Tumor targeting of humanized fragment antibody secreted from transgenic rice cell suspension culture

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Abstract The tumor-associated glycoprotein 72 (TAG 72) has been shown to be expressed in the majority of human adenocarcinomas. In an effort to develop a technique for the safe and inexpensive production of large quantities of anti-TAG 72 humanized antibody fragments (hzAb) as a future source of clinical-grade proteins, we developed a transgenic rice cell suspension culture system. The in vivo assembly and secretion of hzAb were achieved in a transgenic rice cell culture under the control of the rice alpha amylase 3D (RAmy 3D) expression system, and the biological activities of plant-derived hzAb were determined to be quite similar to those of animal-derived antibody. Purified hzAb was shown to bind to the recombinant antigen, TAG 72, and to bind specifically to human LS 174T colon adenocarcinoma cells expressing the TAG 72 antigen, and this binding occurred to the same extent as was seen with animal-derived antibody. Plant-derived hzAb proved as effective as animal-derived antibody in targeting tumors of xenotransplanted LS 174T cells in nude mice. The results of this study indicate that the hzAb derived from plant cell suspension cultures may have great potential for pharmaceutical applications in the development of future cancer therapeutic and diagnostic protocols.

Keywords hzAb · RAmy 3D · TAG 72

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Introduction

Monoclonal antibodies are unique and versatile molecules, and are associated with many applications in research, diagnosis, and the treatment of a variety of diseases, including cancer. Their profound affinity and specificity render them invaluable in both diagnostic and therapeutic applications. However, murine mAbs are rather limited with regard to their utility as therapeutic agents, owing primarily to their short half-life, in addition to their inability to trigger the human anti-mouse antibody (HAMA) response (Colcher et al. 1990).

In this report, we have evaluated the utility of humanized antibody fragments, including Fab and F(ab′)$_2$, which were found to be advantageous in that they offer improved clinical pharmacokinetics, due to their smaller size and reduced immunogenicity (attributable to the absence of an Fc domain; Hudson 1999). These fragments may also evidence more homogeneous tumor penetration, which may circumvent the problems of inaccessibility/poor penetration into tumor masses, and may also display improved specific tumor retention associated with multivalency (Colcher et al. 1999; Olafsen et al. 2004). However, their rapid clearance from the blood can often result in a lower absolute uptake within the tumor. Hence, these antibody fragments are frequently considered more suitable for diagnostic imaging, rather than as therapeutic reagents, particularly because their rapid clearance from background tissues

allows for the early imaging of patients/tumors after the administration of the radioconjugate. A critical step in the evaluation of the potential therapeutic utility of these molecules, then, is the development of a reproducible and efficient technique for large-scale antibody production.

Plants are potentially the most economical system available for the large-scale production of recombinant antibodies (Girard et al. 2006). Like microbes, plant cells are inexpensive to grow and maintain, but because they are higher eukaryotes they can carry out many of the post-translational modifications that occur in human cells (Cabanes et al. 1999). Plant cells are also intrinsically safe, as they neither harbor human pathogens nor generate endotoxins. Plants are capable of synthesizing complex multimeric proteins and glycoproteins, including immunoglobulin (Vaquero et al. 2002). The most important advantage of plant cells over whole plants is that the procedures for the isolation and purification of the product are much simpler (Hellwig et al. 2004), particularly in cases in which the product is secreted into the culture medium. In this work, we have attempted to evaluate the inducible expression of hzAb specific to tumor-associated glycoprotein 72 (TAG 72).

TAG 72 is expressed in the majority of human adenocarcinomas occurring within the colon, ovary, pancreas, breast, and lung, but is not expressed in most normal tissues, with the exception of the endometrium during the secretory phase (Thor et al. 1986). A murine mAb B72.3 that binds specifically to TAG 72 has been approved for in vivo imaging in patients suffering from ovarian and colorectal cancers. A second generation antibody to B72.3, CC49 (Muraro et al. 1988), which has been shown to evidence a higher degree of affinity for TAG 72 than does B72.3, has been shown to efficiently target a variety of carcinomas in clinical trials (Divgi et al. 1995). A recombinant anti-TAG 72 antibody has recently been engineered, and the expressed proteins have been characterized and evaluated for use in both diagnostic and therapeutic applications (Yoon et al. 2006). Despite these recent developments, it is clear that strategies for the treatment of tumor patients will require bulk quantities of the most effective molecules, most notably Fab and F(ab')$_2$.

The α-amylase is an essential enzyme for starch hydrolysis during the germination of cereal grains. The expression of α-amylase genes in germinating cereal grains is induced by both the phytochrome gibberellin and by sugar starvation, and in cultured rice suspension cells they are activated by sugar starvation and repressed by sugar provision (Yu et al. 1991, 1992). R*Amy* 3 and R*Amy* 8 are the two most highly expressed α-amylase genes in sucrose-starved rice suspension cells, with a transcription rate of R*Amy* 3 approximately fourfold that of R*Amy* 8 (Sheu et al. 1996). The R*Amy* 3 promoter and signal sequence were

also utilized for the production of recombinant human α$_1$-antitrypsin (rAAT) and human granulocyte macrophage colony stimulating factor (hGM-CSF) in sucrose-starved rice suspension cells, and functional rAAT and hGM-CSF were secreted into the culture medium, with yields of up to 200 and 129 mg/l (Terashima et al. 2001; Shin et al. 2003), respectively. The R*Amy* 3 promoter and signal sequence employed for the expression of a human serum albumin (HSA) resulted in the accumulation of this recombinant protein in a bioreactor scale culture, with yields of up to 76.4 mg/l (Huang et al. 2005). The rice R*Amy* 3D expression system and rice cell culture represent a powerful system for the production of recombinant proteins as human therapeutic agents.

