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1

REGULAR ARTICLE

Overexpression of alpha enolase in hepatitis C virus-related hepatocellular carcinoma: Association with tumor progression as determined by proteomic analysis

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To identify proteins that could be molecular targets for diagnosis and treatment of hepatitis C virus-related hepatocellular carcinoma (HCV-related HCC), we used a proteomic approach to analyze protein expression in samples of human liver. Twenty-six pairs of tumorous and corresponding nontumorous liver samples from patients with HCV-related HCC and six normal liver samples were analyzed by two-dimensional gel electrophoresis and liquid chromatography-tandem mass spectrometry. One of the numerous spots that showed stronger intensity in tumorous than in nontumorous samples was identified as alpha enolase, a key enzyme in the glycolytic pathway. Expression of this protein increased with tumor dedifferentiation and was significantly higher in poorly differentiated HCC than in well-differentiated HCC. This pattern was reproduced by immunoblot analysis and immunohistochemistry. Expression of alpha enolase also correlated positively with tumor size and venous invasion. These results suggest that alpha enolase is one of the candidates for biomarkers for tumor progression that deserves further investigation in HCV-related HCC.

1 Introduction

Hepatocellular carcinoma (HCC) is one of the most fatal cancers worldwide, with a high incidence in many countries [1]. Infection by hepatitis virus, alcoholic liver dysfunction,

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Abbreviations: AFP, alpha-fetoprotein; **HCC,** hepatocellular carcinoma; **HCV,** hepatitis C virus; **LC,** liver cirrhosis; **NL,** normal liver

autoimmune hepatitis, and exposure to aflatoxin B1 are some recognized causes of HCC. Hepatitis B virus (HBV) and hepatitis C virus (HCV) are the most clearly established risk factors for HCC. Although the properties of these two viruses differ, damage to hepatocytes by chronic viral infection is believed to induce HCC [2, 3]. Persistent infection with hepatitis virus leads to chronic hepatitis (CH) and then to liver cirrhosis (LC). Development of HCC is a multistep process based on histologic changes. HCC tumors are initially well-differentiated (G1 tumor), but with time, they dedifferentiate to moderately (G2 tumor) or poorly (G3 tumor) differentiated types with high proliferation rates. The pathogenesis of HCC is not entirely clear. Molecular biological studies have been helpful in the effort to elucidate this process.

The combination of 2-DE and MS is a proteomic method of high-throughput analysis of protein expression. Several proteomic studies have identified diverse proteins that may be involved in the pathogenic mechanism of HCC [3–12]. In the present study, we analyzed protein expression in 26 pairs of HCV-related HCC samples and corresponding non-tumorous liver samples and in six samples normal liver (NL) to find proteins that might be involved in tumor differentiation and progression.

2   Materials and methods

2.1 Tumor samples

Twenty-six pairs of HCC samples and corresponding non-tumorous liver samples were obtained from patients diagnosed with HCC who underwent surgical liver resection at the Department of Surgery II, Yamaguchi University Hospital. Following resection, samples were frozen immediately and stored at −80°C until use. Written informed consent was obtained from all patients before surgery. None of the patients received any preoperative therapy such as chemotherapy, percutaneous ethanol injection, or transcatheter arterial embolization. The study protocol was approved by the Institutional Review Board for Human Use of the Yamaguchi University School of Medicine. Histologic diagnosis of HCC was made on formalin-fixed, paraffin-embedded sections according to World Health Organization criteria. The HCC samples consisted of six G1, 15 G2, and five G3 tumors. Nontumorous samples consisted of nine CH and 17 LC. Serologically, all patients were positive for HCV antibody and negative for HBs antigen. Clinical characteristics of the patients are shown in Table 1.

2.2 Control liver samples

Normal liver samples were obtained from six patients who underwent hepatic resection for benign liver tumors or metastatic liver tumors due to gastrointestinal cancer. All were negative for both hepatitis B surface antigen and HCV antibody, and the tissues were histopathologically normal.

2.3 Sample preparation

Sample preparation was performed essentially as described previously [9, 12]. Liver samples were homogenized in lysis buffer (50 mM Tris-HCl, pH 7.5, 165 mM sodium chloride, 10 mM sodium fluoride, 1 mM sodium vanadate, 1 mM PMSF, 10 mM EDTA, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 1% NP-40). Suspensions were incubated for 2 h at 4°C, centrifuged at 15 000 × *g* for 30 min at 4°C, and the supernatants were stored at −80°C until use.

Table 1. Clinical characteristics of 26 HCC samples

Differentiation of tumor[a]	G1	G2	G3
Number	6	15	5
Gender male/female	3/3	10/5	3/2
Age (mean)	58–74 (69.3)	50–76 (66.1)	39–78 (64.2)
Histology of nontumorous lesion			
CH	2	7	0
LC	4	8	5
Tumor size (cm) (mean)	1.2–3.2 (2.15)	1.5–13.0 (5.47)	3.4–12.0 (7.28)
Incidence of Serum AFP >20 ng/mL	3/6	10/15	3/5
Incidence of serum PIVKA-II > 40 U/mL	2/6	12/15	4/5
Venous invasion no/yes	6/0	8/7	1/4

a) Assessment based on classification of malignant tumors by the International Union against cancer. G1, G2, and G3 indicate well-, moderately-, and poorly-differentiated HCC, respectively.

AFP, alpha-fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonist-II.

2.4 2-DE and SDS-PAGE

Three hundred micrograms of protein was used for each 2-DE assay. The first-dimensional IEF was performed on 7 cm, immobilized, pH 3–10 linear gradient strips (Amersham Biosciences, Uppsala, Sweden) at 20°C and 50 μA/strip. The strips were rehydrated with 125 μL of sample solution (8 M urea, 2% CHAPS, 0.01% bromophenol blue, 0.56% 2-mercaptoethanol, and 0.5% IPG buffer) for 14 h. IEF was run in three steps: 500 V for 1 h, 1000 V for 1 h, and 8000 V for 3 h. Voltage increases were carried out in a gradient. The second-dimensional run was performed on precast polyacrylamide gels (2-D homogeneous 12.5; Amersham Biosciences) in two steps: 600 V, 20 mA for 30 min and 600 V, 50 mA for 70 min. After electrophoresis, the gels were stained with CBB R-250 (Nacalai Tesque, Kyoto, Japan) for 24 h. Gels were destained with 10% acetic acid in water containing 30% methanol for 30 min and then destained with 7% acetic acid and used for in-gel digestion. Fifty micrograms of protein was used for each SDS-PAGE sample. Sample lysates were mixed with 4 μL of 5 × SDS sample buffer. Samples were denatured for 5 min at 95°C and separated on 10% SDS-PAGE gels. Twenty microliters of lysate was applied to each lane. Electrophoresis was carried out at 15 mA/gel.

2.5 In-gel digestion

CBB dye was removed by rinsing three times in 60% methanol, 50 mM ammonium bicarbonate, and 5 mM DTT for 15 min and twice in 50% ACN, 50 mM ammonium bicarbo-

nate, and 5 mM DTT for 10 min. The gel piece was dehydrated three times in 100% ACN for 30 min and then rehydrated with an in-gel digestion reagent containing 10 µg/mL sequencing-grade modified trypsin (Promega, Madison, WI, USA) in 30% ACN, 50 mM ammonium bicarbonate, and 5 mM DTT. The in-gel digestion was performed overnight at 30°C. The samples were rinsed in 30% ACN, 50 mM ammonium bicarbonate, and 5 mM DTT for 2 h and lyophilized overnight at −30°C.

2.6 Amino acid sequencing by LC-MS/MS

Lyophilized samples were dissolved in 20 µL of 0.1% formic acid and centrifuged at $15\,000 \times g$ for 5 min. Peptide sequencing of identified protein spots was performed by LC-MS/MS with a Spectrum Mill MS Proteomics Workbench (Agilent Technologies, Santa Clara, CA, USA).

2.7 Immunoblot analysis

After SDS-PAGE, fractionated proteins were transferred electrophoretically onto PVDF membranes (Immobilon-P; Millipore, Bedford, MA, USA), and the membranes were blocked overnight at 4°C in TBS containing 5% skim milk. The primary antibody was an anti-enolase polyclonal antibody (1:100, #sc-7455; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Membranes were incubated for 1 h at 4°C, washed four times with TBS containing 0.05% Tween 20, and incubated for 1 h at 4°C with horseradish peroxidase-conjugated secondary antibody (1:2000, #55360; ICN Pharmaceuticals, Aurora, OH, USA). The reaction was visualized with a chemiluminescence reagent (ECL; Amersham Biosciences).

2.8 Immunohistochemistry

Formalin-fixed, paraffin-embedded samples were sectioned at 4 µm thickness. Sections were treated with protease for antigen retrieval and then stained with anti-enolase polyclonal antibody (1:500, #sc-15343; Santa Cruz Biotechnology). Avidin-biotin/3,3'-diaminobenzidine (Ventana Medical Systems, Tucson, AZ, USA) was used for detection, and hematoxylin was used for counterstaining. All procedures were performed automatically with BenchMark IHC/ISH Staining Module (Ventana Medical Systems).

2.9 Statistical analysis

Expression levels of proteins on 2-DE gels were quantified by analyzing the intensity of each spot with ImageMaster 2D Platinum (v5.0 software; Amersham Biosciences). Differences in expression levels between tumorous samples and corresponding non-tumorous samples, and between samples with and without venous invasion were analyzed by Student's *t*-test. Analysis of alpha enolase level in all the histologic groups was performed by ANOVA with Bonferroni-

Dunn test. Correlation of alpha enolase expression with tumor size, serum alpha-fetoprotein (AFP) levels, and serum protein induced by vitamin K absence or antagonist-II (PIVKA-II) levels was determined by Pearson correlation coefficients. $p < 0.05$ was considered significant in Student's *t*-test and Pearson correlation coefficients, and $p < 0.0033$ in ANOVA with Bonferroni-Dunn test.

