production. To our knowledge, this is the first report to describe the use of multimodal cell therapy with PBSCs in a patient with advanced gastric cancer. After immunotherapy, the serum CEA level declined in association with positive DTH skin test results to CEA652 peptide, suggesting that the tumor had responded to immunotherapy (19,20). However, the response was short-lived, with no definitive evidence of tumor shrinkage. Our pilot case study will hopefully encourage the further refinement and development of immune cell therapy in combination with PBSCT for advanced gastric cancer (42).

In conclusion, our study delineated the courses of bone marrow suppression after EAP in 5 patients with chemotherapy-naïve advanced gastric cancer. Sufficient numbers of PBSCs to permit safe transplantation were mobilized and harvested in 4/5 patients studied. Multimodal cell therapy with PBSCs is considered a feasible and promising treatment strategy for advanced gastric cancer. The development of novel intensive treatment strategies combining chemotherapy and immunotherapy will hopefully lead to the complete regression of cancer and prolonged long-term survival in young patients with advanced gastric cancer. Further investigation is warranted.

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Recognition of Epstein-Barr virus-associated gastric carcinoma cells by cytotoxic T lymphocytes induced *in vitro* with autologous lymphoblastoid cell line and LMP2-derived, HLA-A24-restricted 9-mer peptide

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Abstract. Epstein-Barr virus (EBV) is associated with several types of malignancies including Burkitt's lymphoma, Hodgkin's disease, nasopharyngeal carcinoma, and gastric carcinoma. Previous reports have suggested that EBV-related antigen-targeting immunotherapy is one of the promising approaches for the treatment of these malignancies other than gastric carcinoma. EBV-associated gastric carcinoma (EBVaGC) has been shown to express Epstein-Barr virus nuclear antigen 1 (EBNA1) and latent membrane protein 2 (LMP2). In the present study, DNA and mRNA freshly isolated from tumors of patients with gastric cancer were subjected to polymerase chain reaction (PCR) using EBV-specific primers and reverse transcription (RT)-PCR specific for LMP2 transcripts. EBV-specific region was identified in genomic DNA isolated from cancerous tissues in 22% of gastric cancer patients. LMP2 mRNA was also detected in 3 out of these 5 DNA positive samples tested. To investigate the feasibility of specific immunotherapy for EBVaGC, we induced cytotoxic T lymphocytes (CTLs) from peripheral blood lymphocytes using two kinds of antigen-presenting cells (APCs) such as autologous lymphoblastoid cell line (LCL) and LMP2-derived peptide-pulsed dendritic cells (DCs). The cytotoxicity of these CTLs against peptide-pulsed targets was examined by standard ^{51}Cr release assay and interferon (IFN)-γ production assay. We further assessed the recognition of tumor cells endogenously expressing LMP2 by these T cells. T cells induced by peptide-loaded DCs and autologous LCL efficiently lysed peptide-pulsed targets. Furthermore, these T cells could recognize not only tumor cells transfected with LMP2, but also LMP2-positive gastric cancer cells which were successfully isolated and cultured from specimens obtained by surgery. Collectively, sensitization of peripheral blood lymphocytes with LMP2-derived peptide was able to induce CTL response against EBVaGC cells. Thus, EBVaGC is susceptible for the LMP2-targeting immunotherapy.

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Key words: Epstein-Barr virus-associated gastric carcinoma, immunotherapy, $LMP2_{419-427}$ peptide, lymphoblastoid cell line

Introduction

A wide variety of malignancies including Burkitt's lymphoma, Hodgkin's disease (1), undifferentiated nasopharyngeal carcinoma (2,3), post-transplant lymphoproliferative disease (PTLD) (4) and gastric carcinoma are associated with Epstein-Barr virus (EBV) (5,6). EBV-associated gastric carcinoma (EBVaGC) (7-12) has been demonstrated to account for 7-10% of all gastric carcinomas in Japan (13,14). Since there are >100,000 new patients with gastric carcinoma in Japan (15), the number of patients with EBVaGC is considerable.

Like other herpes viruses, EBV induces long-lasting cytotoxic T lymphocyte (CTL) memory (5). In fact, EBV-specific CTL precursors can be reactivated from the peripheral blood of EBV-seropositive individuals by several types of stimulation *in vitro* (5). Thus, to recall and boost T cell responses specific for EBV in EBVaGC patients could lead to the elimination of tumors.

EBV-transformed lymphoblastoid cell line (LCL) and cells from PTLD express all the latent proteins of EBV: six nuclear antigens [Epstein-Barr virus nuclear antigen (EBNA) 1, 2, 3A, 3B, 3C, and LP] and two membrane proteins [latent membrane protein (LMP) 1 and 2] (5). These latent proteins of EBV display hierarchical immunogenicity. Malignant cells in PTLD express the full set of EBV antigens, including the highly immunogenic EBNA3A, 3B, and 3C (16,17). Hence, PTLD is highly immunostimulatory. Rooney *et al* have clearly demonstrated that such EBV-associated disease can be effectively treated by transfer of EBV-specific CTLs that have been generated *in vitro* using donor LCL as stimulator

cells (17). However, the susceptibility of EBV antigens to immunotherapy for EBVaGC remains unclear. Although EBVaGC has been shown to express EBNA1 and LMP2 (5), EBNA1 may not be a suitable target due to its internal Gly/Ala repeat domain encompassed by EBNA1 protein which can prevent EBNA1 from processing by the classical HLA class I pathway (18,19). Thus, we focused on LMP2 as a target molecule for immunotherapy of gastric cancer in the current study.

Some immunodominant LMP2 epitopes restricted by several alleles of human leukocyte antigens (HLA) -class I have been identified. Because HLA-A24 is present in 60% of the Japanese population and in some Caucasians, we chose $LMP2_{419-427}$ peptide (TYGPVFMCL) restricted with HLA-A24 (20). To induce effective CTLs, we used a mixture of $LMP2_{419-427}$ peptide-loaded dendritic cells (DCs) and $LMP2_{419-427}$ peptide-loaded LCL as antigen-presenting cells (APCs) as well as LCL alone. Generated CTLs were tested for cytotoxicity against peptide-pulsed targets and LMP2-expressing tumor cells including primary cultured gastric cancer cells. To our knowledge, this is the first report to show that EBVaGC cells can be recognized by CTLs *in vitro* that are successfully induced with autologous LCL and LMP2-derived peptide.

Materials and methods

Patients and PBMCs from a healthy donor. Peripheral blood mononuclear cells (PBMCs) were isolated from a leukapheresis sample obtained from a healthy donor, FH01 (HLA-A24, B48, B52, DR14, DR15, EBV antibody titers: positive), by Ficoll-Paque (Pharmacia, Piscataway, NJ). Other subjects were 18 Japanese patients aged 55-86 years (13 men, 5 women) who had undergone resection of primary gastric carcinoma. Written informed consent was obtained from the healthy donor and from all patients before surgery.

Cell lines. LCL was generated *in vitro* by transforming B cells from FH01 with the standard EBV isolate B95.8 (21,22), kindly supplied by Dr Kenzo Takada (Department of Tumor Virology, Institute for Genetic Medicine, Hokkaido University, Sapporo, Japan). K562 (chronic myelogenous leukemia) (23) was obtained from the Japanese Collection of Research Biosources (JCRB). 888mel (melanoma cell line, HLA-A24$^+$) (24) was kindly provided by Dr Yutaka Kawakami (Institute for Advanced Medical Research, Keio University School of Medicine, Tokyo, Japan). All cell lines were maintained in RPMI 1640 medium (Nacalai Tesque, Kyoto, Japan) containing 10% heat-inactivated fetal calf serum (Nichirei, Tokyo, Japan), 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma, St. Louis, MO).

Primary culture of gastric carcinoma tissues. The remaining sections of carcinoma tissue after general pathological examinations were cut into small pieces (<1 mm^3), put into plastic dishes, and cultured in RPMI 1640 medium supplemented with 20% heat-inactivated fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. After 10 days, a half of the medium was changed; subsequently the medium was changed once per week. After confluent cultures had developed,

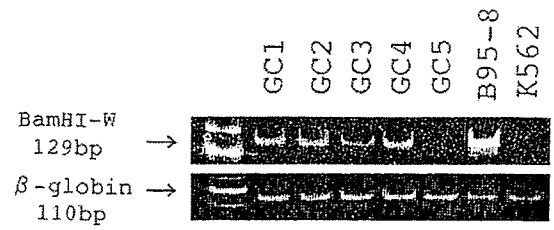

Figure 1. Detection of EBV DNA in gastric carcinoma tissues by PCR analysis. Total cellular DNA extracted from 5 cases (GC1-GC5) of gastric carcinoma and from B95-8 (EBV-positive) and K562 (EBV-negative) cells were subjected to PCR. Arrows indicate the specific PCR products with their predicted sizes. One of the EBV-negative gastric carcinomas, GC5, is shown in the same blot.

the cell cultures were tested for LMP2 expression by immunostaining with rat monoclonal antibodies specific for LMP2 (25) using Histofine Simple Stain MAX-PO kits according to the manufacturer's instructions (Nichirei, Tokyo, Japan). LMP2 antibodies and Plasmid LMP2/pSG5 were kindly supplied by Dr Richard Longnecker (Department of Microbiology-Immunology, Northwestern University Medical School, Chicago, IL).

