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分担研究報告書

同種造血幹細胞移植における移植免疫反応に関与する要因の解析

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研究要旨：同種造血幹細胞移植においてはドナーと患者間のHLA抗原の違いが移植成績に大きな影響を与えている。非血縁者間骨髄移植症例を解析することにより移植免疫反応の解明にはGVHD予防法、ドナーと患者とのHLA適合度を考慮に入れる必要性が明らかになり、同種移植において特異的養子免疫療法を臨床応用する際の症例の選択にための基本情報を得ることができた。

A.研究目的

HLA-A、B、DR血清型適合非血縁移植におけるHLA遺伝子型適合度と移殖免疫反応、生存について解析し、移植免疫反応、とくに抗白血病効果を有するgraft versus host effect解明のための基礎資料ならびにマイナー組織適合性抗原を標的とする養子免疫療法のプロトコル作成のための基本情報を得ることを目的とする。

B.研究方法

非血縁者間骨髄移植症例を対象とし、HLA適合度と急性GVHDならびに生存との関連を解析した。今回はとくにGVHD予防法としてシクロスポリン＋メトトレキセート併用療法とシクロスポリン＋タクロリムス法とを比較した。症例はHLA-A, B,DRB1の遺伝子型が判明している白血病とした。スタンダードリスク白血病は第1寛解期の急性リンパ性白血病と急性非リンパ性白血病と第1慢性期の慢性骨髄性白血病とした。ハイリスク白血病はスタンダードリスクより進行した病期で移植をした白血病とした。

C. 研究結果

HLA不適合症例においてはGVHD予防法としてタクロリムスと短期メトトレキセート併用（FK+MTX）法を用いた症例における重症GVHDの頻度はCSP+MTX法を用いた症例よりも有意に低下していた。一方、HLA-A, B, DRB1適合症例においてはFK+MTX法とCSP+MTX法との間に有意差はなかった。生存について白血病スタンダードリスク、ハイリスク症例ともに、HLA不適合症例ではFK+MTX法がCSP+MTX法に比べ良好な成績を示していたが、HLA適合症例では有意差は認められなかった。

D. 考察

現在までに得られた移植成績（ここでは示されていない）と今回示した成績を考慮した日本骨髄バンクにおけるドナー選択、治療法選択につき考察すると、

a）スタンダードリスクの移植症例は、HLA-A,-B,-DRB1遺伝子型適合ドナーを選択すべきである。可能であれば、HLA-CのKIRリガンド適合（GVHD方向）のドナーを選択することが望ましい。

b）HLA-A,-B遺伝子型適合HLA-DRB1型不適合のドナーの場合にはHLA-C型が適合しておれば、上記HLA-A,-B,-DRのDNA型適合ドナーと

同様に選択可能である。 したがってHLA-C型の検査を実施しHLA-C型が適合していることを確認する必要がある。

c) ハイリスクの移植症例において、HLA-A, -B遺伝子型適合ドナーが見出されず移植まで長く待てない場合にHLA-A, -B遺伝子型不適合ドナーを選択するかどうかは、移植成績を考慮して決定する。この場合に、HLA不適合血縁者間移植や臍帯血移植の選択枝も考えられるが、これら移植法のevidenceレベルを考慮したうえでの治療法の選択が必要であろう。

d) HLA不適合移植においてはGVHD予防法として、FK+MTX法がCSP+MTX法よりも良好な生存率を示したことから、FK+MTX法が推奨される。

E. 結論

本解析により、移植免疫反応の解析にはGVHD予防法、ドナーと患者とのHLA適合度を考慮に入れる必要性が明らかになった。さらに、同種移植において特異的養子免疫療法を臨床応用する際の適用症例の選択のための基本情報を得ることができた。

G. 研究発表

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H.知的財産権の出願・登録状況

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III. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

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IV. 研究成果の刊行物・別刷

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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.
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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.

