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A Humanized Monoclonal Antibody Constructed from Intronless Expression Vectors Targets Human Hepatocellular Carcinoma Cells

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An anti-human hepatocellular carcinoma (HCC) monoclonal antibody, hHP-1, was genetically humanized from a murine monoclonal antibody. In this study, a concept of positional template approach was applied to design the amino acid sequence of hHP-1's variable region, and synthetic DNA fragments for protein expression were produced through overlapping PCR from single strand oligonucleotides. Synthetic DNA fragments and human antibody constant region cDNA were used to construct two CMV promotor-based expression vectors for the antibody light and heavy chains, in which the variable region was connected directly to the constant region without an intron sequence. Completely assembled humanized antibody was successfully expressed in mammalian cells as IgG1 kappa molecules and purified using protein A affinity column. The immunogenicity of the hHP1 was estimated by the amino acid sequence and determined through a HAMA (human anti-murine antibody) serum reaction assay. Results indicated that the immunogenicity of hHP-1 was significantly reduced. *In vitro* binding activity assay showed that the hHP-1 had retained its binding function to a human HCC SMMC-7721 cell-line, without cross binding to other human normal tissues. Immunofluorescence staining showed that hHP-1 had a strong binding activity to SMMC cells. A competitive binding assay showed that the relative binding activity of hHP-1 was approximately 25% binding activity of the original murine antibody. Our results indicate that a humanized antibody could be produced using intronless vectors and expressed as a complete IgG1 kappa antibody. Hence we believe that hHP-1 could be a potential candidate for HCC treatment.

Key Words: **HAMA reaction; hepatocellular carcinoma; humanization; intronless; monoclonal antibody.**

Genetic engineering of biologically active antibody molecules for specifically targeting cancer cells seems to be one of the possible and potential remedies for the treatment of cancers (1, 2). Clinical application of genetically engineered antibodies for cancer treatment has been demonstrated previously (3–6). Nevertheless, most of these anti-cancer antibodies are produced from a murine origin, and hence a human anti-murine antibody (HAMA) reaction is inevitably induced when injected into patients. The HAMA response not only reduces the serum half-life of the antibody (7), making it therapeutically ineffective, but also leads to side effects associated with repetitive administration. The immune complex formed between the murine and the HAMA antibodies can become trapped in capillary beds in the skin, kidney glomerulus, and other locations, causing a potential fatal condition, known as serum sickness (8). For these reasons, the HAMA response limits the effectiveness of using murine antibody in cancer therapy. Humanization of an antibody usually involves genetic manipulation of DNA sequence of its structural components. The simplest way to humanize a murine antibody is to produce a chimeric antibody containing murine variable regions and human constant region. However, the murine variable domains of the chimeric antibody are still immunogenic and able to arouse the patient's immune response, thus it may eventually cause HAMA reaction (9). A reduction of immunogenicity of a murine antibody can be greatly achieved by CDR grafting (10), where only murine CDRs are transferred onto an otherwise completely human framework. Although this method reduces the immunogenicity to a minimum level, the original binding activity could also be completely eliminated since no original framework is involved to support the CDRs (11). In order to recover the original binding function, certain framework residues from the original antibody should be preserved (12). This study uses an advanced concept, known as posi-

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tional template, in the structural design of the desired humanized antibody. In antibody humanization, the positions of the framework residues that could influence the structure of antigen binding site have been determined to construct a positional template table (12). This template indicates the positions of the binding related framework residues that should be kept in the design of a humanized antibody for preserving the binding function. A murine anti-human HCC monoclonal antibody, or HP-1, has been shown to have specifically binding capability to a membrane antigen on several human hepatoma cell-lines, and it could also kill liver cancer cells when coupled with a cytotoxic drug. In this study, we demonstrate that a humanized antibody could be successfully expressed as an IgG1 kappa complete antibody in the absence of a kappa chain intron. This humanized HP-1, or hHP-1, was subjected to a series of binding activity and immunogenicity assays. Our finding provides evidence that the hHP-1 may have the potential for clinical use in HCC treatment.

EXPERIMENTAL PROCEDURES

Cell cultures. Hepatocellular carcinoma cell-line, SMMC-7721 (Shanghai Cell Biology Institute, China) and the Chinese hamster ovary cell-line CHO-K1 (ATCC CCL 61) were cultured in RPMI 1640 (Gibco) and F12 Ham's media (Gibco), respectively. Both culture media were supplemented with 10% fetal bovine serum (Gibco), 5% antibiotic and antimycotic solution (Gibco), and 2 mM/L glutamine (Gibco). The cell cultures were incubated under sterile condition at 37°C with 5% CO_2.

Antibody humanization. Human kappa chain (VK) variable subgroup I, and heavy chain variable (VH) subgroup II of the human consensus framework subgroups were chosen for antibody humanization based on their similarity to the original murine sequence (Fig. 1) (13). The murine framework residues occurring at binding-related positions were kept, but the kappa chain positions 42, 83, and 106 were not retained as murine sequence since they were found to be buried only in a fraction (K42: 4/15; K83: 1/15; K106: 8/15) of the 15 antibody structures examined (12). The three VK positions (14) were considered as low risk positions in humanization that a change of murine-to-human residue should have no influence on binding. The remaining murine framework positions were humanized by replacing the murine amino acid residues with those found at the corresponding positions from the selected human consensus framework subgroups. In addition, a well conserved amino acid residue leucine at the VK framework position 104 (13) was used in the antibody sequence design to avoid an unusual residue placement of alanine found in the original HP-1. Six HP-1 CDRs of both chains were grafted into the hHP-1 variable region. The completed amino acid sequence of the hHP-1 variable region was converted to DNA sequence according to the codons that were most commonly used in antibody gene expression in nature (13). At the 3′ end of the VH (positions 114 and 115 in the Kabat numbering scheme), *GCTAGC* were used for the two amino acid residues, alanine and serine, to provide an *Nhe*I site for sticky ended ligation with the human heavy chain constant domain (CH) cDNA molecule. Another restriction site, *Hin*dIII, was introduced to the 5′ end of the DNA domains in both chains to facilitate cloning into mammalian cell expression vectors. Moreover, certain internal restriction sites, such as *Kpn*I (*GGTACC*) and *Xba*I (*TCTAGA*) were also introduced into the humanized framework DNA sequence by changing the codon usage.