Materials and methods

Construction of the plant expression vector

The hzAb, affinity enhanced antibody, was prepared from humanized anti-TAG-72 monoclonal antibody (AKA) by randomization of amino acid in 95–99 residues of heavy chain complementary determinant regions (HCDRs)-3 using phage displayed library technology (kindly provided by Dr. H. J. Hong at KRIBB). The cDNA for the humanized antibody TAG-72 heavy chain (815-bp) and light chain (685-bp) fragments was cloned into pGEM-T vector (Promega, WI). In order to create restriction sites for cloning, forward and reverse primers containing the *Kpn*I and *Sac*I restriction sites for the heavy chain (5′- TAG GTA CCA GGT CCA GCT AGT GCA GTC T -3′ and 5′- CCG AGC TCT AGG GGT TGA AGT CCC AA -3′) were designed. PCR of the light chain gene was conducted with forward and reverse primers harboring the *Hinc*II and *Sac*I restriction sites (5′- AAG TCG ACA TTG TGA TGA CCC AGT CTC C -3′ and 5′- AAG AGC TCT CTA GAA TTA ACA CTC TCC -3′). Degenerate primers specific to the nucleotide sequences corresponding to each chain of the hzAb, but lacking its signal peptide, were also designed. The resultant 815-bp and 685-bp PCR fragments were cloned into pGEM-T vector (Promega, WI) to generate pMYL200 for the heavy chain and pMYL203 for the light chain. The nucleotide sequence of the cloned gene fragment was determined with an ABI v3.7 DNA sequencer (ABI, Forster City, CA, USA). These DNA fragments harboring the R*Amy* 3D promoter and the signal peptide coding sequence (Toyofuku et al. 1998) were then amplified using the following primers: 5′- GAG CAT GCA CCA CCT GTG CTA GCT ACT CCA CTG -3′ and 5′- AAC TGC AGC CGT GTT CGA TAG TGA GTT G -3′. The PCR product was digested with *Sph*I and *Pst*I and subcloned into the pUC18 multiple cloning sites. The resultant

plasmids, pMYN200 and pMYN203, were digested with KpnI and SacI for the heavy chain and with HincII and SacI for the light chain, in order to excise the gene for the hzAb chain, which was then subcloned into the PstI (blunted with T4 DNA polymerase)—SacI site of pMYN34. The resultant plasmids, pMYN206 and pMYN207, were subsequently digested with HindIII and EcoRI, and the DNA fragments harboring each chain of hzAb with the RAmy 3D promoter and signal peptide coding sequence were inserted into the binary vector.

Rice transformation

Scutellum-derived callus tissue from mature rice seed (Oryza sativa L. japonica cv. Dongjin) was transformed via particle bombardment, as described previously (Sudhakar et al. 1998). Gold particles (0.6 μm) were coated with 10 μg of each of the hzAb plasmids at a 1:1 molar ratio. The method by which the DNA-coated gold particles were prepared for bombardment was described previously (Christou et al. 1991). Four weeks after bombardment, hygromycin-resistant callus tissue was recovered. The callus lines were subcultured twice (at 14-day intervals) on selective media.

Establishment, propagation, and induction of transgenic rice suspension culture

Calli expressing high levels of the transgene were then employed in the establishment of the cell suspension cultures. The calli were cultured on N6 + S medium (Thompson et al. 1986) containing 2,4-dichlorophoenoxyacetic acid (2 mg/l), kinetin (20 g/l), and 3% sucrose for approximately 1.5 months, until friable calli could be obtained. The friable calli were then selected and propagated for an additional 10–15 days. The suspension cell cultures were then established via the transfer of the friable calli to 50 ml of N6 liquid medium in a 300 ml flask. Ten millilitres inocula passed every 9 days for subculturing. The cells were cultured in a shaker at $110 \times g$, and incubated in darkness at 25°C.

To induce hzAb expression, the N6 + S medium was removed for the cell suspension by aspirating, and the cells were transferred to fresh N6-S medium at 10% (weight of wet cells/volume of N6-S medium density). All experiments were performed in duplicate.

Sample preparation, SDS-PAGE and Western blot analysis

The medium from induced rice cells was collected by pouring induced cell suspension through 2–3 layers of myracloth (CalBiochem). Cell clumps were retained by myracloth and relatively clear induced medium was collected in a beaker. The medium was then subjected to filtration through two layers of 8 μm (Whatman paper #2), two layers of 1.2 μm Glass Micro fiber Filters (Whatman NJ, USA), and one 0.45 μm nitrocellulose membrane filter sequentially to obtain hzAb containing medium.

