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Analysis of Naturally Processed Human Histocompatibility Leukocyte Antigen Class I-Bound Peptides from Hepatocellular Carcinoma Tissues *in vivo*

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Naturally processed self-peptides bound to human histocompatibility leukocyte antigens (HLA) class I molecules of human hepatocellular carcinoma tissues (HLA-A2.1, -B44, -B13) *in vivo* were isolated for sequence analysis. Acid-eluted peptides were subjected to reversed-phase high-performance liquid chromatographic separation and single-fraction sequencing was performed by Edman degradation. The peptides were found to be octamers or nonamers and they were derived from the processing of intracellular proteins. Three independent sequences were obtained from HLA-A2.1 molecules. One of the peptides showed sequence homology to the hepatitis B virus (HBV) pre-S protein, one to aldehyde dehydrogenase, and the other to no known protein. Two independent sequences were obtained from HLA-B44, B13 molecules: one showed sequence homology to the human c-abl protein, the other showed no homology to any known protein. A synthetic biotinylated peptide based on the HBV pre-S peptide sequence was confirmed to bind to HLA-A2.1 gene-transfected L cells. These data suggested that peptides potentially recognized by cytotoxic T cells can bind to HLA class I molecules on tumor cells *in vivo*.

Key words: HLA — HLA class I molecule — Bound peptide — Peptide sequencing — Hepatocellular carcinoma

Major histocompatibility complex (MHC) class I molecules are transmembrane polymorphic proteins composed of two polypeptide chains, the heavy chains and β_2-microglobulin. Their primary function is to present endogenous antigenic peptides to $CD8^+$ cytotoxic T lymphocytes (CTLs).[1] The peptides are endogenously synthesized in the cytosol, bound to MHC class I molecules in the endoplasmic reticulum, and transported to the cell surface as class I heavy chain/β_2-microglobulin/peptide trimeric complexes.[2–4] These MHC class I-bound peptides have been partially characterized and have the following common features: (a) they are 8 to 12 amino acids in length[5–7]; (b) they have allele-specific structural motifs[5]; and (c) although extremely heterogeneous, they derive predominantly from a small set of abundantly expressed intracellular proteins. After viral infection, newly synthesized viral proteins are degraded into peptides, some of which bind to MHC class I molecules, and are transported to the surface, rendering the cell susceptible to recognition and lysis by specific CTLs.[8]

Changes in the profile of MHC-bound self-peptides also accompany the *de novo* synthesis of cellular proteins that occurs during differentiation, viral infection and malignant transformation.[9] It is thought that tumor-associated antigens also bind to MHC class I molecules and are recognized by CTLs.[10]

Most known MHC-bound peptides are of viral orgin or are self-peptides derived from normal proteins, based on analyses using *in vitro* materials such as cell lines.[11–13] Little is known about MHC-bound self-peptides derived from human cancerous tissues *in vivo*.

To examine the nature of endogenous peptides associated with human histocompatibility leukocyte antigen (HLA) class I molecules in cancerous tissues *in vivo*, and to compare the peptide binding motifs of *in vivo* cancer cells and *in vitro*-cultured lymphocytes, we have purified HLA class I-bound peptides from surgically resected hepatocellullar carcinoma. Acid-eluted crude peptides were separated by reversed-phase high-performance liquid chromatography (HPLC), and single-fraction sequencing was performed by Edman degradation. One of the derived amino acid sequences showed high sequence homology to hepatitis B virus (HBV). In addition, to confirm that the amino acid sequence was actually derived from HLA class I molecules, binding analysis using synthetic biotinylated peptides was performed.

MATERIALS AND METHODS

Patient and tissue specimen A fresh tissue sample of hepatocellular carcinoma was obtained from a patient

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(HLA-A2.1, -B44, -B13) with a history of hepatitis B infection who underwent hepatectomy at Teine Keijinkai Hospital (Sapporo).

Monoclonal antibodies The following monoclonal antibodies (mAbs) were used as purified IgG: BB7.2 (anti-HLA-A2), 4E (anti-HLA-B framework) and W6/32 (anti-HLA-A, -B, -C).