BamHI-W PCR. DNA samples were extracted from the resected cancer tissues and tested for EBV by *Bam*HI-W polymerase chain reaction (PCR). EBV-specific primers (5'-CCAGACA GCAGCCAATTGTC-3' and 5'-GGTAGAAGACCCCCTCT TAC-3') were used to amplify the 129 bp fragment in the *Bam*HI-W region (26). To test whether total cellular DNA had been extracted, the nucleotide sequence of the human ß-globin gene was amplified with the use of an aliquot of total DNA. B95-8 cells were used as a positive control, and K562 cells were used as a negative control. PCR products were run on 5% polyacrylamide gels. PCR was performed with a KOD Dash PCR kit (Toyobo, Osaka, Japan).

RT-PCR and detection of amplified products. Total RNA was extracted using Isogen reagent according to the manufacturer's instructions (Nippon Gene, Tokyo, Japan). Reverse transcription (RT)-PCR was performed with a BcaBest RNA PCR kit (Takara Bio, Shiga, Japan) (25). LMP2A-specific primers (5'-ATGACTCATCTCAACACATA-3' and 5'-CATGTTAG GCAAATTGCAAA-3') were used for amplification of the 280 bp fragment (26,27). cDNA from tumor tissues and EBV-negative K562 cells were subjected to 35-40 cycles of amplification. cDNA from EBV-positive B95-8 was used as a positive control and subjected to 20-25 cycles of amplification. To confirm that total RNA had been extracted, human ß-tublin mRNA was amplified using an aliquot of cDNA. Specific primers (5'-TGGATCTAGAACCTGGGACCAT-3' and 5'-ACCATGTTGACTGCCAACTTGC-3') were used for amplification of 577 bp in the ß-tublin mRNA, and amplified products were visualized by ethidium bromide staining.

Generation of LCL-induced CTLs. FH01-PBMCs ($2x10^6$) were co-cultured with irradiated (100 Gy) autologous LCL (FH01-LCL) in 24-well tissue culture plates (responder:APC = 40:1). On day 10, the cultures were replated at $2x10^5$ cells per

Table I. The profile of EBVaGC donors.

Donor	Sex	Age	Anatomical subsites[a]	Macroscopic type	Histopathological grading	Staging[b]
GC1	F	69	ML	Borr.2	Well	IV
GC2	M	80	UM	Borr.2	Poorly (solid type)	IV
GC3	M	70	ML	Borr.3	Moderately	IIIb
GC4	M	58	U	0-IIc	Poorly (non-solid type)	Ib

[a]Anatomical subsites: U, upper third; M, middle third; L, lower third (Japanese Classification of Gastric Carcinoma, 13th edition, Japanese Gastric Cancer Association). [b]Stage grouping (TNM Classification of Malignant Tumors, 6th edition, International Union against Cancer).

well. On days 14, 21 and 28, the cultures were stimulated with irradiated FH01-LCL (responder:APC = 4:1), and fresh medium containing interleukin (IL)-2 (25 U/ml) (28,29) was added. Effector cells were assessed for cytotoxic activity after 3 rounds of restimulation.

Generation of $LMP2_{419-427}$ peptide-induced CTLs. PBMCs were separated by adherence to a plastic tissue-culture flask to enrich monocytes. The monocyte-enriched population was then cultured in the presence of 1,000 U/ml granulocyte/macrophage colony-stimulating factor (Genzyme, Minneapolis, MN) and 1,000 U/ml of IL-4 (Genzyme) in RPMI 1640 containing 5% human AB serum (Japan Red Cross Society). After 7 days culture, the cytokine-generated DCs were loaded with 20 μg/ml of $LMP2_{419-427}$ peptide (TYGPVFMCL) (synthesized and provided by Takara Bio, Shiga, Japan) derived from LMP2 in the presence of 3 μg/ml of ß2-microgloblin (Lee BioSolutions, St. Louis, MO) for 4 h and were irradiated (50 Gy). The autologous LCL (FH01-LCL) was also used as APC after $LMP2_{419-427}$ peptide loading (10 μg/ml) and irradiation (100 Gy). FH01-PBMCs ($2x10^6$) were co-cultured with $LMP2_{419-427}$ peptide-loaded DCs and $LMP2_{419-427}$ peptide-loaded FH01-LCL in 24-well tissue culture plates (responder: LCL:DC = 40:2:1). On days 7 and 14, fresh medium, IL-2 (100 IU/ml, Shionogi, Osaka, Japan), and $LMP2_{419-427}$ peptide-loaded (10 μg/ml) FH01-LCL (irradiated) were added to the culture (responder: stimulator = 10:1).

Flow cytometric analysis. Phenotypic characterization of CTLs was carried out by flow cytometry using a FACSCalibur (Becton Dickinson, San Jose, CA) and CellQuest software (Becton Dickinson). Immunofluorescence staining was performed using the following FITC-conjugated monoclonal antibodies (mAbs): anti-CD3 mAb, anti-CD4 mAb, and anti-CD8 mAb (Dako A/S, Denmark).

Measurement of CTL responses in vitro. CTLs were tested for killing activities by standard ^{51}Cr release assay described previously (17). Peptide-pulsed target cells were prepared by incubating the cells with $LMP2_{419-427}$ peptide (20 μg/ml) overnight at 37°C. Target cells were labeled with 200 μCi of ^{51}Cr (Amersham, Piscataway, NJ) per $3x10^6$ cells for 1 h at 37°C, washed in phosphate-buffered saline (PBS), and added to 96-well U-bottom plates at $1x10^4$ cells in a final volume of 200 μl per well. After a 5-h incubation period at 37°C, the

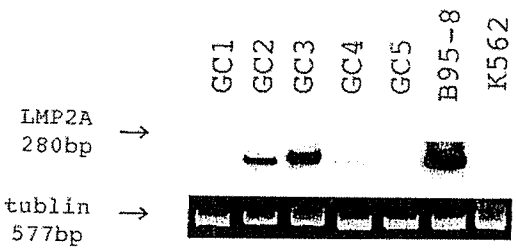

Figure 2. Detection of LMP2A mRNA in gastric carcinoma tissues. Total cellular mRNA was subjected to RT-PCR for LMP2A mRNAs followed by Southern hybridization with ^{32}P-labeled probes. B95-8 and K562 were used as positive and negative controls. Human ß-tubulin mRNA was stained with ethidium bromide. Arrows indicate the specific PCR products with their sizes.

radioactivity in the supernatants was determined using Cobra Model 5002 Gamma Counter (Packard, Meriden, CT). All tests were conducted in triplicate, and the percentage of specific cytotoxicity was defined by the formula: [(experimental ^{51}Cr release - spontaneous ^{51}Cr release)/(maximum ^{51}Cr release - spontaneous ^{51}Cr release)] (17,30).

For interferon (IFN)-γ release, CTLs were co-cultured with target cells for 12 h at 37°C. Culture supernatants were collected for measurement of human IFN-γ release using specific ELISA kits according to the manufacturer's instructions (Biosource, Camarillo, CA).

Results

LMP2 expression in patients with EBV-associated gastric carcinoma. EBV specific region was amplified from genomic DNA extracted from tumors in 4 (GC1-GC4, 3 men and 1 woman) out of 18 patients, indicating that 22% of patients with gastric cancer showing high titer of EBV IgG (viral capsid antigen) were identified to be EBVaGC (Fig. 1, Table I). LMP2 mRNA was detected in 3 of the 5 tumors examined by RT-PCR (Fig. 2). This pattern of the EBV gene expression in gastric carcinoma tissues was consistent with results reported previously (31).