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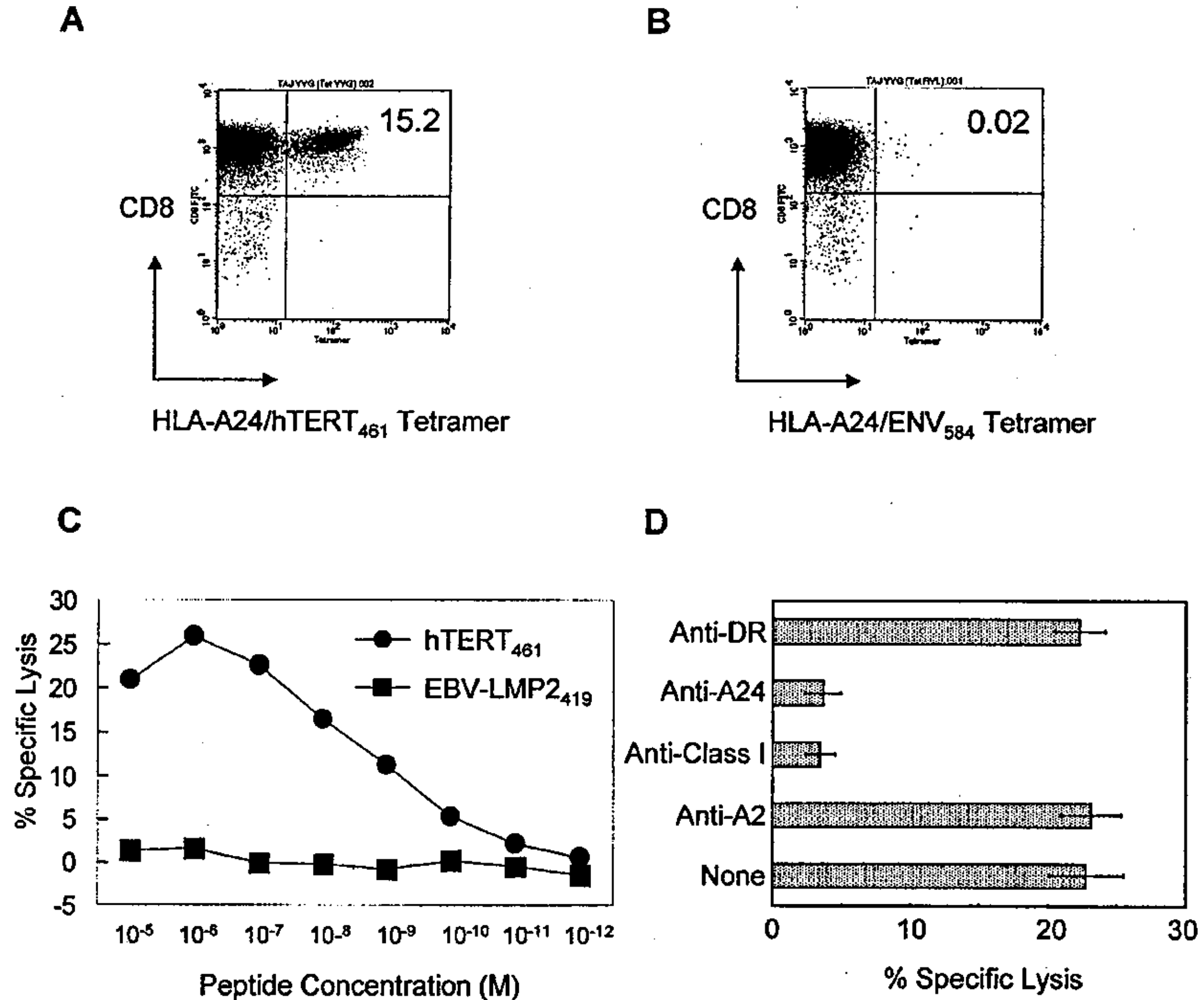

FIGURE 1 – Characterization of polyclonal CTL specific to $hTERT_{461}$. Polyclonal $CD8^+$ T cells after stimulation 4 times were stained with FITC-labeled anti-CD8 antibodies and PE-labeled HLA-A24-tetramers incorporating $hTERT_{461}$ (*a*) or a control peptide, ENV_{584} (*b*), and analyzed by flow 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-$LMP2_{419}$ (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) envelop 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