Immunohistochemistry Dewaxed paraffin sections were immunostained by W6/32 using the streptavidin biotin method (Histofine SAB kit, Nichirei, Tokyo). Sections were counter-stained with hematoxylin.

Immunoaffinity purification of HLA class I molecules To isolate HLA-A2.1, -B44, -B13 molecules, approximately 200 g of hepatocellular carcinoma tissue was used per experiment. The carcinoma tissues were homogenized at 4°C in 1 m*M* $NaHCO_3$ (pH 7.5) and then centrifuged at 1,500*g* for 10 min. The sediment was suspended in 1 m*M* $NaHCO_3$ (pH 7.5) and sequentially centrifuged at 100*g* for 5 min and 1,000*g* for 10 min to isolate the crude membrane fraction. The crude membrane fraction was lysed in phosphate-buffered saline (PBS) containing 1.5% Nonidet-P40, 0.1 m*M* phenylmethylsulfonyl fluoride (PMSF) and 5 m*M* EDTA for 2 h at 4°C. Detergent-insoluble materials were removed by centrifugation at 10,000*g* for 20 min. HLA molecules were purified essentially as described previously.[14] Briefly, HLA molecules were subsequently purified from the supernatant by immunoaffinity chromatography on mAb-coupled Sepharose (CNBr-activated Sepharose 4B, Pharmacia, Sweden) columns at 4°C. The supernatant containing the HLA molecules was passed sequentially through columns of bovine γ-globulin, BB7.2 (anti-HLA-A2) and 4E (anti-HLA-B), at a flow rate of 30 ml/h. The columns were washed with 10 column volumes of PBS containing 0.1% Nonidet-P40, 2 column volumes of PBS, and 2 column volumes of PBS containing 1% octylglucoside. They were eluted with 0.05 *M* diethylamine in 0.15 *M* NaCl containing 1% octylglucoside