Construction of expression vectors. Humanized kappa chain and heavy chain variable domains DNA fragments were synthesized by overlapping PCR using six long overlapping synthetic DNA oligonucleotides. In each DNA fragment construction, the six overlapping oligonucletides were assembled in a two-step reaction. In the first step, each of the six oligonucleotides (5 pmole) was annealed and extended in a 50 μl reaction mixture. In the second step, each of the oligonucleotide primers (50 pmole), were designed to hybridize at the 5′- and 3′-end of the variable domains. The primers including Hv5′: 5′GCCCAAGCTT(*Hin*dIII)CAGGTGCAACTG3′ and Hv3′: 3′CCTAGCTAGC(*Nhe*I)ACTAGAGACAGTG3′ for CHs, and Kv5′: 5′GCCCAAGCTT(*Hin*dIII)GCGCAAGTGCTG3′ and Kv3′: 5′TTTGATCTCCAGTTTGGTCCCTCC3′ for kappa chains, were used to amplify 5 μl PCR product from the first step in a 50 μl reaction mix. The cDNA fragment of the human constant domain was cloned by PCR using degenerate primers [For heavy chain constant domains, HuCH5′: 5′GCGCGGATCCGCTAGC(*Nhe*I)ACCAAGGGCCCATCGGTCTTC3′, and HuCH3′: 5′CCGCTCGAG(*Xho*I)TCATTTACCCGGAGAC AGGGA3′, and for kappa chain constant domain, Huka5′: 5′AGAACTGTGGCTGCACCATCTGTCTTC3′, and Huka3′: 5′CCCG CTCGAG(*Xho*I)TCAACACTCTCCCCTGTTGAAGCTCT3′]. For the heavy chain vector (pHeavy) construction, the humanized variable DNA fragment and the human heavy chain constant cDNA fragment were first digested with *Hin*dIII/*Nhe*I and *Nhe*I/*Xho*I, respectively, and then ligated into a *Hin*dIII/*Xho*I digested vector, pSectag2B/Hygro (Invitrogen). For the kappa chain vector (pKappa) construction, the humanized variable DNA fragment was digested with *Hin*dIII enzyme, the human constant cDNA fragment was first 5′ phosphorylated and digested with *Xho*I enzyme, and then ligated into a *Hin*dIII/*Xho*I digested vector, pSectag2B (Invitrogen). Purified plasmid DNA was subjected to transfection and sequencing. The sequencing result of the variable region was compared with the designed hHP-1 DNA sequence. Point mutations and deletions were corrected by exchanging DNA restriction fragments between different clones before cotransfection into mammalian cells.

Antibody expression in CHO-K1 cell line. Chinese hamster ovary CHO-K1 cells (ATCC) were transfected with two expression vectors (0.75 μg pHeavy and 0.75 μg pKappa) and 5 μl lipofectamine (Gibco) in hybridoma-SFM (Gibco). For stable transformant selection, the selection medium containing 250 μg/ml hygromycin B (Gibco) was added 48 h after transfection. Compact colonies were selected and the supernatants from resistant clones were screened for the secretion of the hHP-1 through a sandwich ELISA. The resistant clone with the highest level of antibody secretion was selected for antibody production in a serum free CHO-S-SFM medium (Gibco). hHP-1 was purified by protein A affinity chromatography from the supernatant harvested from a three-day culture. Approximately 500 ml pooled cell supernatant was centrifuged, filtered, and equilibrated with 1M Tris–HCl (pH 8.0). The treated supernatant was loaded into the protein A column for purification. Purified antibody was stored at −20°C in PBS or at 4°C with 1% bovine serum albumin and 0.02% NaN_3 (Sigma). Twofold serial dilutions of the purified antibody were used for concentration measurement using an ELISA assay for screening and human antibody IgG1 kappa (Sigma) of known concentration was used as a standard. Data of the standard were plotted, and the slope and the Y-intercept of the regression line for calibration were calculated with correlation coefficient not less than 0.99. Data points of the hHP-1 samples within the range of the standard curve were used for calculating the antibody concentration. The purity of the antibody sample and the size of its subunits (the heavy and the kappa chains) were analyzed using SDS–PAGE under non-reducing and reducing conditions, respectively.

Immunofluorescence staining, binding activity assay, competition assay, and HAMA serum reaction assay for humanized HP1 antibody. An immunofluorescence staining assay was performed to confirm the binding specificity of hHP-1 to SMMC cells. SMMC cells were exposed to hHP-1 and HP-1 antibodies, and subsequently a FITC conjugated secondary antibody. The stained images were observed under fluorescence microscopy.

Binding activity of hHP-1 was tested through indirect ELISA. SMMC-7721 cells were grown in a 96-well ELISA plate (Corning) and fixed in 2% paraformaldehyde (Sigma). Normal human tissue samples (Clontech) were coated on an ELISA plate at 5 μg/ml. After blocking with 1% BSA, various concentrations (from 0 to 20 μg/ml) of hHP-1 and a nonspecific human IgG kappa antibody (Sigma) were added. Bound antibody was detected by HRP-labeled mouse anti-human IgG mAb (Pharmingen). Successful coating of the human tissue samples was confirmed using a mouse anti-human cardiac troponin I mAb (HyTest) to target the heart tissue sample.

Three competitor antibodies including hHP-1, a nonspecific human IgG1 kappa antibody (Sigma) and an unlabeled HP-1 antibody were used to compete with a biotin labeled HP-1 (biotin-HP-1) for targeting the SMMC-7721 cells. SMMC cells were seeded, fixed in 2% formaldehyde, and blocked in 1% BSA. Various concentrations (from 0 to 20 μg/ml) of the competitor antibody samples were added, followed by an addition of 5 μg/ml biotin-HP-1. The biotin-HP-1 that bound to the SMMC cells was detected by streptavidin-HRP (Pharmingen).

