naturally processed peptides were isolated from PC9 and LU99 cells, either treated (open circle) or untreated (open diamond) with IFN-γ, and concentrated. Indicated volumes of the peptide solution were pulsed on ^{51}Cr-labeled T2-A24 cells. K3-1-mediated target cell lysis was assessed at an effector-target ratio of 5.

Down-regulation of hTERT expression induced by IFN-γ

Very recently, Lee *et al.*[28] reported that telomerase activity and hTERT expression are attenuated by IFN-γ treatment, mediated by interferon regulatory factor-1 (IRF-1) in human cancer cell lines. As demonstrated in Figure 6, hTERT expression was decreased in LU99, LC99A and QG56-A24 cells after the IFN-γ treatment but not in PC9, 11-18 or MEG-01 cells. Taking into account the uniform up-regulation of immunoproteasome genes, TAPs and HLA-A*2402 molecules, the results strongly suggest that epitope supply to the surfaces of PC9 cells was increased and decreased to those of LU99 cells after IFN-γ treatment. Lee *et al.*[28] reported that induction of IRF-1 was closely correlated with attenuation of hTERT expression induced by IFN-γ treatment. However, IRF-1 induction was observed not only in cell lines such as LU99, LC99A and QG56-A24 where hTERT expression decreased but also in PC9 and MEG-01 where it did not (Fig. 6).

Attenuation of hTERT expression and K3-1-mediated cell lysis of primary lung cancer cells after IFN-γ treatment

Finally, we tested primary adenocarcinoma cells, obtained from a pulmonary fluid sample, to see the impact of IFN-γ treatment on hTERT expression and sensitivity to K3-1. As demonstrated in Figure 7, both hTERT expression and K3-1-medited cell lysis were attenuated after IFN-γ treatment. The results strongly suggest that IFN-γ impacts on hTERT expression and sensitivity to hTERT-specific CTLs *in vivo* as well as *in vitro*.

DISCUSSION

It was earlier reported that an HLA-A24-restricted $hTERT_{461}$-specific CTL could efficiently lyse hematological malignancies.[14] Thus, in our study, we addressed the question whether this epitope-specific CTL could similarly lyse lung cancer cells. An $hTERT_{461}$-specific CTL clone, K3-1, was generated from a healthy donor by repeated peptide stimulation and demonstrated to specifically lyse at least some lung cancer cell lines in an HLA-A24-restricted fashion. However, other examples of HLA-A24-positive lung cancer cell lines were not effectively lysed (Fig. 2*a*, center column), despite possessing telomerase activity. Sequence analysis of hTERT in these lung cancer cell lines revealed no mutation around the epitope (data not shown). Furthermore, K3-1 cytotoxicity against these cell lines was enhanced in the presence of cognate peptide, suggesting an insufficient epitope density. Pretreatment of the cell lines with IFN-γ did not, however, augment the CTL-mediated cytotoxicity. Ayyoub *et al.*[31] reported that an HLA-A2-restricted hTERT peptide 540–548-specific CD8+ T cells did not recognize tumor because of inefficient antigen processing, and we speculate that the epitope $hTERT_{461}$ is not processed and/or presented efficiently in some cell lines for unknown reasons.

IFN-γ plays important roles in the immune response not only to virus infection but also to tumors, up-regulating various genes including HLA class I,[32,33], ER peptide transporters (*e.g.* TAP1, 2),[34,35] proteasome β subunits (*e.g.* LMP2, 7, 10)[36–38] and proteasome regulators (*e.g.* PA28),[39], which contribute to antigen pro-

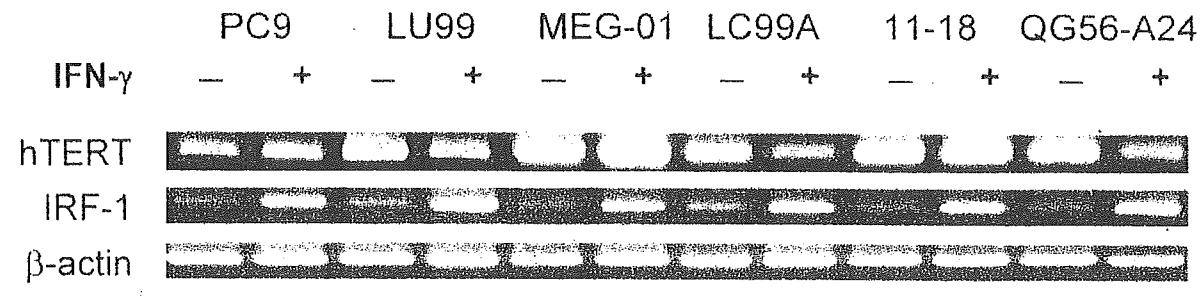

FIGURE 6 – RT-PCR analysis of hTERT and IRF-1 in cancer cell lines. Semi-quantitative RT-PCR analysis was performed using specific primers to hTERT, IRF-1 and β-actin. The mRNAs were isolated from cancer cells either treated or untreated with IFN-γ for 48 hr.