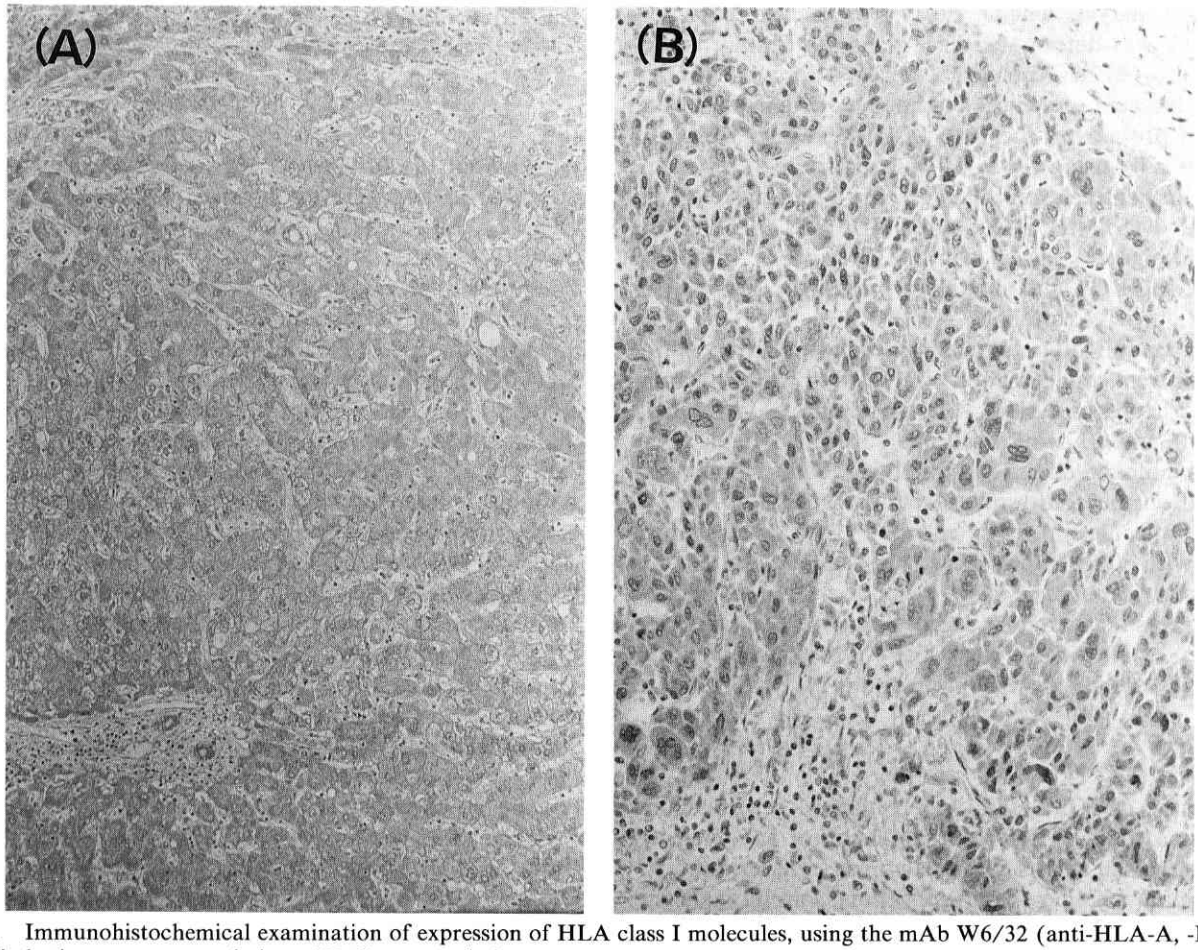

Fig. 1. Immunohistochemical examination of expression of HLA class I molecules, using the mAb W6/32 (anti-HLA-A, -B, -C). (A) Cirrhotic noncancerous lesion. (B) Cancerous lesion.

(pH 11.5). The eluates were immediately neutralized with 0.1 *M* Tris-HCl (pH 6.8), concentrated by ultrafiltration on a Minicon (Amicon, Denvers, MA), and stored at −30°C. Protein content was evaluated by means of a bicinchoninic acid protein assay (Pierce Chemical Co., Rockford IL) and confirmed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE).

Preparation of bound peptides The elution of HLA class I-bound peptides was performed in 10% acetic acid at 37°C for 30 min. The released peptides were separated from proteins by using ultrafiltration on a 3 kDa cartridge (Centricon SR3, Amicon), and stored at −70°C until HPLC separation. The acid-eluted crude peptide fraction was separated by reversed-phase HPLC using a C2/C18 RPC column (Pep-S 4.0×250 mm, Pharmacia) under the following conditions. Buffer A was 0.06% trifluoroacetic acid (TFA) in H_2O and Buffer B was 0.052% TFA in 80% acetonitrile in H_2O. Elution was done with a gradient system of 0 to 20 min, 0 to 19% B; 20 to 150 min, 19 to 57% B. Flow rate: 0.5 ml/min. Absorbance was measured at 210 nm. Fractions were collected and stored at −70°C until sequencing.

Sequence analysis The amino acid sequence of each peptide peak was determined by automated Edman microsequencing (477A; Applied Biosystems, Foster City, CA). The amino acid sequences obtained were compared with those of proteins in the PRF-SEQDP and the Genbank data bases by using the JOIS-F sequence search system.

Synthetic peptides for binding assay Biotinylated peptides HB-1 (STNRQSGRQ) and HB-2 (STDRQSGRQ) were designed based on HBV pre-S p141-149 (STNRQSGRQ), and obtained from Chiron Mimotopes, Inc. (Victoria, Australia).

Binding assay Aliquots of 100 μl of biotinylated peptide (200 μM final concentration) were added to L cell transfectants expressing HLA-A2.1 molecules, L-A2.1 cells (3×10^5), in 100 μl of Dulbecco's modified Eagle's medium and incubated at 37°C for 16 h.[15] The binding assay is based on the ability of exogenous peptides to bind to class I molecules on living cells.[16–18] After further incubation with 50 ng of phycoerythrin-Streptavidin (Becton Dickinson, Mountain View, CA) for 30 min, specific binding was measured by flow cytometry. Each step was followed by two to five washes at 4°C with PBS containing 0.1% bovine serum albumin and 0.1% sodium azide.