For HAMA serum reaction assay, three antibody samples (HP-1, hHP-1, and human IgG kappa) in liquid phase were used to compete with the solid phase HP-1 for binding with human anti-mouse-IgG antibodies. 5 μg/ml of HP-1 was first coated on an ELISA plate, a serum/antibody mixture containing HAMA serum (Type 2SQ, Boehringer Mannheim) and various concentrations of the antibody samples (from 0 to 0. 5 μg/ml) were subsequently added. Human anti-mouse-IgG antibodies from HAMA serum bound to the solid phase HP-1 were detected following the addition of 2.5 μg/ml biotin-conjugated mouse anti-human IgG1 mAb (Pharmingen), and streptavidin-HRP (Pharmingen).

For all three assays mentioned above, protein coating was performed in 0.1 M carbonate buffer (pH 9.2), the incubation steps for both binding and detection were performed in a volume of 50 μl per well at ambient temperature for 1 h. All sera and antibody samples were diluted in a blocking buffer [1% bovine serum albumin in PBS]. The plate was washed six times with PBS between steps. Following detection, OPD substrate solution (Sigma) was added for color development and the absorbance was read at 450 nm.

RESULTS

Amongst the human consensus framework subgroups, the VH framework subgroup II and the VK framework subgroup I were used in the humanization process (Table 1). The VK framework carrying 13 murine amino acid residues was shown to have 84% (67/80) similarity to the human kappa chain frameworks subgroup I. The humanized heavy chain framework carrying 16 murine amino acid residues was shown to have 84% (81/97) similarity to the human heavy chain framework subgroup II. Therefore, the similarity between the murine framework and the human consensus frameworks for both heavy and kappa chains were increased to approximately 84% after humanization. The occurrence frequency of the 29 preserved murine residues (16 from heavy chain and 13 from kappa chain) in the humanized antibody design were obtained from the antibody sequence database (15). In 29 preserved murine residues, eight of them (VH27, VH73, VH76, VH82, VH82a, VH82c, VK3, and VK4) were found to be conserved in other human consensus framework subgroups and 10 of them (VH6, VH48, VH67, VH71, VH78, VK43, VK70, VK78, VK100, and VK104) occurred at a frequency rate not less than 10% in human. In an examination of the antibody sequences contained within the database (13), a human heavy chain framework sequence which had been shown to contain 24 residues, were different from the human heavy chain consensus framework sequence found. For the kappa chain, of 40 complete or almost complete human kappa chain framework sequences, four of them showed more than 13 residues different from the human kappa chain consensus framework sequence. As compared to the hHP-1 framework sequence, which had only 16 heavy chain residues and 13 kappa chain residues different from the human consensus framework sequence, the hHP-1 had a higher sequence similarity to the human consensus framework.

Expression of hHP-1 Antibody

In order to screen a heavy chain expression vector containing a human IgG constant domain subclass I, the heavy chain region from four independent clones were sequenced using a sequencing primer (5′GCATGTGACCTCAGGGGTCCG3′). The subclass of the heavy chain constant region was confirmed by comparing the sequencing results with the conserved human-hinge sequence of different human IgG subclasses (12). The humanized IgG1 heavy chain and the humanized kappa chain DNA fragments as well as the two constant domains cloned from human spleen cDNA were sequenced before cotransfection. For the variable domains synthesized through overlapping PCR, however, five mutations were found in 1114 bases sequenced from four independent kappa chain clones, and 22 mutations were found in 1988 bases sequenced from seven independent heavy chain clones. In addition to mismatches and deletions, the positions of the mutation were not evenly distributed and a “hot spot” region of the mutation was found in the middle part (H165 to H200) of the heavy chain variable domain (Fig. 1). All mutations of the designed sequence of expression vectors were corrected through restriction fragment recombination, and the heavy chain and the kappa chain expression vectors were cotransfected into the CHO-K1 cells. Stable transfectants were selected under hygromycin B, and resistant clones were screened for human IgG kappa antibody secretion by a sandwich ELISA assay. ELISA readings obtained from both negative controls showed similar results as the background level. Amongst the 68 resistant clones selected, 7% (5/68) showed high level antibody secretion (OD450 $>$ 8$\times$ background), and the clone showing the highest expression level (ELISA reading $\times$16 $>$ background level) was selected for hHP-1 antibody production. Purified hHP-1 was subjected to SDS–PAGE electrophoresis to confirm the sample purity. A band showing the size of the completely assembled hHP-1 molecule

TABLE 1

The Humanized HP-1 Variable Domain Amino Acid and DNA Sequence

Kappa chain

```
          10         20         30         40         50         60
5'GCCCAAGCTTGCGCAAGTGCTGACCCAGACACCATCC(AGT)GTGTCTGCA(AGT)GTGGGA(GA
   P  K  L  A  Q  V  L  T  Q  T  P  S  S  V  S  A  S  V  G  D

          70         80         90        100        110        120
TAGG)GTCACCATCAATTGC----------------CDR1------------------TG
 R   V  T  I  N  C  ------------------------------------- W

         130        140        150        160        170        180
GTATCAGCAGAAACCAGGG(AAA)CCTCCCAAAGGCCTGATCTAT--------CDR2-----
 Y  Q  Q  K  P  G   K   P  P  K  G  L  I  Y  -----------------

         190        200        210        220        230        240
----GGAGTCCCATCGCGGTTCAGCGGCAGTGGATCTGGGACACAGTTCACTCTCACCAT
----G  V  P  S  R  F  S  G  S  G  S  G  T  Q  F  T  L  T  I

          250        260        270        280        290        300
CAGC(AGT)GTGCAG(CCA)GAAGAT(TTC)GCCACTTACTACTGT------------CDR3----
 S   S   V   Q   P   E  D   F  A  T  Y  Y  C  --------------------

         310        320        330        340
----------------TTCGGCGGAGGGACC(AAA)CTG(GAGATC)AAA 3'
----------------F  G  G  G  T  K  L  E  I  K
```

