Original Articles

HER2-targeted gold nanoparticles potentially overcome resistance to trastuzumab in gastric cancer

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The authors declare that they have no conflict of interests.

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Abstract

An issue of concern is that no current HER2-targeted therapeutic agent is effective against Trastuzumab (Tmb)-resistant gastric cancer. Gold nanoparticles (AuNPs) are promising drug carriers with unique characteristics of a large surface area available for attachment of materials such as antibodies. Here, we created HER2-targeted AuNPs (T-AuNPs) and examined their therapeutic efficacy and cytotoxic mechanisms using HER2-positive Tmb-resistant (MKN7) or Tmb-sensitive (NCI-N87) gastric cancer cell lines. In vitro, T-AuNPs showed stronger cytotoxic effects than controls against MKN7 and NCI-N87 cells although Tmb had no effect on MKN7 cells. Autophagy played an important role in T-AuNP cytotoxic mechanisms, which was considered to be driven by internalization of T-AuNPs. Finally, T-AuNPs displayed potent antitumor effects against NCI-N87 and MKN7 subcutaneous tumors in in vivo mouse models. In conclusion, HER2-targeted AuNPs with conjugated Tmb is a promising strategy for the development of novel therapeutic agents to overcome Tmb resistance in gastric cancer.

Keywords
Gold nanoparticle; Trastuzumab resistance; HER2; Gastric cancer; Autophagy.

Abbreviations
Tmb, trastuzumab; HER2, human epidermal growth factor receptor 2; AuNP, gold nanoparticle; ECD, extracellular domain; ADCC, antibody-dependent cellular cytotoxicity; PTEN, phosphatase and tensin homolog deleted on chromosome 10; mTOR, mammalian target of rapamycin; T-DM1, trastuzumab emtansine; LPO, lipid hydroperoxide; SPR, surface plasmon resonance, DLS, dynamic light scattering; FCS, fetal calf serum; TEM,
transmission electron microscope; SEM, scanning electron microscope; 3-MA, 3-
Methyladenine; TNF, tumor necrosis factor.
Background

Antibody-based targeted therapy for malignant tumors has been increasingly developed and applied to clinical practice in recent years. Trastuzumab (Tmab) is a humanized monoclonal antibody that binds to the human epidermal growth factor receptor 2 (HER2) and inhibits tumor growth through antibody-dependent cellular cytotoxicity (ADCC) and through interference with HER2 signal transduction that leads to tumor progression (1). Tmab is currently used for patients with HER2-positive breast cancer and gastric cancer and greatly contributes to the prolongation of survival of these patients (2, 3). However, there still remain some issues in HER2-targeted therapy such as acquired resistance to Tmab (4) and application that is limited to only 20-30% of HER2-positive patients with metastatic or recurrent gastric and gastroesophageal junction cancer (2, 5).

Several mechanisms of Tmab-resistance have been reported, such as loss of phosphatase and tensin homolog deleted from chromosome 10 (PTEN) (6), upregulation of PI3K/AKT/ mammalian target of rapamycin (mTOR) and the RAS/RAF/MEK/MAPK pathway (7, 8), loss of p27 expression (9), accumulation of a truncated form of HER2 (p95-HER2) (10), inhibition of Tmab and HER2 interaction by mucin (11) and hyaluronan (12), a substitute signal from other HER family members (EGFR, HER3, HER4) (13), and expression of MET (14, 15) in addition to de novo resistance (16). Novel HER2-targeted therapeutic agents such as trastuzumab emtansine (T-DM1), lapatinib and pertuzumab have already been used clinically for HER2-positive breast cancer with acquired resistance to Tmab, based on successful clinical study of each agent (17-19). For gastric cancer, however, T-DM1 did not show an efficacy benefit over taxane in a global phase 2/3 GATSBY trial (20), and addition of lapatinib did not significantly improve overall survival in either a phase 3 TyTAN trial compared to paclitaxel alone (21) or a phase 3 LOGiC trial compared to the
combination of capecitabine and oxaliplatin (22). Pertuzumab in combination with Tmab and chemotherapy is now being tested in an international phase 3 JACOB trial (23). The combined results indicate that no effective HER2-targeted therapeutic agent currently exists for patients with HER2-positive gastric cancer once resistance to Tmab has been acquired.