3 Results

3.1 Identification of proteins on 2-DE gels

Protein expression was assessed in all tumorous, nontumorous, and NL samples. 2-DE was executed three times for each sample and almost same results were obtained. Hundreds of protein spots were visualized on 2-DE gels, and differences in spot intensities were compared visually and with a computerized approach for each gel. A spot of approximately 48 kDa in mass and a p*I* of 7.0 showed stronger intensity in G3 tumors than in nontumorous samples, G1 tumors, or G2 tumors (Figs. 1, 2). We thought that this protein might be involved in tumor differentiation. The spot was digested and used for MS analysis. This sample provided a good spectrum of amino acid sequences by LC-MS/MS and the protein was identified as alpha enolase, an isoenzyme of enolase, which is a key protein in the glycolytic pathway. The result of LC-MS/MS for the spot is shown in Table 2.

3.2 Alpha enolase expression in HCC

Immunoblot analysis for alpha enolase was performed on each differentiated HCC sample, corresponding nontumorous sample, and NL samples. Repeated analyses showed almost the same results. A slightly increased intensity of the band was identified in G2 tumors, and even more increased intensity was identified in G3 tumors. The intensity was equivalently weak in G1 tumor, nontumorous, and NL samples (Fig. 3). Immunohistochemical examination showed rare staining of cells in NL, in the presence of CH or LC, and in G1 tumors (Figs. 4A–D). Moderate, heterogeneous staining was identified in G2 tumors (Fig. 4E), and strong staining was identified in G3 tumors (Fig. 4F).

3.3 Alpha enolase expression and tumor differentiation

Spot intensity in 2-DE gels was quantified, and the expression level of alpha enolase was analyzed statistically. Spots showing differences in expression levels greater than twofold between tumorous and nontumorous samples were considered upregulated. Upregulation of alpha enolase in each differentiated HCC (G1, G2, and G3 tumors) occurred in 1/6 (17%), 4/15 (27%), and 4/5 (80%) samples, respectively. The numerical values of expression levels were shown in Table 3. G3 tumor samples showed significant upregula-
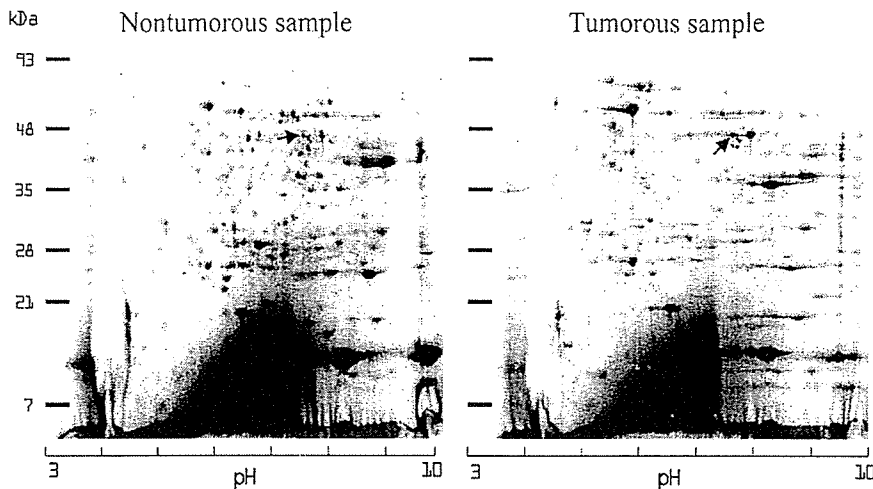
Figure 1. 2-DE pattern of HCV-related HCC sample and corresponding nontumorous sample. Proteins were prepared as described in the Section 2 and were separated on pH 3–10 linear IPG strips and then by 12.5% SDS-PAGE. Gels were stained with CBB R-250. A spot of approximately 48 kDa in mass and a p*l* of 7.0 showed strong intensity in tumorous samples compared to nontumorous samples (arrow).
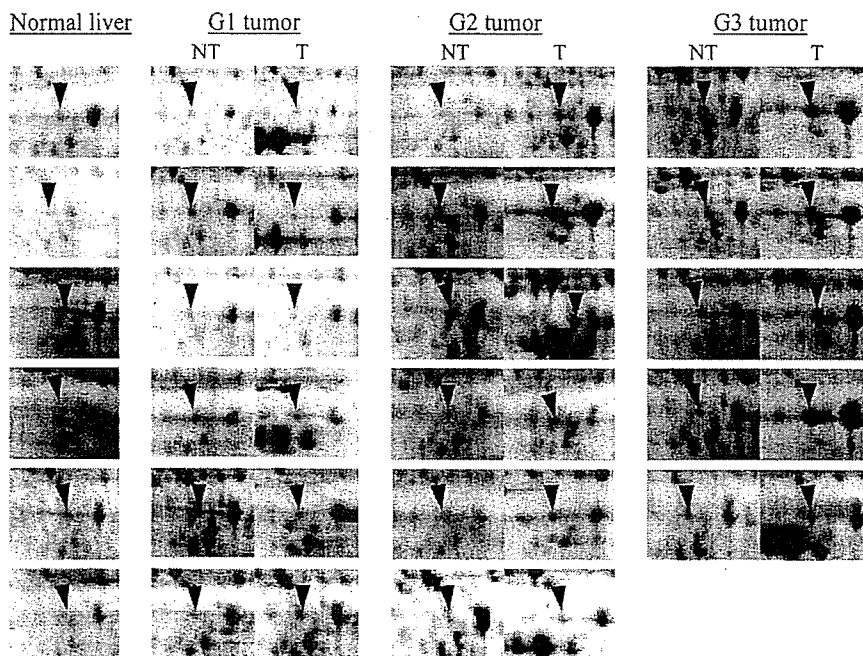

Figure 2. Differences in expression levels of the spot indicated in Fig. 1. Expression levels were greater in tumorous samples of G3 tumors than in other groups. In NL samples, this spot showed low expression, approximately equivalent to that in nontumorous samples. NT, corresponding non-tumorous sample; T, tumorous sample; G1, G2, and G3 tumor indicate well-, moderately-, and poorly differentiated HCC, respectively.

tion when compared with that in corresponding nontumorous samples ($p < 0.05$). In all histologic groups, expression levels of alpha enolase in G3 tumors were significantly upregulated compared to the level in G1 tumors. Alpha enolase in G2 tumors showed a tendency toward upregulation compared to that in G1 tumors ($p = 0.0289$). Alpha enolase in G3 tumors showed a tendency toward upregulation compared to that in G2 tumors ($p = 0.0057$), but the differences were not statistically significant. There was no significant difference in expression of alpha enolase between NL, CH, LC, and G1 tumor samples (Fig. 5).

3.4 Correlation with clinical characteristics

Correlation of alpha enolase expression levels with tumor size, serum AFP levels, serum PIVKA-II levels, and venous invasion was analyzed. Upregulation of alpha enolase was significantly correlated with tumor size ($p < 0.05$, $R^2 = 0.290$) (Fig. 6A). To confirm the significance of the result, two proteins, heat shock 70 kDa protein 1 known to be upregulated in HCC and β-actin known to express constitutively were also assessed. However, none of the two showed significant correlation with tumor size ($R^2 = 0.13$ and 0.002). Patients with venous invasion expressed higher
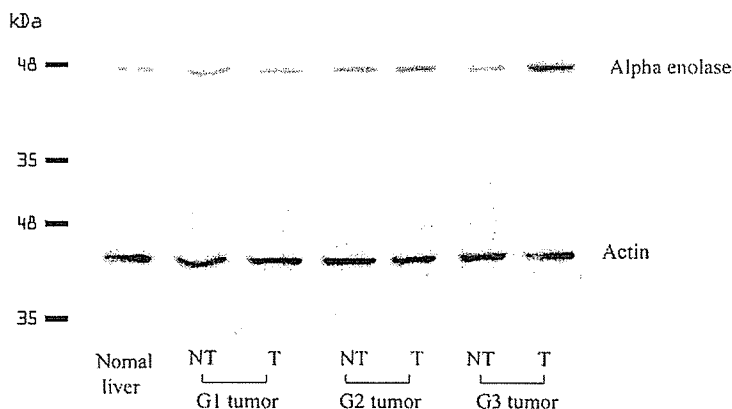
kDa

48 ▬   ～ ～   ～～   ～～～   ～～   ～～   ～～   ▬▬   Alpha enolase

35 ▬

48 ▬

～～   ～～   ～～   ～～   ～～   ～～   ～～   Actin

35 ▬

Normal   NT   T   NT   T   NT   T
liver
         G1 tumor   G2 tumor   G3 tumor

Figure 3. Immunoblot analysis of alpha enolase in NL and in each differentiated HCC. Intensity of the bands was slightly increased in G2 tumor and was further increased in tumorous sample of G3 tumor. The bands of actin are shown as a control. NT, corresponding nontumorous sample; T, tumorous sample; G1, G2, and G3 tumor indicate well-, moderately-, and poorly differentiated HCC, respectively.
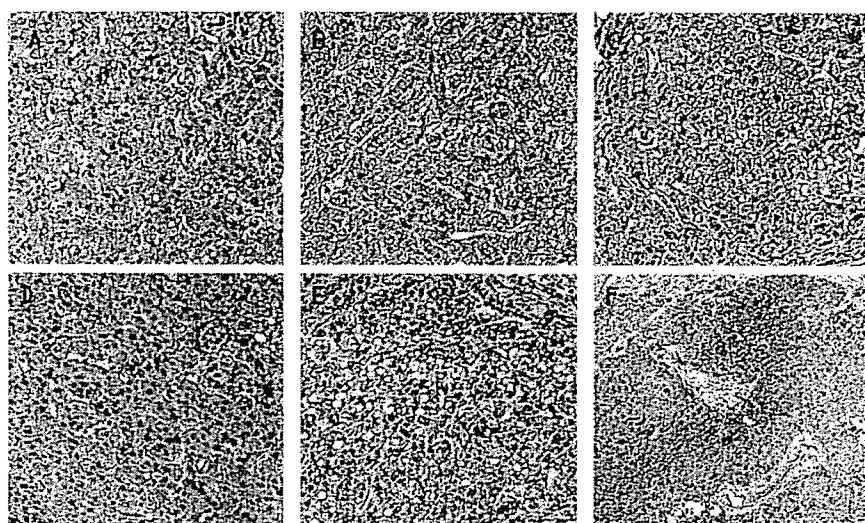

Figure 4. Immunohistochemical evaluation of alpha enolase. NL (A), CH (B), LC (C), and G1 tumor (D) samples showed rare positively stained cells. Moderate, heterogeneous staining was identified in G2 tumor (E) and strong staining was identified in G3 tumor (F). Original magnification, × 40.

levels of alpha enolase than patients without venous invasion (Fig. 6B). No significant correlation was observed between alpha enolase expression and serum AFP or serum PIVKA-II expression.

