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Generation of a Humanized Monoclonal Antibody Against Human Parathyroid Hormone-related Protein and its Efficacy Against Humoral Hypercalcemia of Malignancy

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Abstract. *A humanized monoclonal antibody against parathyroid hormone-related protein (PTHrP) was generated from the mouse monoclonal antibody raised against the peptide corresponding to the N-terminal 34 amino acids of the human PTHrP [(PTHrP(1-34)]. The humanized antibody interacted with the PTHrP(1-34) with a k_D value of 1.90 x 10^{-10} M, and the epitope resides between the amino acids 20 and 30 of the PTHrP. PTHrP(1-34) significantly increased the intracellular cAMP levels in the rat osteosarcoma cells that expressed PTHR1, and the 5 μg/mL or higher concentrations of the humanized antibody almost completely blocked the PTHrP-induced cAMP production even in the presence of 2 μg/mL PTHrP(1-34), demonstrating its ability to fully neutralize PTHrP function. There was no significant difference in the potency of the mouse, chimera, or the humanized antibodies to suppress the PTHrP-induced increase in the intracellular cAMP in ROS cells. Furthermore, at the same doses, the administration of the chimera or the humanized antibody was equally effective in reducing the blood ionized calcium levels of hypercalcemic mice bearing the PAN-7-JCK human pancreatic cancer xenograft or the LC-6-JCK human lung cancer xenograft that secreted PTHrP. Thus, humanized anti-PTHrP may be useful for the treatment of the humoral hypercalcemia of malignancy in humans.*

Hypercalcemia is frequently found in advanced stage cancer patients and deteriorates the quality of life (QOL) (1). Parathyroid hormone-related protein/peptide (PTHrP) was identified as a causative factor of humoral hypercalcemia of malignancy (HHM) (2, 3). The PTHrP protein is synthesized as a 141-amino acid peptide. Peptides consisting of 139 and 173 amino acids are also produced by alternative splicing of the PTHrP mRNA and they all share a common sequence of the N terminal 139 amino acids. Both PTH and PTHrP bind to the parathyroid hormone receptor 1 (PTH1R), a G protein coupled receptor expressing in a wide variety of tissues, and stimulate bone resorption and renal reabsorption of calcium (4). Although the sequence of the N-terminal 34 amino acids between PTHrP and PTH are not identical, fragments of hPTHrP or PTH containing the N-terminal 34 amino acids are fully capable of binding and activating PTH1R (5). Thus, the inhibition of both PTH and PTHrP functions would cause hypocalcemic effects, but selective abrogation of PTHrP function can restore normocalcemic conditions in HHM patients.

Therapies for HHM have focused on the impairment of osteoclastic bone resorption, and bisphosphonates, specific inhibitors of osteoclasts, have been used as the standard drug in clinical practice (6). However, the clinical efficacy of bisphosphonate drugs is still limited, and increasing doses of bisphosphonate cause renal toxicity. 22-oxa-1,25-$(OH)_2D_3$ (OCT) inhibited the expression of PTHrP at a transcriptional level (7, 8) and was efficacious against cancer-induced hypercalcemia in tumor-bearing animals (9). However, OCT cannot be easily applied to HHM treatment due to the development of refractoriness and also to the potential calcium property of the D hormone analogues.

On the other hand, it was demonstrated that both polyclonal and monoclonal antibodies that neutralized PTHrP exerted anti-hypercalcemic and anti-cachextic effects in animal models (10-12). In an attempt to develop a non-immunogenic therapeutic agent for the treatment of HHM, we generated a humanized anti-PTHrP monoclonal antibody by the CDR (complementarity determining region) grafting method from the mouse monoclonal antibody

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Key Words: Humanized monoclonal antibody, epitope, parathyroid hormone-related protein, hypercalcemia.

Table I. *Primers used for PCR.*

Primer	Nucleotide sequence
MHC2	aaatagcccttgaccaggca
MLC	ggatcccgggtcagrggaaggtggraaca
MBC1HGP1	gtctaagcttccaccatggggtttgggctgagctgggttttcctcgttgctcttttaagaggtgtccagt gtcaggtgcagctggtggagtctgggggaggcgtggtccagcctgggaggtccctgag
MBC1HGP2	ctaccaccactactaatggttgccacccactccagccccttgcctggagcctggcggacccaagacatg ccatagctactgaaggtgaatccagaggctgcacaggagagtctcagggacctcccaggctgg
MBC1HGP3	accattagtagtggtggtagttacacctactatccagacagtgtgaaggggcgattcaccatctccag agacaattccaagaacacgctgtatctgcaaatgaacagcctgagagctgaggacac
MBC1HGP4	tgttggatccctgaggagacggtgaccagggttccctggccccagtaagcaaagtaagtcatagtag tctgtctcgcacagtaatacacagccgtgtcctcagctctcag
MBC1HVS1	gtctaagcttccaccatggggtttgggctg
MBC1HVR1	tgttggatccctgaggagacggtgaccagg
MBC1LGP1	acaaagcttccaccatggcctggactcctctcttcttcttttgttcttcattgctcaggttctttctccca gcttgtgctgactcaatcgccctctgcctctgcctccctgggagcctcggtcaagctcac
MBC1LGP2	ctgtggcttccatcttgcttaagtttcatcaagtaccgagggcccttctctggctgctgctgatgccattc aatggtgtacgtactgtgctgactactcaaggtgcaggtgagcttgaccgaggctcc
MBC1LGP3	agcaagatggaagccacagcacaggtgatgggattcctgatcgcttctcaggctccagctctggggc tgagcgctacctcaccatctccagcctccagtctgaggatgaggctgacta
MBC1LGP4	cttggatccgggctgacctaggacggtcagtttggtccctccgccgaacaccctcacaaattgttcctt aattgtatcacccacaccacagtaatagtcagcctcatcctcaga
MBC1LVS1	acaaagcttccaccatg
MBC1LVR1	cttggatccgggctgacct

r=a or g

raised against the human PTHrP(1-34), whose amino acid sequence was conserved in mouse and rat PTHrP. The humanized anti-PTHrP antibody recognized the amino acids between 20 and 30 of PTHrP and was fully capable of neutralizing PTHrP. In addition, the efficacy of the humanized anti-PTHrP antibody against hypercalcemia in human tumor xenograft models was equivalent to those of the mouse and chimera anti-PTHrP antibodies.