Heavy chain

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          10         20         30         40         50         60
5'GCCCAAGCTTCAGGTGCAACTG(CAG)CAGTCAGGACCTGGCCTAGTG(AAA)CCCTCACAG(AC
   P  K  L  Q  V  Q  L   Q  Q  S  G  P  G  L  V   K  P  S  Q   T

          70         80         90        100        110        120
C)CTGTCCATCACCTGCACAGTCTCTGGTTTCTCACTAAAT------CDR1-----TGGAT
  L  S  I  T  C  T  V  S  G  F  S  L  N  ---------------W  I

         130        140        150        160        170        180
TAGGCAGTCTCCAGGAAAGGGTCTAGAGTGGCTGGGA-----------------------
 R  Q  S  P  G  K  G  L  E  W  L  G  -----------------------

         190        200        210        220        230        240
--CDR2-------------------AGACTGACCATC(AGT)AAGGACAATTCC(AAA)AGCCA
-------------------------R  L  T  I  S  K  D  N  S  K  S  Q

          250        260        270        280        290        300
AGTT(TCC)TTC(AAG)ATGAACAGTCTG(ACCGCTGC)TGACACAGCC(GTC)TATTATTGTGCCAG
 V   S   F   K   M   N  S  L   T  A  A  D  T  A   V   Y  Y  C  A  R

         310        320        330        340        350        360
------------------CDR3---------------TGGGGCCAAGGTACCCTGGTCAC
-------------------------------------W  G  Q  G  T  L  V  T