Nanotechnology, which has progressed rapidly in recent years, greatly contributes to progress in medical fields including cancer therapy through improvement in drug delivery systems. Nano-sized (10-200 nm) particles tend to accumulate more in tumor than in normal tissues since tumor tissues are characterized by greater leakiness of the tumor vasculature and lack of effective lymphatic drainage compared to normal tissues. This process is known as the enhanced permeability and retention (EPR) effect and is widely recognized as important for effective drug delivery (24). Gold nanoparticles (AuNPs) are one of the novel nanomaterials that have been developed as therapeutic agents as well as contrast agents for diagnosis (25). AuNPs have unique characteristics of high in vivo stability and a large surface area that is available for the attachment of various molecules such as antibodies, peptides and nucleic acids (26, 27), and they reportedly possess cytotoxic potential through induction of autophagy and oxidative stress (28, 29).

In the present study, we created novel HER2-targeted AuNPs by conjugating Tmab onto the surface of AuNPs (T-AuNPs) and we examined their antitumor effect and cytotoxic mechanisms using some gastric cancer cell lines including a Tmab-resistant cell line in vitro and in vivo. We further tested T-AuNPs on a HER2-negative gastric cancer cell line with overexpression of HER2 mediated by an adenovirus vector (30, 31). This attempt to employ AuNPs for delivery of a therapeutic antibody is a novel approach for the treatment of gastric cancer. Thus, the findings of this study may be a breakthrough in the development of novel HER2-targeted therapeutic agents for gastric cancer that can overcome the major obstacle of
Tmab-resistance, which many HER2-targeted agents have failed to overcome.

Methods

Synthesis of trastuzumab-conjugated gold nanoparticles (T-AuNPs)

T-AuNPs were synthesized basically according to the protocol outlined by Kumar et al. (32). Briefly, Tmab (Roche Ltd., Basel, Switzerland) was attached to the surface of 50 nm AuNPs (Sigma-Aldrich, St. Louis, MO, USA) via a linker (SensoPath Technologies, Bozeman, MT, USA) that consisted of a short polyethylene glycol chain terminated at one end by a hydrazide moiety and at the other end by two thiol groups. Finally, 5 kD methoxyPEG-SH (Creative PEGWorks, Chapel Hill, NC, USA) was added to the antibody/NP conjugates to cap any remaining bare surfaces of the AuNPs. AuNPs conjugated with anti-rabbit IgG antibodies (IgG-AuNPs), AuNPs covered only by PEG (C-AuNPs), unconjugated Tmab (Tmab) or the mixture (not conjugated) of Tmab and C-AuNPs (Tmab+C-AuNPs) were used as controls in this study.

The surface plasmon resonance (SPR) of synthesized AuNPs was measured using a spectrophotometer (ND-1000; NanoDrop, Wilmington, DE, USA) to confirm successful surface modification of AuNPs, which was proved by a few nanometer peak wavelength shift in SPR. The size and surface charge (zeta potential) of the synthesized AuNPs were measured by dynamic light scattering (DLS) (Malvern Zetasizer Nano-ZS; Sysmex, Netherlands).

Cell lines and cell cultures

The human gastric cancer cell lines MKN7 and MKN74 were purchased from the Japanese Collection of Research Bioresources (JCRB) Cell Bank (Osaka, Japan), and another human gastric cancer cell line NCI-N87 was purchased from the American Type Culture
Collection (ATCC, Manassas, VA, USA). The human lung fibroblast line NHLF was purchased from Lonza (formerly Cambrex, Walkersville, MD, USA). The three gastric cancer cell lines were cultured in RPMI medium supplemented with 10% fetal calf serum (FCS), and NHLF cells were cultured in FBM medium supplemented with 10% FCS. No cell line was cultured for more than 5 months following resuscitation. Cell authentication was not performed by the authors.

Recombinant adenovirus

A replication-deficient adenoviral vector expressing the extracellular and transmembrane domains of HER2 (Ad/HER2-ECD) was constructed, expanded, and purified as described previously (30). Ad/HER2-ECD was used in this study to overexpress HER2-ECD in MKN74, a HER2-negative gastric cancer cell line.

Cell viability assay

MKN7, MKN74, NCI-N87 and NHLF cells, seeded in 96-well plates (1×10³ cells/well) (n=5), were treated with phosphate-buffered saline (PBS), C-AuNPs, Tmab, T-AuNPs or Tmab+C-AuNPs, and cell viability was determined 3 days after treatment using the Cell Proliferation Kit II (XTT) (Roche Molecular Biochemicals) according to the manufacturer’s protocol. The amount of Tmab attached to the surface of T-AuNPs was adjusted so that it was theoretically equivalent to that of free Tmab. The viability of PBS-treated cells was considered as 100%.