Materials and Methods

Generation of the chimera anti-PTHrP antibody. The cDNA for the mouse anti-PTHrP antibody was cloned by RT-PCR from the hybridoma cells #23-57-137-1 that produced an antibody against PTHrP(1-34) (11, 13). RNA was extracted with a Quick Prep mRNA Purification Kit (Pharmacia) and used as the template for the cDNA synthesis. cDNA synthesis was carried out with the AMV RV transcriptase and the primers for the H (MHC2) and L (MLC) chains. The *Hin*dIII-*Bln*1 DNA fragment containing the variable region of the light chain was cloned at the *Hin*dIII-*Bln*1 cleavage site of Cλ/pUC19, in which the constant region of the light chain of human IgGλ was cloned (14), to generate MBC1L(λ)/pUC19. Then, MBC1L(λ)/pUC19 was digested with *Eco*RI, and the DNA fragment containing the variable region of the light chain of the murine anti-PTHrP antibody linked to the constant region of the light chain of the human IgGλ was cloned at the *Hin*dIII-*Pvu*II cleavage site of pCHO1 that harbored the promoter for the human elongation factor EF1α gene (15).

The *Eco*RI-*Sma*I DNA fragment containing the variable region of the heavy chain was cloned at the *Eco*RI-*Sma*I cleavage site of pUC19, generating MBC1Hv/pUC19. Then, the *Apa*I-*Bam*HI DNA fragment containing the constant region of the heavy chain of human IgGγ1 (16) was ligated at the *ApaI-Bam*HI cleavage site of MBCHv/pUC19. The resulting plasmid was digested with *Bam*HI and *Eco*RI, and the *Bam*HI-*Eco*RI DNA fragment containing the variable region of the heavy chain linked to the constant region of the heavy chain of human IgGγ1 was cloned at the *Bam*HI-*Eco*RI cleavage site of pCHO1 (15).

The PCR was carried out for 30 cycles of consecutive reactions at 94˚C for 45 sec, 60˚C for 45 sec and 72˚C for 2 min.

Generation of the humanized anti-PTHrP antibody. The complementarity determining regions (CDRs) derived from the variable regions of the mouse antibody were grafted into an appropriate framework (FR) of variable regions in the human heavy and light chains as described (17, 18). Amino acid sequences of the variable regions of the heavy and light chains were compared with all the human antibodies available in public databases, and the sequences of S31679 (PIR protein sequence database) and U03868 (GenBank) were selected as the template for the human heavy and light chains, respectively (19, 20). The variable regions of the heavy chain of the humanized antibody were designed by grafting CDRs of the mouse antibody to FRs of the human antibody S31679 and were generated by PCR. PCR was carried out for 5 cycles with primers, MBC1HGP1, MBC1HGP2, MBC1HGP3 and MBC1HGP4, and an additional 30 cycles after adding the MBC1HVS1 and MBC1HVR1 primers. The resulting DNA fragments were cloned in pUC19, generating hMBC1Hv/pUC19. After confirming the nucleotide sequences, the *Eco*RI-*Bam*HI DNA fragment was ligated at the *Eco*RI-*Bam*HI cleavage site of pCHO1, generating hMBC1HcDNA/pCHO1.

In order to design the light chain of the humanized antibody, FR1, FR2 and FR3 in the variable regions of the mouse antibodies were grafted to those of HSU03868 and FR4 of the mouse antibody was grafted to that of S31679. CDRs of the human antibody were generated by PCR: 5 cycles with primers MBC1LGP1, MBC1LGP2, MBC1LGP3 and MBC1LGP4, and an additional 30 cycles after adding the MBC1LVS1 and MBC1LVR1 primers. The resulting DNA fragments were cloned at the *Bam*HI-*Hin*dIII cleavage site of pUC19. After confirming the nucleotide sequences, the *Hind*III-*Bln*I DNA fragment was ligated at the *Hin*dIII-*Bln*I cleavage site of Cλ/pUC19, generating hMBC1Laλ/pUC19. The *Eco*RI fragment encoding the light chain of the humanized antibody was excised and ligated at the *Eco*RI site of pCHO1 to generate hMBC1Lxλ/pCHO1, where x represents the version of the antibody. Each version of the antibody was generated by introducing a mutation by a specific primer and PCR. Sequences of the primers used for PCR are listed in Table I.

Expression and purification of antibodies. The chimera and the humanized antibodies were expressed in CHO cells. The plasmid harboring the cDNAs for heavy and light chains of the chimera and humanized antibodies were co-transfected into the CHO cells by electroporation, and clones producing the highest level of the antibodies were selected by enzyme-linked immunosorbent assay (ELISA) for human IgG. The antibodies were collected from the media of the cells cultured in α-MEM supplemented with 2% ultra-low IgG fetal bovine serum for 3–4 days, and they were purified by protein A affinity column chromatography. The mouse antibody was also collected from the culture media of the hybridoma cells #23-57-137-1 and was purified by protein A affinity column chromatography. The concentrations of antibodies were determined by ELISA with human or mouse $IgG_1\lambda$ as a standard.