         370        380
TGTCTCT(AGT)GCTAGCTAGG 3'
 V  S   S   A  S  *
```

Note. The framework residues that are found only in the original murine HP-1 framework are in *italic*. The murine CDRs are shown as doted lines (- - -). The framework residues only found in the human consensus framework subgroups are in brackets. The other framework residues that are conserved in both the murine HP-1 and the human consensus frameworks are in black. The flanking restriction sites for cloning (*Hin*dIII, AAGCTT, and *Nhe*I, GCTAGC), and the internal restriction sites. *Xba*I (TCTAGA) and *Kpn*I (GGTACC) are underlined.

was detected (Fig. 2a, lane B). When β-mercaptoethanol (Sigma) was added, the heavy and kappa chains were shown to have molecular weights of approximately 50 and 25 kDa, respectively, which were similar to the human IgG kappa antibody (Fig. 2a, lanes C and E). The hHP-1 antibody sample was quan-

a

	Amino acid sequence of kappa chain frameworks
Murine HP-1 kappa chain	*AQVL*T Q*T*PS*P* *V*SA*A*V G*GT*VT I*N*C WYQQK PG*QP*P K*G*LIY GVPSR FSGSG SGT*Q*F TLTIS *GV*Q*C*E D*A*ATY YC FG*G*GT *EAVV*K
Human kappa chain subgroup I	**DIQM**T Q**S**PS**S** **L**SA**S**V G**DR**VT I**T**C WYQQK PG**KA**P K**L**LIY GVPSR FSGSG SGT**D**F TLTIS **SL**Q**P**E D**F**ATY YC FG**Q**GT **KVEI**K
Humanized HP-1	*AQVL*T Q*T*PS**S** *V*SA**S**V G**DR**VT I*N*C WYQQK PG**K***P*P K*G*LIY GVPSR FSGSG SGT*Q*F TLTIS **S***V*Q**P**E D**F**ATY YC FG*G*GT **K***L***EI**K

b

	Amino acid sequence of heavy chain frameworks
Murine HP-1 heavy chain	QVQL*K* *Q*SGPG LV*Q*PS Q*S*LS*I* TCTVS G*F*S*LN* WIRQ*S* PGKGL EW*L*G R*L*TI*T* *K*D*N*S*R* *SQVFF* *EMNSL* *QPK*DT A*I*YYC AR WGQGT LVTVS *E*
Human Heavy chain subgroup I	QVQL**Q** **E**SGPG LV**K**PS Q**T**LS**L** TCTVS G**G**S**VS** WIRQ**P** PGKGL EW**I**G R**V**TI**S** **V**DT**S**K NQ**FSL** **KLS**S**V** **TAA**DT A**V**YYC AR WGQGT LVTVS **S**
Humanized HP-1	QVQL**Q** *Q*SGPG LV**K**PS Q**T**LS*I* TCTVS G*F*S*LN* WIRQ*S* PGKGL EW*L*G R*L*TI**S** *K*D*N*S**K** *SQVSF* **K***MNSL* **TAA**DT A**V**YYC AR WGQGT LVTVS **S**

c

Position	**Mutation (CK)**		**Position**	**Mutation (CH)**
K112	Deletion		H36	T→G, T→C
K215	Deletion		H132	C→A
K216	Deletion		H147	A→T
K249	Deletion		H148	G→T, G→A X 2
K279	G→C		H167	A→G
			H171	G→T, G→A X 3
			H175	A→G
			H184	C→G
			H187	C→T
			H188	A→C, A→T X 2
			H190	C→T, C→A X2
			H193	Deletion

FIG. 1. Comparison of the framework sequence of the original HP-1, human consensus framework subgroups, and humanized sequence design: (a) The kappa chain and (b) the heavy chain framework sequences of the murine HP-1. The sequences found in both the murine and the selected human consensus frameworks are shown. The sequence only found in the murine HP-1 is in *italic* and the sequence only found in the human consensus frameworks is in **bold**. In each framework sequence, the four rows showing the four different framework regions (FR1, FR2, FR3, and FR4) that flank the three CDRs. (c) A summary of the sequencing results of humanized variable domain: Five mutations were found in the 1114 bases sequenced from four independent clones in the kappa chain variable domain. For the heavy chain, mutations occurred 22 times within the 1988 bases sequenced from seven independent clones. The coupled columns in the left and right show the positions and types of mutation found in the sequenced kappa chains and heavy chains, respectively.

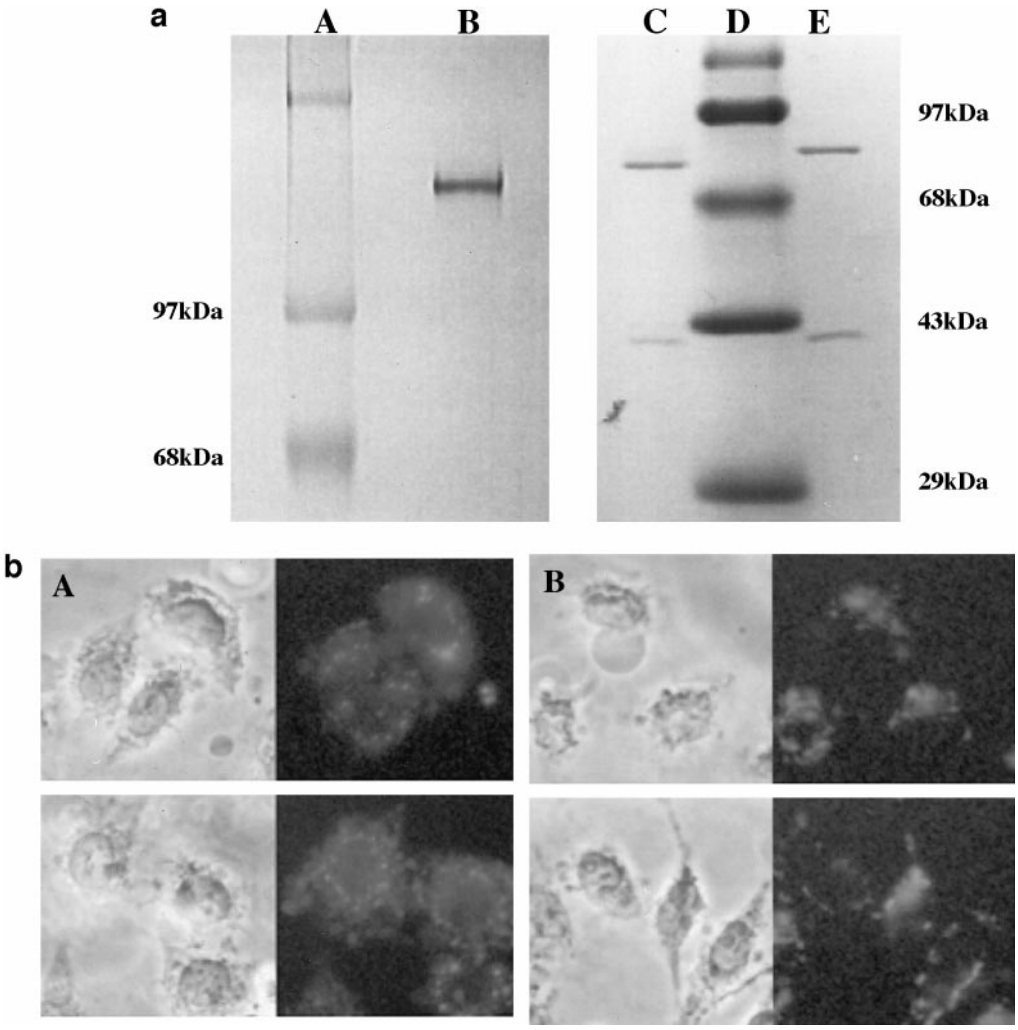

FIG. 2. (a) SDS–PAGE analysis of purified hHP-1 antibody samples. Lane A, Molecular weight marker; Lane B, completely assembled hHP-1 molecule in 8% polyacrylamide gel under a nonreducing condition; Lane C, heavy chain and kappa chain of 1 μg hHP-1 under reducing condition; Lane D, molecular weight marker; Lane E, 1 μg human IgG kappa in 12% polyacrylamide gel under reducing condition. (b) Immunofluorescence binding of hHP-1 and murine HP-1 to SMMC-7721 cells. Left: Phase-contrast image stained cells. Right: cells antigen stained with the antibody samples (A: hHP-1 or B: murine HP-1) and FITC-conjugated secondary antibodies. Both negative controls stained with the secondary antibodies or stained with a nonspecific human IgG kappa antibody did not show any fluorescence (not shown in figure). This figure is shown in monochrome.

tified through a sandwich ELISA assay using human IgG kappa antibody sample of known concentrations as the standard for calibration, and the expression level of the secreted hHP-1 was approximately 100 μg from one liter supernatant of a three days culture.

Immunofluorescence Staining, Binding Assay, Competition Assay, and HAMA Serum Reaction Assay for hHP-1 Antibody

Results from the immuno-staining assay indicated that when the same concentration of hHP-1 and murine HP-1 was separately applied to SMMC cells, fluorescent images were observed. However, the image stained with hHP-1 apparently gave a stronger fluorescent signal (Fig. 2b).

hHP-1 was tested for its binding activity towards a SMMC-7721 cell line through an indirect ELISA. A human IgG1 kappa antibody without any defined binding properties was used as a negative control. In Fig. 3, the binding absorbance of the hHP-1 increased as the antibody concentration increased, and no significant change was observed in the negative control. A competition assay was performed to test whether the hHP-1 had the ability to target the antigen of the original HP-1, and to compare the relative binding affinity change before and after the humanization process. In order to demonstrate the level of competition, a binding inhibition curve was constructed. ELISA readings obtained with biotin-HP-1 added only (i.e., without any competitors) was calculated as zero percent inhibition.

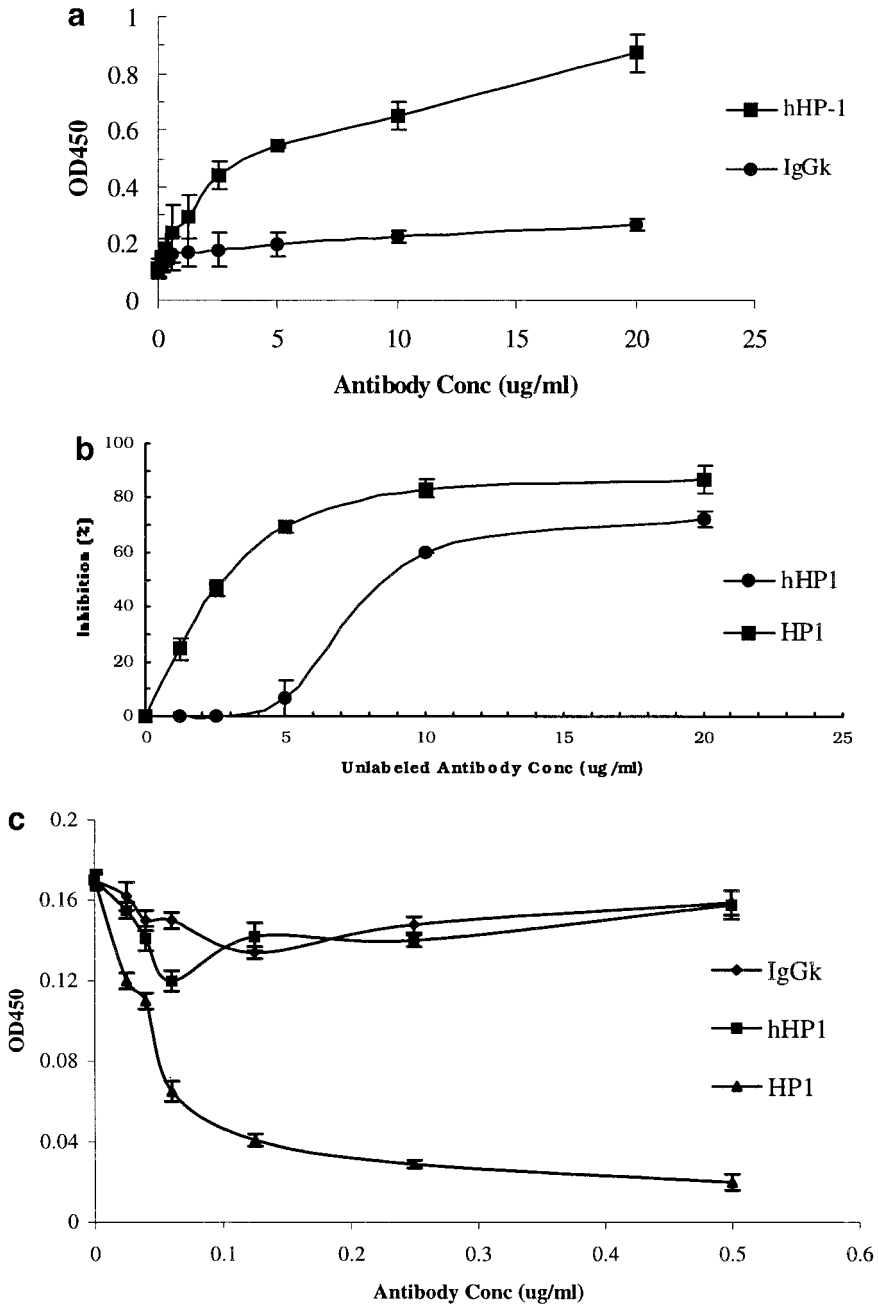