The samples were then separated on 10% SDS-PAGE (Laemmli 1970) under non-reducing and reducing conditions, and then transferred to Hybond-C Extra transfer membranes (Amersham Pharmacia; Towbin et al. 1979). The membranes were then incubated in blocking solution [5% (w/v) non-fat dried milk in TBST buffer (20 mM Tris-Cl, pH 7.5, 500 mM NaCl, and 0.05% Tween 20)], followed by goat anti-human mAb [F(ab′)₂-specific] conjugated with alkaline phosphatase (Promega, WI) for detection of the hzAb chains. Chinese Hamster Ovary (CHO) cell-derived antibody (provided by Dr. HJ Hong) was utilized as a positive control.

Purification of hzAb

The supernatants filtrated from the transgenic rice cell culture were diluted to a concentration of 1 in 10 with equilibration buffer (20 mM sodium phosphate, pH 7.0), then applied to a Hi Trap Protein G affinity column (Amersharm Pharmacia; 5 ml bed volume, 1.6×2.5 cm) which had been pre-equilibrated with equilibration buffer (100 ml) at a flow rate of 1.5 ml/min. After loading, the column was washed with equilibrated buffer (20 column volume) until a baseline absorbance of 280 nm was achieved. Elution was then conducted stepwise with 0.1 M glycine-HCl (pH 2.7). The eluted fractions were dialyzed against PBS buffer (pH 7.4) after the pH had been raised to neutrality via the addition of 1 M Tris–HCl (pH 9.0), then stored at 4°C. In order to separate Fab and F(ab′)₂, gel filtration chromatography was conducted on a Sephadex G75 column (Amersham Pharmacia) with a mobile phase of 20 mM sodium phosphate buffer, pH 7.0, at a flow rate of 1 ml/min. The concentrations of the purified Fab and F(ab′)₂ proteins were spectroscopically determined using extinction coefficients at 280 nm of $\varepsilon = 1.53$ and $\varepsilon = 1.48$ for a 1 mg/ml solution in a 1 cm cell (Gill and von Hippel 1989).

Binding analyses of hzAb

The binding of the transgenic rice cell culture-secreted hzAb to the TAG 72-positive bovine submaxillary gland mucin (BSM) (Type I-S; Sigma, St. Louis, MO) was evaluated via ELISA. In order to determine the reactivity of the hzAb to TAG 72, 1 μg per well of BSM was coated on the individual wells of 96-well plates (Nunc Maxisorp ImmunoTM plates, Rochester, NY). These plates were

blocked with 5% BSA in PBS for 1 h at 37°C, and then washed with PBST buffer (0.01% Tween 20 in PBS). The test samples were incubated for 2 h at room temperature in twofold serial dilutions. After washing, 100 μl of alkaline phosphatase-conjugated anti-human IgG (H + L specific, Promega, WI) diluted to 1:3,000 in PBS, were added per well. The plates were then incubated for 2 h at 37°C. The *p*-nitrophenyl phosphate tablets were used as the substrates, and the colorimetric reaction was allowed to proceed for 10 min at room temperature in darkness. Absorbance was read at a wavelength of 405 nm, using a Microplate reader.

A TAG 72-expressing human colon carcinoma cell line; LS 174T (ATCC CL188) was employed in this experiment. The human osteosarcoma cell line, HOS, was used as a negative control because it expresses a noncross reactive antigen even though it is highly homologous to TAG 72. Each of the cell lines were washed in phosphate buffered saline (PBS) and detached via 10 min of incubation with 10 mM EDTA in PBS. The cells were washed in 2% fetal bovine serum (FBS) in PBS. A total of 1×10^6 cells were incubated for 2 h in 1 ml of PBS containing 1:10 diluted antibodies (original concentration was 100 μg/ml) at 4°C. Subsequently, the cells were washed twice in cold PBS buffer with FBS and incubated for 2 h on ice with PE-conjugated goat anti-human IgG antibody (Santa Cruz, CA). The cells were then washed twice in cold PBS containing 0.5% bovine serum albumin, and then incubated for 10 min with fixing solution (1% paraformaldehyde in PBS) at 4°C. Finally, the cells were analyzed using a FACScan system (Becton Dickinson, Mountain View, CA), using CellQuest software for Macintosh. Data were obtained via the analysis of 10,000 cells.

Radiolabeling of hzAb

The Iodo-Bead method (Pierce Biotechnology, Rockford, IL) was used for the radiolabeling of the purified hzAb antibodies with [125]I (Amersham Pharmacia) or [123]I (Korea Atomic Energy Research Institute). The radiolabeled antibodies were purified via gel filtration on a PD-10 column (Amersham Pharmacia) and sterilized via filtration (0.22 μm, Millipore Billerica, MA). The radiolabeling yield and radiochemical purity were determined with Instant thin layer chromatography-silica gel (Gelman Scientific) in the stationary phase and 70% methanol in the mobile phase.

Biodistribution and tumor imaging of hzAb

Female athymic mice (BALB/c-nu/nu; 5–6 weeks old; 17–23 g) were obtained from Japan SLC, Inc. The tumors were grown after s.c. injection of 5×10^6 human colon adenocarcinoma cells (LS 174T) into the left thigh. After 14 days, ^{125}I-labeled antibodies (20 μg per 740 KBq) were injected into the tail vein of the athymic mice bearing the LS 174T tumors. For each time point, a group of three mice was sacrificed for the collection and weighing of blood, tumors, and organs. Radioactivity was assessed using a gamma scintillation counter. The percentage of the injected dose per gram of tissue (% ID/g) was calculated for each organ.