4 Discussion

The aim of this study was to identify proteins that could be molecular targets for diagnosis and treatment of HCV-related HCC. Our 2-DE and LC-MS/MS proteomic analyses of 26 HCC samples revealed that alpha enolase expression was upregulated in G3 tumors compared with that in G1 and G2 tumors, and expression levels were positively correlated with tumor size and venous invasion.

Alpha enolase is an isoenzyme of enolase, a key protein that catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate in the glycolytic pathway. Three enolase isoenzymes have been identified. The alpha form is present in most tissues and embryonically, the beta form is expressed

in muscle tissues, and the gamma form, also known as neuron-specific enolase (NSE), is found only in neuronal tissues. Alpha enolase has been detected not only in the cytoplasm but also at the membrane surface. Although the mechanism of surface expression and the orientation on the membrane are not well understood, it is known that the C-terminal amino acid, lysine, is exposed at the cell surface and is involved in binding to plasminogen, which is then activated and converted to plasmin [13, 14]. Plasmin, stabilized at the cell surface, induces fibrinolysis [15, 16].

In cancer cells, the rate-determining enzyme for glycolysis is converted to an isoenzyme different from that in normal cells and the capability of glycolysis is increased because of increased cell proliferation. Overexpression of alpha enolase, the embryonal form, could be part of this mechanism. In response to upregulated alpha enolase expression, the fibrinolytic system is inordinately accelerated. Consequently, increased local fibrinolysis may contribute to cancer cell invasion and metastasis. This is consistent with our results showing upregulated alpha enolase expression in

Table 2. The result of LC-MS/MS for the spot

Start-end residues	MH+ calculated (Da)	m/z measured (Da)	z	Sequence
9–27	2154.072	719.09	3	EIFDSRG NPTVEVDL FTSK
64–79	1690.986	846.27	2	AVEHINKT IAPALVSK
64–79	1690.986	564.68	3	AVEHINKT IAPALVSK
120–131	1259.711	630.89	2	AGAVEK GVPLYR
132–161	3011.570	1004.98	3	HIADLA GNSEVILPV PAFNVING GSHAGNK
183–196	1597.907	533.62	3	IGAEVYH NLKNVIK
406–419	1691.897	846.93	2	YNQLLRIE EELGSK
406–419	1691.897	565.02	3	YNQLLRIE EELGSK
412–421	1103.595	552.49	2	IEEELGSKAK
426–433	959.543	480.44	2	NFRNPLAK

The matched peptides cover 26% of the protein.

Table 3. Expression of alpha enolase in each differentiated HCC

	NT		T		p-value	Occurrence of upregulation
G1[a]	0.053	0.014	0.073	0.021	N.S.	1/6 (17%)
G2[b]	0.108	0.020	0.169	0.030	N.S.	4/15 (27%)
G3[c]	0.118	0.028	0.302	0.066	$p < 0.05$	4/5 (80%)

Values are shown as mean ± SD (arbitrary unit).
a), b), c) G1, G2, and G3 indicate well-, moderately-, and poorly-differentiated HCC, respectively.
NT, Nontumorous samples; T, tumorous samples; N.S., Not significant.
Expression levels of proteins on 2-DE gels were quantified by analyzing the intensity of each spot with ImageMaster 2-D Platinum. Spots showing differences in expression levels greater than two-fold between tumorous and nontumorous samples were considered upregulated. Differences in expression levels between tumorous samples and corresponding nontumorous samples were analyzed by Student's *t*-test.

Figure 5. Quantitative analysis performed by ANOVA with Bonferroni-Dunn test. $p < 0.0033$ was considered significant. Comparing all histologic groups, expression of alpha enolase in G3 tumors was significantly upregulated compared to that in G1 tumors. Alpha enolase in G2 tumors showed a tendency towards upregulation, compared to that in G1 tumors ($p = 0.0289$), and alpha enolase in G3 tumors showed a tendency towards upregulation compared to that in G2 tumors ($p = 0.0057$). However, these differences were not statistically significant. There was no significant difference among NL, CH, LC, and G1 tumor samples. NL, normal liver; CH, chronic hepatitis; LC, liver cirrhosis; G1, G2, and G3 indicate well-, moderately-, and poorly-differentiated HCC, respectively.

Overexpression of alpha enolase has been reported in a cervical cancer cell line [18, 19] and a colon cancer cell line [20], in endometrial hyperplasia and adenocarcinoma [21], in breast cancer [22, 23], and in Alzheimer's disease [24]. It has also been reported in an HCC cell line [25]. However, there have been reports of downregulation of alpha enolase expression in non-small cell lung cancer [26] and of downregulation of alpha enolase expression in HBV-HCC and non-B, non-C HCC, albeit at a low frequency [10]. The investigators used samples of differentiation grades I and II of the Edmondson-Steiner grading system, which are comparable to G1 and G2 tumors; this might explain these differences. Additional studies are necessary to confirm expression differences in HBV-HCC.

Transcriptome analysis of HCV-related HCC samples showed significant upregulation of alpha enolase in G3 tumors ($p < 0.05$) (unpublished data). The expression level of the mRNA and that of the encoded protein do not necessarily correlate because of the effects of mRNA lability, post-translational modification, and degradation of proteins. In the case of alpha enolase, however, mRNA levels do correlate with protein levels in HCV-related HCC.

Molecular biological techniques have developed rapidly in recent years and will play essential roles in clarifying the pathogenesis of HCV-related HCC. The overexpression of alpha enolase in G3 tumors and the positive correlation with tumor size and venous invasion suggest that alpha enolase is

patients with large tumors. Alpha enolase has also been described as a stress protein induced by hypoxia [17]. In large tumors, oxygen is relatively decreased in the central region. Thus, it is reasonable that alpha enolase is upregulated in large tumors.
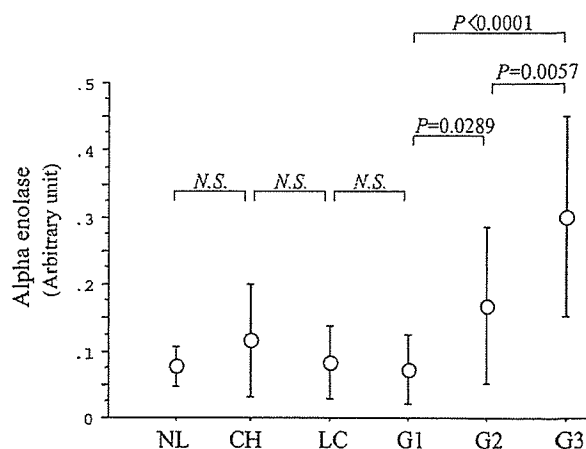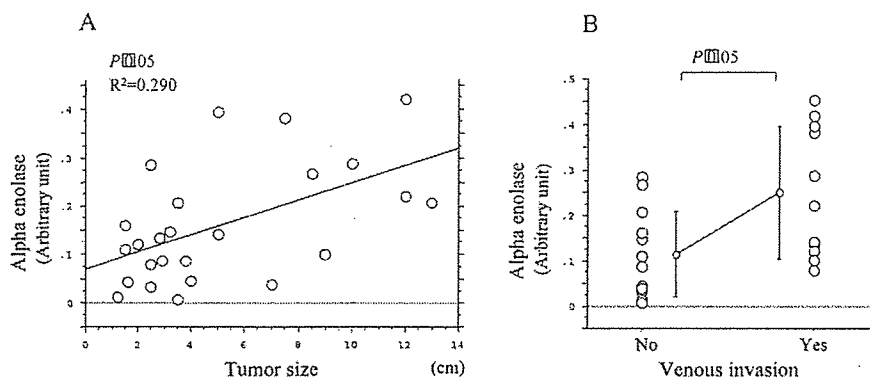
A

B

Figure 6. Relation between alpha enolase expression, tumor size, and venous invasion. (A) Upregulation of alpha enolase was significantly correlated with tumor size ($P < 0.05$, $R^2 = 0.290$). (B) Patients with venous invasion expressed high levels of alpha enolase compared to patients without venous invasion.

one of candidates for biomarkers for tumor progression that deserve further investigation and may constitute a target for gene and protein therapy in HCV-related HCC.

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Tumor secreting high levels of IL-15 induces specific immunity to low immunogenic colon adenocarcinoma via CD8+ T cells

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Abstract. Although interleukin (IL)-15 augments innate and acquired immunities, IL-15 expression is controlled at the levels of transcription, translation and intracellular trafficking. We constructed plasmid vectors encoding the murine mature-IL-15 cDNA linked to an Igκ leader sequence and full-length murine IL-15 cDNA to evaluate the efficacy of the mature-IL-15 vector. Weakly immunogenic colon 26 cells were transfected with the above-mentioned vectors or with empty vector (mock). Transfectants with mature-IL-15 produced significantly higher levels of IL-15 than did transfectants with full-length IL-15. When injected into syngeneic BALB/c mice, transfectants secreting high levels of IL-15 were rejected completely. Depletion of natural killer cells or CD4+ T cells did not affect the growth of transfectants. In contrast, transfectants treated with anti-CD8 antibody re-grew 1 month later after implantation. These findings indicate that CD8+ T cells are required for complete rejection of the tumor. Gene therapy with transfectants expressing mature-IL-15 containing the Igκ leader sequence may be useful as a tumor vaccine.