FIG. 3. (a) Binding activity of hHP-1 to SMMC-7721 cells. SMMC cells were incubated with various concentrations of hHP-1, and the negative control, a human IgG1 kappa antibody. Absorbance was read at 405 nm. The curve was constructed by plotting the mean absorbance values against the antibody concentrations. Error bars indicate the range of absorbency at each data point. (b) Binding competition of biotin-labeled murine HP-1 against unlabeled hHP-1 and murine HP-1. A fixed concentration of the biotin-HP1 antibody sample was used to compete with both the unlabeled hHP-1 and unlabeled murine HP-1 for binding to SMMC-7721 cells. Following detection with streptavidin-HRP (which could only detect the bound biotin-murine HP1 through streptavidin-biotin interaction), the absorbance was read at 450 nM. The range of the data is shown by error bars. Nonspecific human IgG kappa antibody showed 0% biotin-HP-1 binding inhibition at all concentrations (data not shown). (c) An inhibition curve showing HAMA reaction assay. Solid phase murine HP-1 was incubated with a mixture containing HAMA serum and various concentrations of the three liquid phase antibody samples (murine HP-1, humanized HP-1, and human IgG1 kappa). The curve was constructed by plotting the mean absorbance values against the antibody concentration in the serum/antibody mixture. Absorbance was measured at 405 nm. Error bars indicate the range of absorbance at each data point.

The reading of 100% inhibition was the background reading from the wells containing no biotin-HP-1. Results showed that both hHP-1 and the HP-1 antibodies could compete with the biotin-HP-1 for binding to SMMC cells, and the binding inhibition levels increased with increased competitor antibody concentra-

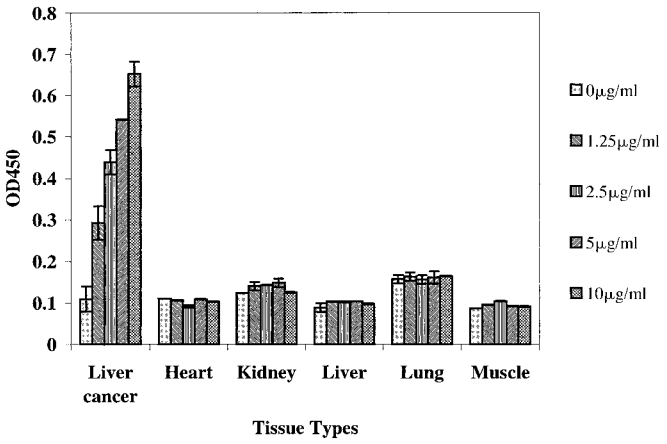

FIG. 4. A comparison of binding activity of hHP-1 to SMMC-7721 cells and human tissue samples. A bar chart was constructed by plotting the mean absorbance values against the antibody concentrations, and the reading at various concentrations of hHP-1 were indicated by patterned bars. Error bars indicate the range of the data.

tions (Fig. 3). hHP-1 antibody required a concentration of approximately four times higher than the HP-1 to compete against the biotin-HP1 for 50% binding inhibition. The results suggest that the hHP-1 antibody could target the same antigen as the original HP-1 antibody with approximately 25% of original binding affinity.

HAMA serum reaction assay was used to estimate the immunogenicity of the hHP-1. ELISA readings obtained from the wells incubated with a mixture of HAMA serum and the liquid phase HP-1 decreased with increased concentrations of liquid phase HP-1. These results therefore indicate that HP-1 interacted with the HAMA serum antibody and inhibited the interaction between the solid phase HP-1 and the HAMA serum antibody. Alternatively, the ELISA reading for the other two liquid phase antibodies (hHP-1 and human IgG kappa) at various concentrations did not show any significant inhibitory effects (Fig. 3c).

Binding Activity of hHP-1 to Liver Cancer Cells and Other Human Tissue Samples

hHP-1 was also tested for its binding activity to different normal human tissue samples (including heart, kidney, liver, lung, and muscle) via an indirect ELISA assay, and a significant increase in absorbency readings was observed in liver cancer samples stained with hHP-1 (Fig. 4). This indicates that the hHP-1 showed a negative response to normal human tissue samples, but had specific binding activity towards HCC SMMC-7721 cells.

DISCUSSION

A humanized antibody that could specifically target human liver cancer cells, and less immunogenic to humans than the original murine antibody was constructed in this study. Based on protein sequence analysis and the HAMA serum assay, the hHP1 appeared to be much less immunogenic than the original murine HP-1. Our protein expression system demonstrated that hHP-1 could be successfully expressed as an IgG1 kappa complete antibody in the absence of a kappa chain intron when a CMV promoter was used. In this study, our approach of using the human consensus framework sequence as a humanization template rather than a human consensus sequence was different from others (15–16). The advantage of using an individual sequence is that a sequence of highest homology to the murine sequence can be selected, and so the number of murine framework residues grafted into the humanized antibody would be minimized. This assumes that an individual human antibody is not immunogenic to humans. However, this assumption may not strictly be correct because the process of antibody affinity maturation in B-cells can change the framework sequence of an antibody, and create an immunogenic epitope that does not exist in the original germline human antibody. Therefore, individual antibody sequence after affinity maturation could be immunogenic to humans. In contrast, the human consensus framework sequence is the most commonly found framework sequence in the human population, and would theoretically to be least immunogenic to humans. In our case, VK subgroup I (VKI) and VH subgroup II (VHII) were selected as humanization templates among the human consensus framework subgroups because they are most similar to the HP-1 frameworks (VKI, 69% similarity; VHII, 71% similarity). When these two subgroups were used as the humanization template, only 29 murine residues (13 from VK and 16 from VH) were present in the humanized framework. As a result, the framework sequence similarity to the human consensus framework sequence was increased from ~70% to ~84%. In addition to protein sequence analysis, we used HAMA serum reaction assay for estimating the immunogenicity of hHP-1. As HAMA serum contains human anti-mouse antibodies that recognize the antigenic regions of a murine antibody, the serum can be used to detect the remaining antigenic epitopes in a humanized antibody. Thus this assay should be more reliable than solely using protein sequence analysis alone.