RESULTS

Expression of HLA class I molecules in hepatocellular carcinoma tissues Reduced expression of MHC class I molecules often results from malignant transformation.[19] To examine whether the hepatocellular carcinoma tissue is available for isolating HLA class I molecules, dewaxed paraffin sections were stained with W6/32 mAb using the streptavidin biotin method. Since this cancerous tissue was stained similarly to cirrhotic noncancerous tissue (Fig. 1), HLA class I molecules were present in this tissue.

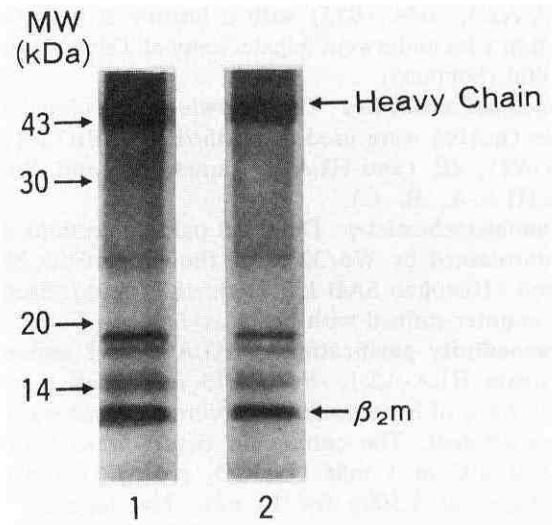

Fig. 2. SDS-PAGE analysis of HLA class I molecules purified from hepatocellular carcinoma tissues. HLA class I molecules were purified by immunoaffinity chromatography using the mAbs BB7.2 and 4E, and purified class I molecules were analyzed by SDS-PAGE on a 13.5% acrylamide gel followed by silver staining. Lane 1, HLA-A2.1 molecules; lane 2, HLA-B44, B13 molecules. β_2m, β_2-microglobulin.

Purification of HLA class I molecules HLA class I molecules were purified by immunoaffinity chromatography using the mAbs BB7.2 (anti-HLA-A2) and 4E (anti-HLA-B) from the detergent lysate of hepatocellular carcinoma tissue. HLA class I molecules were eluted from the affinity matrix and a 1-μg aliquot was analyzed by SDS-PAGE on 13.5% acrylamide gel followed by silver staining. As shown in Fig. 2, heavy chain and β_2-microglobulin were detected as 45 kDa and 12 kDa bands.[20] The final yields of HLA-A2.1 molecules and HLA-B44, B13 molecules were 1.9 mg and 1.8 mg, respectively.

Purification and sequencing of self-peptides derived from HLA-A2.1 molecules The HLA-A2.1-bound peptides of hepatocellular carcinoma tissue were separated by reversed-phase HPLC. The resulting profile (Fig. 3) showed marked clustering of peaks between 10 and 45 min, corresponding to 10 and 27% acetonitrile concentrations in the eluates, respectively. Single-fraction sequencing was performed by Edman degradation. The three peptides obtained (Table I) were assumed to be nonamers or octamers since little signal was found beyond degradation cycle 8 or 9. The sources of these peptides were examined by searching the data bases. Of

these three sequences, peaks 5 and 11 were identified as fragments of HBV pre-S p141-149 (86% homology) and aldehyde dehydrogenase p5572-5595 (71% homology), respectively. The origin of the other sequence, peak 8 was not identified. The peak at 23 min could not be sequenced by Edman degradation, because it contained large amounts of amino acids and organic chemicals. The above sequences do not conform with consensus allele-specific motifs for HLA-A2.1, which specify Ile or Leu at position 2, and Val or Leu at the carboxyl terminus as anchor positions.[5]