Tumor imaging was conducted with the LS 174T tumor-bearing mice, which had been injected into the tail vein with ^{123}I-labeled antibodies (20 μg/740 KBq), and tumor images of the radiolabeled antibodies were acquired at 2, 6, and 24 h post injection, using a gamma camera (TRIAD TXLT-20, Trionix, OH, USA).

Results

hzAb construct

A 60 independently transformed rice calli were regenerated. Of these, six were initially screened and tested positive via Western blotting of the induced cell extracts of transgenic rice cells. The highest producer line based on Northern and Western blot analyses was selected for further analysis (data not shown). To confirm secretion of hzAb from transgenic rice cell suspension culture, Western blot analysis was conducted (Fig. 1). After induction, hzAb was secreted into the medium with other native rice proteins, principally the α-amylases. As a positive control, the hzAb Fab fragment antibody generated from the CHO cell line is shown (lane PC). In this sample, with the exception of the expected 25 kDa, the production of dimers and higher aggregates of hzAb is not evident. Plants expressing hzAb evidenced higher molecular weight hzAb bands at around 30 kDa, which slightly differed from the predicted MW of hzAb (Fig. 1, lane T), but these bands were not present in the wild-type culture supernatants (Fig. 1, lane N). This difference in size might be caused by slightly different conformation such as different glycosylation in animal and plant cell. The slight higher weight bands was also detected in the rice callus extracts, which indicates that the plant expressed hzAb existed almost wholly in those two forms. When the plant-derived hzAb was diluted to yield a 25 kDa band of intensity equal to the positive control sample, the doublet bands corresponding to hzAb were still clearly detectable in the rice callus extracts (data not shown).

Purification of hzAb

For the affinity purification of the hzAb proteins, the transgenic rice cells producing hzAb were cultured in sucrose-free medium and the culture supernatant were
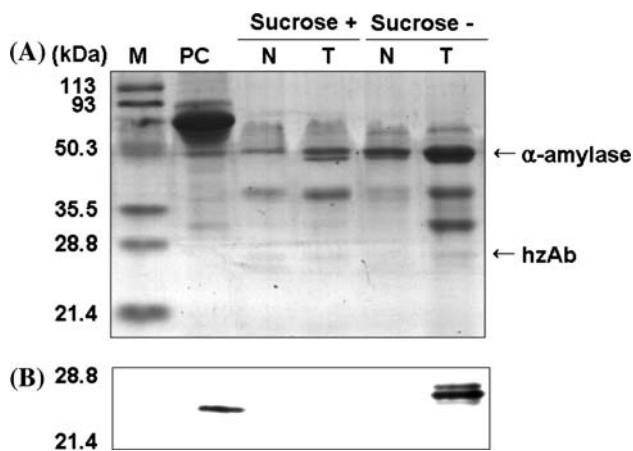
Fig. 1 SDS-PAGE (**A**) and Western blot (**B**) of culture supernatants of hzAb-transformed rice suspension cells. Culture supernatants were run on a 10% polyacrylamide gel under reducing conditions and probed with anti-human mAb. Lanes M: protein molecular weight standards. Lane PC: culture supernatants of CHO cells expressing Fab. Lane N: culture supernatants of wild-type rice suspension cells. Lanes T: culture supernatant of transgenic rice cell expressing hzAb (20 µl of culture supernatants). S+/−: culture medium with/without sugar

subjected to an affinity chromatography on a Hi Trap Protein G HP column (Proudfoot et al. 1992). The quality of the purified antibody was analyzed by 10% SDS-PAGE with silver staining and Western blot analysis on nonreducing conditions. The identities of approximately 40 and 120 kDa bands were confirmed by Western blotting using anti-human mAb [F(ab′)2-sepecific] antibody (Fig. 2). Recovery of protein was determined after concentration using absorbance measurements at 280 nm (Table 1). Ninety seven percent of the total proteins were recovered

after protein G affinity column. The proportion of Fab compared to F(ab′)2 was 3–7 indicating more than double amount of F(ab′)2 to Fab are present.

Further analysis of the purified sample on Sephadex G 75 gel filtration column (Fig. 2) revealed that samples from concentrated F(ab′)2 as a major peak at an apparent molecular weight of 120 kDa and from the remaining protein at 40 kDa being Fab (Fig. 2). After separation by Sephadex G 75 gel filtration column, Fab and F(ab′)2 are in the ratio of 6:4. This result showed that F(ab′)2 fragment were more easily degraded during gel filtration than Fab fragment.