Lipid Hydroperoxide (LPO) assay

Oxidative stress was assessed by measuring lipid peroxidation in cells. MKN7 and
NCI-N87 cells (n=5) that were treated with PBS, C-AuNPs, Tmab, T-AuNPs or Tmab+C-AuNPs, were harvested at 48 hours after treatment. Lipid hydroperoxides in chloroform extracts of sonicated samples were examined using a Lipid Hydroperoxide (LPO) Assay Kit (Caymen Chemicals, Ann Arbor, MI, USA) according to the manufacturer’s instructions. The level of LPO in PBS-treated cells was considered as 100%.

Western blot analysis

Proteins extracted from whole-cell lysates were electrophoresed on 6-10% SDS-polyacrylamide gels and were transferred to Hybond-polyvinylidene difluoride transfer membranes (GE Healthcare UK Ltd.). The membranes were incubated with primary antibodies against poly (ADP ribose) polymerase (PARP), microtubule-associated protein light chain 3 (LC3), AKT, phospho-AKT, mTOR, phospho-mTOR, HER2, phospho-HER2, EGFR (Cell Signaling Technology, Danvers, MA, USA), PTEN (Santa Cruz Biotechnology, Dallas, TX, USA) and β-actin (Sigma-Aldrich), followed by peroxidase-linked secondary antibody. The Amersham ECL chemiluminescence system (GE Healthcare UK Ltd.) was used to detect the peroxidase activity of the bound antibody. Equal loading of samples was confirmed by β-actin analysis.

Dark field microscopy

MKN7 cells seeded in two-well chamber slides were treated with T-AuNPs or PBS for 24 hours. Cells fixed in 1% paraformaldehyde were observed using a microscope (BX50) (Olympus, Tokyo, Japan) equipped with an U-DCD dark-field condenser.

Confocal fluorescent microscopy
MKN7, NCI-N87 and MKN74 cells that were seeded in a 35-mm glass base dish were treated with Alexa Fluor 647 (ThermoFisher Scientific, Waltham, MA, USA)-labelled T-AuNPs or Alexa Fluor 647-labelled Tmab for 4, 12 or 24 hours. After addition of Lysotracker® (Cell Signaling Technology) to stain lysosomes, the cells were analyzed using a confocal laser scanning microscope (Fluoview FV10i) (Olympus).

Electron microscopy

T-AuNPs were imaged using a transmission electron microscope (TEM) (H-7650) (Hitachi, Tokyo, Japan) and a scanning electron microscope (SEM) (S-4800) (Hitachi). T-AuNPs labeled with 10 nm gold-conjugated goat anti-mouse IgG were imaged by immunoelectron microscopy using the H-7650 TEM. Cellular uptake of T-AuNPs into MKN7 cells was observed at 24 hours after treatment.

In vivo experiments

NCI-N87 (3 × 10^6 cells/mouse) and MKN7 (5 × 10^6 cells/mouse) cells were injected subcutaneously into the flanks of 8-week-old female BALB/c nude mice and 8-week-old female NOD/SCID mice, respectively. When tumors reached a diameter of around 5-10 mm, the NCI-N87-implanted mice were divided into 5 groups (PBS, C-AuNPs, Tmab, T-AuNPs and Tmab+C-AuNPs) (n=5-7) and the MKN7 mice into 3 groups (PBS, Tmab and T-AuNPs) (n=5-7), and the mice were treated intratumorally 3 times in one week. The doses of T-AuNPs, C-AuNPs and Tmab were 5.0 × 10^9 particles, 5.0 × 10^9 particles and 1.4 μg, respectively. The perpendicular diameter of each tumor was then measured twice a week up to day 31, and tumor volume was calculated with the following formula: tumor volume (mm^3) = a × b^2 × 0.5, where a is the longest diameter, b is the shortest diameter, and 0.5 is a
constant to calculate the volume of an ellipsoid. In another experiment, NCI-N87 subcutaneous tumors harvested at 3 days after the last (3rd) treatment were immunohistochemically stained for LC3 to evaluate induction of autophagy. Mice were housed in a specific pathogen-free environment in the Department of Animal Resources of Okayama University. All animal experimental protocols were approved by the Institutional Animal Care and Use Committee of Okayama University.