Primary culture of tumor cells obtained from patients. Primary cultures of LMP2 mRNA-positive tumor cells derived from EBV-positive patients (GC2 and GC3) were obtained by the

Figure 3. Microscopic state of GC3 cells. GC3 was derived from resected stomach tissue from a patient with moderately differentiated adenocarcinoma. GC3 cells had a flat structure (bar, 100 μm).

above described method. As shown in Fig. 3, morphological examination indicated that these cells were epithelioid and irregular in size, with large hyperchromatic vesicular nuclei suggesting malignant features of cells. Furthermore, these cells were stained with anti-LMP2 antibody by immunohistochemical study (Fig. 4A). FACS analysis showed that GC3 cells were positive for HLA-A24. 888mel stably transfected with LMP2 cDNA (LMP2-888mel) was also stained by anti-LMP2 antibody (Fig. 4B and C). Thus, GC3 and LMP2-888mel were used as target cells expressing LMP2 endogenously for CTL assay in the following experiments.

Generation of CTLs specific for LMP2. To induce CTLs specific for LMP2, we first cocultured PBMCs with irradiated autologous EBV-transformed LCL (LCL-induced CTL). LCL is known to be a potent APC which can generate effector CTLs by stimulating resting memory $CD8^+$ T cells. After 3 rounds of stimulation, LCL-induced CTLs efficiently lysed autologous LCL (FH01). Although the recognition of tumor cells expressing LMP2 (LMP2-888mel) was observed, its activity was relatively low (Fig. 5A). Since LCL does not seem to induce primary CTL response because of the lack of second signals through the costimulatory molecules, we next used DCs as an APC to make CTL potent enough to kill tumor targets. A mixture of DCs pulsed with $LMP2_{419\text{-}427}$, a HLA-A24-restricted peptide derived from LMP2, and LCL was used as a stimulator in this experiment. T cells induced by these APCs (TYG-LCL-induced CTLs) were then restimulated with peptide-loaded LCLs. Phenotypic analysis of effector cells obtained after two rounds of restimulation showed that the percentage of $CD3^+$, $CD8^+$ and $CD4^+$ cells were 93.3±3.9, 70.5±6.2 and 21.6±9.7 respectively. TYG-LCL induced CTLs had 1.5 times higher cytotoxic activity against both peptide-pulsed cells and targets expressing LMP2 endogenously (LCL, LMP2-888mel) (Fig. 5B).

Recognition of primary cultured tumor cells by CTLs. Since established cell lines showing autonomic growth may behave differently from the primary cultured cells, it seems to be

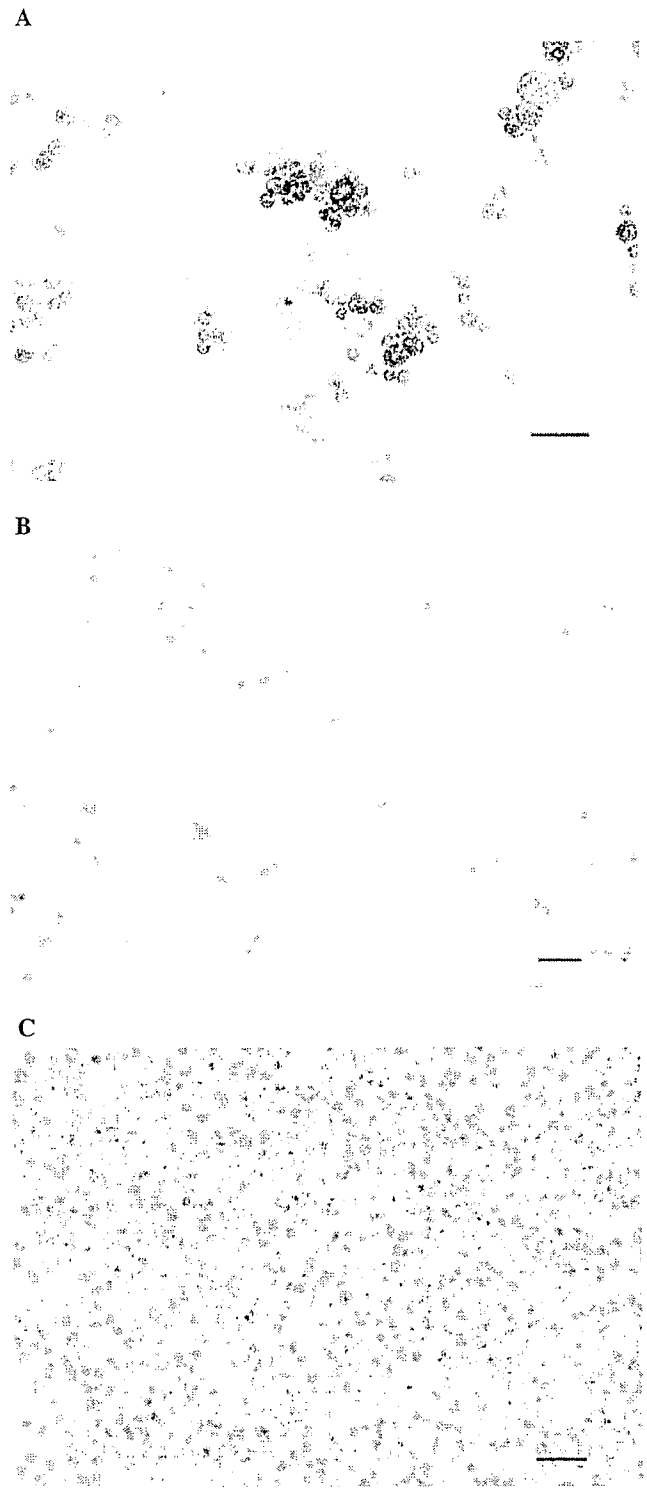

Figure 4. Immunohistochemical staining of LMP2 (bar, 100 μm). GC3 cells were showing weakly positive LMP2 staining at the cell membrane (A). 888mel-LMP2 was positive staining of LMP2 (B), but 888mel was negative (C).

important to investigate the recognition of tumor cells freshly isolated from patients by T cells. Therefore, we tested the recognition of GC3 cells obtained and cultured from surgically resected specimens of patients with EBVaGC, by TYG-LCL-

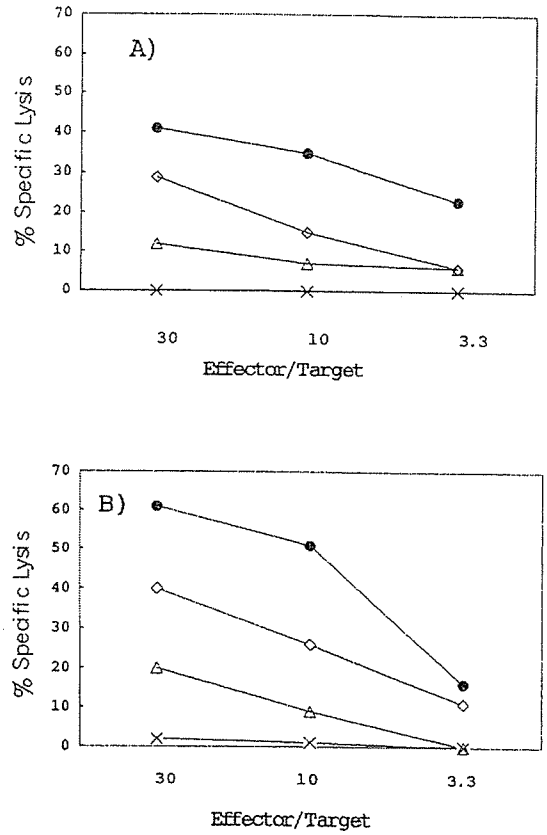

Figure 5. The use of LCL and peptide-pulsed DCs as APCs augments the cytotoxic activity of CTLs. (A), T cells were primed with autologous LCL. After 3 rounds of restimulation, effector cells were tested for cytotoxic activity against FH01-LCL (●), 888mel (×), LMP2-888mel (◊), and $LMP2_{419-427}$ peptide loaded 888mel (Δ). (B), T cells were induced by autologous LCL and $LMP2_{419-427}$ peptide-pulsed DCs. Effector cells were tested for cytotoxic activity against FH01-LCL (●), 888mel (×), LMP2-888mel (◊), and $LMP2_{419-427}$ peptide loaded 888mel (Δ).