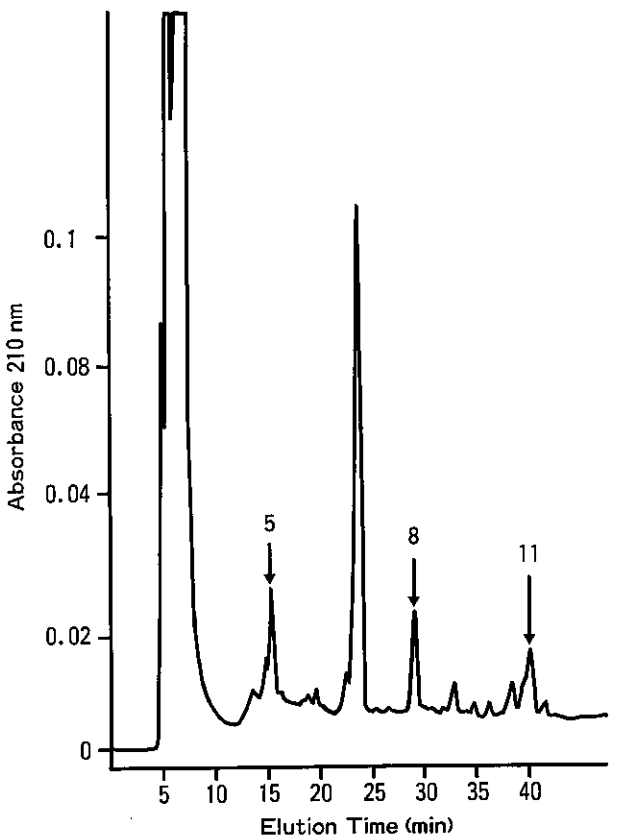

Fig. 3. Reversed-phase HPLC separation of HLA-A2.1-bound peptides isolated from hepatocellular carcinoma tissues was performed using a C2/C18 column. Elution times of 10 and 45 min correspond to 10 and 27% acetonitrile in eluates, respectively.

Purification and sequencing of self-peptides derived from HLA-B44, B13 molecules Similarly, self-peptides eluted from HLA-B44, B13 molecules were separated by reversed-phase HPLC. The resulting profile (Fig. 4) showed marked clustering of peaks between 10 and 40 min, corresponding to 10 and 25% acetonitrile concentrations in the eluates, respectively. Single-fraction sequencing was performed. The two peptides obtained (Table I) were assumed to be nonamers, since little signal was found beyond degradation cycle 9. The sources of the peptides were examined by searching the data bases. Peak 3 was identified as a fragment of human c-abl p793-800 (86% homology), but peak 5 was not identified.

Binding analysis The peptide of peak 5 derived from HLA-A2.1 molecules was found to contain Asp or Asn at position 3 by automated Edman microsequencing. Thus, to examine whether the amino acid sequence which has Asp or Asn at position 3 was actually derived from HLA-A2.1 molecules, two biotinylated synthetic peptides, HB-1 (STNRQSGRQ) and HB-2 (STDRQSGRQ) were synthesized. These peptides were tested for binding to HLA-A2.1 gene-transfected L cells, L-A2.1 cells and the specific binding was measured by flow cytometry. When biotinylated HB-1 peptide was added to L-A2.1 cells, the fluorescence signal shifted to the right compared with the background signal in the absence of biotinylated peptide (Fig. 5). In contrast, no shift was obtained upon incubation in the presence of biotinylated HB-2 peptide (Fig. 5), indicating that this peptide did not bind to L-A2.1 cells. These results indicated that the HB-1 peptide bound to L cell transfectants expressing HLA-A2.1 molecules, and the peptide of peak 5 derived from HLA-A2.1 molecules had Asn not Asp at position 3.

Table I. Amino Acid Sequences of Eluted Peptides and Their Homology

Fraction	Sequence[a]									Homology
	1	2	3	4	5	6	7	8	9	
HLA-A2.1										
5	S	T	B	X	Q	S	G	X	Q	HBV pre-S p141-149
8	X	S	K	K	F	D	Q	S	Q	No match
11	X	Q	Y	T	S	R	M	I		Aldehyde dehydrogenase p5572-5595[b]
HLA-B44, B13										
3	A	A	D	D	X	F	K	D		c-abl p793-800
5	A	A	I	G	Y	M	X	K		No match

a) B, N (Asn) or D (Asp); X, Undetermined amino acid.
b) Proposed human protein source with sequence homology to the aldehyde dehydrogenase gene sequence.