Statistical analysis

All data are expressed as means ± SD. Differences between groups were examined for significance with Student’s t-test. P values <0.05 were considered significant.

Results

Characteristics of synthesized T-AuNPs

After the synthesis of T-AuNPs (Figure 1A) and C-AuNPs, the characteristics of these particles were analyzed using DLS, spectrophotometry and electron microscopy. DLS analysis showed that the size of the T-AuNPs was 85.39 ± 0.68 nm and that the surface was negatively charged with 39.43 ± 0.85 mV (Supplementary Table S1). The size and the Z potential of the C-AuNPs and the naked AuNPs were 94.12 ± 0.32 nm and -43.70 ± 0.10 mV, and 78.60 ± 0.34 nm and -52.83 ± 0.93 mV, respectively. Spectrophotometry revealed a few nanometer peak wavelength shifts in SPR between the AuNPs and the T-AuNPs (Figure 1B), which indicated successful conjugation of Tmab onto the surface of the AuNPs. TEM and SEM images showed that T-AuNPs that were 50-80 nm in size were monodispersed in the medium with colloidal stability (Figure 1C, 1D), and immunoelectron microscopic images showed that several Tmabs, which were labeled with 10 nm AuNP, were successfully
conjugated onto the surface of the AuNPs (Figure 1E, 1F).

Cytotoxic activity of T-AuNPs against gastric cancer cell lines

Unlike NCI-N87, which is a Tmab-sensitive HER2-positive cell line, MKN7 is known to be Tmab-resistant despite high HER2 expression. This resistance is due to low Tmab capacity to bind to HER2 (33) and to an activated EGFR signaling pathway in these cells although the PTEN expression level was similar to that in the NCI-N87 and other cell lines (Figure 2A). When the half maximal (50%) inhibitory concentration (IC50) dose of C-AuNPs, Tmab and T-AuNPs was examined for NCI-N87 cells, T-AuNPs showed 4.5-fold higher cytotoxic activity than C-AuNPs and 6-fold higher activity than Tmab (Figure 2B). This means that T-AuNPs are capable of bringing the same cytotoxic activity as Tmab with a 6-fold smaller amount of Tmab on NCI-N87 cells. The IC50 dose of T-AuNPs on NCI-N87 cells was $4.6 \times 10^9$ particles/ml and a dose of Tmab included in this concentration of T-AuNPs was 1.3 μg/ml, and these concentrations were used in subsequent in vitro experiments. In cell viability assays that compared T-AuNPs with controls of PBS, C-AuNPs, Tmab, and Tmab+C-AuNPs, T-AuNPs exhibited significantly higher cytotoxicity compared to the controls towards the HER2-positive MKN7 and NCI-N87 cell lines, while they exhibited little cytotoxicity towards the HER2-negative MKN74 and NHLF cell lines (Figure 2A, 2C). It is especially noteworthy that T-AuNPs showed a potent cytotoxic effect towards the Tmab-resistant MKN7 cell line, while, predictably, Tmab did not show any effect on MKN7 cells. These results suggested that T-AuNPs possessed an appropriate HER2-based tumor selectivity and exerted a potent cytotoxic effect through unique mechanisms that differ from those of the non-conjugated Tmab.
Cytotoxic mechanisms of T-AuNPs

In terms of autophagy as a potential cytotoxic mechanism of T-AuNPs, western blot analysis showed that T-AuNPs induced strong LC3-II expression in MKN7 and NCI-N87 cells compared to the controls (Figure 3A). T-AuNPs also inhibited p62 expression in MKN7 and NCI-N87 cells (Supplementary Figure S1). This autophagy induction by T-AuNPs was also proved by the finding that addition of 3-Methyladenine (3-MA), an autophagy inhibitor, significantly blocked the induction of autophagic cell death by T-AuNPs (Figure 3B). In terms of apoptosis, no increase in the cleaved form of PARP was observed in MKN and NCI-N87 cells (Figure 3A). LPO assays showed that T-AuNPs induced significantly stronger oxidative stress in MKN7 cells compared to C-AuNPs and Tmab, while a tendency towards oxidative stress induction by T-AuNPs was observed in NCI-N87 cells although it was not statistically significant compared to controls (Figure 3C). The influence of T-AuNP treatment on downstream proteins of HER2 signaling such as AKT and mTOR was examined by western blot analysis, which showed that T-AuNPs inhibited the phosphorylation of AKT in both MKN7 and NCI-N87 cells and inhibited the phosphorylation of mTOR in NCI-N87 cells, but not in MKN7 cells (Figure 3D). These results indicated that induction of autophagy was a cytotoxic mechanism of T-AuNPs in both MKN7 and NCI-N87 cells, while T-AuNP induction of oxidative stress and the influence of T-AuNPs on the HER2 signaling pathway varied with the type of cell line used.