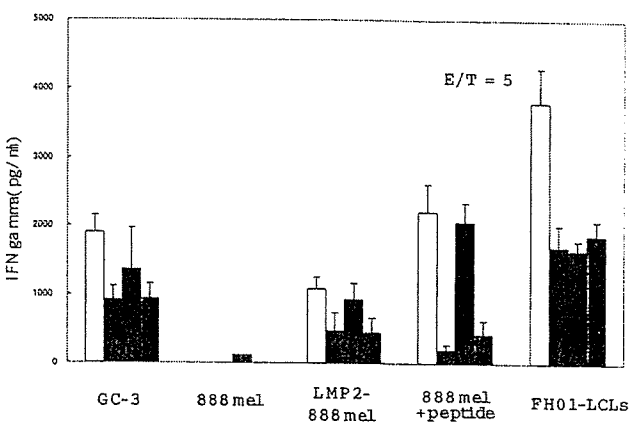

Figure 6. The recognition of TYG-LCL-induced CTLs against the cells of GC3 by IFN-γ determination assay. CTLs were stimulated *in vitro* with target cells with or without (open bar) monoclonal antibodies specific for HLA class I (hatched bar), class II (dotted bar) or HLA A24 mAb (solid bar). Culture supernatant were harvested overnight and evaluated for IFN-γ levels by ELISA (in pg/ml; mean ± SEM of triplicate samples). Error bars show the standard deviation.

induced CTLs. As demonstrated in Fig. 6, the TYG-LCL-induced CTLs produced ~1,900 pg/ml of IFN-γ in response to GC3 cells as well as 888mel-LMP2 whereas it produced <50 pg/ml in response to 888mel (LMP2 negative). The production of IFN-γ was partially abolished by anti-class I and anti-HLA-A24 mAbs in the blocking assay (Fig. 6). In contrast, the recognition of peptide-pulsed 888mel was blocked almost completely by anti-HLA-A24 mAb.

Discussion

The goal of this study was to assess the possibility of LMP2-targeting immunotherapy for EBVaGC by measuring the cytotoxicity of CTLs induced by LCL and peptides derived from LMP2 against primary cultured gastric cancer cells as well as tumor cell lines, which express LMP2 antigen. Principally, strong CTL responses toward EBNA3A, 3B, and 3C subset of virus proteins will occur in the healthy virus carriers (16,17). However, if one considers many EBV-associated malignancies, only a limited group of antigens such as EBNA1 and LMP2 are known to be expressed on tumor cells. Because LMP2 is the only EBV transcript expressed by B cells from EBV-positive healthy donors, it is reasonable to use LCL as APCs for inducing CTL competent for killing tumors (32). Indeed, quite a few epitope peptides have been identified by stimulating T cells with peptide-loaded LCLs (20). It has also been shown that cross-priming of CTLs using DCs loaded with dead LCLs induced an expansion of $CD8^+$ T cells specific for EBNA3A and LMP2. Consistent with these previous reports, our study showed that *in vitro* sensitization of PBMCs by LCL gave rise to $CD8^+$-dominant CTLs showing cytotoxic activity against LCL. These CTLs recognized tumor cells transfected with LMP2 cDNA (LMP2-888mel) (Fig. 5), whereas 888mel, non-transfectant for LMP2, was not lysed by them. Since LCL has the potential to generate T cells able to kill tumors, vaccination with LMP2-expressing cells mimicking LCL is considered to be a useful strategy to induce tumor immunity *in vivo*.

Peptide-based approach has also been investigated in EBV-related tumors. $LMP2_{419-427}$, a 9-mer peptide derived from LMP2, has been identified to be an epitope for $CD8^+$ T cells (29). A mixture of LCL and DCs pulsed with the peptide was used as a stimulator in this setting. Generated CTLs (TYG-LCL-induced CTLs) showed 1.5 times higher activity not only to LCL but also to LMP2-transfected tumor (LMP2-888mel) as compared to CTLs induced by LCL alone. This result suggests that the use of an epitope peptide in addition to LCL is one of the options to get a more robust CTL response. Because LCL is considered to be able to stimulate only resting memory cells due to the lack of second signal through costimulatory molecules, the use of DCs as professional APCs might enhance CTL response by priming naive T cells specific for peptide. However, since peptide alone (peptide-pulsed DC without LCL) did not exert full response (data not shown) comparable to that induced by LCL, further examinations into the role of the peptide in generating CTL in this method are needed.

Although there have been several reports regarding the generation of CTLs able to recognize autologous tumor cells, they are limited to certain types of tumors including melanoma (33), breast cancer (34,35) and ovarian cancer (35). There have

been no reports that indicated the recognition of primary cultured autologous gastric cancer cells by CTLs so far, presumably because it is difficult to get a primary tumor cell culture and T cells at the same time. In the present study, TYG-LCL-induced CTLs produced substantial amount of IFN-γ in HLA-A24 restricted manner when they were incubated with a peptide-pulsed target. This CTL could also recognize cells expressing LMP2 endogenously (LMP2-888mel). Furthermore, they recognized primary cultures of tumor cells (GC3) obtained from patients with EBVaGC. Since this recognition was not blocked by HLA-A24 mAb completely, TYG-LCL induced CTLs may include certain clones which are specific for some different epitopes endogenously expressed on LCL other than that specific for $LMP2_{419-427}$ peptide. Indeed, FH01, the donor for PBMC, shares the same HLA-class I molecules as B52 besides A24 with 888mel (24). Thus, some unknown epitopes restricted by B52 might be involved in this recognition of tumor by T cells.

In conclusion, LMP2-expressing tumor cells including primary cultured gastric cancer cells from EBVaGC were successfully recognized by CTLs induced *in vitro* with LCL and HLA-A24-restricted peptide derived from LMP2. EBVaGC is suggested to be susceptible to the LMP2-targeting immunotherapy. Therefore, vaccination using cells endogenously expressing LMP2 along with the peptide or adoptive immunotherapy using T cells induced by LCL and the peptide are promising approaches for the treatment of HLA-A24 positive EBVaGC.

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免疫学

新しい癌の体外循環治療技術の開発

New technology of direct hemoperfusion for cancer therapy

背景

担癌患者の血中には癌の進展に伴い種々の特異的・非特異的免疫抑制物質が増加し，それに伴い抗腫瘍細胞性免疫能は低下する[1]．これらの免疫抑制物質を十分に除去した後に免疫療法あるいは化学療法を行えば，治療効果を増強しうることが期待できる．このような理論的背景に立脚し，1976年以降1980年代にかけて欧米およびわが国において，進行癌患者に対する治療として血漿交換による免疫抑制物質除去療法が試みられた[2-4]．本療法は一定の治療効果が認められたにもかかわらず，血漿置換液として大量に使用される血液製剤に起因する感染の危険性，有用な血漿成分の破棄，コストの問題などが山積し，普及には至らなかった．また，当時の癌免疫療法と化学療法の発達程度の未熟さが，この治療法のさらなる改良への気運を妨げてきた．

免疫抑制物質吸着性極細繊維カラムを用いた，血漿交換を伴わない癌体外循環治療技術の開発

近年の樹状細胞療法を中心とした癌の免疫細胞療法の進歩は著しく，また一方，癌の進展に伴う免疫抑制動態の詳細も明らかにされた．すなわち，癌患者の血中に増加してくるTGF-β，IL-6，VEGFなどのサイトカイン(表1)は，じかに癌の進展に関与するのみならず，細胞性免疫能を抑制することにより癌の進展をさらに助長する[5-9]．また，患者に悪液質をもたらし，化学療法の副作用増強にも関与し，その用量規定因子にもなっている．

著者らはこれら免疫抑制性サイトカインの制御を目的として，血漿交換を伴わない血液吸着療法としての癌体外循環治療技術の開発を行っている(図1)．すでにTGF-β吸着剤として特定のアミノ基を官能基として固定化した多孔質のポリスチレン系極細繊維を開発・同定し，それを充填した体外循環治療カラムが，他治療を組み合わせない単独治療においても担癌ラットの腫瘍増殖を有意に抑制し生存期間を延長することを明らかにした[10]．また，ヒト癌性胸腹水を用いた吸着実験で，同吸着剤がTGF-β以上にVEGF，IL-6を強く(90%以上)吸着除去することを明らかにしている．

本開発技術は，繊維工学，高分子化学の癌治療への応用であり，繊維の製造工程において抗サイトカイン抗体などの薬剤を使用せず，安全で簡便な体外循環治療を繰り返し施行できるという利点を有している．

表1　担癌の進行に伴い血中に増加する免疫抑制物質

transforming growth factor-β (TGF-β)
immunosuppressive acid protein (IAP)
interleukin-6 (IL-6)
prostaglandin E_2 (PGE_2)
vascular endothelial growth factor (VEGF)
etc.