ELISA assay. The binding specificity of the antibody was determined by an ELISA with 96-well plates. The indicated concentrations of the chimera and the humanized antibodies were added to the wells that had been coated with the indicated concentrations of the peptides purchased from QIAGEN (Tokyo, Japan) and incubated at room temperature for 1 h. After washing the wells with a buffer containing 10 mM Tris-HCl (pH7.0), 150 mM NaCl and 0.1% Tween 20, alkaline phosphatase-conjugated goat anti-human IgG was added. After incubation at room temperature for 1 h followed by washing with a buffer containing 10 mM Tris-HCl (pH7.0), 150 mM NaCl and 0.1% Tween 20, phosphatase substrate was added to the wells and the optical density at 405/620 nm, which represents the alkaline phosphatase activity, was measured.

Kinetic analysis by BIACORE. The purified antibodies were diluted with HBS containing 10 mM HEPES (pH 7.4), 0.15 M NaCl, 3.4 mM EDTA and 0.005% surfactant P20. Affinity of the antibodies to the N-terminal 35 amino acid of PTHrP was determined with BIACORE according to the method reported by Karlsson *et al.* (21). The carboxylated matrix on the sensor surface was activated by the injection of a solution containing 0.05 M N-hydroxysuccinimide (NHS) and 0.2 M N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), followed by an injection of 80 mM 2-(2-pyridinyldithio)ethaneamine (PDEA). Then, [Cys^{35}]-PTHrP(1-35) dissolved in 10 mM acetate buffer (pH 5.0) at a concentration of 5 μg/mL was loaded and coupled to the sensor surface. After blocking the remaining active groups on the sensor surface with a buffer containing 0.1 M formate (pH 4.3), 50 mM l-cysteine and 1 M NaCl, the sensor surface was washed with 10 mM HCl to remove non-covalently bound reagents. For the association and dissociation analyses, antibodies and HBS were loaded at the flow rate of 20 μL/min.

The BIACORE-upgrade system, Sensor Chip CM5, HBS, amine-coupling kit and PDEA thiol-coupling kit were purchased from BIACORE AB (Sweden). The synthetic peptide [Cys^{35}]-PTHrP(1-35) was purchased from Sawady Technology (Tokyo, Japan).

Determination of the ability of the antibody to neutralize PTHrP. The neutralizing activity of the humanized anti-PTHrP antibody was measured by rat osteosarcoma cell line ROS 17/2.8-5 (ROS) cell assay (22). ROS cells were seeded onto a 24-well plate at a density of 1 x 10^5 cells/well. After cultivation at 37˚C for 4 days, the cells were washed and incubated in the F-12 medium containing 10 mM HEPES and 0.5 mM isobutylmethylxanthine (IBMX) at 37˚C for 30 min. Then, 2 ng/mL PTHrP(1-34), with or without the indicated anti-PTHrP antibody, was added to the cells, and the cells were further cultured at 37˚C for 30 min. Intracellular cyclic adenosine-monophosphate (cAMP) was extracted from the cells with 95% ethanol / 3 mM HCl, dried under nitrogen gas at 55˚C and dissolved in 3 mM HCl. The concentration of cAMP in 3 mM HCl was determined with an enzyme immunoassay kit (Single Range cAMP EIA kit; PerSeptive Diagnostics, Cambridge, USA). ROS 17/2.8-5 cells were obtained from Riken Cell Bank (Tsukuba, Japan) and maintained in F-12 medium containing 10% FCS.

Animal models for HHM. A human pancreatic cancer cell line (PAN-7-JCK) and a lung cancer cell line (LC-6-JCK) were purchased from the Central Institute for Experimental Animals (Kawasaki, Japan). The PAN-7-JCK and LC-6-JCK cells were maintained *in vivo* in nude mice. To generate HHM models, tumors transplanted in nude mice were excised, and small pieces of tumor tissues (about $10mm^3$) were subcutaneously transplanted into six-week-old male nude mice (BALB/cA Jcl-nu) (for PAN-7-JCK) or nude rats (F344/N Jcl-rnu) (for LC-6-JCK). Mice and rats displaying blood ionized calcium (iCa) levels at least 0.5 mmol/L higher than the normal control animals (normal range 1.3-1.4 mmol/L) were used as HHM models in this study. The mice and rats used in this study were purchased from CLEA (Tokyo, Japan).

Treatment of animals with anti-PTHrP antibodies. The HHM rats and mice were intravenously (*i.v.*) administered PBS (vehicle control), chimera anti-PTHrP antibody, or humanized anti-PTHrP antibody at the indicated doses. Both the normal control group and the treated groups consisted of 7 animals (for nude mice) or 12 animals (for nude rats). Concentrations of iCa in the blood were measured before administration (day 0) and on the indicated days after the administration of the antibody. Animals were freely given food and water throughout the study.

Blood was collected by retroorbital puncture (mice) or tail vein puncture (rats), and the concentration of iCa was measured by the electrode method using the 634 Ca^{++}/pH analyzer autoanalyzer (Bayer, MA, USA).