Intracellular uptake and localization of T-AuNPs

Intracellular uptake of T-AuNPs was roughly assessed using dark field microscopy, and was then more finely assessed down to the level of the intracellular localization of T-AuNPs using TEM and confocal fluorescence microscopy. In dark field microscopy, more gold spots
were observed in the cells treated with T-AuNPs than in the cells treated with the controls, indicating that the T-AuNPs were more efficiently internalized into the cells or at least more T-AuNPs were bound to the cell surface at 24 hours after treatment compared to controls (Figure 4A). TEM showed that T-AuNPs were indeed internalized into MKN7 cells at 24 hours after treatment, some of which were located in the cytoplasm surrounded by vesicles and some of which were scattered in the cytoplasm without any surrounding vesicles (Figure 4B). The cellular location of Tmab was then monitored after treatment with Tmab or T-AuNPs by using confocal microscopy and Tmab labeled with a red fluorescent dye. Stronger red fluorescence was observed on the surface of NCI-N87 cells than on the surface of MKN7 cells at each time point (Supplementary Figure S2), which proved that Tmab-binding capacity to HER2, as previously reported, was inhibited on MKN7 cells despite a relatively high HER2 expression level (33). In both cell lines, while many of the Tmabs that were internalized into cells after binding to the cell surface colocalized with lysosomes (yellow color in the merged image), some T-AuNPs that were internalized into cells interestingly seemed to exist independently in the cytoplasm and were not merged with lysosomes (the color remained red in the merged image) (Figure 4C, 4D). These findings suggested that T-AuNPs were efficiently internalized into cells and that this HER2-dependent internalization may be involved in the cytotoxic mechanisms of T-AuNPs. In particular, T-AuNPs that are scattered in the cytoplasm without lysosomal encapsulation may play an important role in strong cytotoxic induction.

Combined therapy of T-AuNPs with artificial HER2 overexpression on HER2-negative gastric cancer cells

Another challenge in terms of HER2-targeted therapy of gastric cancer besides the
acquisition of Tmab-resistance, is the low HER2-positve rate (20-30%). To overcome this problem, combination therapy of T-AuNPs with HER2 overexpression mediated by Ad/HER2-ECD in the HER2-negative MKN74 gastric cancer cell line was tested (Figure 5A). Ad/HER2-ECD expresses only the extracellular domain of HER2, which theoretically does not activate the HER2 signaling pathway that leads to tumor progression. Cell viability assays showed that T-AuNPs became effective for cell growth inhibition after HER2 overexpression on MKN74 cells and suppressed cell growth significantly compared to the controls although Tmab did not exhibit any difference in growth inhibition efficacy between parental MKN74 and HER2-overexpressing MKN74 cells (Figure 5B). Confocal fluorescence microscopy showed that T-AuNPs were effectively internalized into the cytoplasm in HER2-overexpressing MKN74 cells, especially at 24 hours after treatment, whereas T-AuNPs were not internalized into the parental MKN74 cells even though they appeared to be attached to the cell surface (Figure 5C, 5D). These findings suggested that T-AuNPs acquired growth inhibition efficacy towards HER2-negative MKN74 cells after HER2-ECD overexpression and that this cytotoxic activity was mediated by internalization of T-AuNPs into cells and not by inhibition of the HER2 signaling pathway.

Antitumor effects of T-AuNPs on subcutaneous mouse tumor models

Finally, the antitumor effects of T-AuNPs were evaluated using subcutaneous mouse NCI-N87 and MKN7 tumor models. Intratumoral injection of T-AuNPs significantly suppressed the growth of NCI-N87 tumors compared to the controls including Tmab and Tmab+C-AuNPs ($p<0.05$) (Figure 6A), and also significantly suppressed the growth of Tmab-resistant MKN7 tumors ($p<0.05$) for which Tmab showed no antitumor effect (Figure 6B). Immunostaining of NCI-N87 subcutaneous tumors for the autophagy marker LC3-II
showed that T-AuNPs strongly induced autophagy compared to the controls (Figure 6C, 6D). These results proved that T-AuNPs also exerted potent antitumor effects in in vivo tumor models through induction of autophagy.