Introduction

Interleukin (IL)-15 is a 15-kDa cytokine (1,2) that uses the ß and γ chains of the IL-2 receptor (R) for signal transduction and shares biologic activities with IL-2. In particular, IL-15 promotes proliferation and activities of T, B and natural killer (NK) cells (3,4) and is a potent inducer of lymphokine-activated cytotoxic activity against tumor cells (5-7).

However, IL-15R contains a unique α chain that has a higher affinity and broader tissue distribution than that of the IL-2Rα chain (8,9). Indeed, there are several significant differences in the molecular and cellular features of IL-2 and IL-15 (10). IL-15 shows stronger mediation of NK and

NKT cell differentiation and survival *in vivo* in comparison with IL-2 (11,12). Moreover, IL-15 can selectively promote proliferation and long-lasting survival of memory CD8+ T cells, in contrast to IL-2, which inhibits proliferation of CD8+ memory T cells (13-15). In addition, doses of IL-15 required to induced severe hypotension and pulmonary vascular leak syndrome were six times higher that those of IL-2, resulting in a higher therapeutic index for IL-15 (16). Such findings have led to a renewed clinical interest in IL-15.

Though IL-15 mRNA is expressed in a broad normal organization and tumor cells (1), it has been difficult to detect IL-15 protein in supernatants of many cells that express IL-15 mRNA (17). IL-15 expression is controlled at the levels of transcription, translation, and intracellular trafficking (18-20). Although there was a 4- to 5-fold increase in translation of IL-15 mRNA with the alternative short signal peptide in comparison with that of the wild-type 48-aa signal peptide (17,21,22), only IL-15 containing the 48-aa signal peptide is secreted (21). We previously reported that highly immunogenic tumor (Meth-A) cells transfected with IL-15 containing the 48-aa signal peptide can elicit an anti-tumor immune response (23). However, in a preliminary study we found that these anti-tumor effects were not present in the weakly immunogenic colon 26 cells.

In the present study, we constructed plasmid vectors encoding the murine mature-IL-15 cDNA linked to a high efficiency Igκ leader sequence and evaluated the efficacy of high IL-15-producing tumor. We report that high IL-15 production caused complete rejection of weakly immunogenic colon 26 cells and induced long-lasting CD8+ T cell-mediated specific anti-tumor immunity.

Materials and methods

Reverse transcription-PCR and primers. RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR) were carried out as described previously with some modifications (24). Briefly, cells (5×10^6) were lysed in 1 ml of TRIzol reagent (Life Technologies, Grand Island, NY), and total cellular RNA was isolated according to the manufacturer's instructions. One microliter of total RNA (1 μg) was added to 19 μl of RT-mixture (Takara, Ohtsu, Japan). After mixing, the samples were incubated at 30°C for 10 min, 55°C for 30 min, 95°C

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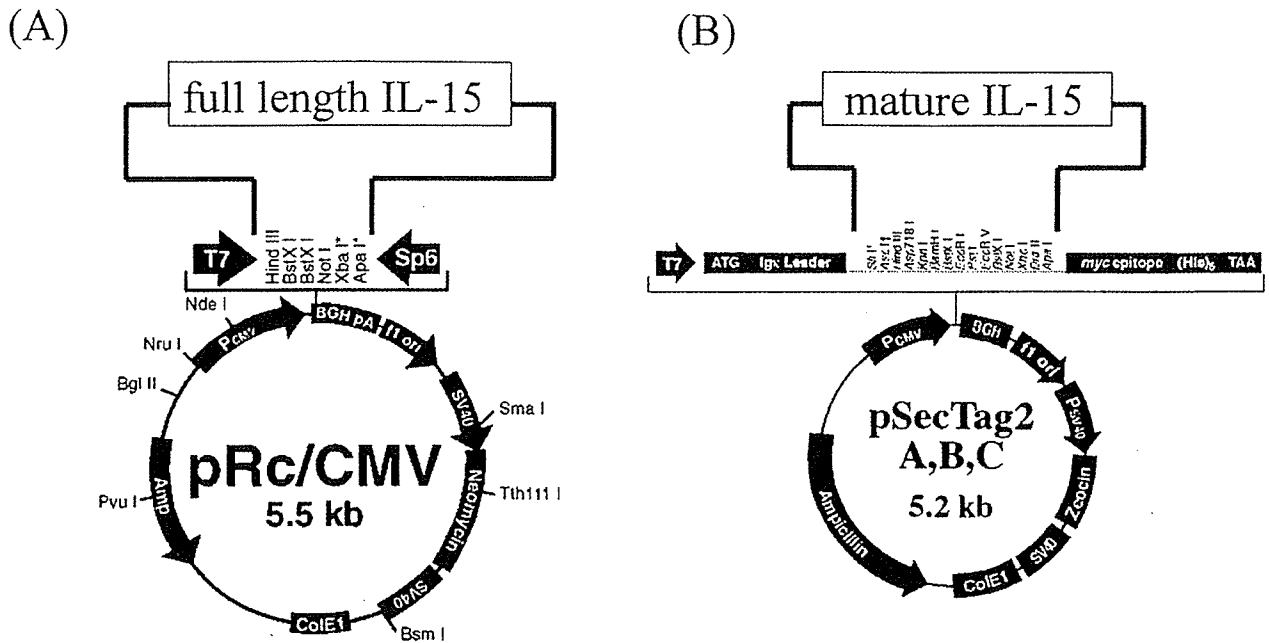E-mail: hazama@yamaguchi-u.ac.jp

(A)

(B)

Figure 1. (A), Illustration of full-length IL-15 expression vector. (B), Illustration of mature-IL-15 expression vector.

for 5 min, and 4°C for at least 5 min. PCR-mixture (80 μl) (Takara) containing 100 nM primers was added to each RT reaction. PCR amplification consisted of 34 cycles of denaturation at 94°C for 1 min, annealing at 58 or 68°C for 1 min, and extension at 72°C for 2 min. The annealing temparature was 68°C for vector construction and 58°C for mRNA detection.

The primer sequences for murine full-length IL-15 vector construction were sense, 5'-AGCTGCGGCCGCATGAAAA TTTTGAAACCATAT-3' and antisense, 3' primer 5'-AGCT AGTCTAGATCAGGACGTGTTGATGAACAT-3'. Those for murine mature-IL-15 vector construction were sense, 5'-CCTCGTGAATTCGCCAACTGGATAGATGTAAGA-3' and antisense, 5'-TACACACTCGAGTCAGGACGTGTTGA TGAACAT-3'. Primers for detection of murine IL-15 mRNA were sense, 5'-TAGATATAAGATATGACCTGGA-3' and antisense, 5'-TGTTGAACATTTGGACAAT-3', and those for detection of murine ß-actin mRNA were sense, 5'-TCGA CAACGGCTCCGGCATGT-3', and antisense, 5'-GCTGAT CCACATCTGCTGGAA-3'. The expected sizes of the PCR products were 513, 372, 330, and 1046 bp, respectively. The PCR products were separated by electrophoresis on 1% agarose gels and visualized by staining with ethidium bromide and UV transillumination.

Murine full-length-IL-15 expression vector (Fig. 1A). A murine IL-15 cDNA was isolated by RT-PCR from total RNA of lipopolysaccharide-stimulated murine spleen cells. The PCR fragment was cloned into the *NotI/XbaI* site of the pRC/CMV eukaryotic expression vector (Invitrogen, San Diego, CA). The nucleotide sequence was confirmed by sequence analysis with the T7 promoter.

Murine mature-IL-15 expression vector (Fig. 1B). A mature-IL-15 cDNA was isolated by PCR with the full-length IL-15

expression vector as a template. The PCR fragment was cloned into the *EcoRI/XhoI* site of the pSecTag2B eukaryotic expression vector (Invitrogen), which contains the Igκ leader sequence. The nucleotide sequence was confirmed by sequence analysis from the T7 promoter.

Tumor cells and transfection. Colon 26 cells (25), a murine colon adenocarcinoma cell line derived from BALB/c mice, were maintained in RPMI 1640 (Life Technologies) medium supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin G, and 100 μg/ml streptomycin. Sub-confluent cultures in 100-mm petri dishes were transfected with 5 μg of murine full-length or mature-IL-15 expression plasmid or vector alone with Lipofectamine reagent (Life Technologies) according to the manufacturer's instructions. G418 (100 μg/ml) (Life Technologies), which selects for the pRC/CMV vector, and Zeocin (100 μg/ml) (Invitrogen), which selects for the pSecTag2B vector, were added to the cells 48 h later. G418- or Zeocin-resistant clones were isolated and expanded in culture medium containing 100 μg/ml of G418 or Zeocin as appropriate.

Bioassay for IL-15 production by transfectants. Because an ELISA kit for murine IL-15 is not available, the activity of IL-15 in culture supernatants was quantified with the CTLL-2 bioassay as descrived previously (23). Briefly, each transfectant line (1x10⁶ cells/ml) was cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum without G418 or Zeosin for 48 h. Culture supernatants were collected by centrifugation for 5 min at 400 g and filtered (0.22 μm) prior to bioassay. IL-15 and IL-2 dependent CTLL-2 cells (4000 cells/well) in 96-well flat-bottom microtiter plates were incubated with culture medium supplemented with 5x10⁻⁵ M 2-mercaptoethanol in the presence of culture super-natant with or without anti-murine IL-15 monoclonal anti-

body (G277-3588, PharMingen, San Diego, CA). After 20-h incubation, 50 μg of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) (Chemicon International, Temecula, CA) was added to each well, and the reaction was allowed to incubate for an additional 4 h at 37°C. Isopropanol with 0.04 N HCl (100 μl) was then added to each well. Color development at a wavelength of 540 nm was monitored with an ELISA reader (SLT Labinstruments, Austria). Serial dilutions of murine recombinant IL-15 (BioSource, Camarillo, CA) were used as a standard.