表2　免疫繊維カラムの目的・用途(進行固形腫瘍患者を対象として)

① 細胞免疫療法との連動による効果増強
② 化学療法との併用による効果増強→難治性癌(膵癌，スキルス胃癌など)を対象とした癌治療用医療器材としての承認
③ 末期癌患者の悪液質(QOL)改善
④ 患者血清の *ex vivo* 処理による細胞培養用自己血清の調製

臨床応用へ向けて

この現在開発中の癌治療用体外循環カラムを“免疫繊維カラム”と著者らは仮称しているが，現在継続中の動物実験における有効性確認，安全性確認を経た後に，近々臨床試験を開始する予定である(図2)．それには，表2に列挙したように種々の目的，用途が考えられる．癌治療用医療機材として

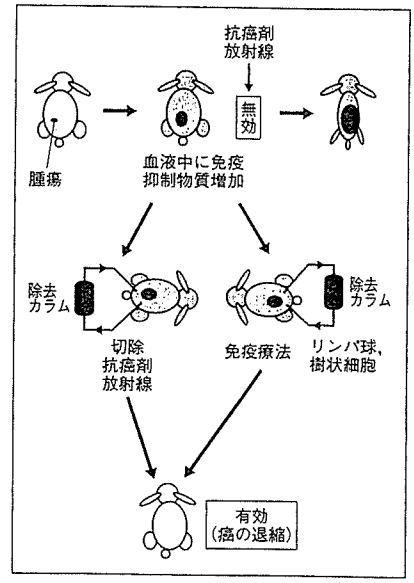

図1　免疫抑制物質除去カラム(免疫繊維カラム)を用いた血漿交換を伴わない癌体外循環治療技術
除去カラムはアミノ基含有ポリスチレン繊維の不織布を充填したもの．

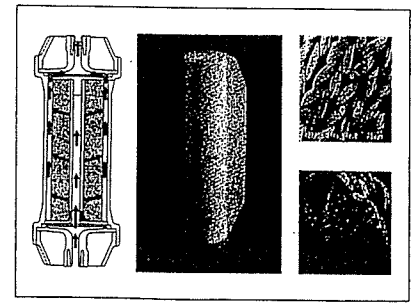

図2　免疫繊維カラム

の承認を最終目標とするため，進行癌患者を対象としてその単独治療による免疫賦活効果(血中免疫抑制性サイトカインの除去と細胞性免疫能の回復)を確認した後に，膵癌，スキルス胃癌などの難治性固形癌(これらの癌の進展にはTGF-β，VEGFがともに深く関与)を対象疾患とし，化学療法との併用による臨床試験を展開していければと考えている．

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癌・腫瘍学

アポトーシス誘導抗ErbB-2抗体による癌治療

Apoptosis inducing anti-ErbB-2 antibody for cancer therapy

癌に対する治療法としてモノクローナル抗体を用いた治療が注目を集めており，血液疾患に対する抗体治療はすでに臨床のなかで重要な位置を占めつつある．しかし，固形癌に関しては乳癌に対するtrastuzumab以外にはいまだFDAで認可された抗体は存在していないという現状である．

このような情況のなかで，著者らが独自に作成した消化器癌に過剰発現を多く認める受容体型チロシンキナーゼであるErbB-2に対するモノクローナル抗体CH401が，これまで報告されている抗ErbB-2抗体による抗腫瘍効果の作用機序とはまったく異なるアポトーシスの誘導であることが明らかになった．さらに，このアポトーシスに至る機序にMAPキナーゼの関与が示唆されるデータが出つつある．

本稿ではこのモノクローナル抗体CH401の作用機序解析を通して消化器癌に対して，CH401を用いる有用性について述べたい．

ErbB-2と癌

ErbB-2はレセプター型のチロシンキナーゼファミリーに属する癌遺伝子産物であり，遺伝子の増幅，蛋白の過剰発現は種々の癌において報告されている．乳癌では遺伝子増幅は10～33%，過剰発現は17～37%，卵巣癌では前者が20～26%，後者が32%，胃癌においてはそれぞれ8～25%，12～55%，大腸癌では3～7%，27～56%などと報告されている．

現在まで，ErbB-2に対する阻害剤や特異的抗体が種々開発され，治療研究が行われており，これらの薬剤による抗腫瘍効果が報告されてきている．実際，これまでにさまざまな抗ErbB-2抗体が作製され，そのひとつのtrastuzumabは現在FDAで認可している唯一の固形癌に対する抗体である．これらの抗ErbB-2抗体は抗体単独で，抗腫瘍効果を発揮するが，その作用機序はおもにErbB-2のダウンレギュレーションであることがすでに示されている[1]．

抗ErbB-2モノクローナル抗体CH401によるアポトーシスの誘導

著者らもこれまで数種類の抗ErbB-2モノクローナル抗体を作製してきた．そのうちの1種CH401がErbB-2過剰発現癌細胞にアポトーシスを誘導することを明らかにしてきた[2](図1)．このことはこれまで報告されているtrastuzumabを含むほとんどの抗ErbB-2抗体による抗腫瘍効果の機序とはまったく異なるものであった．このため，つぎにこのアポトーシスの機序に関する検討を行った．

ErbB-2からの増殖シグナルはおもにMAPキナーゼとPI3キナーゼ-Aktの経路に伝達されることが明らかになっている．そこで，MAPキナーゼおよびPI3キナーゼ-Aktに与える影響を検討した．さらに，アポトーシスの実行分子であるcaspaseの活性に与える影響を検討した．

MAPキナーゼ(ERK/JNK/p38)およびPI3キナーゼ-Akt経路の活性変化

まず，MAPキナーゼについてその活性の変化について検討した．増殖に関与するErkに関して，その活性はCH401添加8時間後より低下を認め，一方，アポトーシスに関与するJNKおよびp38については抗体添加2時間後から著明な活性上昇が認められた．以上よりアポトーシスの誘導に，各MAPキナーゼの関与が想定された．さらに，PI3キナーゼ-Aktの経路については，CH401添加後，Aktの活性は低下し，この変化もアポトーシスの誘導に関与していることが明らかになった．

つぎに，caspaseについて検討を行った．代表的な3種類のcaspase(caspase-3,8,9)に関して，CH401処理後の活性変化について検討を

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Original article

Titration of serum p53 antibodies in patients with gastric cancer: a single-institute study of 40 patients

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Abstract

Background. **Alterations of the *p53* tumor suppressor gene are the most commonly observed genetic abnormalities in many different types of human malignancies. The accumulation of mutant *p53* often leads to the production of p53 antibody (p53-Ab) in the sera of patients with various cancers. To evaluate the clinical implications of serum p53-Abs in patients with gastric cancer, we compared p53-Abs with conventional tumor markers such as carcinoembryonic antigen (CEA) and carbohydrate antigen (CA)19-9.**

Methods. **Serum samples were obtained preoperatively from 40 patients with histologically confirmed gastric adenocarcinoma, including 28 (70%) patients in stage Ia. The serum p53-Abs were assessed by enzyme-linked immunosorbent assay, using a new version of a highly specific, quantitative p53-Abs Kit (MESACUP Kit II).**

Results. **p53-Abs were detected in 6 (15%) of 40 patients with gastric cancer, including 3 patients with early gastric cancer. Seven (17.5%) of the 40 patients were positive for CEA in serum. However, none of 7 patients with high CEA levels were positive for p53-Abs. No significant correlation of p53-Abs with patient age, sex, pathological parameters, tumor markers such as CEA and CA19-9, or poor survival (P = 0.116) was observed.**

Conclusion. **Although we employed the latest version of the p53-Abs Kit, the sensitivity of serum p53-Ab in gastric cancer patients was relatively low. No correlation was found between the presence of p53-Ab and the staging of cancer or survival. However, serum p53-Ab was detectable in patients with gastric cancer even in the early stages of disease. In addition, it may be independent of CEA and CA19-9.**

Key words Gastric cancer · p53 Antibody · Tumor marker

Introduction

Conventional tumor markers such as carcinoembryonic antigen (CEA), carbohydrate antigen (CA)19-9, squamous cell carcinoma antigen (SCC-Ag), tissue polypeptide antigen (TPA), and cytokeratin fragment (CYFRA)21-1, are not suitable for the screening or monitoring of patients with malignant tumors, because of low sensitivity and specificity. It has been suggested that oncogenes and tumor suppressor genes and their products may be useful in biochemical tests for cancer [1]. The tumor suppressor *p53* gene, located on chromosome 17p13.1, frequently undergoes mutation in the genesis of human cancer [2]. The frequency of *p53* mutations in all malignant tumors was reported to be at least 50% [3, 4]. The mutated *p53* gene leads to the synthesis of a mutant protein with a longer than normal half-life, and massive overexpression of the protein products [5, 6]. The accumulation of mutant p53 protein has been found to be immunogenic in cancer patients and to result in the production of p53 antibody (p53-Ab) in serum [7].

The p53-Ab in the sera of cancer patients can be detected by immunoprecipitation or Western blotting, or by enzyme-linked immunosorbent assay (ELISA) [7–10]. Circulating p53-Abs in patients have been reported for various types of carcinomas [9, 10], including breast cancer, hematopoietic malignancy, esophageal cancer, colon cancer, ovarian cancer, lung cancer, pancreatic cancer, and gastric cancer [11–15]. Several studies have demonstrated that the p53-Ab in sera served as an early marker of malignant disease, as an indicator for monitoring patients with malignant tumors during treatment, and as a prognostic factor for patients with several types of tumors [11, 16–18]. Because these studies attempted to evaluate the clinical value of p53-Ab under different conditions, the role of p53-Ab in patients with malignant tumors has not been clearly established yet.