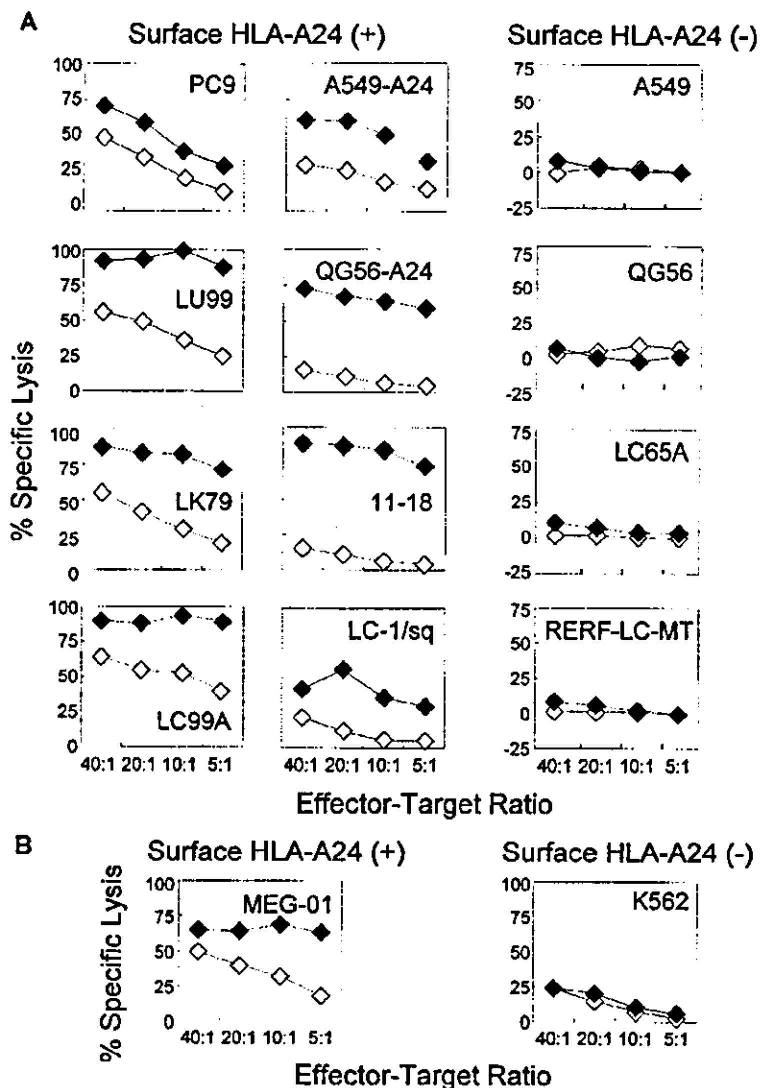

FIGURE 2 – Cytotoxicity of $hTERT_{461}$-specific CTL clone, K3-1 against cancer cell lines. (a) Cytotoxicity of $hTERT_{461}$-specific CTL clone K3-1 for 8 lung cancer cell lines with positive surface HLA-A24 expression and 4 with negative surface HLA-A24 expression as target cells (see Table I). The 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

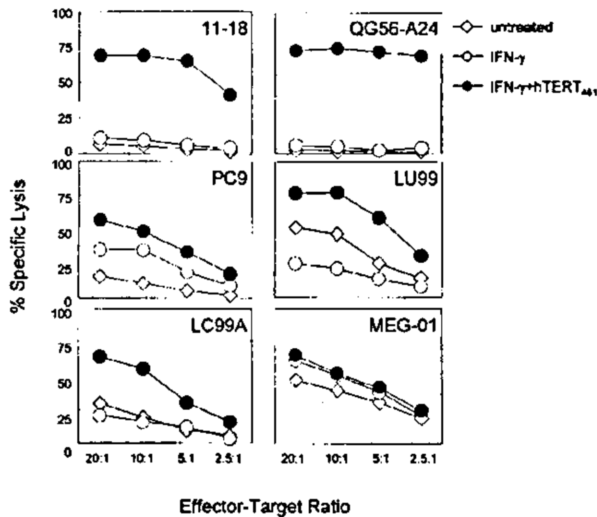

FIGURE 3 – K3-1-mediated lung cancer cell lysis induced by IFN-γ treatment. Cytotoxicity of the $hTERT_{461}$-specific CTL clone K3-1 against HLA-A24-positive lung cancer cells determined with (open circle) or without (open diamond) IFN-γ pre-treatment (100 U/ml for 48 hr). Cytolysis of IFN-γ treated 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-$LMP2_{419}$ (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.