Mucin binding of hzAb

The functional activity of plant-derived hzAb was evaluated via mucin binding ELISA. All transgenic rice suspension culture supernatants tested demonstrated binding to soluble mucin in a concentration-dependent manner. Figure 3 shows the mean and standard deviation of the highest producer cell line as compared to a titration curve for CHO cell-generated Fab. There was quite big variation in the level of expression in the first screening. However, the six cell lines, which were used in this experiment, were selected from the first screening for highly producing cell lines and their expression levels are with relatively small variation. The absorbance values of plant-derived hzAb were significantly lower than those of the Fab positive control, but the hzAb (3 µg/ml) purified from the transgenic rice cell culture with the highest intensity of hzAb protein bands evidenced significantly greater absorbance ($P < 0.05$) up to a dilution of 1:500. No binding of culture supernatants of the rice suspension cells to
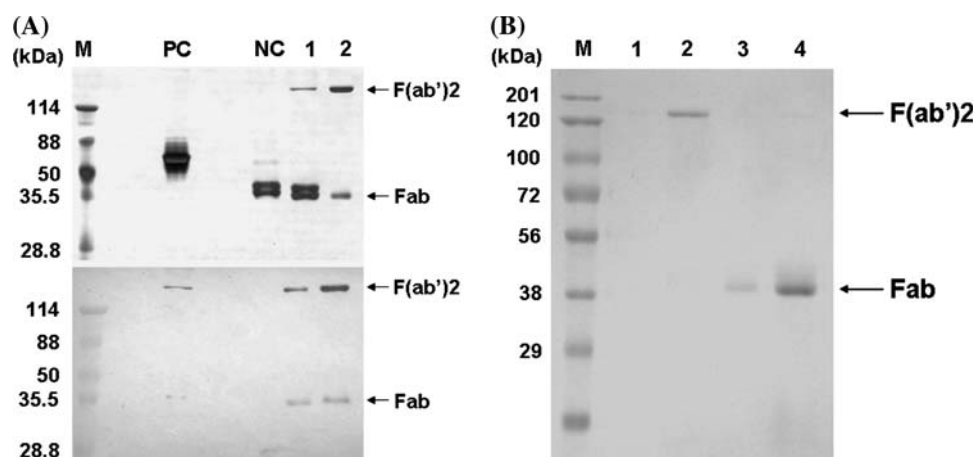

Fig. 2 Non-reducing SDS-PAGE and Western blot analysis of the hzAb purified by using Protein G affinity column (**A**) and Sephadex G 75 gel filtration column (**B**). The protein were separated electrophoretically on nonreducing 10% polyacrylamide gel. Lane M: protein molecular weight standards. Lane PC: culture supernatants of CHO cells expressing Fab. Lane NC: culture supernatants of wild-type rice suspension cells. (**A**) Lane 1: culture supernatant of transgenic rice

cell expressing hzAb (20 µl of culture supernatants). Lane 2: the purified hzAb (30 ng, 0.3 µg/ml) by protein G affinity column. (**B**) Lane M: protein molecular weight standards. Lanes 1, 3: Before concentration, the purified hzAb separated by using Sephadex G 75 gel filtration column. Lanes 2, 4: After concentration, the purified hzAb separated by using Sephadex G 75 gel filtration column

Table 1 Purification of hzAb from cultured medium

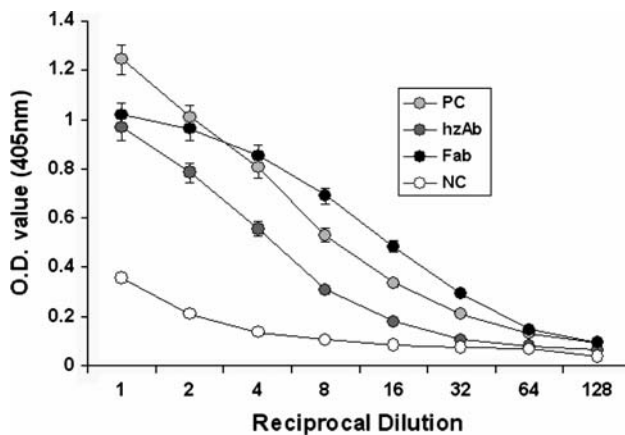| Fraction	hzAb	F(ab')2 (mg)	Fab	Recovery (%)
Transgenic rice cell supernatant	30			
Flowthrough	0			
Protein G affinity column		20.37	8.73	97
Sephadex G 75 size filtration column		5.2	7.8	63.8

Fig. 3 Mucin binding of culture supernatants of transgenic rice suspension cells measured by ELISA. Culture supernatants from CHO cells expressing Fab (100 ng/ml) and their twofold dilutions were added to the 100 μg mucin coated plate. The hzAb purified from culture supernatant of transgenic rice cell expressing hzAb by Protein G affinity column and Fab separated from purified hzAb by Sephadex G 75 gel filtration column (3 μg/ml) and NC were coated to the plate, respectively

a control protein, BSA, was detected. The culture supernatants of the wild-type rice suspension cells did not evidence binding to mucin, thereby confirming that all activity was attributable to the hzAb generated by the plants, and was not caused by cross-reactive native plant proteins.

Anti-TAG 72 activity of hzAb

In the analysis of in vitro specific binding activity, hzAb was applied to FACS with the LS174T human colon carcinoma cell line and human osteosarcoma cell (HOS) was used as a negative control cell line (Fig. 4). The figure revealed that peak shift for plant hzAb was as much as that for animal hzAb when allowed reacting with the LS174T cells, and the detected level was similar to that observed with animal hzAb. No reactivity of either hzAb with the HOS cells could be detected.