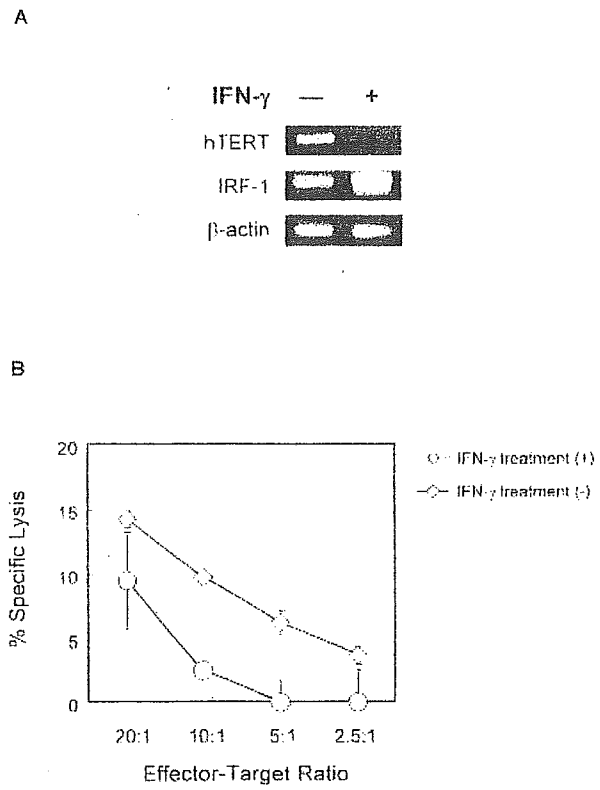

FIGURE 7 – Impact of IFN-γ treatment on primary lung cancer cells. (*a*) Freshly isolated adenocarcinoma cells from an HLA-A24-positive patient was treated or untreated with IFN-γ for 48 hr. Semi-quantitative RT-PCR analysis was performed using specific primers to hTERT, IRF-1 and β-actin. (*b*) Cytotoxicity of the $hTERT_{461}$-specific CTL clone K3-1 was determined with (open circle) or without (open diamond) IFN-γ pre-treatment (100 U/ml for 48 hr). Assays were performed at the indicated effector-target ratios. Bars indicate standard deviations.

cessing and presentation. In general, IFN-γ treatment is believed to enhance the presentation of peptides in the context of HLA class I molecules on the surface of target cells, leading to more efficient recognition by CTL. In our study, however, K3-1-mediated lysis of PC9, LU99, LC99A and MEG-01 cells was differentially affected by IFN-γ pre-treatment (Fig. 3). In addition, CTL assays using acid extracted peptides from cells indicated that the epitope was increased on PC9 but not LU99 cells after IFN-γ treatment (Fig 5*b*).

Paradoxical effects of IFN-γ on CTL recognition have in fact been reported. One example is induction of immunoproteasomes by IFN-γ treatment, with destruction of RU1-specific CTL epitopes and loss of CTL recognition.[40] In our study, immunoproteasomes did not appear to cleave the peptide within the $hTERT_{461}$ because the K3-1-mediated cytotoxicity against PC9 or MEG-01 cells was enhanced after IFN-γ treatment. In addition, LCL have been shown to express immunoproteasomes constitutively,[41] as here confirmed by Western blot analysis (Fig. 4*c*). Some telomerase-positive LCL were efficiently lysed by K3-1 in an HLA-A24-restricted fashion (data not shown), suggesting that immunoproteasomes do not destroy the $hTERT_{461}$ epitope. Another paradoxical effect of IFN-γ is mediated by inhibitory natural killer cell receptors expressed on the effector cells inducing inhibitory signaling.[42] Such receptors bind to several HLA-class I molecules, which could be upregulated by IFN-γ, thus executing the inhibitory effect of CTL-mediated target cell lysis after treatment with IFN-γ. Indeed, Malmberg *et al.*[43] reported that IFN-γ treatment inhibited lysis of ovarian cancer cells by specific CTL *via* a CD94/NKG2A-dependent mechanism. However, this could be excluded in the present case because LU99 cells treated with IFN-γ were efficiently lysed after being pulsed with cognate peptide (Fig. 3), and surface expression of CD94 on K3-1 was not detected by flow cytometric analysis (data not shown). In addition, inhibitory natural killer cell receptors, such as p58.1/KIR2DL1 or p58.2/KIR2DL2/3, were not found to be expressed on K3-1 (data not shown).