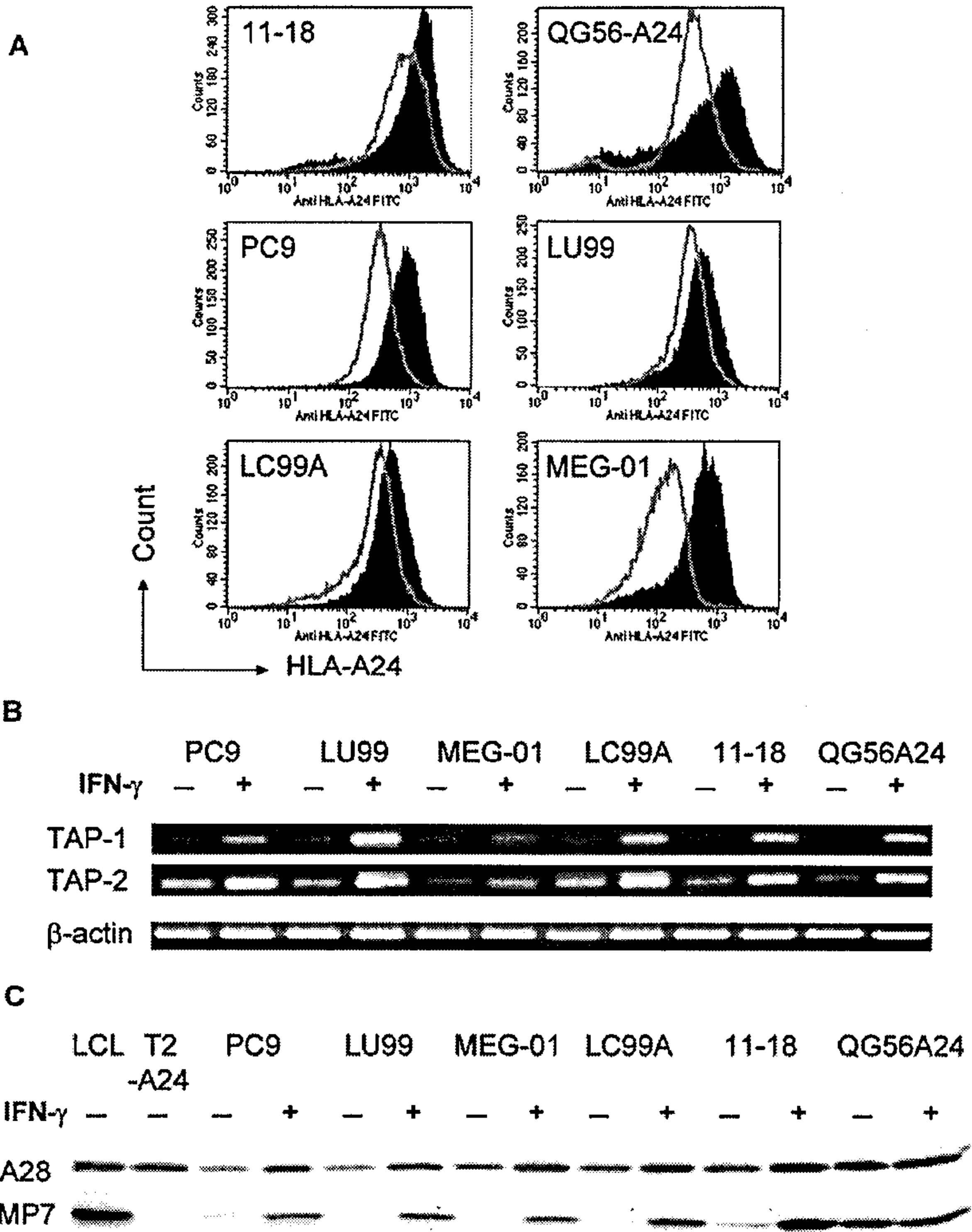

FIGURE 4 – Effects of IFN-γ on the regulation of molecules which play roles in antigen processing and presentation. (*a*) Results for surface HLA-A24 expression with (black shadowed) and without (lined) IFN-γ treatment analyzed by flow cytometry. Surface expression of HLA-A24 molecule was examined by 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.