Results

Generation of the humanized anti-PTHrP antibody. By grafting the CDRs of the mouse antibody into FRs of the human antibodies S31679 and U03868, we created 22

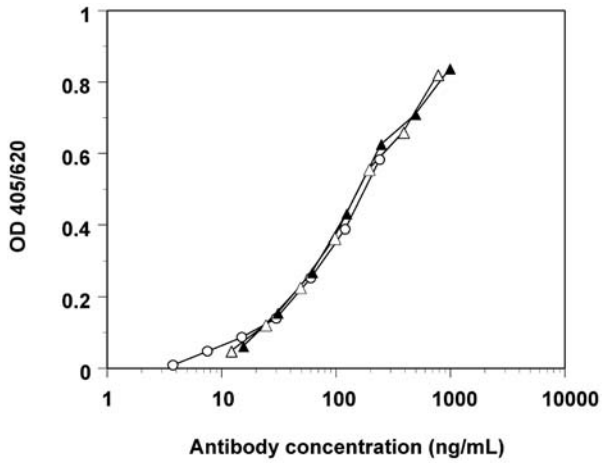

Figure 1. *Binding activities of the chimera and the humanized antibodies to human PTHrP(1-34). Ninety-six-well plates were coated with 1 μg/mL PTHrP(1-34), and PTHrP(1-34) was incubated with the indicated concentrations of the chimera antibody (chimera), the humanized antibody version q (hu-q), or the humanized antibody version r (hu-r). After washing and the addition of an alkaline-phosphatase-conjugated second antibody, amounts of the antibodies bound to PTHrP(1-34) were determined by measuring the alkaline phosphatase activities. Optical densities at 405/620 nm, which represents alkaline phosphatase activity, are shown. ○: chimera antibody, ▲: hu-q antibody, △: hu-r antibody.*

versions of humanized anti-PTHrP antibodies. To select the optimal humanized antibody, we first examined the binding activities to PHTrP(1-34) of the 22 antibodies by ELISA and found that further amino acid substitutions within the FRs of the heavy chain did not significantly increase the binding activity to PTHrP(1-34), whereas those of the light chain augmented it. Among the 22 humanized antibodies, two versions of the antibodies, designated hu-q and hu-r, appeared to have binding activities similar to those of the mouse (not shown) and chimera antibodies (Figure 1). The nucleotide sequencing of the hu-q and hu-r antibodies revealed that they were different by only one amino acid located in the FR2 of the light chain (Figure 2). Neither the hu-q nor the hu-r antibody reacted with PTH at concentrations of 10 μg/mL (not shown).

Next, we determined the affinities of hu-q and hu-r to the N-terminal portion of PTHrP using BIACORE. The chimera antibody interacted with [Cys^{35}]-PTHrP(1-35) with an association rate (k_{ass}) of 1.29 x 10^6 $M^{-1}s^{-1}$ and a dissociation rate (k_{dss}) of 1.46 x 10^{-4} s^{-1}, giving rise to the K_D value of 1.13 x 10^{-10} M (Figure 3). Although the hu-q and hu-r antibodies showed similar binding activities to PTHrP(1-34) in ELISA, kinetic analysis by BIACORE revealed small but significant differences in the binding of the hu-q and hu-r antibodies to PTHrP(1-34). The hu-q antibody interacted with PTHrP(1-34) with a k_{ass} value of 1.02 x 10^6 $M^{-1}s^{-1}$ and a k_{dss} value of 1.94 x 10^{-4} s^{-1}, and the hu-r antibody with a k_{ass} value of 1.23 x 10^6 $M^{-1}s^{-1}$ and a *Kdss* value of 2.85 x 10^{-4} s^{-1}. Accordingly, the K_D values of the hu-q and the hu-r antibodies to PTHrP(1-34) were 1.90 x 10^{-10} M and 2.32 x 10^{-10} M, respectively. Thus, it appears that, although the hu-q and hu-r antibodies have a similar association rate constant to PTHrP(1-34), the dissociation rate constant to PTHrP(1-34) of the hu-q antibody was lower than that of the hu-r antibody. We also determined the K_D value of the mouse antibody to PTHrP and found that it was 1.02 x 10^{-10} M, which was more or less the same as that of the chimera antibody (1.13 x 10^{-10} M).

Ability of the humanized anti-PTHrP antibody to neutralize PTHrP. Next, the hu-q and hu-r antibodies were tested for their abilities to neutralize PTHrP (Figure 4). The ability of the antibody that neutralizes PTHrP was examined by measuring the amounts of cAMP in ROS cells that expressed PTH1R. The addition of 2 ng/mL PTHrP(1-34) to the culture media of the ROS cells led to a significant increase in the intracellular concentration of cAMP. Both murine and chimera antibodies inhibited the PTHrP(1-34)-induced increase in cAMP levels in a dose-dependent manner; there was no significant increase in the intracellular cAMP in the presence of 5 μg/mL or higher concentrations of the antibodies (Figure 4). The hu-q and hu-r antibodies were also effective in abrogating the increase in cAMP. However, the ability of the hu-q antibody to neutralize the human PTHrP was slightly stronger than that of the hu-r antibody especially at lower concentrations (Figure 4). This is consistent with the result that the K_D value of the hu-q antibody to PTHrP(1-34) was smaller than that of the hu-r antibody. None of the hu-q, hu-r, or chimera antibodies significantly affected the cAMP production induced by PTH even at the concentration of 10 μg/mL (not shown).

Epitope recognized by the hu-q antibody. The potency of the hu-q antibody to neutralize PTHrP function was slightly higher than that of the hu-r antibody and was almost equivalent to those of the mouse and chimera antibodies. Based on these findings, we selected the hu-q antibody as the candidate antibody for clinical application. To further characterize the hu-q antibody, we performed epitope mapping by binding assay with various peptides derived from PTHrP(1-34). As shown in Figure 5, the amount of the hu-q antibody bound to PTHrP(5-34) or PTHrP(10-34) was the same as that bound to PTHrP(1-34). The hu-q antibody could also bind to PTHrP(15-34) and PTHrP(20-34), but the amounts of the hu-q antibody bound to PTHrP(15-34) and PTHrP(20-34) was about 53% and 8.5%, respectively, of that which was bound to PTHrP(1-34). Further deletion from the N-terminus of PTHrP(1-34) abrogated the antibody binding; the amount of the hu-q antibody bound to PTHrP(25-34) was only 1.5% of that of PTHrP(1-34). Because the length of the peptide might