We demonstrated that hTERT expression itself was attenuated in the LU99 cells after IFN-γ treatment, resulting in inefficient recognition by the hTERT-specific CTL. The same attenuation was observed in primary lung cancer cells obtained from a lung cancer patient (Fig.7). A few reports revealed that IFN-γ reduces the expression of tumor antigens, such as MART-1/Melan A[44] or murine gp70.[45] With regard to these antigens, IFN-γ may promote immune-escape of tumors because these are not necessary for tumor proliferation. However, it is of particular interest to consider the effects of IFN-γ on telomerase activity in cancer immunity. IFN-γ may exert an anti-tumor influence primarily by suppressing hTERT transcription, resulting in limited proliferative potential. If such hTERT suppression is no longer occurring by whatever mechanism, such as IRF-1 gene inactivation as observed in some cancer cells,[46] IFN-γ might increase hTERT epitope processing and presentation leading to augmented susceptibility to specific CTL, as shown in PC9 cells (Figs. 3 and 5). Thus, the effects of IFN-γ on tumor cells through modulation of hTERT expression can be considered to feature a "fail safe" mechanism for efficient anti-tumor activity due to its impact on innate and adaptive immunity. With regard to clinical application, immunotherapy for malignant tumors using hTERT-specific CTL has unique advantages. hTERT-specific CTL not only kill tumor cells through the recognition of epitopes expressed on their surfaces but also produce and release IFN-γ *in situ*. Indeed, Le Poole *et al.*[44] reported that examination of melanoma lesions by quantitative reverse transcriptase-polymerase chain reaction revealed up to 188-fold more abundant IFN-γ transcripts produced by T cells when compared to control skin. In such circumstances, hTERT expression of tumor cells could be downregulated, resulting in suppressed tumor growth. However, some HLA-A24 positive lung cancer cells with hTERT expression were not efficiently recognized by hTERT-specific CTL, probably because of low epitope density on the cell surface. The effects of hTERT-specific CTL against such tumor cells might thus be limited. Downregulation of K3-1-mediated lysis was less pronounced with LC99A cells, although there was clear attenuation of hTERT transcription after IFN-γ treatment (Figs. 3 and 6). The reason is unclear but it could be speculated that more efficient processing and/or presentation might compensate for any shortage of hTERT proteins.

A previous study revealed that hTERT transcription may be decreased after IFN-γ treatment through induction of IRF-1.[28] Our study also confirmed downregulation of hTERT expression after IFN-γ treatment in 3 of 6 cell lines examined and primary lung cancer cells from a patient, in parallel with IRF-1 induction. However, in PC9, MEG-01 and 11–18 cells where hTERT expression did not decrease, IRF-1 was also induced. These equivocal findings for IRF-1 might be related with functional inactivation of the IRF-1 gene[46,47] or deletion or mutation of putative IRF-1 binding sites in the hTERT promoter. Alternatively, other yet-to-be identified third party molecules that cooperate with IRF-1 might be inactivated. Further studies are required to clarify the

mechanisms underlying the effect of IFN-γ upon down-regulation of hTERT expression.

In conclusion, we propose here a mechanism of attenuated CTL-mediated lysis of tumor cells through hTERT down-regulation induced by IFN-γ. Our study indicates that hTERT-specific CTL-based immunotherapy could be effective in patients with lung cancers which present relevant epitopes.

ACKNOWLEDGEMENTS

Valuable discussion and suggestions by Dr. T. Tsurumi, Dr. M. Yasukawa and Dr. T. Kiyono are highly appreciated. We are very grateful to Y Matsudaira and H Tamaki for their secretarial help. We also greatly appreciate Dr. M. Seto and Dr. T. Takahashi for generously donating cancer cell lines.