In vivo tumor targeting study of hzAb

The tumor-targeting ability of the hzAb and whole IgG were evaluated in athymic mice harboring human colon adenocarcinoma xenografts. The $^{125}$I-IgG and ^{125}I-hzAb were injected into the mouse model, and their biodistribution characteristics were assessed at 4, 24, 48, and 72 h postinjection. The %ID/g of the tumor and normal tissues are shown in Fig. 5. The hzAb F(ab')$_2$ and whole IgG evidenced tumor localization, peaking at 6 and 24 h, with tumor uptakes higher with the whole IgG than with the hzAb fragments at all tested time points. In addition, the quantity of ^{125}I-hzAb fragments accumulated within the tumor decreased in a time-dependent manner, whereas the quantity of ^{125}I-whole IgG within the tumor was largely maintained throughout the test period. At 4 h postinjection, the tumor uptake of ^{125}I-Fab and ^{125}I-F(ab')$_2$ was approximately 29% and 39%, respectively, of that of ^{125}I-IgG (7.875%ID/g). Maximum tumor uptakes of 3.260 and 1.438%ID/g were achieved at 6 h for hzAb F(ab')$_2$ and hzAb Fab, respectively. This is probably attributable to the short plasma half-life and the lower binding affinity of the hzAb fragments, as compared to that of the whole IgG. These results indicated that the hzAb fragments generated from the transgenic rice cell suspension cultures evidenced significant binding affinity, both in vitro and in vivo, as compared with the native IgG.

Gamma camera images of the ^{123}I-labeled hzAb fragments and IgG were acquired at 2, 6, and 24 h postinjection in the TAG 72-expressing LS 174T xenograft tumors. Images acquired from representative mice at 24 h are shown in Fig. 6. At 2 and 6 h post-injection, the visualization of tumor uptake by all the preparations was obscured, as the result of the prominent signals observed in the blood and kidneys (data not shown). By 24 h, the images of ^{123}I-labeled hzAb F(ab')$_2$ in the athymic mice harboring human colon adenocarcinoma xenografts (Fig. 6, middle) demonstrated less activity in the tumor tissues than was observed with the whole ^{123}I-labeled IgG (right). By way of contrast, the images of ^{123}I-labeled hzAb Fab (left) proved unsatisfactory due to extensive kidney activity, as compared to hzAb F(ab')$_2$ and the whole IgG (Fig. 5). In all of the above studies, consistent results were obtained within the mouse groups.

Discussion

The fast-moving field of recombinant antibody engineering and expression continuously opens up new opportunities,

Fig. 4 FACS analysis of hzAb on the human colon carcinoma cell. Both forms of hzAb were applied to different cell lines with anti-human IgG conjugated PE. NC: treatment of PE-goat anti-human IgG antibody only. Animal hzAb: incubation of culture supernatants of CHO cells expressing Fab (300 ng, 2 μg/ml). Plant hzAb: incubation of culture supernatants of the purified hzAb (150 ng, 3 μg/ml) from the transgenic rice suspension cells. LS 174T: human colon carcinoma cell line expressing TAG 72 antigen. HOS: human osteosarcoma cell line used as a negative control
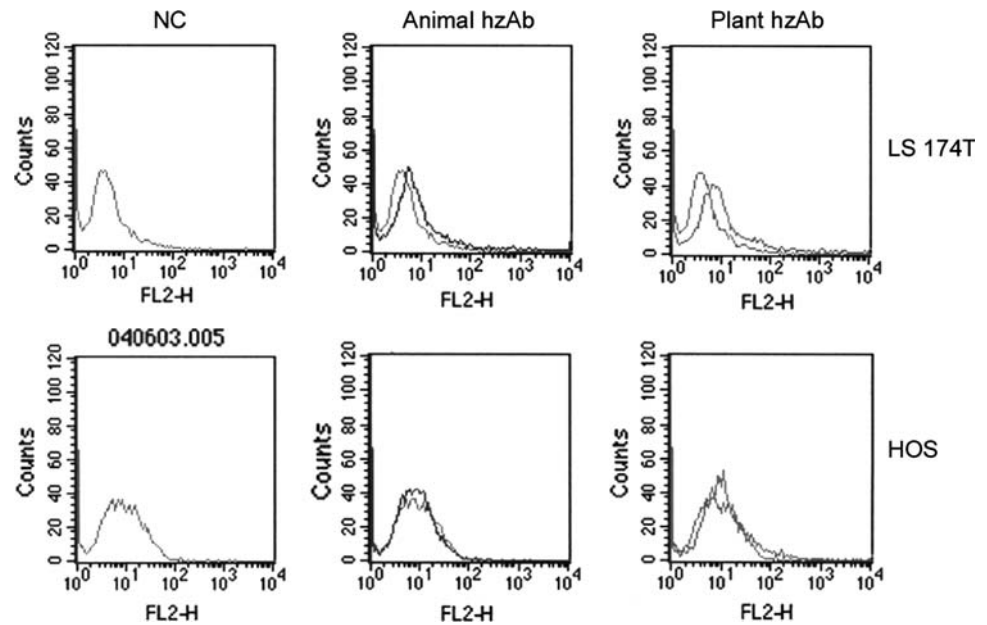
not only for the medical sciences, but also for applied and fundamental agronomic research. Many reports to date describe expression of model antibody molecules, or antibodies rose against endogenous plant antigens such as phytochrome A (Owen et al. 1992) and ABA (Artsaenko et al. 1995), or viral proteins (Tavladoraki et al. 1993). The production of a therapeutic full-size antibody in tobacco has also been reported (Ma et al. 1995).

The other plant-derived antibody currently in phase II clinical trials is CaroRx, a chimeric secretory IgA/G produced in transgenic tobacco plants (Ma et al. 1995, 1998; Larrick et al. 2001). An antibody against carcino-embrionic antigen (CEA) has recently been expressed in rice (Stoger et al. 2000) and rice cell culture (Torres et al. 1999).