A) Heavy chain

	FR1			CDR1	FR2		CDR2	
	1	2	3		4	5		6
Kabat No.	1234567890123456789012345678 90			12345	67890123456789		012A3456789012345	
murine	EVQLVESGGDLVKPGGSLKLSCAASGFTFS			SYGMS	WIRQTPDKRLEWVA		TISSGGSYTYYPDSVKG	
S31679	QVQLVESGGGVVQPGRSLRLSCAASGFTFS			SYAMH	WVRQAPGKGLEWVA		VISYDGSNKYYADSVKG	
huMBC1-H	QVQLVESGGGVVQPGRSLRLSCAASGFTFS			SYGMS	WVRQAPGKGLEWVA		TISSGGSYTYYPDSVKG	

	FR3			CDR3	FR4
	7	8	9	10	11
Kabat No.	6789012345678901 2ABC345678901234			567890A12	34567890123
murine	RFTISRDNAKNTLYLQMSSLKSEDTAMFYCAR			QTTMTYFAY	WGQGTLVTVSA
S31679	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAR			ESRGDY	WGQGTLVTVSS
HuMBC1-H	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAR			QTTMTYFAY	WGQGTLVTVSS

B) Light chain

	FR1		CDR1	FR2	CDR2
	1	2	3	4	5
Kabat No.	12345678901234567890123		4567A8901234	567890123456789	01234ABCD56
murine	QLVLTQSSS-ASFSLGASAKLTC		TLSSQHSTYTIE	WYQQQPLKPPKYVMD	LKQDGSHSTGD
HSU03868	QLVLTQSPS-ASASLGASVKLTC		TLSSGHSSYAIA	WHQQQPEKGPRYLMK	LNSDGSHSKGD
hMBC1-L	QLVLTQSPS-ASASLGASVKLTC		TLSSQHSTYTIE	WHQQQPEKGPRYLMK	LKQDGSHSTGD
hu-q	----------	----------	----------	-Y--------K---D	----------
hu-r	----------	----------	----------	-Y----------D	----------

	FR3				CDR3	FR4
	6	7	8	9	10	
Kabat No.	789012345678901234567890123456 78				9012345ABCD67	890123456A789
Mouse L	GIPDRFSGSSSGADRYLSISNIQPEDEAMYIC				GVGDTIKEQFVYV	FGGGTKVTVLGQP
HSU03868	GIPDRFSGSSSGAERYLTISSLQSEDEADYYC				QTWGTGI	
hMBC1-L	GIPDRFSGSSSGAERYLTISSLQSEDEADYYC				GVGDTIKEQFVYV	FGGGTKLTVLGQP
hu-q	----------I-				----------	----------
hu-r	----------I-				----------	----------

Figure 2. *Amino acid sequences of the humanized anti-PTHrP antibody. Amino acid sequences of the FRs and CDRs in the heavy (A) and light (B) chains of the murine anti-PTHrP(1-34) (hybridoma #23-57-137H) were compared to those of S31679, HSU03868 and humanized antibodies. The heavy chain (huMBC1-H) and light chain (huMBC1-L) of the humanized antibodies were generated by grafting the FRs of the mouse antibody into those of S31679 and HSU03868. In the light chain of version q (hu-q) and version r (hu-r) of the humanized antibodies, only the amino acids that differed from those in huMBC1-L are indicated.*

affect the binding of the hu-q antibody to the peptides, we also examined the binding of the hu-q antibody to PTHrP(5-20), PTHrP(10-25) and PTHrP(15-30), each of which consists of 15 amino acids. The amounts of the hu-q antibody bound to PTHrP(5-20), PTHrP(10-25) and PTHrP(15-30) were 0.5%, 3% and 9.3%, respectively, of that of PTHrP(1-34). These results indicate that the amino acids between 20 and 30 of the PTHrP are essential for the binding of the hu-q antibody.

Effects of the humanized anti-PTHrP antibody on blood iCa concentration. The fact that the ability of the hu-q antibody to neutralize PTHrP(1-34) was almost the same as that of mouse and chimera antibodies prompted us to examine the effect of the hu-q antibody on HHM *in vivo*. The efficacy of the humanized anti-PTHrP antibody against HHM was examined in nude mice carrying the human PAN-7-JCK xenograft (PAN-7 HHM mice). The PAN-7 HHM mice