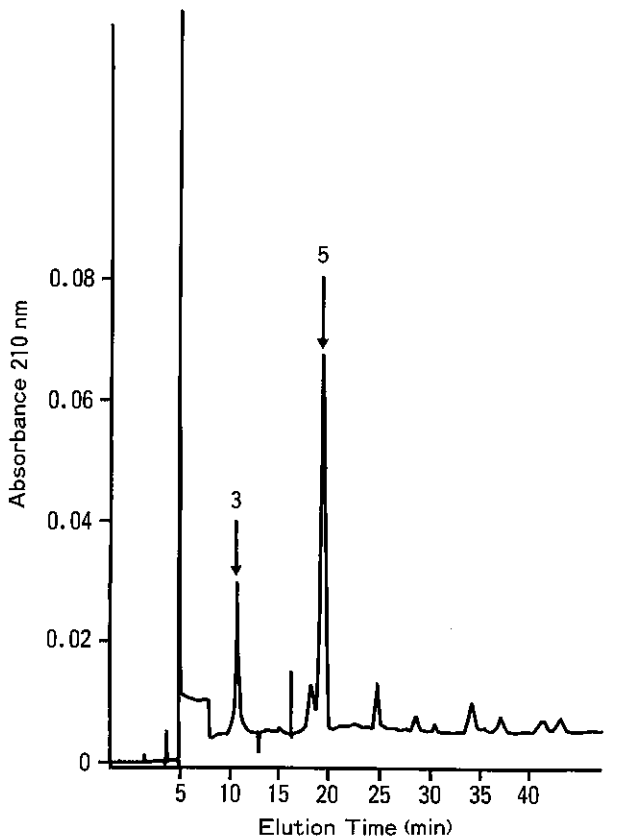

Fig. 4. Reversed-phase HPLC separation of HLA-B44,B13-bound peptides isolated from hepatocellular carcinoma tissues was performed using a C2/C18 column. Elution times of 10 and 45 min correspond to 10 and 27% acetonitrile in eluates, respectively.

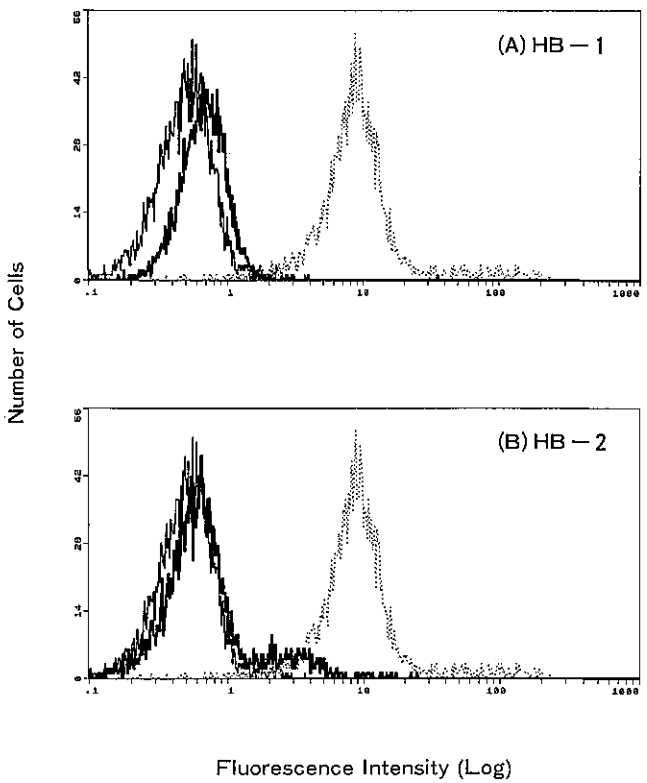

Fig. 5. Binding of the biotinylated peptides to HLA class I molecules on the cell surface of HLA-A2.1 gene-transfected L cells, L-A2.1 cells. The expression of HLA-A2.1 molecules was estimated by using biotinylated mAb BB7.2 (dotted lines). Background fluorescence in the absence of biotinylated peptide (thin lines). (A) Binding of HB-1 to L-A2.1 cells (thick lines). (B) Failure of binding of HB-2 to L-A2.1 cells. The fluorescence signal (thick lines) essentially coincided with background levels (thin lines).

