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 $1.90 \times 10^{-10}$ 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)$_2$D$_3$ (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-cachectic 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.
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 HincII-Bln1 DNA fragment containing the variable region of the light chain was cloned at the HincII-Bln1 cleavage site of CpUC19, in which the constant region of the light chain of human IgG1 was cloned (14), to generate MBC1L(pUC19). Then, MBC1L(pUC19) was digested with EcoRI, 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 human IgG1 was cloned at the HincII-PvuII cleavage site of CpCHO1 that harbored the promoter for the human elongation factor EF1α gene (15).

The EcoRI-Sma1 DNA fragment containing the variable region of the heavy chain was cloned at the EcoRI-Sma1 cleavage site of pUC19, generating MBCHv(pUC19). Then, the ApaI-BamHI DNA fragment containing the constant region of the heavy chain of human IgG1 (16) was ligated at the ApaI-BamHI cleavage site of MBCHv(pUC19). The resulting plasmid was digested with BamHI and EcoRI, and the BamHI-EcoRI DNA fragment containing the variable region of the heavy chain linked to the constant region of the heavy chain of human IgG1 was cloned at the BamHI-EcoRI 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.

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 HincII-Bln1 DNA fragment containing the variable region of the light chain was cloned at the HincII-Bln1 cleavage site of CpUC19, in which the constant region of the light chain of human IgG1 was cloned (14), to generate MBC1L(pUC19). Then, MBC1L(pUC19) was digested with EcoRI, 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 human IgG1 was cloned at the HincII-PvuII cleavage site of pCHO1 that harbored the promoter for the human elongation factor EF1α gene (15).

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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 HSC05368 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 MBCLVS1 and MBCLVR1 primers. The resulting DNA fragments were cloned at the BamHI-HindIII cleavage site of pUC19. After confirming the nucleotide sequences, the HindIII-BstI DNA fragment was ligated at the HindIII-BstI cleavage site of Cα/pUC19, generating hMBC1Lα/pUC19. The EcoRI fragment encoding the light chain of the humanized antibody was excised and ligated at the EcoRI site of pCHO1 to generate hMBC1Lα/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 chromatography. The concentrations of antibodies were determined by ELISA with human or mouse IgG1 as a standard. Chromatography. The concentrations of antibodies were 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 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-pyridyldithio)ethanethiolamine (PDEA). Then, [Cys53]-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 CMS, HBS, amine-coupling kit and PDEA thiol-coupling kit were purchased from BIACORE AB (Sweden). The synthetic peptide [Cys53]-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-J-7C) and a lung cancer cell line (LC-6-JCK) were purchased from the Central Institute for Experimental Animals (Kawasaki, Japan). The PAN-J-7C 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/cJcl-nu) (for PAN-J-7C) or nude rats (F344/N Jcl-rcu) (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 fromCLEA (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
versions of humanized anti-PTHrP antibodies. To select the optimal humanized antibody, we first examined the binding activities to PTHrP(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 [Cys35]-PTHrP(1-35) with an association rate ($k_{ass}$) of 1.29 x 10^6 M$^{-1}$s$^{-1}$ and a dissociation rate ($k_{diss}$) 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 $k_{diss}$ 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 $K_D$ 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
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...
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 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 BLACORE, in which [Cys35]-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.
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).
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.

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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.

References