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The impact of *EGFR* mutations on gefitinib sensitivity in non-small cell lung cancer

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Gefitinib (ZD1839, Iressa®; AstraZeneca) has produced objective tumor responses and symptom improvement in some patients with non-small cell lung cancer. In clinical trials, 12–18.4% of patients had a rapid and often dramatic clinical response, and a subset analysis of the Iressa Dose Evaluation in Advanced Lung Cancer (IDEAL)-1 and -2 trials demonstrated that female gender and adenocarcinoma were associated with a higher response to gefitinib. However, analysis from clinical trials have not found a relationship between epidermal growth factor receptor (EGFR) expression and response in patients receiving gefitinib. Recently, three studies have identified mutations affecting the EGFR in lung cancer from patients who respond to gefitinib. *EGFR* gene mutations were common in lung cancer from 'never smokers' and were associated with sensitivity of tumors to gefitinib. Furthermore, it has been reported that the phosphatidylinositol 3-kinase/Akt signaling pathway plays a critical role in the antitumor effects of gefitinib. Although *EGFR* mutations do not fully explain the clinical benefit, the data regarding *EGFR* mutations may help to define the patient population that will most likely benefit from EGFR tyrosine-kinase-targeted therapies.

Keywords: Akt, EGFR, *EGFR* mutation, epidermal growth factor receptor, gefitinib, gefitinib sensitivity, non-small cell lung cancer, NSCLC, tyrosine kinase inhibitor

future medicine

The epidermal growth factor receptor (EGFR, also known as ErbB1 or HER1) is a transmembrane receptor tyrosine kinase (TK) of the HER family (HER2/neu [ErbB2], HER3 [ErbB3], and HER4 [ErbB4]), and is activated by the extracellular binding of a variety of ligands [1]. The receptor plays an important role in the proliferation and metastasis of tumor cells [1], and several human cancers (e.g., cancers of the upper aerodigestive tract, colon, pancreas, breast, ovary, bladder, kidney, and gliomas) display EGFR RNA and/or protein overexpression [2-10]. Current molecularly targeted agents aim at inhibiting specific pathways and key molecules that are involved in tumor growth and progression, while sparing normal cells. Examples of such agents include trastuzumab, which is a monoclonal antibody that targets the HER2/neu receptor protein in breast cancer, and imatinib mesylate, a small molecule receptor tyrosine kinase inhibitor (TKI) that targets Bcr/abl and c-Kit in chronic myelogenous leukemia and gastrointestinal stromal tumors, respectively [11,12]. Depending on the specific molecule targeted and the mechanism of inhibition, these agents may offer novel clinical benefits compared with the outcomes of cytotoxic chemotherapy, or at least comparable benefits with reduced general toxicity and improved convenience.

The TK activity of EGFR has received considerable attention as a target for cancer therapy [1,13-15]. In recent clinical trials, the selective and orally active EGFR TKI gefitinib (ZD1839, Iressa®; AstraZeneca) has produced objective tumor responses and symptom improvement in some patients with non-small cell lung cancer (NSCLC) who had previously received chemotherapy [16,17]. Partial clinical responses to gefitinib have been observed most frequently in females, non-smokers and patients with adenocarcinomas [16-18]. In addition, a significant variability in the response to gefitinib has been noted, with higher responses seen in Japanese patients when compared with a predominantly European-derived population (27.5 versus 10.4%) [16]. The EGFR TKIs are the first class of oral targeted therapies to produce such responses in advanced NSCLC. However, two large, multinational Phase III studies of gefitinib in combination with standard platinum-based chemotherapy regimens in the first-line treatment of patients with NSCLC did not show any added advantage [19,20].

The molecular mechanisms underlying sensitivity to gefitinib were unknown. However, three recent studies have identified mutations affecting the EGFR present in lung cancer tissues from patients who responded to gefitinib. These findings may help select patients who can benefit from treatment with gefitinib in the future. This review will concentrate on the agent most advanced in clinical development, the EGFR TKI gefitinib, and discuss the predictive factors for response to gefitinib.