**Discussion**

No effective HER2-targeted therapeutic agent currently exists for metastatic gastric cancer with acquired resistance to Tmab, which is in contrast to breast cancer for which novel HER2-targeted agents such as T-DM1, lapatinib and pertuzumab are all in clinical use. Gastric cancer is the third leading cause of death and the fifth most common malignancy in the world, half of which occurs in Eastern Asia (34). Since Tmab therapy in combination with chemotherapy is the first treatment option for patients with HER2-positive metastatic gastric cancer and most of these patients eventually become refractory to Tmab, the development of novel therapeutic agents that are effective for Tmab-refractory gastric cancer is of great urgency. In this study, we used an original approach to develop such an agent by using AuNPs with Tmab conjugated on their surface, which can cause cytotoxic effects additional to those mediated by Tmab alone. AuNPs have recently emerged as an attention-grabbing nanomaterial in medical fields because of their unique properties such as their ease of synthesis and surface modification.

In terms of the safety of AuNPs in humans, AuNPs can be considered relatively safe because gold compounds have a long history (more than 80 years) of being used as therapeutic drugs for patients with rheumatoid arthritis, which may facilitate clinical application of AuNP-containing materials (35). While no AuNP-containing drug has yet been approved for clinical use, CYT-6091, which consists of recombinant human tumor necrosis factor alpha (rhTNF) covalently linked to the surface of a pegylated colloidal gold
nanoparticle, was the first cancer therapeutic agent that used AuNPs. A phase I dose escalation clinical trial of CYT-6091 for advanced stage cancer patients recently proved that rhTNF formulated as CYT-6091 was safely administered systemically to patients at doses of rhTNF that were previously shown to be toxic, without appreciable side effects (36).

Regarding the quality assessment of T-AuNPs after the conjugation process in our study, estimation of the number of Tmabs that were attached to an AuNP was initially determined by visual observation using immunoelectron microscopy. Based on this analysis it was estimated that three or four Tmabs at most were attached to the surface of an AuNP. Although it is uncertain whether this number is the true value or is an underestimate, for the most part these created T-AuNPs exerted strong HER2-dependent cytotoxic activity on gastric cancer cell lines in in vitro assay. Notably, T-AuNPs showed potent cytotoxic activity not only towards the Tmab-sensitive NCI-N87 cell line, but also towards the Tmab-resistant MKN7 cell line, and this effect was proven to be brought about through induction of autophagy, which is consistent with previous reports of AuNPs (28). While this autophagy induction was considered to be caused by the internalization of T-AuNPs, TEM and confocal fluorescence microscopy revealed the interesting finding that some T-AuNPs were scattered in the cytoplasm independently, without being encapsulated by lysosomes. This phenomenon may play a role in the strong cytotoxic activity of T-AuNPs although further investigation is needed to clarify this point. HER2 internalization itself upon the presence of Tmab is actually controversial (37, 38). On the other hand, it is unclear whether induction of oxidative stress is a universal cytotoxic mechanism of T-AuNPs because a significantly strong induction of oxidative stress by T-AuNPs was observed in MKN7 cells, but not in NCI-N87 cells. It is also uncertain how T-AuNPs regulate HER2 downstream molecules such as AKT and mTOR because T-AuNPs downregulated the phosphorylation of AKT in both MKN7 and NCI-N87
cells but only downregulated the phosphorylation of mTOR in NCI-N87 cells and not in MKN7 cells. Indeed, the influence of T-AuNPs on oxidative stress and the HER2 signaling pathway may be cell-dependent. In terms of apoptosis, there was no evidence that T-AuNPs induced apoptosis although other antibody-conjugated gold nanoparticles targeting the EGFR reportedly induced apoptosis in addition to autophagy in non-small lung cancer cells (29). Further investigation is needed to clarify the cytotoxic mechanisms of T-AuNPs.