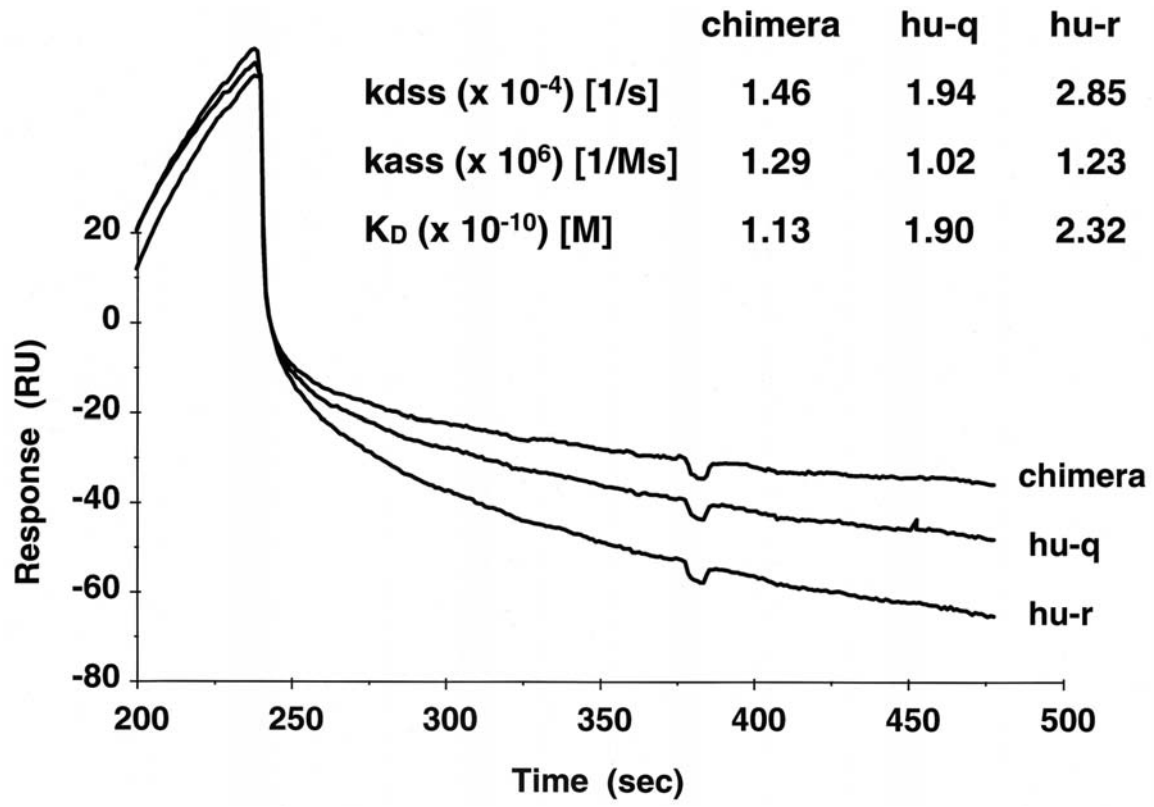

Figure 3. *Kinetic analysis of the binding of the chimera and the humanized antibodies to the human PTHrP(1-34). Affinities to the N-terminal 35 amino acid of PTHrP of the chimera, and version q (hu-q) and version r (hu-r) of the humanized antibodies were determined with BIACORE, in which [Cys^{35}]-PTHrP(1-35) was coupled to the sensor surface. The antibodies and HBS were loaded with a flow rate of 20 μL/min. kass: association rates, kdss: dissociation rates, K_D: dissociation constant.*

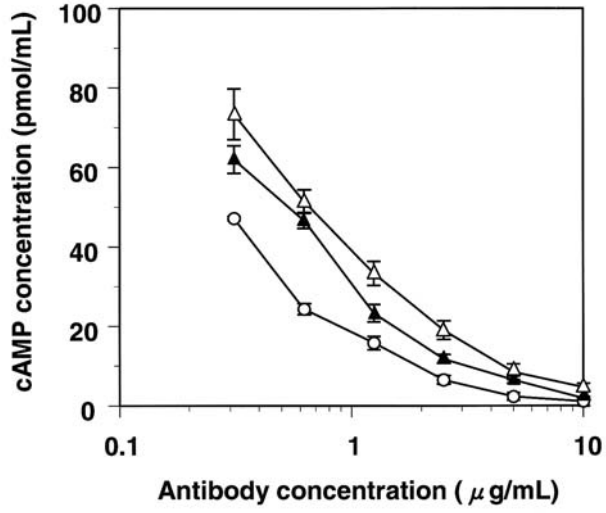

Figure 4. *Abilities of the chimera and the humanized antibodies to neutralize the human PTHrP(1-34). One hundred thousand ROS cells were seeded on a 96-well plate and cultured at 37˚C. After 3 days, the cells were washed with PBS and incubated in 0.5 mM isobutylmethylxanthine at 37˚C for 30 min. Then, 2 ng/mL PTHrP(1-34) together with the indicated concentrations of the chimera antibody (chimera) or the humanized antibody version q (hu-q) or version r (hu-r) was added to the cells and the cells were further cultured at 37˚C for 30 min. Intracellular cyclic adenosine-monophosphate (cAMP) was extracted from the cells, and the amounts of cAMP were determined by an enzyme immunoassay kit. Concentrations of cAMP are indicated as means with standard deviations. ○: chimera, ▲: hu-q, △: hu-r.*

displayed a higher level of blood iCa concentration within 14 days after tumor transplantation. At 21 days post tumor transplantation, the mean concentration of the blood iCa in the PAN-7 HHM mice was 2.18±0.04 mM, which was significantly higher than the control nude mice (1.35±0.02 mM). Single administration of the humanized anti-PTHrP antibody lowered the blood iCa concentration of the PAN-7 HHM mice; the blood iCa level of the mice that received the hu-q antibody was very close to that of the normal control mice 4 days after administration (Figure 6). The efficacy of the hu-q antibody