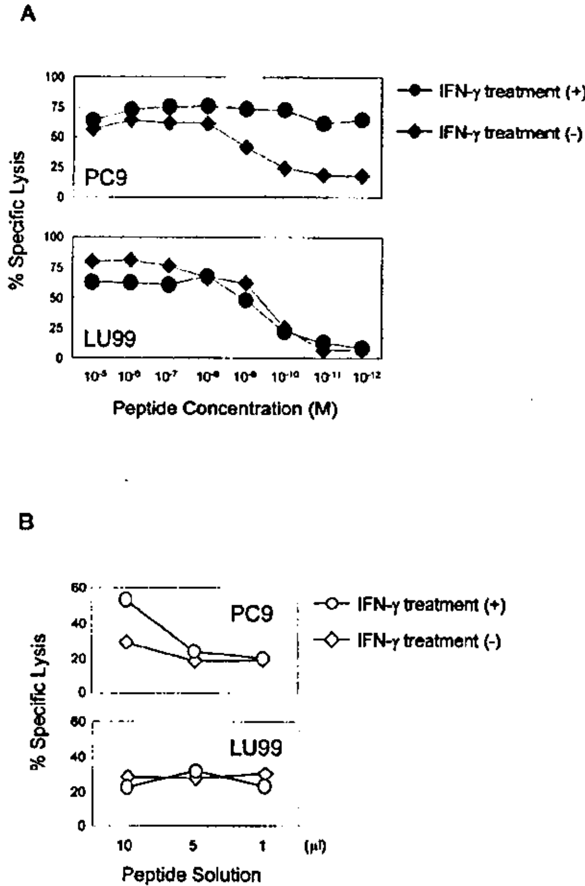

FIGURE 5 – The effects of IFN-γ on susceptibility of PC9 and LU99 cell lines to K3-1. (*a*) Cytotoxicity of the $hTERT_{461}$-specific CTL clone K3-1 against HLA-A24-positive lung cancer cells, PC9 or LU99, either treated (closed circle) or untreated (closed diamond) with 100 U/ml IFN-γ for 48 hr, as 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-

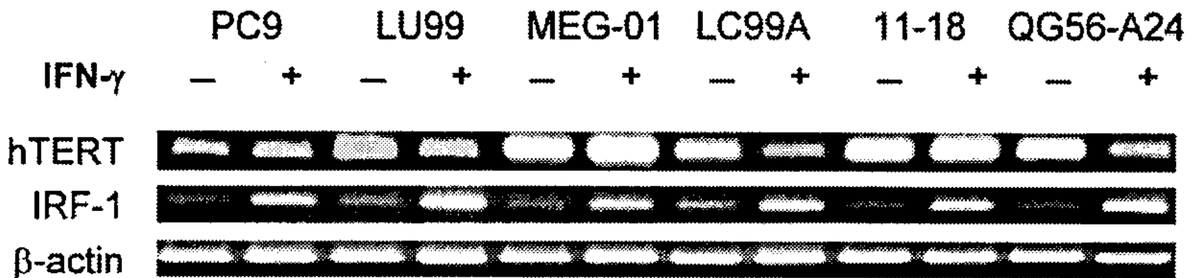

FIGURE 6 – RT-PCR analysis of hTERT and IRF-1 in cancer cell lines. Semi-quantitative RT-PCR analysis was performed using specific primers to hTERT, IRF-1 and β-actin. The mRNAs were isolated from cancer cells either treated or untreated with IFN-γ for 48 hr.