Animal studies. Seven-week-old female BALB/c mice were purchased from Japan SCL (Hamamatsu, Japan). Mice were inoculated with freshly prepared suspensions of tumor cells at a concentration of 2×10^6 cells/ml. Animals were inoculated with a total of 5×10^5 cells; all were inoculated subcutaneously in the right lower abdominal quadrant with a 27-gauge needle. Tumor volumes were measured in mm^3 with a venire caliper and calculated according to the following formula: a x b^2/2, where a is the larger and b is the smaller of the two dimensions. All animal experiments were conducted in accordance with the guidelines of the Animal Care and Use Committee of Yamaguchi University School of Medicine.

Re-challenge with parental cells (colon 26) and Meth-A. Sixty days after disappearance of the initial implant of mature-IL-15/colon 26 cells, 10 mice were injected with 5×10^5 parental colon 26 cells in the previously uninjected side, left lower abdominal quadrant. Meth-A cells, which were derived from a methyl-cholanthrene-induced fibrosarcoma in BALB/c mice (26), were also injected into both nonimmunized (n=10) and immunized (n=10) mice.

In vivo depletion of NK, CD4$^+$ and CD8$^+$ T cells. Depletion of NK, CD4$^+$ and CD8$^+$ T cells was carried out as described previously (27). Briefly, to deplete NK cells, 200 μl of a 1:15 dilution of anti-asialo GM1 antibody (Wako Fine Chemicals, Osaka, Japan) in phosphate-buffered saline (PBS) or control rabbit serum (diluted 1:15 in PBS) was injected intra-peritoneally into mice 2 days prior to tumor challenge and 5, 7 and 11 days after tumor challenge. Monoclonal anti-bodies against CD4$^+$ cells (GK1.5) and CD8$^+$ cells (2.43) (both purchased from American Type Culture Collection, Rockville, MD) or HBSS (Gibco-BRL) (control) were injected intraperitoneally (1.0 mg) into mice (n=6) to deplete subsets of immune cells 3 days before and once each week after the inoculation of tumor cells. Flow cytometric analysis was per-formed with an EPICS XL (Beckman Coulter, Fullerton, CA) to verify 95% depletion of specific cell subsets in the spleen after the administration of depleting antibodies. Tumor volume was recorded twice a week.

Histologic evaluation for immune cells infiltrating into tumor tissues. On days 7 and 14 after inoculation, tumors were dissected, fixed in 10% neutral buffered-formalin, and embedded in paraffin. Sections (4 μm) were stained with hematoxylin and eosin. For immunohistochemical staining, tissues were embedded in OCT compound (Ames Division, Miles Laboratories, Elkhart, IN), snap-frozen in liquid nitrogen, and stored at -80°C. Acetone-fixed 6-μm cryostat
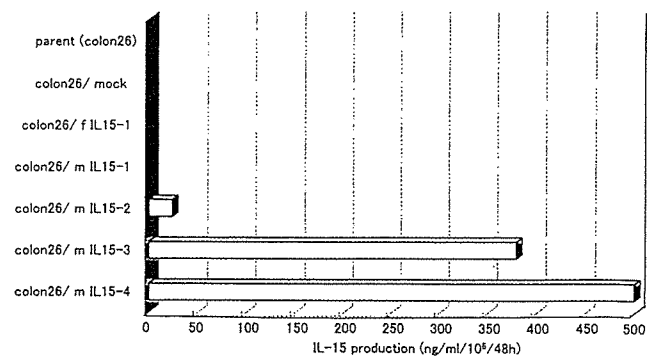

Figure 2. Production of IL-15 by transfectants was confirmed by CTLL-2 proliferation assay. Levels of IL-15 produced by mature-IL-15 transfectants (clones 1, 2, 3 and 4) were between 0 and 500 ng/10^6 cells/48 h, whereas full-length IL-15 transfectants and mock transfectants did not produce IL-15.

sections were blocked with goat serum and then immuno-stained with optimal dilutions of the following rat mAbs: L3/T4 (CD4, Becton Dickinson, Franklin Lakes, NJ) and KT15 (CD8, Serotec, Sapporo, Japan). Slides were then sequentially incubated with biotinylated goat anti-mouse IgG (Zymed laboratories, South San Francisco, CA) and ABComplex (Dako, Tokyo, Japan). Each incubation step lasted at least 30 min and was followed by a 10-min wash with PBS. Sections were then incubated with 0.03% H$_2$O$_2$ and 0.06% 3,3-diaminobenzidine for 2-5 min, rinsed with tap water, and counterstained with hematoxylin.

Statistical analysis. Statistically significant differences were evaluated with Student's t-test. A value of P<0.05 was considered statistically significant. Results are presented as mean ± SE.

Results

Expression of IL-15 mRNA by transfectants. Four independent G418-resistant full-length IL-15 clones (named colon 26/fIL-15-1, 2, 3 and 4) and four independent Zeocin-resistant mature-IL-15 clones (named colon 26/mIL-15-1, 2, 3 and 4) were isolated and expanded. RNA was isolated, and RT-PCR was performed. Three of 4 (75%) clones (clones 1, 2 and 4) expressed full-length IL-15 mRNA, and 4 of 4 (100%) clones expressed mature-IL-15 mRNA. IL-15 mRNA was not detected in colon 26 cells transfected with empty vector or parental colon 26 cells (data not shown).

Bioassay for IL-15 produced by transfectants (Fig. 2). Production of IL-15 protein was confirmed by CTLL-2 pro-liferation assay. IL-15 levels in the supernatants of mature-IL-15 transfectants (10^6 cells/48 h) were 0 ng, 24 ng, 380 ng and 500 ng for clones 1, 2, 3 and 4, respectively. The bio-activity of each culture supernatant was neutralized completely by 10 μg/ml anti-IL-15 antibody. Colon 26 cells transfected with vector expressing full-length IL-15, mock transfected cells and parental colon 26 cells did not produce bioactive IL-15.
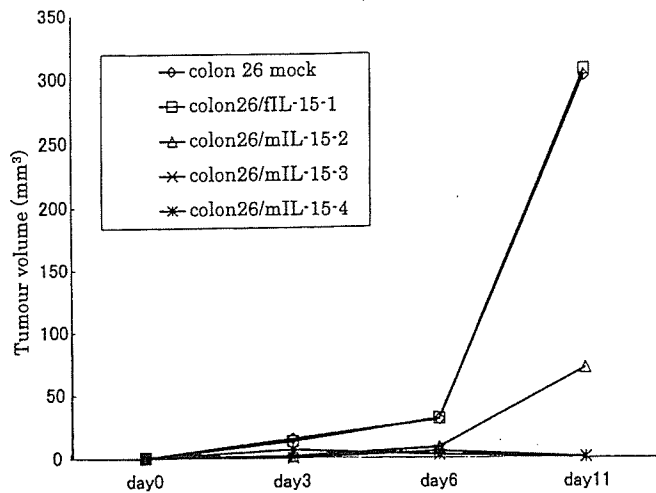
Figure 3. Tumorigenicity of full-length IL-15, mature-IL-15 (clones 1, 2, 3 and 4) and mock transfectants were examined by subcutaneous injection into BALB/c mice. Mean tumor volumes of clones 3 and 4 were significantly smaller on day 11 (P<0.01), whereas mock transfectants and clone 1 grew progressively. Each SEM was <10%.
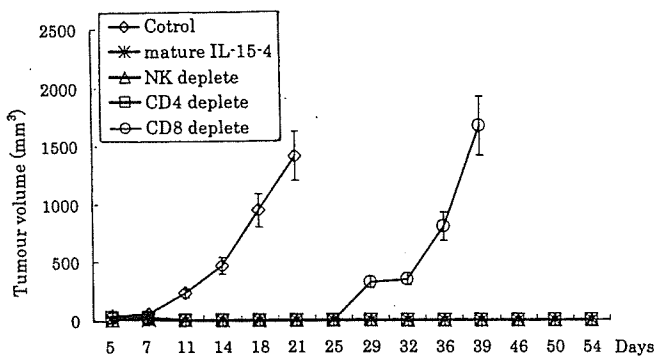

Figure 4. Depleted mice and control mice (HBSS only) were implanted with colon 26/mIL-15-4 cells. There was no difference in tumor growth between the NK-depleted mice, CD4+-depleted mice, and control mice. Tumors in mice treated with anti-CD8+ antibodies was initially rejected but grew rapidly approximately 1 month after transplantation.

Inhibition of tumor growth in vivo (Fig. 3). Transfection of colon 26 cells with full-length or mature-IL-15 expression vector did not alter the growth properties of the cells *in vitro* as assessed by doubling time or morphology (data not shown) in comparison with parental or mock transfected cells. The tumorigenicity of full-length IL-15, mature-IL-15, and mock transfected cultures were examined by subcutaneous injection into BALB/c mice. The mean tumor volumes of the mature-IL-15, -3, -4 were significantly reduced (n=7), whereas mock transfectants grew progressively (n=7). Full-length IL-15 transfectant showed growth similar to that of mock transfected cells.

Re-challenge with parental colon 26 cells and Meth-A. We next examined whether primary rejection of IL-15 transfectants led to protective immunity. Sixty days after the disappearance of the initial mature-IL-15/colon 26 implants, immunized

mice (n=7) were injected with 5x10^{5} parental colon 26 cells in the lower left abdomen. Seven non-immunized mice were injected in the same manner as controls. Tumor rejection was observed in all immunized mice, whereas colon 26 cells grew progressively in nonimmunized mice (data not shown). To confirm the specificity of the protective immunity, 5x10^{5} Meth-A cells were injected into both nonimmunized and immunized mice. There was no significant difference in growth of implanted Meth-A cells between nonimmunized and immunized mice (data not shown).