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Gastric cancer is widely prevalent in the world and is one of the leading malignancies in terms of incidence and cause of cancer death in East Asia and South America. In Japan, the mortality rate of gastric cancer is showing a decreasing trend, reflecting advances in medical technology, such as early detection and treatment with an endoscope. It is necessary to evaluate the clinical usefulness of new early diagnostic markers of malignancies (for example, in gastric cancer) which could be found in the early stage of tumorigenesis. In this regard, reports on p53-Ab in the sera of patients with gastric cancer have not been adequate [15, 19–22].

In this study, we examined 40 patients with gastric cancer, including 28 (70%) patients in the early stages of the disease, for the presence of circulating antibodies against the tumor suppressor protein p53 and we examined these findings in relation to conventional tumor markers, tumor characteristics, and the clinical status of the patients. The serum levels of p53-Abs were assessed by ELISA, using a new version of a highly specific, quantitative p53-Ab Kit [23].

Patients and methods

Patients

Forty patients with primary gastric cancer who underwent gastric resection at the Department of Surgery, Division of Digestive Surgery, Kyoto Prefectural University of Medicine, Kyoto, Japan, between July and December 2000 were enrolled in this study. Written informed consent was obtained from each patient. No patients had received preoperative radiotherapy or chemotherapy. There were 28 (70%) male and 12 (30%) female patients, with an average age of 60.6 years (range, 28–86 years).

Serum and tumor samples

Serum samples were collected from each patient before and 28 days after surgery. Samples were stored at −80 °C until they were assayed. After resection, the tumor specimens were subjected to routine processing for the control of resection margins; also, exact histological investigation included an evaluation of staging in accordance with the International Union Against Cancer (UICC)/TNM classification.

Enzyme immunoassay for serum p53 antibodies

Serum p53-Ab levels were assessed by (ELISA) with the anti-p53 EIA Kit II (MESACUP anti-p53 Test; Medical and Biological Laboratories (MBL), Nagoya, Japan). In brief, the samples were added, for 1 h at 37 °C, to microtiter wells coated with wild-type human p53 protein or a control protein to detect nonspecific interactions. After washing, a peroxidase-conjugated goat antihuman immunoglobulin G that binds p53-Ab was applied for 1 h at 37 °C. Then substrate solution was added for 30 min at 37 °C. After the addition of stop solution, color development was assessed by measuring absorption at 450 nm, using a photospectrometer. Levels of p53-Abs were determined from a calibration curve constructed from the specific signals of standards. The cutoff value for serum p53-Abs was 1.3 U/ml. The specificity of this assay is greater than 95.5% [23].

CEA and CA19-9 assays

Serum CEA concentrations were measured with an immunoradiometric assay, using a CEA RIABEAD Kit (Abbott Japan, Tokyo, Japan). Serum CA19-9 concentrations were also measured with an immunoradiometric assay, using a CA19-9 RIA Kit (TFB, Tokyo, Japan). According to the manufacturers, the cutoff values for serum CEA and CA19-9 were 2.5 ng/ml and 37 U/ml, respectively.

Statistical analysis

Fisher's exact test, Student's *t*-test, and the Mann-Whitney *U*-test were used to determine the significance of differences between two groups. Survival curves were plotted using the Kaplan-Meier method. The logrank test was adopted to compare two groups. Cox regression analysis was performed to determine which factors would be useful in predicting overall survival. A P value of less than 0.05 was considered significant.

Results

Detection of serum p53 antibody in gastric cancer

We tested serum samples from 40 patients with gastric cancer for the presence of p53-Abs. Six (15.0%) of the 40 patients were positive for serum p53-Abs: the mean age of this group was 63 years (range, 40–77 years), and the male/female ratio was 2:1. The other 34 (85.0%) patients were negative for serum p53-Abs: their mean age was 60.2 years (range, 28–86 years), and the male/female ratio was 2.4:1. Based on the UICC/TNM classification, 3 of the 6 p53Ab-positive patients were in stage Ia; none of the 6 patients was in stage IV, but p53-Abs were also detected at stages II, IIIa, and IIIb. No significant differences between the p53Ab-positive and -negative groups were observed in age, sex, or tumor staging (Table 1).

We analyzed the histopathological factors of tissue type, tumor invasion, lymph node metastasis, and dis-

Table 1. Correlation between the presence of serum p53 antibody (Ab) and clinicopathological features in gastric cancer

Variables	Total	Serum p53 antibody		*P* value
		Positive	Negative	
Number of patients	40	6	34	
Age (years)	60.6	63	60.2	0.64
Sex (M:F)	2.3:1	2.0:1	2.4:1	0.88
Stage				
Ia	28	3	25	
Ib	3	0	3	
II	4	1	3	0.3
IIIa	1	1	0	
IIIb	2	1	1	
IV	2	0	2	

Table 2. Correlation between the presence of serum p53-Ab and histopathological findings in gastric cancer

	Serum p53 antibody		*P* value
	Positive	Negative	
Tissue type			
Differentiated	2	21	0.272
Undifferentiated	4	13	
Tumor invasion			
Mucosa or submucosa	3	25	0.363
Deeper than submucosa	3	9	
Lymph node metastasis			
Negative	3	31	0.111
Positive	3	3	
Distant metastasis			
Negative	6	33	0.909
Positive	0	1	

tant metastasis. Four (66.7%) of the 6 p53Ab-positive patients had histologically undifferentiated adenocarcinomas, compared to 13 (38.2%) of the 34 p53Ab-negative patients (P = 0.272). Three (50%) of the 6 patients with lymph node metastasis were positive for serum p53-Ab, whereas only 3 (8.8%) of the 34 patients without lymph node metastasis were positive (P = 0.111). There were no significant differences in these factors between the groups who were positive and negative for p53-Ab (Table 2).

Sensitivity of serum CEA, CA19-9, and p53 antibody in gastric cancer

The correlation between the presence of serum p53-Ab and the two tumor markers CEA and CA19-9 was analyzed. The sensitivities of CEA and CA19-9 in this study were 17.5% (7/40) and 10% (4/40), respectively. The 7 patients positive for CEA did not express p53-Abs, and CEA was not detected in any p53-Ab-positive patients (Table 3).

Table 3. Correlation between the presence of serum p53-Ab and tumor markers in gastric cancer

	Serum p53 antibody		*P* value
	Positive	Negative	
CEA			
Positive	0	7	0.426
Negative	6	27	
CA19-9			
Positive	1	3	0.762
Negative	5	31	

We analyzed the sensitivity of serum p53-Ab and CEA according to stage based on the UICC/TNM classification. Three (10.7%) of the 28 patients in stage Ia were positive for serum p53-Ab, whereas none (0%) of these 28 patients was positive for CEA. In stage IV, both patients were positive for CEA, but neither was positive for p53-Ab (Table 4).

Three (50%) of the 6 p53Ab-positive patients became negative postoperatively, while 5 (71.4%) of the 7 CEA-positive patients became negative postoperatively (Table 4).

Detection of serum p53 antibody in stage Ia gastric cancer

We focused on the stage-Ia patients to investigate the clinical usefulness of the levels of serum p53-Ab as a marker for the early detection of gastric cancer. Table 5 demonstrates that only p53-Ab was positive in patients with stage Ia gastric cancer, whereas CEA and CA19-9 were not positive. No significant differences between the p53Ab-positive and -negative groups were observed in regard to tissue type or tumor invasion.

Survival rates

The 4-year survival rates for patients with sera that was positive or negative for CEA, CA19-9, and p53-Ab are shown in Table 6. The median follow-up time for all 40 patients was 31.7 months (range, 1–48 months). The 4-year survival rate was 82.9% for the p53Ab-negative patients and 60% for the p53-Ab-positive patients. However, there was no significant difference in the rate of survival between the p53-Ab-positive group and the p53Ab-negative group (P = 0.116). In contrast, the overall survival of patients positive for CEA was significantly shorter than that in the CEA-negative patients (P = 0.0008) (Table 6).