In the present study, we report for the first time the production of the humanized antibody specific for the TAG 72 in the transgenic rice cell suspension culture system. The antigen binding activity of the recombinant hzAb was tested; hzAb was produced from induced *Oryza sativa* rice calli and purified by protein G affinity chromatography. The hzAb was measured for its antigen binding specific activity in vitro and in vivo. In a mouse model, we investigated the efficiency of hzAb to act as a potential radio-pharmaceutical for the treatment of cancer.

Fab and F(ab')$_2$ are different in Ag binding avidity because of their different structure. Fab and F(ab')$_2$ can be used as diagnostic agent or therapeutic drug depending on their Ag binding affinity because they have different half life to tumor in radio-immunotherapy. In order to investigate the possibility to use plant-based Fab and F(ab')$_2$ as radio-immunotherapeutic agent, they were examined separately.

Here, we selected an inducible rice R*Amy* 3D expression system to enhance the productivity of hzAb by sucrose starvation (Huang et al. 2005). This R*Amy* 3D expression system combines the high expression levels due to the strong inducible R*Amy* 3D promoter with the advantages of plant expression systems in general (Horn et al. 2004) and the low protease activity for the recombinant protein stabilization. Shin et al. (2003) showed that transgenic rice cell suspension culture that produced hGM-CSF and yielded 1,000-fold higher amount of biologically active hGM-CSF than our previous expression system, which utilized tobacco cell suspension cultures and the CaMV 35S promoter. This observation was explained by the increased production of hGM-CSF due to induction in sucrose starved condition. As observed in the culture medium, large portion of total secreted protein of hGM-CSF facilitated downstream purification. We therefore introduced the R*Amy* 3D promoter and signal sequence in our recombinant hzAb construct upstream of the sequence encoding the R*Amy* 3D signal peptide as well as the mature hzAb.

The present study shows that it is possible to take advantage of the weak affinity of protein G for hzAb fragments to purify both recombinant Fab and F(ab')$_2$ of hzAb from the transgenic rice cell suspension culture. One native protein G affinity chromatography step yielded the exclusive recovery of hzAb with a purity of 97% after elution with 0.1 M glycine-HCl (pH 2.7). Furthermore, the 120 kDa F(ab')$_2$ dimer seemed to be highly enriched in the purified hzAb sample as shown in Supplement Fig. 1 lane T, as the 50 kDa Fab monomer was detectable on the Western blot when hzAb was loaded onto a SDS-PAGE in non-reducing condition. It is likely that the F(ab')$_2$ dimer containing two binding site shows a higher affinity for
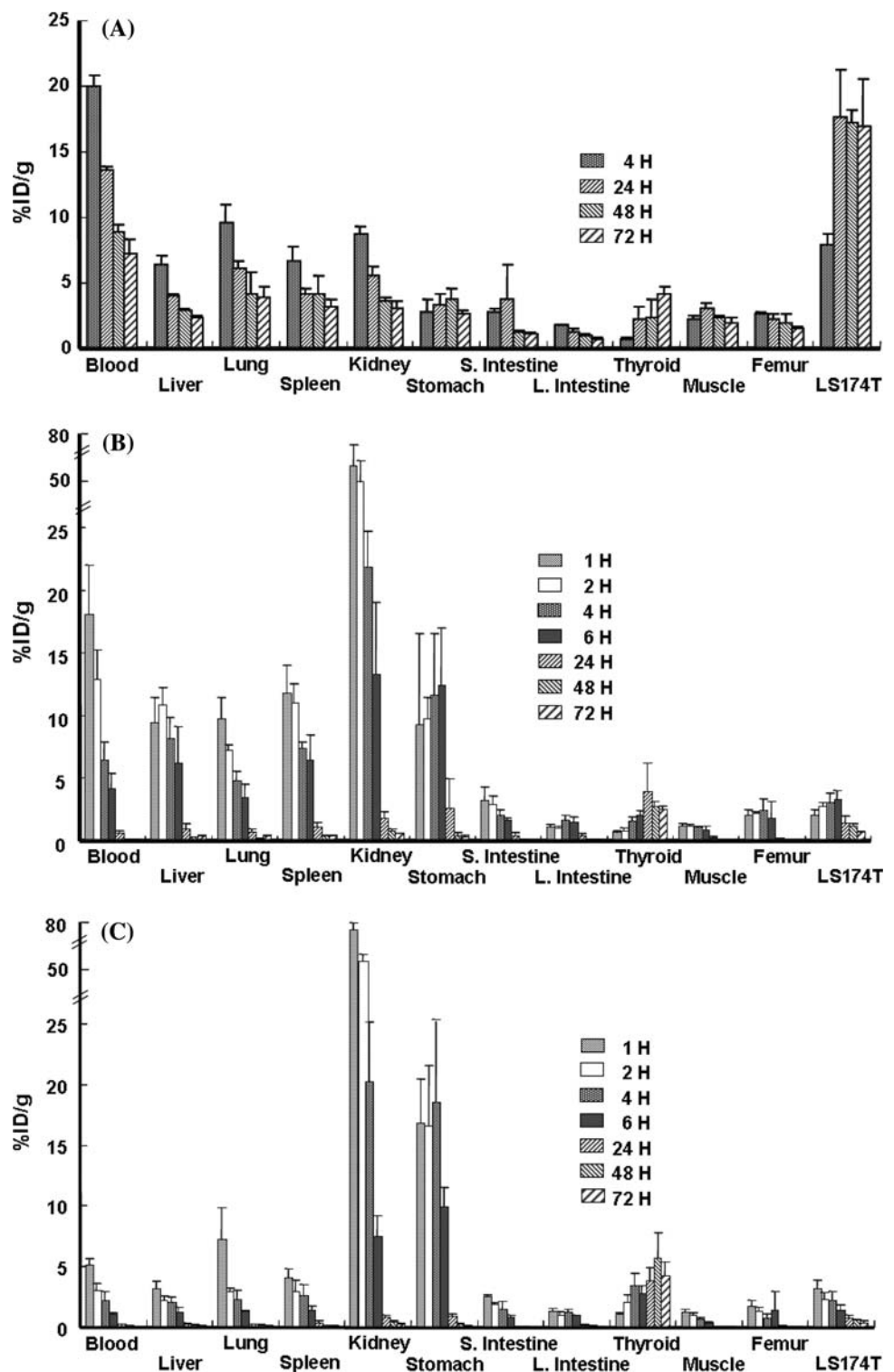
binding to the protein G Sepharose than the Fab monomer. This result for the highly enriched F(ab′)₂ dimer visualized in lane T of Supplement Fig. 1, indicated that rice cultured cell favors the correct assembly of hzAb and that our purification procedure via protein G Sepharose does not stabilize the dimer and monomer structures of plant derived hzAb.