Effects of anti-asialo GM1, anti-CD4 and anti-CD8 antibodies on growth of mature-IL-15 clones (Fig. 4). Depleted mice and control mice were inoculated with mature-IL-15-expressing cells. There was no difference in tumor growth among the NK-depleted group (n=6), CD4+-depleted group (n=6) and the control group (n=7). Tumors in mice treated with anti-CD8 antibody were initially rejected completely but grew rapidly approximately 1 month after transplantation in comparison with those in mice treated with HBSS (control).

Histology at the site of tumor cell injection (Fig. 5). To characterize the host cellular responses augmented by IL-15 production, histological analysis of the injection site was performed 14 days following the injection of tumor cells. Immunohistochemical analysis of the site of injection of mature-IL-15 transfectants (clone 4) revealed infiltration of CD4+ and CD8+ lymphocytes and Mac-1-positive monocytes. This was not observed with mock transfectants.

Discussion

The anti-tumor effects of IL-15-transfected tumor cells have been described. Two areas have been the focus of recent studies, the low efficiency signal peptide of IL-15 and the induction of anti-tumor effector cells by IL-15.

Both murine and human IL-15 contain an unusually long 48-aa signal peptide, and an alternative short 21-aa signal peptide is also found in human (17,21) and an alternative 26-aa form is present in mice (22). We previously reported that highly immunogenic tumor (Meth-A) cells transfected with the human IL-15 containing the 48-aa signal peptide can elicit local and systemic T cell-dependent immunity (23). However, secretion of bioactive IL-15 was low, and these anti-tumor effects were not evident with weakly immunogenic colon 26 cells (Figs. 2 and 3). Although Kimura *et al* (28) reported the efficacy of Meth-A cells transfected with the alternative form of murine IL-15, which produces relatively large amounts of intracellular IL-15, they did not examine the efficacy with weakly immunogenic tumor cells.

We previously reported that replacement of the endogenous IL-18 leader sequence with the Igκ signal peptide caused efficient secretion of bioactive IL-18 protein (27). We constructed plasmid vectors containing the murine mature-IL-15 linked to the Igκ leader sequence and evaluated the efficacy of high IL-15-producing tumor. Tumorigenicity of mature-IL-15 transfectants of colon 26 cells was decreased in proportion to the level of IL-15 secretion. The clone with the highest production of IL-15 was rejected completely when injected subcutaneously (Figs. 2 and 3).
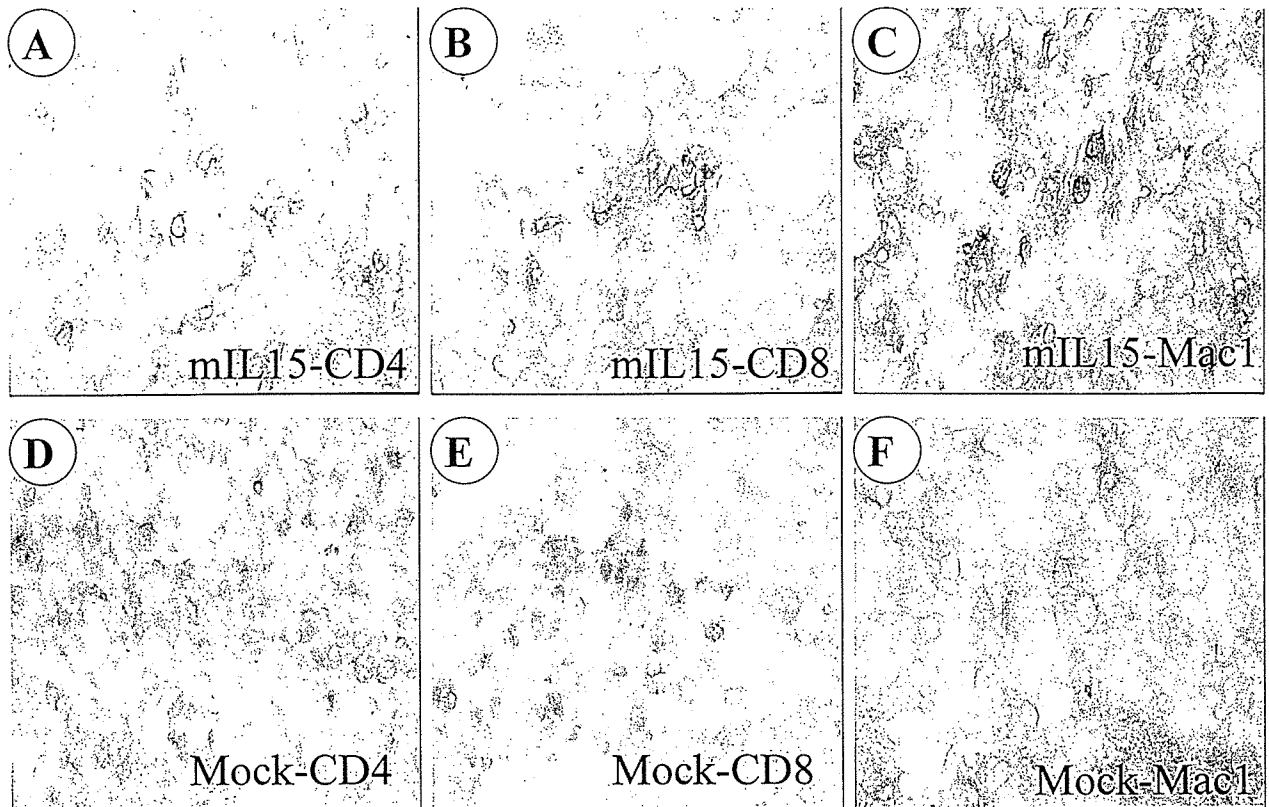
Figure 5. Immunohistochemical analysis of tumor implantation sites in BALB/c mice 14 days after subcutaneous injection of colon 26/mIL-15-4 cells (A-C) and mock transfectants (D-F). Staining with L3/T4 (CD4) (A), KT15 (CD8) (B) and Mac-1 (CD11b) (C) revealed infiltration of CD4+ and CD8+ lymphocytes and Mac-1-positive monocytes, respectively. Such infiltration was not observed in mice treated with mock transfectants (D-F).

Fourteen days after subcutaneous injection of colon 26/mIL-15-4 cells infiltration of CD4+ and CD8+ lymphocytes and Mac-1-positive monocytes was observed at the site of injection (Fig. 5). These findings were supported by previous reports that described the function of IL-15 in proliferation and functional activation of T, B and NK cells (3,4) and monocytes (29). To further clarity of anti-tumor mechanisms, we performed *in vivo* depletion of NK cells, CD4+ and CD8+ T cells. The anti-tumor effects of colon 26/mIL-15-4 cells were partially abrogated by treatment with anti-CD8+ antibodies but not by depletion of NK cells or CD4+ T cells (Fig. 4). In CD8+ T cell-depleted mice, colon 26/mIL-15-4 cells disappeared temporarily, but re-grew 1 month after implantation. These results indicate that inoculation of IL-15-secreting tumor cells may mediate initial anti-tumor effects through CD8+ T cells, CD4+ T cells, NK cells and a variety of immunocompetent cells and that long-lasting specific immunity is mediated only through CD8+ T cells. However, Meazza *et al* (30) reported that TS/A tumor cells, which secrete high levels of IL-15, reduced tumorigenicity, and that depletion of CD8+ T cells or NK cells abrogated the efficacy of IL-15. This inconsistency may be due to differences in IL-15 between murine and human. Although murine and human IL-15 cross-react, there is only 73% amino acid identity between murine and human IL-15 (1). Therefore, it may be more appropriate to use murine IL-15 in murine studies. Indeed, Yajima *et al* (31) reported that murine IL-15 transgenic mice have anti-

tumor activity against MHC class I-negative and -positive malignant melanoma through augmented NK activity and cytotoxic T-cell response, respectively. The present study is the first to show that murine tumor cells secreting high levels of murine IL-15 can mediate complete rejection of weakly immunogenic tumor cells and induce long-lasting specific anti-tumor immunity.

These results suggest that IL-15 is important in tumor immunity and that IL-15 may be an excellent candidate for a tumor-vaccine adjuvant for boosting CD8+ memory T cells as therapy for weakly immunogenic human cancers.

Acknowledgements

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MUC1 Peptide Vaccination in Patients with Advanced Pancreas or Biliary Tract Cancer

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Abstract. *Background: To evaluate the immunogenicity of MUC1 peptide vaccine in advanced pancreatic and bile duct cancers, a phase I clinical trial was conducted. Materials and Methods: A 100-mer MUC1 peptide consisting of the extracellular tandem repeat domain and incomplete Freund's adjuvant were subcutaneously administered to 6 pancreatic and 3 bile duct cancer patients at weeks 1, 3 and 5 and doses ranging from 300 to 3000 µg. Circulating intracytoplasmic cytokine-positive CD4$^+$ T cells and anti-MUC1 IgG antibodies were measured before and after vaccination. Results: There were no adverse events, except for mild reddening and swelling at the vaccination site. In 8 patients eligible for clinical evaluation, 7 had progressive disease and 1 stable disease with a tendency for increased circulating anti-MUC1 IgG antibody after vaccination. Conclusion: This phase I clinical trial revealed the safety of a vaccine containing 100-mer MUC1 peptides and incomplete Freund's adjuvant.*

MUC1 is a type I transmembrane glycoprotein with an extracellular domain composed of a polypeptide core containing multiple tandem repeats of a 20 amino acid sequence with numerous carbohydrate chains (1). The autoimmunogenicity of MUC1 was first shown by inducing HLA-unrestricted cytotoxic T lymphocytes (CTLs) against the tandem repeat region (2), which was confirmed by subsequent investigations (3-5). Thereafter, Domenech *et al.* demonstrated the presence of HLA-restricted CTLs against

the tandem repeat sequence (6). The nanomer peptide STAPPAHGV, which corresponds to residues 9-17 of the 20 amino acid repeat sequence, was found to have significant binding affinity to several class I alleles, including HLA-A1, A2, A3 and A11, and to be able to elicit a MUC1-specific CTL response in an A11$^+$ cancer patient. On the other hand, a humoral immune response to MUC1 was also revealed (7, 8) and circulating antibodies against the tandem repeat peptides were detected in various cancers (9, 10). These findings suggested the potential application of MUC1 in cancer immunotherapy and led to clinical trials of a MUC1 peptide vaccination (11-14).