While T-AuNPs possess the intriguing properties described above, the current T-AuNPs definitely have several limitations. One major limitation is the inability to systemically deliver the T-AuNPs to tumor tissue. When we examined the biodistribution of T-AuNPs after systemic administration via the tail vein in the subcutaneous NCI-N87 tumor mouse model, most of the AuNPs had accumulated in the liver and only a few AuNPs were observed in tumor tissue (data not shown). One of the reasons for this inability to systemically deliver AuNPs to tumor tissues is considered to be due to the size of the AuNPs. In this study, we employed 50 nm AuNPs based on a previous report that stated that 40- and 50-nm gold nanoparticles coated with antibodies demonstrated the greatest effect in terms of internalization of nanoparticles after binding to the cell surface (39). However, the efficiency of drug delivery from the blood circulation to the targeted cells was not considered in that report. It may be therefore better to reduce the size of the nanoparticles, which would decrease liver trapping and increase their distribution to tumor tissues, although nanoparticles smaller than 10 nm are rapidly excreted from the kidney (40, 41). AuNPs around 20 nm in size may be suitable for achieving better delivery of T-AuNPs to tumor tissues after systemic administration (42). Another limitation of this study may be the lack of experiments regarding the effect of T-AuNPs in an environment with a working immune system. Since one of the cytotoxic mechanisms of antibody-based therapy is ADCC, and since the influence
of AuNPs on tumor immunity is totally unknown, some experiments using T-AuNPs in an environment with a working immune system such as in immune-competent mice should lead to the discovery of new knowledge regarding AuNPs.

In the present study, we demonstrated that our created T-AuNPs exerted a strong cytotoxic effect on both Tmab-sensitive and Tmab-resistant gastric cancer cells through autophagy that was induced by internalization of the T-AuNPs into cells in vitro and in vivo. In addition, we also proved that T-AuNPs could become effective even towards HER2-negative gastric cancer cells when combined with HER2-ECD overexpression on those cells. Although the current T-AuNPs are clearly not the best HER2-targeted agent for clinical use due to an inability to systemically deliver them to tumors, the concept of using AuNPs for Tmab delivery has the potential to forge a new path in the development of novel HER2-targeted therapeutic agents that are expected to overcome acquired resistance to Tmab.

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Figure Legends

Figure 1. Characteristics of the synthesized T-AuNPs

(A) Schematic illustration of T-AuNPs. (B) The amount of light absorbed by unprocessed AuNPs and synthesized T-AuNPs was measured using a spectrophotometer. (C) A TEM image of T-AuNPs. (D) An SEM image of T-AuNPs. (E) A low-magnification immunoelectron microscopic image of T-AuNPs. (F) A high-magnification immunoelectron microscopic image of T-AuNPs. Scale bars, 100 nm.

Figure 2. Cytotoxic activity of T-AuNPs towards gastric cancer cell lines

(A) Expression levels of HER2, EGFR and PTEN in MKN7, NCI-N87, MKN74 and NHLF cells were examined on western blot analysis. (B) The viability of NCI-N87 cells was assessed using an XTT assay at 72 hours after treatment with several doses of C-AuNPs, Tmab, or T-AuNPs. Dose-response curves are shown. Doses are described based on a dose (μl) of each agent added in a 96-well plate in this experiment. The intersection of each curve with the horizontal dashed line shows the IC50 dose of each treatment. (C) The viability of the four cell lines, MKN7, NCI-N87, MKN74 and NHLF, was assessed using an XTT assay at 72 hours after PBS, C-AuNPs (4.6 × 10⁹ particles/ml), Tmab (1.3 μg/ml), T-AuNPs (4.6 × 10⁹ particles/ml) or Tmab+C-AuNPs treatment. Note that although MKN7 and NCI-N87 are both HER2-positve, MKN7 is Tmab-resistant. **, P<0.01. ***, P<0.001.

Figure 3. Cytotoxic mechanisms of T-AuNPs

(A) Western blot analysis of PARP and LC3 in whole cell lysates of MKN7 and NCI-N87 cells collected at 6 hours after treatment with PBS, C-AuNPs (4.6 × 10⁹ particles/ml), Tmab (1.3 μg/ml), T-AuNPs (4.6 × 10⁹ particles/ml) or Tmab+C-AuNPs. Cleaved PARP (C-PARP)
and LC3-II expression indicate induction of apoptosis and autophagy, respectively. (B) The viability of MKN7 and NCI-N87 cells was assessed using an XTT assay at 72 hours after T-AuNP (4.6 × 10⁹ particles/ml) treatment with or without addition of 3-MA (200 nM), an autophagy inhibitor, prior to T-AuNP treatment. *, P<0.05. ***, P<0.001. (C) LPO assay of MKN7 and NCI-N87 cells treated with PBS, C-AuNPs (4.6 × 10⁹ particles/ml), Tmab (1.3 µg/ml), T-AuNPs (4.6 × 10⁹ particles/ml) or Tmab+C-AuNPs for 48 hours. The LPO level reflects the level of oxidative stress. **, P<0.01. (D) Western blot analysis of p-AKT, AKT, p-mTOR and mTOR in whole cell lysates of MKN7 and NCI-N87 cells collected at 3 hours after each treatment.