Table 4. Correlations between sensitivity of serum CEA and p53 Ab according to clinical stage

	Serum p53 antibody	CEA	*P* value
Stage			
Ia	10.7% (3/28)	0% (0/28)	0.49
Ib	0% (0/3)	66.7% (2/3)	0.19
II	25% (1/4)	50% (2/4)	0.56
IIIa	100% (1/1)	0% (0/1)	0.31
IIIb	50% (1/2)	50% (1/2)	1
IV	0% (0/2)	100% (2/2)	0.12
Negative conversion post-surgery	50% (3/6)	71.4% (5/7)	0.52

Table 5. Correlation between the presence of serum p53-Ab, histopathological findings, and tumor markers in stage Ia patients

	Serum p53 antibody		
	Positive	Negative	*P* value
Tissue type			
Differentiated	2	19	0.853
Undifferentiated	1	6	
Tumor invasion			
Mucosa	2	15	0.906
Submucosa	1	10	
CEA			
Positive	0	0	0.49
Negative	3	25	
CA19-9			
Positive	0	0	0.49
Negative	3	25	

Table 6. Association between 4-year survival rates and tumor markers in patients with gastric cancer

	Survival rate (%)	*P* value
CEA		
Positive	25.7	0.0008
Negative	92.3	
CA19-9		
Positive	50	0.118
Negative	85.4	
p53-Ab		
Positive	60	0.116
Negative	82.9	

However, Cox regression analysis of all factors listed in Tables 1 and 2 revealed that lymph node metastasis, but not p53 Ab or CEA, was an independent prognostic factor in gastric cancer ($P < 0.05$).

Discussion

At present, there is no satisfactory tumor marker for the diagnosis or monitoring of malignant disease. It is expected that a new biological marker which shows high sensitivity and specificity and can be used with relative ease will be established.

p53-Ab is an autoantibody induced by mutation of the *p53* tumor suppressor gene, and has been detected in the sera of patients with various types of cancers. Since its initial description more than 20 years ago, the usefulness of serum p53-Ab in patients with various cancers has been reported [9–15]. Gastric cancer remains a major cause of cancer-related deaths in the world. Serum CEA is generally used for the diagnosis and monitoring of gastric cancer, but only a limited proportion of patients benefit. Therefore, potential new biological markers, such as p53-Ab, E-cadherin, or hepatocyte growth factor (HGF) for patients with gastric cancer, have received attention [24–26]. Because gastric cancer can be diagnosed at an early stage by endoscopy, it is suitable for testing a potential biological marker for early diagnosis. Nevertheless, only a small number of reports regarding the evaluation of p53-Abs in the sera of patients with gastric cancer have been published to date [15, 19–22].

The present study demonstrated that, in 15% (6 of 40) of patients, gastric cancer was detectable by p53-Ab ELISA assay preoperatively. This is comparable with previous observations in patients with gastric cancer [15, 19–23]. No significant correlation between p53-Abs and either tumor stage, tissue grade of differentiation, depth of tumor invasion, lymph node metastasis, or distant metastasis was observed. The positive rate for CEA and CA19-9 in the sera of patients with gastric cancer was 17.5% (7 of 40) and 10% (4 of 40), respectively, which is similar to results reported by other groups [27, 28]. Most interestingly, the 6 patients positive for p53-Abs did not show high levels of CEA, and only 1 patient positive for p53-Ab showed a high CA19-9 level. The presence of p53-Ab was not associated with serum CEA

or CA19-9 ($P = 0.426$ and $P = 0.762$, respectively). It was supposed that p53-Ab might be an independent marker of CEA or CA19-9. The positivity rate for the diagnosis of gastric cancer increased to 32.5% when p53-Ab and CEA were combined in this study.

Because alterations in the *p53* gene result in an accumulation of the protein in tumor cells, the presence of serum p53-Ab was described as an early event that could predate the diagnosis [29]. Our results demonstrated that, of 28 patients with stage Ia gastric cancer tested preoperatively, 3 were positive for p53-Ab in serum, whereas none was positive for serum CEA or CA19-9. A *p53* mutation may be not only an advanced-stage phenomenon but may also be an early event of carcinogenesis. Several studies have reported that p53-Ab can be found in the serum of individuals at high risk of developing cancer, including heavy smokers and workers exposed to vinyl chloride [16, 29, 30]. In contrast, no association between *p53* abnormalities (overexpression/mutation) and *Helicobacter pylori* infection was found in patients with gastric adenocarcinoma; therefore, mutations of the *p53* gene do not seem to be a predominant event in gastric carcinogenesis [31]. These contradictory findings might be explained by a report that 39.1% of patients with gastric cancer positive for p53-Ab in sera had tumor tissues that stained negative for p53 protein [19].

Although there have been several reports that the presence of p53-Ab in serum was a prognostic factor for patients with various types of malignancies, the prognostic value of p53-Abs in patients with gastric cancer is still controversial [15, 19, 21]. We did not find a significant correlation between the presence of p53-Abs in the sera of patients with gastric cancer and overall survival, despite the finding that the 4-year survival rate was about 20% higher in the p53-Ab-negative patients than that in the -positive patients. On the other hand, high levels of CEA could be associated with prognosis. However, Cox regression analysis revealed that lymph node metastasis, but not p53 Ab or CEA, served as an independent prognostic factor in gastric cancer in this series.

The p53-Abs circulating in patients with various types of cancer can be detected by several methods, including immunoprecipitation, Western blotting, and ELISA [7–10]. Because none of these methods give satisfactory rates of detection, further improvement is needed. We employed the latest version of an ELISA kit, which has the advantage of quantitative analysis, for the detection of p53-Ab. Using this assay, Shimada et al. [23], in a multiinstitutional study, reported 20.4% positivity for p53-Abs in 1085 patients with 15 types of malignant tumors, and they determined a cutoff value of 1.3 U/ml with over 95.5% specificity by analyzing serum samples of 205 healthy controls. This assay could thus contribute to achieving high true-positive rates with low false-positive rates. Recently, a new protocol for the rapid and sensitive detection of p53-Abs in serum by immunomagnetic electrochemiluminescence (IM-ECL) was developed [32]. Further study will be needed to fully elucidate the importance of this detection method.

Here, we measured circulating p53-Ab levels in the sera of 40 patients with gastric cancer using a new version of the p53-Abs ELISA kit. The presence of p53-Ab was demonstrated in 15% (6 of 40) of the patients with gastric cancer preoperatively. No correlation was found between the presence of p53-Ab and the staging of cancer or survival. However, circulating p53-Ab was detectable in patients with early-stage gastric cancer, and was independent of the currently available tumor markers CEA and CA19-9.

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特集　Biotherapy Frontier 2005

癌の細胞療法—変遷と今後の展開—

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要旨　固形癌に対する免疫細胞療法の変遷と問題点を概説し，今後の方向性の一つとして現在われわれが取り組んでいる，完全閉鎖系体外循環による免疫細胞療法の開発研究について言及した。

固形癌に対する免疫細胞療法は，樹状細胞の *in vitro* 誘導と数々の腫瘍拒絶抗原の解明を契機に，培養T細胞を用いた受動免疫療法から樹状細胞を用いた能動免疫療法へと1990年代後半に大きく流れを変えた。しかし，よくデザインされた臨床試験でその有効性が客観的に評価された報告は未だごくわずかである。今後は造血幹細胞移植や化学療法を併用した集学的細胞療法の開発研究が盛んになるであろうが，抗腫瘍効果が不十分であれば普遍性，安全性，経済性の面から，真の癌医療として定着していくのは困難であろう。

in vitro での細胞処理が必要な免疫細胞療法は，治療に至るまでの労力，経費ともに莫大でなおかつ培養過程における微生物汚染の危険性を避けて通れない。したがって，われわれはポリスチレン系極細繊維を充填された2種類の癌治療用体外循環カラムの開発研究を行っている。一つは免疫抑制性サイトカインを血中から効率的に吸着除去することを目的とし，もう一つは免疫賦活剤を固相化することにより血中の免疫担当細胞を直接活性化することを目的としている。安全に，簡便に，繰り返し施行可能な完全閉鎖系体外循環による免疫細胞療法の新しい治療体系の構築をめざしている。

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Present Status and Future Prospect of Immune-cell Therapy for Solid Cancer

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Summary

Immune-cell therapy for solid cancer is still a challenge. Adoptive immunotherapy with cultured killer T lymphocytes and interleukin-2 have been actively tried since the middle of the 1980's. However, the mainstream has become dendritic cell-based, tumor-specific active immunotherapy since the latter half of the 1990's. Despite much effort, immune-cell therapy has not yet achieved an adequate anti-tumor effect comparable with that of chemotherapy. In addition, there are many problems with immune-cell therapy which needs *in vitro* cell processing. It is labor- and resource-consuming, and cannot avoid the risk of microbial contamination. In view of these problems, we started research on a new immunotherapy technique with direct hemoperfusion. We have succeeded in developing a direct hemoperfusion column for cancer immunotherapy which consists of extra-fine synthetic fibers removing immunosuppressive cytokines from the peripheral blood of advanced cancer patients. This column can be an effective immunotherapy technique in conjunction with immune-cell therapy or chemotherapy. We are now planning a pre-marketing clinical trial of this column.