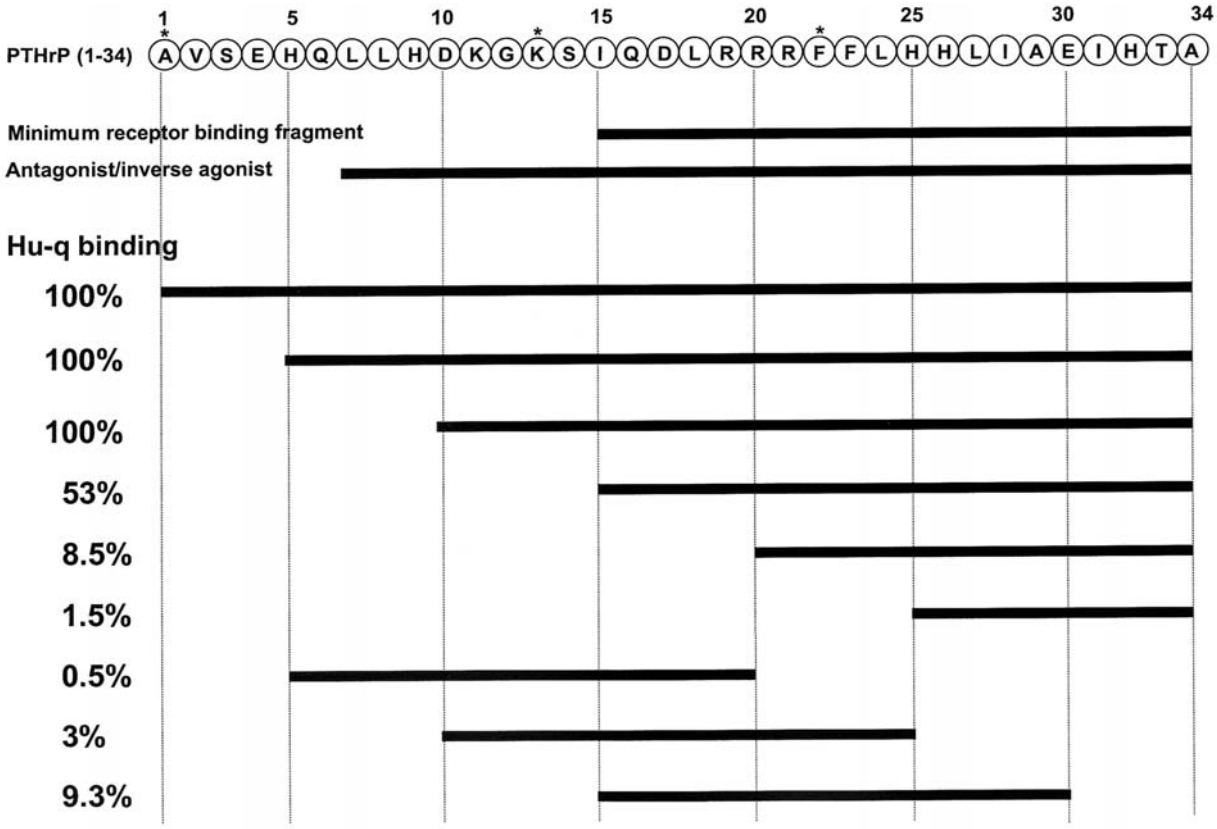

Figure 5. *Region of the human PTHrP recognized by the humanized antibody. The binding activity of the humanized antibody version q (hu-q) was examined with the peptides corresponding to the indicated part of the human PTHrP. The 96-well plates were coated with 2 μM of the indicated peptides, and the peptides were incubated with the 200 μg/mL of the humanized antibody version q (hu-q). After washing and the addition of the alkaline-phosphatase-conjugated second antibody, the amounts of the antibodies bound to the indicated peptides were determined by measuring optical densities at 405/620 nm, which represents alkaline phosphatase activity. Binding activities of the hu-q antibody to the indicated peptides are shown as the percentage of that to PTHrP(1-34).*

against HHM was almost equivalent to that of the chimera antibody: at the same doses, the chimera and the hu-q antibodies lowered the blood iCa concentration to the same extent (Figure 6).

Decrease in the blood iCa level by the hu-q antibody also occurred in another HHM model, and the anti-hypercalcemic effect of the antibody became more evident when the hu-q was repeatedly administered. Nude rats carrying the human LC-6-JCK xenograft (LC-6 HHM rats) also displayed higher levels of blood iCa (2.35±0.07 mM) as compared to the normal control rats (1.40±0.01 mM). The LC-6 HHM rats that received repeated administration of the hu-q antibody at the dose of 3 mg/kg maintained a blood iCa level very close to that of the normal control rats without showing any signs of toxicity (Figure 7).

Discussion

In this study, we selected two different humanized anti-PTHrP antibodies, hu-q and hu-r, by ELISA with PTHrP(1-34). Kinetic analysis with BIACORE revealed that the K_D value of the hu-q antibody to PTHrP(1-34) was slightly lower than that of the hu-r antibody, presumably due to a smaller dissociation rate from the antigen. The hu-q and hu-r antibodies have a similar association rate constant to PTHrP(1-34), but the dissociation rate constant to PTHrP(1-34) of the hu-q antibody was lower than that of the hu-r antibody. Different dissociation rates of the hu-q and hu-r antibodies were also reflected in their ability to neutralize PTHrP. The potency of the hu-r antibody to inhibit PTHrP-dependent cAMP production in ROS cells was weaker than that of the hu-q antibody especially at lower concentrations. From this result, we selected the hu-q antibody as the candidate for clinical applications. The hu-q and the hu-r antibodies share the same heavy chain, but the hu-q antibody harbors a lysine substitution for arginine in the FR2 of the light chain. Thus, it seems likely that the positive charge of arginine in the FR2 plays an important role in the binding of the antibody to PTHrP(1-34).