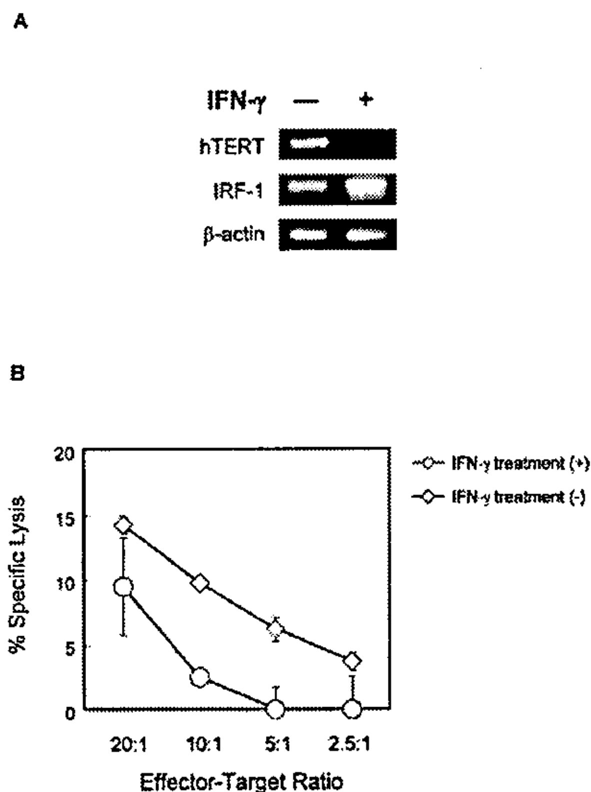

FIGURE 7 – Impact of IFN-γ treatment on primary lung cancer cells. (*a*) Freshly isolated adenocarcinoma cells from an HLA-A24-positive patient was treated or untreated with IFN-γ for 48 hr. Semi-quantitative RT-PCR analysis was performed using specific primers to hTERT, IRF-1 and β-actin. (*b*) Cytotoxicity 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.

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Identification of an epitope from the epithelial cell adhesion molecule eliciting HLA-A*2402-restricted cytotoxic T-lymphocyte responses

Key words:
autoimmunity; CTL; dendritic cells; epithelial cell adhesion molecule; HLA-A24; immunotherapy

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Abstract: Because the epithelial cell adhesion molecule (Ep-CAM) is expressed in almost all carcinomas and human leucocyte antigen (HLA)-A*2402 is the most common allele in many ethnic groups, including Japanese, the identification of peptide sequences, which elicit HLA-A*2402-restricted Ep-CAM-specific cytotoxic T-lymphocyte (CTL) responses, would facilitate specific immunotherapy for various histological types of carcinomas. An epitope was identified through the following steps: (i) computer-based epitope prediction from the amino acid sequence of Ep-CAM, (ii) major histocompatibility complex (MHC) stabilization assay to determine the affinity of the predicted peptide with HLA-A*2402 molecules, (iii) stimulation of $CD8^+$ T cells with peptide-pulsed dendritic cells and (iv) testing the CTL specificity by means of enzyme-linked immunospot (ELISPOT) assays, CTL assays and MHC/peptide-tetramer staining. Peripheral $CD8^+$ T cells of four of five healthy donors after three rounds of stimulation with the peptide $Ep\text{-}CAM_{173-181}$ (RYQLDPKFI) secreted interferon-γ in ELISPOT assays when exposed to the peptide. A CTL clone specific to the peptide efficiently lysed Ep-CAM-expressing cancer cell lines in an HLA-A*2402-restricted fashion. Endogenous processing and presentation of the peptide in a lung cancer cell line were confirmed by means of cold target inhibition assays. The CTL clone was also lytic to normal bronchial epithelial cells but to a lesser extent at low effector: target ratios. All these data suggest that the peptide-specific CTL responses may play some roles both in anti-cancer and autoimmune reactions. The peptide should prove useful to study anti-Ep-CAM CTL responses among population possessing HLA-A*2402.

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Cytotoxic T-lymphocytes (CTLs) have become widely accepted as important players in resistance to cancer. Although various CTL epitopes of tumour-associated antigens have been identified so far (1, 2), the search for additional epitopes continues, because the expression of tumour antigens is heterogeneous among tumours of various histological origins, various patients and between individual lesions. From the clinical point of view, molecular characterization of

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additional tumour antigens is crucial for successful immunotherapy, because immunoselection of antigen-negative tumour cell variants has been observed during peptide vaccination (3–5).

Epithelial cell adhesion molecule (Ep-CAM), also referred to as EGP-2, 17-1A, GA733-2, KSA or PE-35 (6–10), was originally reported as a serologically defined surface antigen, highly expressed on many carcinomas of diverse histological origins, such as colon (11), lung (12), head and neck (13) and breast tumours (14), but with limited expression by normal epithelial cells (15, 16). Its function is to mediate Ca^{2+}-independent homotypic cell–cell adhesion. Because of its intensive and uniform expression in a variety of human tumours, Ep-CAM has become one of the most attractive targets for immunotherapy with monoclonal antibodies, or even for gene therapy (17). Treatment of a series of patients suffering from Dukes' C colorectal carcinoma with a monoclonal antibody against Ep-CAM, namely 17-1A, has been found to reduce mortality and recurrence (18, 19). Recently, it was reported that HLA-A*0201-restricted Ep-CAM-derived peptide-specific CTLs can lyse epithelial tumour cells but not normal cells (20, 21). Immunotherapy using such epitope peptides has potential efficacy.