The first study, by Goydos *et al.*, demonstrated the safety of a vaccine composed of a synthetic MUC1 peptide with 5 repeats of the 20 amino acid sequence and BCG (11). Karanikas *et al.* then reported the results of a clinical trial with the MUC1 peptide of 5 repeats fused with mannan in 25 patients with advanced breast, gastric or colorectal cancer (12). They detected large amounts of IgG$_1$ anti-MUC1 antibodies in 13 of the 25 patients, and could induce HLA-A2-restricted CTLs, but a significant CTL response was only seen in 2 out of 10 patients tested. Gilewski *et al.* reported the results of a vaccination with the MUC1 peptide consisting of 1.5 repeats conjugated with keyhole limpet hemocyanin (KLH) together with the immune adjuvant QS-21 in 9 breast cancer patients (13). High IgM and IgG antibody titers against the MUC1 peptide were detected; however, there was no evidence of T cell activation. Another of their studies, using a 106-amino-acid-long MUC1 peptide conjugated with KLH plus QS-21 in 6 breast cancer patients, again showed that the T cell response against the MUC1 peptide was minimal and inconsistent (14). These clinical data suggested that the tandem repeat peptide of MUC1 could be useful for inducing anti-MUC1 antibodies rather than CTLs.

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Key Words: MUC1, peptide, vaccine, pancreas cancer.

Recently, von Mensdorff-Pouilly *et al.* have reported that a positive test result for both IgG and IgM antibodies in pretreatment serum was associated with significant disease-specific survival in stage I and II breast cancer patients (15). We also revealed that circulating anti-MUC1 IgG antibody was a favorable prognostic factor for cancer of the pancreas (16). These results suggest that the antibodies might protect the host against cancer progression. In this study, we attempted a phase I clinical trial of a 100-mer MUC1 tandem repeat peptide with incomplete Freund's adjuvant in patients with advanced pancreatic or bile duct cancer.

Materials and Methods

Trial eligibility. Five patients with inoperable pancreatic cancer, 2 with recurrent disease of bile duct cancer, 1 with recurrent disease of pancreatic cancer and 1 with inoperable bile duct cancer were enrolled in this study. They were required to have computed tomography (CT) or magnetic resonance imaging (MRI) for evaluating clinical stage or recurrent disease. The eligibility criteria were as follows: age of 85 years or less, serum creatinine of less than 1.4 mg/dl, bilirubin of less than 1.5 mg/dl, platelet count of 100,000/μl or more, hemoglobin of 8.0 g/dl or more, and total WBC of 3000/μl or more. Hepatitis B surface antigen and Hepatitis C antibody were negative in all patients. The patients were untreated for at least 4 weeks before entry into the study, and had to have an Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0 to 2 at the time of entry. Patients with evidence of other serious illness, immunosuppression, or autoimmune disease were excluded. Treatment of the enrolled patients was carried out at Yamaguchi University, Japan, from June 2000 through March 2004.

All patients were required to comprehend and sign an informed consent form approved by the Institutional Review Board of Yamaguchi University School of Medicine.

Vaccine preparation and administration. The MUC1 peptide, consisting of 100 amino acids (5 repeats) of the extracellular tandem repeat domain, was synthesized at the Peptide Synthesis Facility, University of Pittsburgh (Dr. O. J. Finn, Pittsburg, PA, USA), in accordance with the U.S. FDA Good Laboratory Practice Regulations and the Japanese GLP Standard. Montanide ISA-51 (incomplete Freund's adjuvant) was manufactured by Seppic, Inc. (Paris, France) and supplied in glass ampoules containing 3 ml of sterile adjuvant solution.

An appropriate amount of MUC1 peptide was diluted with sterile 0.9% NaCl solution and added in a 1:1 volume to Montanide ISA-51 and then mixed using a stopcock and two glass syringes for 5 min. The resulting emulsion was injected, using a glass syringe, subcutaneouly into the frontal thigh in a volume of 1 ml. Alternative thighs were used for a total of 3 injections, which were done 2 weeks apart. Skin tests were performed using 50 μg of the peptide in 0.9% NaCl solution injected intradermally in a volume of 100 μl using a 1-ml disposable syringe. The injection site was observed at 15 min and 48 h. For patients who requested the additional administration of MUC1 peptides, vaccination was repeated with monitoring for adverse events.

Evaluation of adverse events and clinical response. All adverse events were evaluated by the National Cancer Institute-Common

Toxicity Criteria (NCI-CTC) version 2.0 (17) at every vaccination. All known sites of disease were evaluated by CT scan before and after 3 vaccinations. Patients were assigned to a response category according to the response evaluation criteria for solid tumors, given in a revised version of the WHO criteria published in June 1999 in the WHO Handbook for reporting results of cancer treatment.

Intracellular cytokine assays. Peripheral blood samples were collected and the proportions of $CD4^+$ T cells producing intracellular cytokines were determined using flow cytometry, as reported previously (18). In brief, peripheral blood samples were collected by venapuncture into syringes containing sodium heparin anticoagulant. Phycoerythin-cyanine 5 (PC5)-conjugated anti-CD3 monoclonal antibody (mAb) and energy-coupled dye (ECD)-conjugated anti-CD4 mAb were purchased from Coulter Immunology (Hialeath, FL, USA). Fluorescein isothiocyanate (FITC)-conjugated anti-IFN-γ mAb, phycoerythrin (PE)-conjugated anti-IL4 mAb and FITC/PE-conjugated control mAbs were purchased from Becton Dickinson (San Jose, CA, USA). PE-conjugated anti-interleukin (IL)-6 mAb and anti-IL-10 were purchased from R & D (Minneapolis, MN, USA) and PharMingen (San Diego, CA, USA), respectively. The proportions of CD3/CD4-positive lymphocytes producing IFN-γ, IL-4, IL-6 or IL-10 were measured using flow cytometry according to the instructions of the reagent manufacturer (Becton Dickinson). Briefly, 1 ml blood samples were treated immediately with 10 μg/ml of Brefeldin A (BFA) (Sigma Chemical, St. Louis, MO, USA) to block cytokine secretion, keeping the products within cells, and were kept at ambient temperature. Cell surfaces were stained with anti-CD3 and anti-CD4 mAbs. The red cells were lysed with 1 x FACS Lysing Solution (Becton Dickinson) for 10 min at room temperature. After washing with phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and NaN_3, the cells were permeabilized with 0.5 ml of 1 x FACS Permeabilizing Solution (Becton Dickinson) for 10 min at room temperature. After two washes, the cells were incubated with optimal concentrations of anti-IFN-γ, anti-IL-4, anti-IL-6 or anti-IL-10 mAb. Samples were analyzed on an EPICS/XL flow cytometer (Coulter Electronics, Inc., Hialeath, FL, USA), and the data were analyzed using a System II software program (Coulter Electronics). The percentages of cytokine-producing $CD4^+$ T cells were calculated. Negative control reagents were used to verify the staining specificity of the experimental antibodies and to serve as a guide for setting markers to delineate positive and negative populations.

ELISA assays. An enzyme immunoassay for detecting antibodies was performed, as described previously (16). Briefly, the MUC1 peptide was coated onto 96-well microtiter plates (ASAHI TECHNO GLASS Corporation, Japan) at 100 μg/ml in PBS (pH 7.4) at 4°C for 12 h. The plates were washed with PBS, and non-specific binding sites were blocked with 3% HAS/PBS at 37°C for 1 h. The plates were then incubated with patient sera diluted 1:40 in 1% HSA/PBS at 37°C for 1 h. After washing with 0.05% Tween-20/PBS, they were incubated with the second antibody, a horseradish peroxidase-conjugated mouse anti-human IgG (DAKO Corporation, Carpinteria, CA, USA) diluted 1:5000 in 1% HSA/PBS, and washed with PBS. Substrate reaction using *O*-phenylenediamine dihydrochloride (DAKO) was determined at 492 nm in an autoreader (Labsystems, Helsinki, Finland). An anti-MUC1 mAb

Table I. *Patient characteristics and clinical response.*

Patient	Age/sex	Disease[a]	Prior therapy[b]	Dose of peptide (mg)	No. of vaccines received	Clinical response[c] (mos.)
1	77/M	PC	none	300	4	PD
2	66/M	BC	S	300	7	n.e.
3	58/F	BC	S, C, R	300	3	PD
4	65/M	PC	S	1000	3	PD
5	51/M	PC	R	1000	3	PD
6	57/M	PC	R	3000	3	PD
7	54/M	BC	none	3000	3	PD
8	49/M	PC	none	3000	3	SD (3)
9	56/M	PC	R	3000	3	PD

[a]PC, pancreas cancer; BC, biliary tract cancer.
[b]S, surgery; C, chemotherapy, R, radiotherapy.
[c]mos; months; PD, progressive disease; SD, stable disease; n.e., not evaluated.

E29 (DAKO) was used as a positive control. All of the serum samples were simultaneously measured in triplicate using one 96-well plate to compare each optical density (OD) value.

Results

Patient characteristics and clinical responses. Nine patients with advanced cancer of the pancreas or bile duct were enrolled in this phase 1 clinical study of a MUC1 peptide vaccination. The detailed characteristics of the patients are shown in Table I. The mean age of the patients was 59.2 years (range: 49-77 years). Six patients were in an inoperable state and 3 had recurrent diseases after surgery. The dose of MUC1 peptides ranged from 300 to 3000 μg; as no apparent toxicity was observed in patients 4 and 5 with a dose of 1000 μg, the highest dose (3000 μg) was started from patient 6.