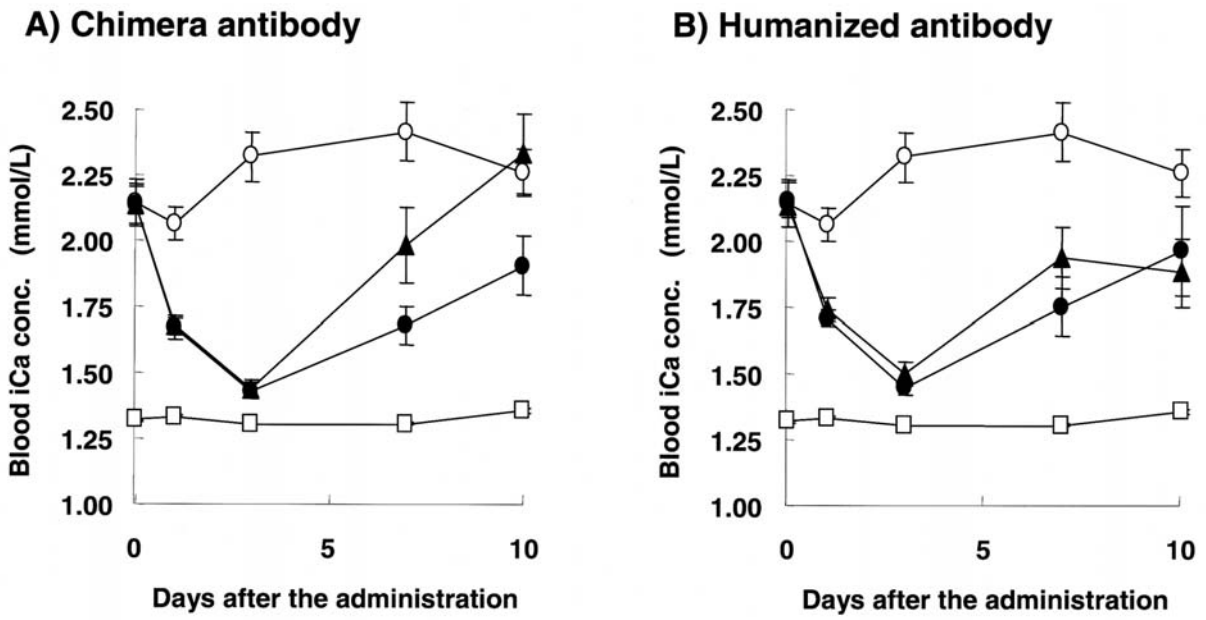

Figure 6. *Effects of the chimera and the humanized antibodies on HHM in mice bearing the PAN-7-JCK xenograft. The cells of the human pancreatic carcinoma PAN-7-JCK were subcutaneously transplanted into nude mice. After the mice developed hypercalcemia, 10 or 30 μg per mouse of the chimera antibody (A) or humanized antibody (B) version q (hu-q) was intravenously administered. Blood iCa concentrations were determined 0, 1, 3, 7 and 10 days after the administration. The vehicle control mice received the same volume of PBS; the non-tumor-bearing mice did not receive any treatment. ○: Vehicle control, ▲: 10 μg/mouse of antibody, ●: 30 μg/mouse of antibody, □: non-tumor-bearing nude mice (no administration). The blood iCa levels are shown as the mean with standard error of the 7 mice.*

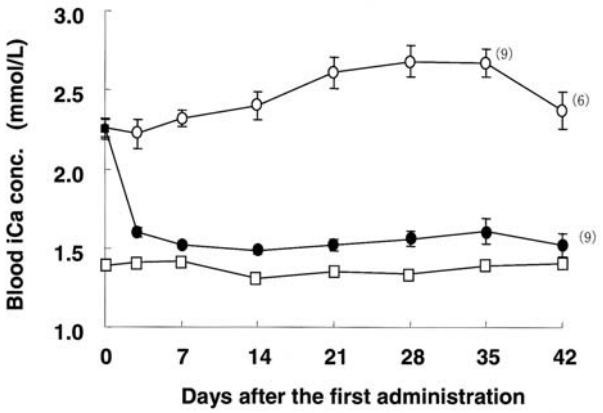

Figure 7. *Effects of the humanized antibody on HHM in rats bearing the LC-6-JCK xenograft. Cells of the human large cell lung carcinoma LC-6-JCK were subcutaneously transplanted to nude rats. After rats developed hypercalcemia (about 60 days after transplantation), the humanized antibody version q (hu-q) was administered once a week for 6 times at the dose of 3 mg/kg. The blood iCa concentrations were determined 0, 3, 7, 14, 21, 28, 35 and 42 days after the first administration. The vehicle control rats received the same volume of PBS; the non-tumor-bearing rats did not receive any treatment. The blood iCa levels are shown as mean values of the surviving rats (12 rats for all symbols without a number or the indicated number of rats for symbols with a number).*
○: Vehicle control (PBS), ●: hu-q 3 mg/kg/weeks, □: non-tumor-bearing nude rat

The amino acids between 20 and 30 of PTHrP appeared to be essential for the binding of the hu-q antibody. The binding activity of the hu-q antibody to PTHrP(15-30) and to PTHrP(20-34) was more or less the same, but it was about one fifth that of to PTHrP(15-34). Thus, though not essential, amino acids between 15 and 20 and those between 30 and 34 of PTHrP also contribute to the antibody binding, *e.g.*, for the appropriate 3-D structure to be recognized by the antibody. PTHrP(15-34) has been shown to be the minimal receptor binding fragment, whereas PTHrP(7-34) acted as an antagonist to PTH1R (23). Furthermore, the crosslink of PTHrP and PTH1R demonstrated that the Ala1, Lys13 and Phe23 of PTHrP directly interacted with PTH1R, and mutation analysis revealed that the Trp substitution for Phe23 conferred the ability of PTHrP to bind to PTH2R (24). These facts indicate that the region between amino acids 15 and 34 of PTHrP is essential for the binding to PTH1R and that the Phe23 of PTHrP plays an important role in the selectivity and binding to PTH1R. Thus, the hu-q antibody is likely to block the function of PTHrP by masking its receptor binding region.

The hu-q antibody was capable of reducing the blood iCa level in mouse and rat HHM models. Single administration of the same doses of the chimera or the hu-q antibody equally reduced the blood iCa level. The potency of the hu-q antibody in reducing the blood iCa level was more or less

the same as that of the chimera antibody. In addition, animals with HHM sustained a normal level of blood iCa levels without showing any signs of toxicity when they received repeated administrations of the hu-q antibody. Therefore, the hu-q antibody can be beneficial for cancer patients with HMM.

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