Using a bioinformatic approach, in the present study, we first predicted seven peptide sequences in Ep-CAM, which might bind to HLA-A*2402 molecules, the most common allele in Japanese (more than 60%) and also present in persons of European descent (nearly 20%). Specific CTL was successfully induced in four of five healthy donors by using Ep-$CAM_{173-181}$ (RYQLDPKFI) and a $CD8^+$ CTL clone specific to this peptide showed cytotoxicity against HLA-$A24^+$ Ep-CAM^+ but not HLA-A24 cancer cells. Cold target inhibition assays suggested that the peptide was naturally processed and was presented on the surfaces of HLA-$A24^+$ Ep-CAM^+ cancer cells. The fine specificity of the peptide-specific CTL was extensively studied and the results were discussed in the light of anti-cancer and anti-self cellular immunity.

Materials and methods

Donors and cell lines

The study design and purpose, which had been approved by the Institutional Review Board of Aichi Cancer Center, Nagoya, Japan, were explained fully to all donors. Peripheral blood was obtained from five HLA-A24-positive healthy donors and peripheral blood mononuclear cells (PBMCs) were isolated by means of centrifugation on a Ficoll-Paque (Pharmacia, Piscataway, NJ) density gradient.

Human cancer cell lines – LU99, HSC-2, MKN28, MKN45 and COLO320DM cells – were purchased from the Japanese Collection of Research Bioresources (Tokyo, Japan) and LC-1/sq from RIKEN Cell Bank (Tsukuba, Japan). LC-1/sq cells were maintained in 45% RPMI 1640 medium (Sigma, St Louis, MO) and 45% Ham's F12 medium (Sigma) supplemented with 10% fetal calf serum (FCS) (Life Technologies Limited, Auckland, New Zealand), L-glutamine, penicillin and streptomycin. COLO320DM and MKN28 were maintained in Dulbecco's modified Eagle medium (Sigma) with the same supplements. The other cancer cell lines were cultured in RPMI1640 medium with the same supplements (referred to as complete medium). HLA-A24-positive, normal human bronchial epithelial cells, designated as NHBE, were cultured according to the manufacturer's recommendations (CC2540, Clonetics Corp, BioWhittaker, Walkersville, MD). The HLA-A*2402 transfectants – T2-A24, QG56-A24 and A549-A24 – were established and were cultured as previously described (22, 23).

Reverse transcription polymerase chain reaction

Using a GenElute mRNA Miniprep kit (Sigma Chemical Co., St Louis, MO), total RNA was extracted from cultured cell lines. Gene-specific oligonucleotide primers were synthesized at Proligo (Kyoto, Japan) and were used in order to evaluate the mRNA expression of Ep-CAM. Forward and reverse primers used were as follows: ATG GCG CCC CCG CAG GTC CT and TTA TGC ATT GAG TTC CCT ATG CAT CTC ACC. Reverse transcription polymerase chain reaction (RT-PCR) was performed by using a thermal cycler (Perkin-Elmer, Wellesley, MA) and products were analysed by means of 1.5% agarose gel electrophoresis with ethidium bromide visualization.

Western blot analysis

Western blot analysis was performed as described previously (24) with slight modifications. Briefly, aliquots of 130-μg protein from the post-nuclear supernatant of the cell lysate were applied to 12% SDS-PAGE and were blotted onto Immobilon-P membranes (Millipore Corporation, Bedford, MA). After probing with a monoclonal antibody specific to Ep-CAM (clone 323/A3, Laboratory Vision, Fremont, CA), followed by peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) (Zymed, San Francisco, CA), proteins were visualized with the help of an ECL Western blot detection system (Amersham Biosciences, Buckinghamshire, UK).

Synthetic peptides

In order to identify potential HLA-A24-binding peptides within Ep-CAM (accession number M33011), we employed a computer-based program accessed through the World Wide Web site BioInformatics & Molecular Analysis Section (BIMAS) HLA peptide-binding predictions (available at http://bimas.dcrt.nih.gov/molbio/hla_bind/). Most peptides were synthesized with a Cleaved PepSet from Mimotope