10.1517/17410541.1.1.27 © 2004 Future Medicine Ltd ISSN 1741-0541

Gefitinib

EGFR is a ubiquitous 170-kDa membrane-spanning glycoprotein composed of an amino-terminal extracellular ligand-binding domain; a hydrophobic transmembrane region; a cytoplasmic domain that contains the TK domain; and a carboxy-terminal region that contains critical tyrosine residues and receptor regulatory motifs [21]. Ligand binding leads to the formation of a variety of activated ErbB receptor homo- and heterodimer complexes. Dimerization produces structural changes in the intracellular portion of the receptor that activate the TK domain. The enzymatic activity of EGFR TK transfers phosphate moieties from ATP to specific tyrosine residues in the cytoplasmic tail of the EGFR protein. These phosphorylated tyrosines serve as the docking sites for a number of signal transducers and adaptor molecules that initiate a plethora of signaling pathways, resulting in cell proliferation, differentiation, migration, adhesion, protection from apoptosis, and transformation (**Figure 1**).

Among the multiple signal transduction pathways activated by the EGF family, the mitogen-activated protein kinase (MAPK; ERK-2) pathway is one of the most relevant; it regulates cellular processes, such as gene transcription and proliferation, by activating a variety of substrates located in the cytosol, nucleus, and plasma membrane [22-24]. Another important signal transduction pathway activated by the EGF family of receptors is the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway, which mediates cell survival [25,26].

Different approaches to inhibiting EGFR have resulted in a number of EGFR-targeted agents in clinical development, including small molecule EGFR TKIs, monoclonal antibodies, vaccines, immunotoxins, and recombinant ligand–toxin fusion proteins. Small molecule EGFR TKIs act by blocking the ATP-binding site of the EGFR TK enzyme inside tumor cells. Based on this mechanism of action, EGFR TKIs have the potential to inhibit all mechanisms of EGFR TK activation, including constitutively activating mutations and receptor crosstalk. EGFR TKIs were designed to selectively inhibit EGFR TK relative to other kinase enzymes present in normal tissues [27].

Gefitinib is a low-molecular-weight (447 kDa) quinazoline derivative that specifically inhibits the activation of EGFR TK through the competitive binding of the ATP-binding domain of the receptor. The selectivity of the compound for EGFR was demonstrated against HER2 TK and the two vascular endothelial growth factor (VEGF) receptors KDR TK and Flt-1 TK, and a $\geq$ 100-fold difference in IC_{50} was noted for EGFR compared with the other TKs. Similarly, gefitinib did not inhibit the activity of the serine threonine kinases raf, MEK-1, and MAPK [28]. Compared with anti-EGFR monoclonal antibodies, such as cetuximab, gefitinib offers the advantages of oral bioavailability and once-daily treatment. The drug was effective in preclinical models for inhibiting the growth of a variety of human tumor cell lines, including lung, colorectal, oral, and prostate, suggesting the potential for its broad applicability in the treatment of solid tumor types [29]. Furthermore, lung tumor xenografts have been inhibited by gefitinib alone or in combination with chemotherapy agents [29]. Gefitinib has been shown to potentiate the antitumor effects of most cytotoxic agents, including platinum-based chemotherapy agents in preclinical models with cell lines sensitive to EGFR inhibition [29,30]. In addition, gefitinib has shown activity against NSCLC xenografts in combination with taxanes, doxorubicin, and antifolates [29,30].