Variable Domain DNA Synthesis

A humanized variable domain is a new sequence that does not exist in nature. Overlapping PCR is a method widely used in antibody engineering to synthesize a full-length humanized variable domain DNA fragment (14, 17–18). In the past, in addition to using proofreading DNA polymerase, several methods have been used to minimize the problem of the high error rate in the

overlapping PCR reaction (19–20). A convenient approach to construct a correct sequence is to exchange DNA restriction enzyme fragments. If the required DNA fragments containing the correct sequence were not available, site-directed mutagenesis should be performed in the final step to correct the remaining mistakes. Based on the consideration that the restriction sites available in a small (~350 bp) variable domain DNA fragment are limited, and our sequence design, additional restriction enzyme sites [for example, GG**G**AC**T** → GG**T**AC**C** (*Kpn*I)] have been introduced into the variable domain by changing genetic codon usage and leaving the amino acid sequence unchanged.

In creating a mammalian cell expression system for hHP1, separate kappa- and heavy-chain expression vectors were constructed. Both vectors contained a leader peptide to direct the antibody molecule into the secretory pathway, making it possible for the humanized antibody to be purified directly from the culture supernatant. For the heavy chain vector, because both human IgA and IgE constant domains also have the two conserved amino acids (Ala-Ser) at the N-terminal (12), the antibody class can therefore be changed to human IgA or IgE to perform different biological functions for other antibody application, such as IgE mediated mast-cell activation against helminthic infection (21). This cloning approach using an *Nhe*I site in the heavy chain vector can be applied to express any antibodies with the desired biological activity via simple cloning steps, and is therefore useful in antibody humanization and other antibody engineering work.

Unlike in the heavy chain constant domain, the first two amino acids (Arg-Thr) in a human kappa chain constant domain cannot be used to create any common restriction sites for cloning by changing the genetic codon usage. The joining between the variable and the constant domain could be performed through either RNA splicing in mammalian cell (22), or direct joining through blunt-end ligation. When a kappa chain variable domain is directly joined to the constant domain in an immunoglobulin (Ig) promotor driven expression vector, it has been reported that no antibody could be expressed from the mammalian cells (22). One possible explanation for the failure in using an Ig promotor to mediate antibody expression in the absence of intron may be the lack of a specific intronic Ig enhancer located within the major intron between the kappa chain variable and constant regions. Without the specific Ig enhancer, the Ig promotor may fail to direct kappa chain transcription and so the expression pathway would be blocked at the transcription level. Previously, RNA splicing was the commonly used method to collect the kappa chain variable domain and the constant domain in a kappa chain expression vector, no matter whether it consisted of an Ig promotor driven expression vector, such as pAG4270 (22) or a viral promotor driven expression vector, such as CHMV-V_LLys-K_R (23). However, considering that cloning of a large intronic fragment from genomic DNA is a tedious and time consuming process, and that manipulating the small size of an intronless cDNA is more convenient than the large size genomic DNA. We decided to use the second approach i.e., direct joining through blunt-end ligation for the hHP-1 expression, and constructed a cytomegalovirus (CMV) promoter driven kappa chain expression vector with directly joined variable and constant domains. In contrast to previous studies which no detectable antibody obtained from the expression system using Ig promotor driven intronless expression vectors (22), the results of sandwich ELISA confirmed that the CMV promotor expression system used in our system was capable of supporting antibody expression without the kappa chain intron sequence. Nevertheless, the expression level is relatively low (100 μg per liter) comparing with the antibody expression level normally achieved using a stable transfected CHO-K1 cell-line. Nonetheless, similar result of low antibody expression level was also reported with a cDNA expression system driven by a viral promotor P_{SV40} (24). This low expression level suggests that the kappa chain intron may contribute to the high antibody yield obtained in the CHO-K1 cell-line, and a possible function of the intron sequence is to stabilize the RNA molecule (25). A possible way to increase the expression level in an intronless expression system is to increase the transcription level by using adenovirus early protein (E1A and E1B proteins) that can enhance transcription for CMV promotor (26). This can be accomplished by introducing the adenovirus E1a or E1b gene into the genome of a stable transfected cell-line through an additional transfection step. A similar transactivation approach has been applied to enhance antibody expression from CHO cells (27).