Figure 4. Intracellular uptake and localization of T-AuNPs

(A) MKN7 and NCI-N87 cells were observed using dark field microscopy at 24 hours after treatment with PBS, C-AuNPs (4.6 × 10⁹ particles/ml), IgG-AuNPs (4.6 × 10⁹ particles/ml), T-AuNPs (4.6 × 10⁹ particles/ml) or Tmab+C-AuNPs. Scale bar, 20 µm. (B) MKN7 cells were observed using TEM at 24 hours after T-AuNP treatment. The top right panel is a magnified picture of the indicated square in the left panel. The bottom right panel is a magnified picture of a different area. Red arrowheads show T-AuNPs located in the cytoplasm that are surrounded by vesicles. Red arrows show T-AuNPs that are scattered in the cytoplasm without surrounding vesicles. Scale bar in the left panel, 2 µm. Scale bar in the right panels, 1 µm. (C) MKN7 and NCI-N87 cells were observed using confocal fluorescence microscopy at 4, 12 and 24 hours after treatment with Tmab (1.3 µg/ml) or T-AuNPs (4.6 × 10⁹ particles/ml). Tmab and T-AuNPs were labeled with a red fluorescent dye, and lysosomes were labeled with a green fluorescent dye. White arrowheads show Tmab that had internalized into cells and colocalized with lysosomes (yellow color upon merging). White
arrows show T-AuNPs that had internalized into cells and were independently located in the cytoplasm (color remained red upon merging). Scale bar, 20 µm. (D) Area index of red and yellow inside the cells, not on the surface, at 24 hours in Figure 4C was quantified by the Image J software.

Figure 5. Combined therapy of T-AuNPs with artificial HER2 overexpression in HER2-negative gastric cancer cells

(A) Western blot analysis of HER2 expression in whole cell lysates of parental MKN74 cells and MKN74 treated with Ad/HER2-ECD for 12 hours. (B) Cell viability of parental MKN74 cells and of HER2-overexpressing MKN74 cells was assessed using the XTT assay at 72 hours after treatment with PBS, C-AuNPs (4.6 × 10^9 particles/ml), Tmab (1.3 µg/ml), T-AuNPs (4.6 × 10^9 particles/ml) or Tmab+C-AuNPs. ***, P<0.001. (C) Parental MKN74 and HER2-overexpressing MKN74 cells were observed using confocal fluorescence microscopy at 4, 12 and 24 hours after treatment with Tmab (1.3 µg/ml) or T-AuNPs (4.6 × 10^9 particles/ml). Tmab and T-AuNPs were labeled with a red fluorescent dye, and lysosomes were labeled with a green fluorescent dye. White arrows show T-AuNPs that had internalized into cells and were independently located in the cytoplasm (color remained red upon merging). Scale bar, 20 µm. (D) Area index of red and yellow inside the cells, not on the surface, at 24 hours in Figure 5C was quantified by the Image J software.

Figure 6. Antitumor effects of T-AuNPs on subcutaneous tumor models

(A) NCI-N87 subcutaneous tumors in BALB/c nude mice were intratumorally treated with PBS, C-AuNPs (5.0 × 10^9 particles), Tmab (1.4 µg), T-AuNPs (5.0 × 10^9 particles) or Tmab+C-AuNPs 3 times in one week (black arrows), and tumor volume was then monitored
up to day 31. *, *P*<0.05. (B) MKN7 subcutaneous tumors in NOD/SCID mice were intratumorally treated with PBS, Tmab (1.4 μg) or T-AuNPs (5.0 x 10^9 particles) 3 times in one week (black arrows), and tumor volume was monitored up to day 31. *, *P*<0.05. (C) NCI-N87 tumors harvested 3 days after three treatments with PBS, C-AuNPs (5.0 x 10^9 particles), Tmab (1.4 μg), T-AuNPs (5.0 x 10^9 particles) or Tmab+C-AuNPs were immunohistochemically stained for LC3. Scale bar, 100 μm. (D) LC3 intensity was quantified in three randomly selected fields of each group in the Figure 6C by the Image J software and statistically analyzed. *, *P*<0.05. **, *P*<0.01.