It was difficult to draw any definitive results from this small-scale phase 1 study with regards to clinical responses and prognostic factor analysis. Nevertheless, the available results might be relevant from the point of view of developing a suitable peptide vaccine. In 9 patients who received MUC1 vaccinations, 8 were eligible for clinical evaluation. Of these, a stable disease (SD) in 1 patient (patient 8) and progressive diseases (PD) in 7 patients were diagnosed 2 weeks after the last vaccination (Table I). Patient 8 was diagnosed with SD by sequential CT scans and measurements of a tumor marker, CA19-9. The clinical response of patient 2 was unclear because recurrence was masked by bacterial cholangitis and subsequent liver abcesses during the observation period after vaccination. Patients 1 and 2 were vaccinated more than 3 times, to comply with their request.

Adverse events. All 9 patients were evaluated for adverse events according to the NCI-CTC (17). The vaccinations

were generally well tolerated without hematological toxicity or symptoms of any autoimmune diseases. In all patients, mild reddening, swelling and itching at the vaccination site were observed, for which treatment was not required, and skin tests against MUC1 peptides were negative.

Immunological responses. Immunological responses could be evaluated in 7 out of 9 patients. Intracellular cytokine-positive CD4$^+$ T cell (%) and circulating anti-MUC1 antibody levels before and after vaccination are shown in Figure 1. IL-10 is a Th2 cytokine and IL-6 stimulates the proliferation of antibody-producing cells. In 5 out of 7 patients, both IL-10 and IL-6-producing CD4$^+$ T cell counts tended to decrease after vaccination (Figure 1a). Intracellular IFN-γ or IL-4-positive CD4$^+$ T cells were always under detectable levels (data not shown). The titer of circulating anti-MUC1 IgG antibodies also showed decrease or no change in 5 out of 7 patients. However, it tended to increase in the patient who showed SD for 3 months (Figure 1b).

Discussion

This phase I clinical trial revealed the safety of a vaccine containing 100-mer MUC1 peptides and incomplete Freund's adjuvant in advanced pancreas and bile duct cancer patients. The only adverse event observed was mild reddening and swelling at the vaccination site. A skin test against the MUC1 peptide before vaccination was negative in all patients. Although 1 pancreatic cancer patient showed SD with a modest increase of circulating anti-MUC1 IgG titer after vaccination, 7 other evaluable patients were PD, and the circulating cytokine-producing CD4$^+$ T cell and anti-MUC1 IgG levels tended to decrease in most patients. It seems that these results reflect a rapid progression of
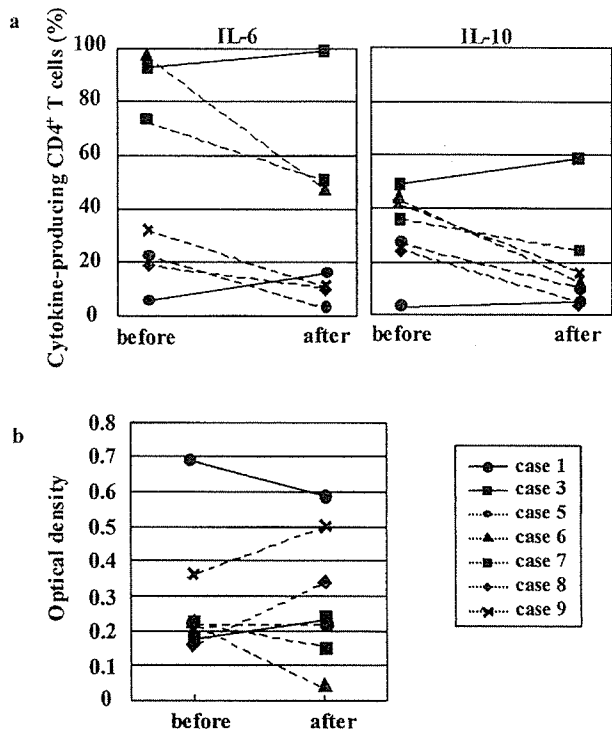
Figure 1. *Intracytoplasmic cytokine and anti-MUC1 IgG antibody assays before and after vaccination. (a) The effect of vaccination on the percentage of IL-6- or IL-10-producing CD4+ T cells in peripheral blood. (b) The effect of vaccination on circulating anti-MUC1 IgG antibody levels. All samples were measured simultaneously in each assay. Results are shown as the mean from triplicate wells.*

that soluble MUC1 is not transported to late endosomes or MHC class II compartments for processing and binding to class II MHC (26). These suggest that the reduction of tumor burden, which leads to decreased immunosuppressive factors including MUC1, could be essential to cancer therapy with a peptide vaccine.

Ramanathan *et al.* recently reported the results of a phase I study of a MUC1 vaccine in patients with resected (n=15) or locally advanced (n=1) pancreatic cancer without prior chemotherapy or radiotherapy (27). Their MUC1 peptide was the same one as used in our study. Escalating doses of the peptide (100, 300, 1000 and 3000 μg) were admixed with SB-A2 and administered intramuscularly every 3 weeks for 3 doses. Two of 15 resected patients are alive and disease-free at follow-up of 32 and 61 months. Both patients were at stage T3N1M0 at surgical operation. Immunological parameters including delayed-type hypersensitivity, circulating CD8+ T cell's number, the serum level of anti-MUC1 antibody and the cytokine (IFN-γ or IL-4) production of peripheral blood T cells were improved after vaccination in some patients. They observed an almost total suppression of the T cell's ability to make either IFN-γ or IL-4 in every patient before vaccination, which corresponds to our present results, but the production of cytokines increased significantly after vaccination in 5 patients. These findings suggested the importance of reduced tumor burden for peptide vaccine therapy in pancreatic cancer. A phase I study of MUC1 peptide vaccination for resected pancreatic cancer is now being prepared in our departments.

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advanced pancreatic or bile duct cancer and the presence of a profound immunosuppressive status in those patients.

Pancreatic and biliary tract cancers are two of the worst cancers with regards to 5-year survival rates (19, 20). In pancreatic cancer, several mechanisms for escaping immune surveillance have been shown, including the secretion of immunosuppressive cytokines such as IL-10 and TGF-β, local hindrance of tumor infiltrating lymphocytes (TILs) and loss of the signal transducing CD3ζ chain of TILs (21). On the other hand, we have revealed that MUC1 is involved in the metastatic ability of pancreatic cancer cells (22) and is a poor prognostic factor for cancer of the pancreas (23). Recently, Monti *et al.* demonstrated that MUC1 mucins derived from pancreatic cancer cells suppress the maturation of dendritic cells, resulting in low immunostimulatory functions and the IL-10^{high}IL-12^{low} cytokine secretion phenotype of dendritic cells (24), suggesting that MUC1 *per se* could be a potent immunosuppressive factor. In this context, the findings of Hiltbold *et al.* should be noted. They showed that the efficiency of MUC1 processing by dendritic cells and the resulting strength of CTL activity were inversely correlated with the degree of MUC1 glycocylation (25), and

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Immunological evaluation of personalized peptide vaccination for patients with pancreatic cancer

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Abstract. The prognosis of pancreatic cancer is extremely poor, and development of new treatment modalities is needed. One such treatment could be specific immunotherapy. To evaluate safety and immunological responses, we conducted a phase I study of personalized peptide vaccination for pancreatic cancer patients (n=11). Namely, pre-vaccination peripheral blood mononuclear cells were screened for their reactivity *in vitro* to each of 14 or 16 peptides in HLA-A24+ or -A2+ patients, and only the reactive peptides (maximum: 4) were vaccinated *in vivo*. This regimen was generally well tolerated, although inflammatory reactions at the injection site were observed in 7 patients. Delayed-type hypersensitivity to peptides used for vaccination was observed in 7 patients. Increased cellular and humoral immune responses to at least one of peptides used for vaccination were observed in the post-vaccination PBMCs and sera from 4 of 8 patients and 4 of 10 patients tested, respectively. The 6- and 12-month survival rates for patients who received >3 vaccinations (n=10) were 80% and 20%, respectively. Due to tolerability and capability of inducing specific immunity, further development of personalized peptide-based immunotherapy for pancreatic cancer patients is warranted.

Introduction

Patients with pancreatic cancer (PC) have a poor prognosis, and for this reason, PC is considered to be one of the deadliest types of malignancy. The median survival time (MST) after diagnosis is <12 months, with a 5-year survival rate of approximately 3-5% (1,2). There is no standard therapy for advanced PC, although many chemotherapeutic agents have been used in clinical trials in the past two decades (3-6). Among these chemotherapeutic agents, gemcitabine (GEM) is somewhat clinically effective, but the MST is still <6-9 months. Therefore, development of new treatment modalities is needed, one such treatment could be a peptide-based specific immunotherapeutic approach, as recent advances in tumor immunology have resulted in the identification of many tumor-associated antigens and epitopes recognized by HLA-class-I-restricted cytotoxic T lymphocytes (CTLs) from various cancers, including PC (7-10). However, clinical trials using those peptides have rarely demonstrated major clinical responses (11-13). This failure could be due to an insufficient induction of anti-tumor responses by these vaccine regimens, under which the peptide-specific memory T cells were not measured in pre-vaccination peripheral blood mononuclear cells (PBMCs). We have reported that personalized vaccinations based on pre-vaccination measurement of peptide-specific CTLs in the circulation induced potent anti-tumor immune responses in patients with cancers, such as lung, gastric, colorectal, prostate, and gynecologic cancers (14-19). Moreover, we previously reported that PC cells expressed tumor-associated antigens that encoded the peptides used for those clinical studies (20). Peptide-specific CTL precursors were also detectable in the majority of PC patients (20). In this report, we describe the safety and the immune responses to personalized peptide vaccination of PC patients.

Patients and methods

Patients and eligibility criteria. The Institutional Review Boards of Yamaguchi University and Kurume University approved this clinical protocol (#2031). Complete written informed consent was obtained from all of the patients at the time of enrollment. According to the protocol, the patients were required to be positive for HLA-A24 or -A2. All patients were clinically confirmed to have PC. Eligibility criteria included the following: age of ≤85 years, serum creatinine of <1.4 mg/dl,

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