Although both expression vectors contained their own dominant selection markers (hygromycin for the heavy chain and zeocin for the kappa chain), we found that the stable transfectant singly selected under hygromycin selection alone was capable of synthesizing both the kappa and heavy chains for a complete antibody expression. It seems to be a general rule that heavy chain single selection, but not the light chain, is sufficient to maintain a complete heterogeneous antibody expression in mammalian cells even if the heavy chain and the light chain were cloned in separate expression vectors. An explanation for the heavy chain single selection phenomena is based on the fact that unassembled heavy chains are generally not secreted (in contrast to light chains) but are retained in the endoplasmic reticulum. The unassembled heavy chains can bind to an endoplasmic reticulum protein (28), and when these chains become accumulated at a certain level, they become toxic to the cell (29). Thus, the expression of heavy chain itself is acting as a selective pressure for light chain expression. When the heavy

chain is being expressed, the light chain must also be expressed to prevent the cytotoxic effect from the non-secreted heavy chain, otherwise cytotoxic effect due to accumulation of the heavy chain would occur. Although there is no disadvantage in using double selection, this single selection approach should be applied to reduce the production cost.

Binding Activity of Humanized HP1

The binding activity of hHP-1 was confirmed by several binding assays. Although some previous studies have reported that the binding activity of a humanized antibody could be as strong as the original murine antibody (14, 18), results of reduced binding activity similar to ours have been commonly found in humanization process. In the two designs of a humanized anti-carcinoma antibody constructed by Roguska (30), the binding activity was reduced to approximately 10 and 30% of the HP-1, respectively. The binding activity maintained in the final design of a humanized anti-vascular endothelial growth factor antibody was about 50% of the original antibody and the affinity maintained in the first 11 designs ranged from 0–50% (11). The change of protein sequence from murine to human may account for the affinity reduction. The protein sequence of the hHP-1 variable region was designed according to the positional template constructed by Padlan (12), and this construction is based on the common structural features found in antibodies (e.g., in almost all antibodies, the conserved residue Trp in heavy chain position 47 is involved in the interaction between light chain CDRs and heavy chain framework). The positional template includes approximately 100 binding related framework positions and in theory, it is expected that humanizing the framework residues at positions not included in the positional template should not influence the binding activity. However, this expectation is not absolutely correct and it has been found that humanizing a murine residue at positions not included in the positional template, such as H82b (31) and H75 (11) may affect the binding activity. Thus, in some exceptional cases, framework positions not included in the positional template may also contribute to the binding function and these exceptions are not predictable. We consider that somatic mutation may account for the explanation of these exceptional cases. Somatic mutation is a process that occurs in affinity maturation of B-cells, and it causes changes of the variable domain sequence and introduces some rare occurring residues into the frameworks, which may contribute to the binding function. Previous studies have indicated that humanizing a rare occurring residue; such as lysine at position H94 (11) and proline at position H7 (32) could cause an affinity loss. When somatic mutation occurs, and introduces a rare occurring residue in a framework position that is not originally involved in binding (i.e., excluded in the positional template), the positional template concept could not predict the potential negative impact on binding as the residue is changed from a murine residue to a human residue. We hypothesize that the potential influence of the rare occurring amino acid in binding may account for the affinity reduction of hHP-1. In hHP-1, 23 murine framework residues were changed to human residues. Amongst the changed murine residues, seven residues (H75R, H85K, H113E, K10P, K80C, K104A, and K105V) rarely occur at the positions according to the antibody sequence database (13). Based on the potential importance of the rare occurring residues in binding process (11, 32), it is expected that by changing these residues back to murine residues in the humanized antibody is a possible way to restore the binding activity.

Potential Clinical Application of Humanized Antibodies in Cancer Therapy

Humanized antibodies have been recently used in clinical trials for human cancer treatment (1–3). As an IgG1 molecule, the hHP-1's long serum half-life allows it to be retained in the human body for a long period before degradation. Hence it could have the potential to control extra-hepatic metastasis by killing cancer cells in the blood circulation. Since hHP-1 contains a human IgG1 constant region that may be effective for recruiting human immune effector function, including complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC) (33). Therefore hHP-1 can be used not only as a carrier molecule for toxin delivery (such as conjugated scFv and Fab), but also could potentially serve as a cancer-killing molecule. In summary, the application of positional template approach is proven to be a promising model for antibody sequence design, and the production a humanized antibody using an intronless vector sequence can be achievable. Our results also support that hHP-1 has binding specificity towards HCC cells and it could be potentially useful in the HCC treatment.

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