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はじめに

癌の免疫細胞療法の臨床研究の歴史は，熱狂と落胆の繰り返しである。1980年代後半に開始されたT細胞移入による受動免疫療法は，1990年代後半には樹状細胞（dendritic cell，DC）を用いた能動免疫療法へと大きく流れを変えたが，未だ総じて満足な抗腫瘍効果を達成し得えていない。生体が有する免疫細胞の力で癌を治すことは誰しもが願う究極の理想であるが，煩雑で高価な“細胞療法”という治療形態が，普遍性・経済性の面から時代のニーズに合致するのか否かという問いかけを，今後は無視できないであろう。抗腫瘍効果が十分でなければなおさらである。

本稿では固形癌に対する免疫細胞療法の変遷と問題点を概説し，今後の方向性の一つとして現在われわれが取り組んでいる，完全閉鎖系体外循環による免疫細胞療法の開発研究について言及する。

I. 癌の免疫細胞療法—受動免疫療法から能動免疫療法へ—

固形癌に対する免疫細胞療法の臨床応用開始の契機は，T細胞増殖因子であるインターロイキン（interleukin，IL）-2の発見とそのリコンビナント製剤としての利用開始にまでさかのぼることができる[1]。T細胞やナチュラル・キラー（natural killer，NK）細胞を*in vitro*でIL-2存在下に培養すると，NK非感受性の腫瘍細胞を広範に非特異的に傷害することのできるリンフォカイン活性化キラー（lymphokine-activated killer，LAK）細胞へと増殖を伴う分化を遂げる[2]。このLAK細胞を用いた受動免疫療法である養子免疫療法（adoptive immunotherapy，AIT）が，IL-2をアジュバントとして併用しつつ1980年代後半より様々な固形癌に対して試みられた。当初欧米においてメラノーマや腎癌に対してその有効性が示されたが[3]，消化器癌を中心とした他癌腫に対する治療効果は，本邦を中心に精力的にその臨床研究が展開されたとおり，肝転移や癌性胸腹膜炎に対する局所療法を除けば極めて弱かった[4,5]。その後，より腫瘍特異性の高い腫瘍浸潤リンパ球（tumor infiltrating lymphocyte，TIL）やサイトカイン遺伝子導入リンパ球を用いたAITの臨床研究が再びメラノーマや腎癌に対して欧米で積極的になされ，これら免疫原性が高い腫瘍に対するAITに関する多くの知見が集積された[6]。しかし，効果を期待するためには，*ex vivo*でIL-2や抗CD3抗体存在下にエフェクターであるT細胞を10^{10}～10^{11}個のオーダーにまで多量に培養・増殖させる必要があるAITは，後述するように臨床応用上多くの問題点を内包していた。

その後，1994年にSallustoらにより末梢血単球から*in vitro*で顆粒球マクロファージコロニー刺激因子（granulocyte/macrophage colony-stimulating factor，GM-CSF）とIL-4存在下に，生体内で最も強力な抗原提示細胞であるDCの誘導が可能なことが明らかにされ[7]，DCを用いた能動免疫療法（DCワクチン療法）への道が開かれた。また，ほぼ時を同じくして，メラノーマのみならず種々の悪性腫瘍に発現する腫瘍拒絶抗原が分子レベルで明らかにされはじめたことが，この流れに拍車をかけた[8]。すなわち，1990年代後半から癌の免疫細胞療法の流れは，培養T細胞を用いた受動免疫療法からDCを用いた能動免疫療法へ大きくシフトした[9]。

世界中で数多くのグループが，DCと種々の形態の腫瘍抗原との組み合わせで，様々な癌腫に対しDCワクチン療法の臨床研究・臨床試験を手掛けてきたが，以下に列挙するとおり未だ十分に明らかにされていない多くの問題点がある[10]。

1. DCの至適投与細胞数は？

GM-CSFとIL-4で誘導される単球由来DCは，増殖を伴わない分化を遂げるため，培養に供する末梢血単核細胞のせいぜい5％前後しか回収されず，増殖を伴う分化T細胞を用いるAITと大きく異なる[11]。phase I試験でDCの投与細胞数を

段階的に増やし，その効果を検討している報告も散見されるが，投与細胞数と免疫学的効果・腫瘍縮小効果の間には，有意な相関を認めないとする報告が多い[12,13]。

2.　成熟DCか未成熟DCか?

McIloyらは，メラノーマを対象としたDCワクチン療法の総数10の臨床試験の167例をメタアナリシスし，腫瘍縮小を誘導する規定因子を検討した結果，患者の年齢，性別，化学療法歴の有無，DCの投与量，投与ルートなどは有意な因子ではなく，TNF-αを用いたDCの成熟化のみが腫瘍の縮小を誘導する唯一の規定因子であったと結論付けている[12]。これと同一の見解を示す報告は多く，DCワクチン療法の効果発現において成熟DCが強く関与していることはほぼ間違いなさそうである[14,15]。

3.　DCと組み合わせる最適な腫瘍抗原の形態は?

腫瘍抗原として特定のHLA-class I分子に結合するペプチドやtumor lysate，wholeの腫瘍細胞をDCと融合させる方法（DC-tumor hybrid）などが用いられているが，どれが最も優れるかは結論がでていない。メラノーマにおいて最も多くの腫瘍拒絶抗原ペプチドが同定され臨床応用も多くなされているが，ペプチドよりtumor lysateが優れるという報告もある[16]。DCと腫瘍細胞の融合はポリエチレングリコールを用いる方法と電気融合法があるが，効率的に真のDC-tumor hybridを作製することは容易ではない。最近Hayashi，Shimizuらは，融合効率の高い画期的な電気融合法をマウスの系で開発し，DC-tumor hybridがDCワクチン療法における最適の抗原形態であることを報告しているが[17,18]，DC-tumor hybridは現時点においては，他抗原に匹敵する臨床効果を誘導しているとはいい難い[19]。

4.　DCの至適投与ルートは?

静脈内投与で免疫学的効果，臨床効果を誘導し得たとする報告もあるが[20]，皮内・皮下投与が一般的である。手技的に煩雑であるが，リンパ節内投与は最近でもよい治療成績が散見される[21,22]。

5.　最適な免疫学的効果判定方法は?

従来DCワクチン療法は臨床応用開始初期のphase I試験として試みられてきたため，安全性とともに治療前後の当該抗原に対する免疫学的応答性の変化が多く検討されてきた。これには簡便な皮内テストから煩雑な*in vitro*のassayまで種々の方法があるが，臨床試験を施行している施設間の技術格差が大きいため，信頼できる情報が乏しい。また，免疫学的効果が得られてもそれが腫瘍の縮小に結び付いているのはごくわずかの症例である[23]。今後はsurrogate endpointである免疫学的効果は付随的なものとし，化学療法と同様のprimary endpointである，腫瘍縮小効果と生存期間の延長効果を厳密に評価していくべきと考えられる。

いずれにしても，DCワクチン療法に限らずすべての*in vitro*での細胞処理を必要とする免疫細胞療法は，治療を成立させるための労力と経費が莫大であるため，以上のような問題点を解明・解決していくために，本邦でも多施設共同臨床研究の推進が望まれる。一方，2004年6月の米国臨床腫瘍学会（ASCO）において，転移性メラノーマに対するfirst-line therapyとして，標準的化学療法（ダカルバシン）と複数のメラノーマ抗原ペプチド（MAGE1，MAGE3，gp100，Melan-Aなど）をパルスした成熟DCを用いた免疫療法の効果を比較したヨーロッパでの多施設共同第Ⅲ相臨床試験の結果が報告された[24]。2001年1月～2003年6月までの間に全108例が登録されたが，奏効率（化学療法群：5.5%，DC群：3.8%），全生存期間，無増悪生存期間ともに両群で有意差なしという結果であった。本発表は，学会当日まで抄録内容が一切公開されないlate breaking abstractとして大きな期待と関心を集め，われわれも当日会場に居合わせたが，超満員のフロアからは落胆のため息があちらこちらから聞かれた。労力，経費ともに莫大なDCワクチン療法が，メラノーマにおいてでさえ従来の化学療法の効果を凌駕できなかったこの結果は，今後の癌の免疫細胞療法の開発研究の趨勢に影響を及ぼさざるを得ないであろう。

II.　免疫細胞療法の新しい流れ

1.　免疫細胞療法としての同種造血幹細胞移植

造血幹細胞移植は，造血器腫瘍に対する骨髄破