As observed by Sharp and Doran (2001) for the monoclonal antibody IgG in tobacco cell culture, our secreted hzAb fragments migrated on SDS-PAGE at reducing
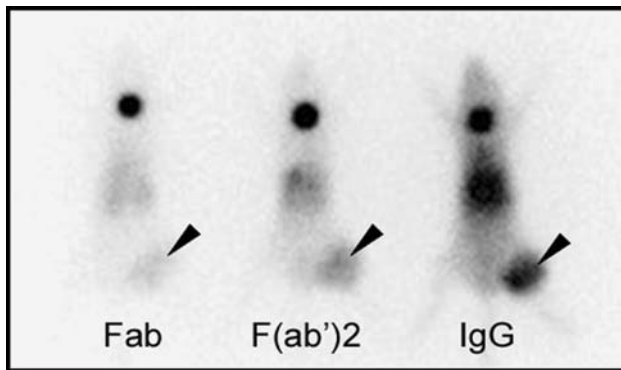
Fig. 6 Images of athymic mice bearing LS-174T human colon carcinoma xenografts injected with ^{123}I-labeled antibodies. Autoradiography was conducted 24 h after administration. ^{123}I-labeled hzAb Fab: left. ^{123}I-labeled hzAb F(ab′)$_2$: center. ^{123}I-labeled IgG: right. *Arrows* point to the tumors

condition (about 30 kDa) than expected from its calculated molecular weight of 25 kDa.

Nicotiana tabacum plants infected with the viral RNA transcribed from the scFv T84.66 constructs yielded ∼5 mg/kg fresh leaf weight (FLW) as deduced from the I_{50} values determined by competition ELISA (Vaquero et al. 2002). In contrast, Ma et al. (1995) obtained up to 200–500 μg IgA/G per gram FLW and 5–8% of total soluble protein (TSP) produced in transgenic *N. tabacum* leaves. Transgenic tobacco plant expressing a tumor-specific scFv gene under the control of TMV coat protein promoter yielded up to 60 μg/ml of intercellular medium (McCormick et al. 1999). Stoger et al. (2000) obtained up to 1.5–29 μg scFv T84.66 per microgram rice using scFv gene including nucleotide coding for the KDEL retention signal and by driving the expression of hzAb under the control of CaMV 35S promoter in transgenic rice plants.

In our study, maximum amount of hzAb obtained in the rice cell culture medium with the Ramy3D expression system was 30 mg/l and about 2% of total secreted protein (TSP) in 10 day by inducted culture, and this showed that plant cell cultures are significantly more efficient than the transgenic animal system.

Recombinant hzAb was tested for its biological activity in a mucin antigen binding ELISA (Fig. 3). Recombinant hzAb produced from the transgenic rice cell suspension culture bound specifically to cells expressing membrane-bound TAG 72 on their surface (Fig. 4). This proves that the plant derived hzAb fragments were present as monomer and dimer that is essential for its antigen specificity as only correct folded hzAb fragments are biologically active and capable of binding to the mucin antigen.

In our in vivo experiments, hzAb fragments showed high radio immunolabeled binding activities, especially about the effects on biodistribution and imaging of hzAb

on tumor. Unlike IgG, hzAb fragments showed rapid clearance from the blood pool and provided a homogenous tumor penetration compared to that of IgG against tumor and a significantly lower percent of injected dose in tumor. This is because of short plasma half-life and lower binding affinity (Colcher et al. 1999). The hzAb fragments showed an ideal reagent for diagnostic applications on account of its excellent tumor penetration and low background. Therefore, hzAb fragments in our tumor targeting experiment may be better regents for imaging purpose compared with IgG.

In this study, we have shown that biologically active, soluble antibody can be produced at high yields in transgenic rice cells. To the best of our knowledge, this is the first study to determine the diagnostic and therapeutic potential of anti-TAG 72 humanized fragment antibodies generated in plant cell cultures.

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