DISCUSSION

The repertoire of self-peptides presented by MHC class I molecules is influenced by alterations in protein metabolism that occur during viral infection,[8] cellular differentiation[9] and malignant transformation. In this report, we have analyzed self-peptides bound to HLA class I molecules of human hepatocellar carcinoma tissues *in vivo*.

Since little is known about MHC-bound self-peptides obtained from *in vivo* materials, especially tumor tissue, we used fresh human cancerous tissues to isolate HLA class I molecules.[21] When isolating HLA molecules from cancerous tissue, there is a possibility of contamination with molecules isolated from noncancerous tissues, such as fibroblasts, endothelial cells, and mononuclear cells. It is thought that acid-eluted materials from them may influence peptide sequencing. However, any such eluates from noncancerous tissues would be present in very small amounts, and HPLC separation showed no significant peak that might affect purification of the peptides and/or microsequencing.

Most analyses of HLA class I-bound peptides have been performed using *in vitro*-cultured lymphoblastoid cell lines, and it has been shown that the bound peptides were derived from intracellular proteins.[11–13] There is a possibility that peptides specific for hepatocytes and hepatocellular carcinoma cells bind to HLA class I molecules. Our data indicated that the peptide derived from HLA class I molecules of hepatocellular carcinoma showed homology to HBV. It is thought that this peptide was derived from the processed HBV protein of hepatocellular carcinoma tissues, in which viral genomes integrated during past infection were expressed.[22, 23]

The amino acid sequences derived from HLA-A2.1 molecules have allele-specific motifs for HLA-A2.1,

which specify Ile/Leu at position 2, and Val/Leu at carboxyl terminus as anchor positions.[5] The peptides eluted from HLA-A2.1 molecules of hepatocellular carcinoma tissues did not have allele-specific motifs for HLA-A2.1 molecules. Recently, it has been reported that the amino acid residue at position 3 is important for the interaction with the binding groove of HLA-A2.1 molecules as a secondary anchor.[24] The peptide derived from HLA-A2.1 molecules, which showed homology to HBV, had Asn or Asp at position 3 by Edman sequencing. A biotinylated synthetic peptide which had Asn, not Asp, at position 3 bound to HLA-A2.1 gene-transfected L cells in binding assays. X-ray crystallographic studies showed that the side chain of residue 3 of the peptide fitted into pocket D within the binding groove of MHC class I molecules.[25,26] Our data suggest that the side chain of the amino acid residue of position 3 interacts with pocket D within the binding groove of HLA-A2.1 molecule by forming a hydrogen bond, although the peptide has no allele-specific motifs.

Two independent peptides were derived from HLA-B44, B13 molecules. They do not have allele-specific motifs for HLA-B44, which specify Glu at position 2 and Arg/Leu at position 9 as anchor positions.[27] Allele-specific motifs for HLA-B13 molecules have not been described. Therefore, to confirm whether the peptides were actually derived from HLA-B13, B44 molecules, it is necessary to examine the binding of these isolated peptides to HLA-B44, B13 molecules.

MHC class I-restricted $CD8^+$ CTLs recognize the class I/peptide complexes. Recent studies have revealed that tumor-associated peptides recognized by CTLs bound to MHC class I molecules.[21,28] Further, the c-myc oncoprotein bound to HLA class I molecules.[19] Here, we isolated a human c-abl oncoprotein-homologous peptide from hepatocellular carcinoma *in vivo*. It is implied that MHC class I-bound peptide derived from oncogene products, c-myc and c-abl, may also be recognized by the appropriate T cell receptors (TCRs) *in vivo*.

We have isolated and sequenced five HLA class I-bound peptides derived from human hepatocellular carcinoma. These peptides might have some association with the carcinogenesis and/or the immune response of the hepatocellular carcinoma *in vivo*. The analysis of these peptides derived from human carcinoma tissues may be helpful for developing a peptide vaccine as a strategy for cancer immunotherapy in the future.

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