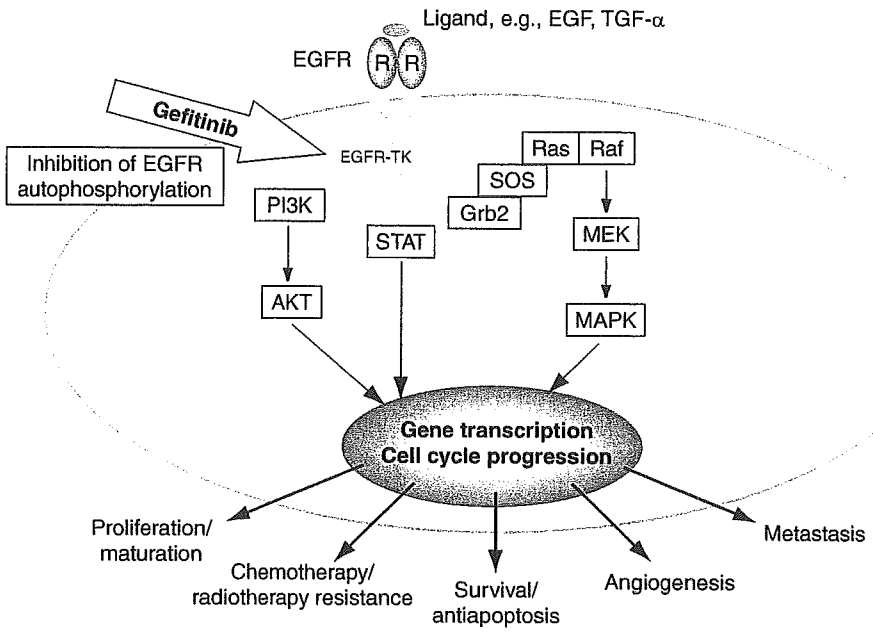
Pharmacodynamic studies indicate that gefitinib blocks cell cycle progression in the G1 phase by upregulating $p27^{Kip1}$, a cell cycle inhibitor, and downregulating c-fos, a transcriptional activator that is prominent in EGFR-mediated signaling [31]. Elevated levels of $p27^{Kip1}$ block cell cycle progression in the G1 phase of growth, thus sustaining the hypophosphorylated state of the retinoblastoma (*RB1*) gene product, which is necessary to keep cells from progressing in the cell cycle [32]. The inhibition of tumor growth seen with gefitinib is also accompanied by decreases in VEGF, basic fibroblast growth factor (bFGF), and TGF-α, which are all potent inducers of tumor angiogenesis [30]. Thus, gefitinib may also inhibit tumor growth by interfering with angiogenesis.

These observations suggest that by inhibiting the EGFR TK, gefitinib treatment alters the expression levels of key molecules in tumor cells that are important for stimulating proliferation, cell cycle progression, tumor angiogenesis, metastasis, and inhibition of apoptosis.

EGFR gene mutations

Recently, three clinical studies have identified mutations affecting the EGFR in lung cancers from patients who respond to gefitinib. These reports identified somatic mutations in the

Figure 1. The mechanism of action of gefitinib signal transduction blockage through EGFR tyrosine kinase.



EGF: Epidermal growth factor; EGFR: Epidermal growth factor receptor; Grb2: Growth factor receptor-binding protein 2; MAPK: Mitogen-activated protein kinase; PI3K: Phosphatidylinositol 3-kinase; R: Receptor; STAT: Signal transducers and activators of transcription; TGF: Transforming growth factor; TK: Tyrosine kinase.

EGFR gene in NSCLC patients that showed a clinical response to gefitinib. The mutations were short, in-frame deletions or substitutions clustered around the region encoding the ATP-binding pocket of the receptor's TK domain.

Lynch *et al.* [33] used primary lung tumor samples from responders to gefitinib versus non-responders to search for mutations in *EGFR*. They found that heterozygous gain-of-function mutations were clustered within the TK domain of the EGFR (the active site where gefitinib binds) in tumor specimens analyzed from eight of nine patients who were sensitive to gefitinib. Of these patients, four had in-frame deletions within exon 19 and three had amino acid substitutions within exon 21. Matched normal tissue from these patients did not contain the mutations and no mutations were seen in seven patients who had not responded to gefitinib. Paez *et al.* [34] screened Japanese and American patients with NSCLC for mutations restricted to the activation loop (A-loop) of a large subset of all human membrane receptor TKs. They also examined samples from patients treated with gefitinib for mutations in *EGFR* and found mutations similar to those in the study by Lynch *et al* [33].

Both papers showed a correlation of the mutations with certain patient characteristics, which, in turn, correlated with the subset of patients who respond to gefitinib. In the study by Paez *et al.* [34], these mutations were more frequent in female and Japanese patients, as well as in patients with adenocarcinoma compared with other histological types of NSCLC. This report showed that 15 (29%) out of the 58 Japanese patients were heterozygous for the *EGFR* mutations in their tumor tissue but not in their normal tissue, compared with only 1 patient out of 61 from the USA. The result corresponds to the findings of the first Iressa Dose Evaluation in Advanced Lung Cancer (IDEAL-1) trial, which showed a 27.5% response rate in Japanese patients [16]. An association was also seen in both studies between the presence of mutations and gefitinib sensitivity in female patients with bronchioloalveolar carcinoma (a subtype of adenocarcinoma) who were not current smokers, further confirming the subset of patients known to respond to gefitinib. In addition, Pao *et al.* [35] showed that adenocarcinomas from 'never smokers' (patients who had smoked < 100 cigarettes in their lifetime) comprised a distinct subset of lung cancers, frequently containing mutations within the TK domain of