INTRODUCTION

G-protein-coupled receptors (GPCRs), the largest superfamily of membrane receptors in the human genome, transduce extracellular signals to the intracellular space through binding of their cognate ligands. Intracellular signaling events triggered by conformational changes in GPCRs and interactions with intracellular proteins regulate numerous cellular functions, such as growth, motility, and differentiation. Because of the critical role of GPCRs in numerous biological functions, they are involved in the progression and prognosis of a variety of diseases and are the targets of ~35% of all commercialized drugs.

Endothelin receptor type A (ET_{A}), a class A G-protein-coupled receptor (GPCR), is involved in the progression and metastasis of colorectal, breast, lung, ovarian, and prostate cancer. We overexpressed and purified human endothelin receptor type A in Escherichia coli and reconstituted it with lipid and membrane scaffold proteins to prepare an ET_{A} nanodisc as a functional antigen with a structure similar to that of native GPCR. By screening a human naïve immune single-chain variable fragment phage library constructed in-house, we successfully isolated a human anti-ET_{A} antibody (AG8) exhibiting high specificity for ET_{A} in the β-arrestin Tango assay and effective inhibitory activity against the ET-1-induced signaling cascade via ET_{A} using either a CHO-K1 cell line stably expressing human ET_{A} or HT-29 colorectal cancer cells, in which AG8 exhibited IC_{50} values of 56 and 51 nM, respectively. In addition, AG8 treatment repressed the transcription of inhibin βA and reduced the ET_{A}-induced phosphorylation of protein kinase B and extracellular regulated kinase. Furthermore, tumor growth was effectively inhibited by AG8 in a colorectal cancer mouse xenograft model. The human anti-ET_{A} antibody isolated in this study could be used as a potential therapeutic for cancers, including colorectal cancer.

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Expression and purification of human ET₄₄, mouse ET₄₄, and membrane scaffold protein-1

Human ET₄₄ (hET₄₄), mouse ET₄₄ (mET₄₄), and membrane scaffold protein-1 (MSP-1) proteins were expressed and purified as described in the literature.  E. coli BL21(DE3) harboring pETp9-hET₄₄ or pP9-mET₄₄ (for pP9-derived plasmids), or pET28a-MSP-1 (for pET28a-derived plasmids) was inoculated in Luria-Bertani (LB) medium supplemented with 100 μg/ml ampicillin (Millipore Sigma, Burlington, MA, USA) and 50 μg/ml kanamycin (Millipore Sigma, Burlington, MA, USA) and cultivated for 16 h at 37°C and 250 rpm. Then, 100-fold dilutions of overnight-grown cells were inoculated in LB medium supplemented with the same antibiotics and incubated at 37°C until the absorbance of the culture broth at 600 nm (OD₆₀₀) reached 0.6. After the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) (0.5 mM for hET₄₄ and mET₄₄. 1 mM for MSP-1) and incubation under specific culture conditions (25°C for 16 h for hET₄₄ and mET₄₄. 30°C for 4 h for MSP-1) to induce protein expression, cells were harvested by centrifugation at 8000×g and disrupted using a microfluidizer (Microfluidics, Westwood, MA, USA). To prepare endothelin receptors (hET₄₄ and mET₄₄), the resulting lysates were centrifuged at 12,000×g for 20 min, and the supernatants were ultracentrifuged at 100,000×g for 1.5 h to recover the membrane fractions from the pellets. After the membrane fractions were dissolved in 0.5% sarkosyl and centrifuged at 30,000×g for 30 min to remove insoluble aggregates, the recovered supernatants were bound to Ni-NTA agarose (Qiagen, Germantown, MD, USA) equilibrated with Buffer A (25 mM Tris–HCl and 1 mM phenylmethylsulfonylfluoride (pH 7.8)). After the resin was washed with 20 column volumes (CV) of Buffer A supplemented with 20 mM imidazole, the resin-bound proteins were eluted using 5 CV of Buffer A supplemented with 300 mM imidazole. Then, the eluents were loaded onto a PD-10 desalting column (Cytiva, Marlborough, MA, USA) to remove excess imidazole, and the buffer was exchanged with 25 mM Tris–HCl (pH 7.8) containing 10% glycerol. The purified endothelin receptors (hET₄₄ and mET₄₄) were stored at −80°C before use. To prepare MSP-1 proteins, cell lysates were centrifuged at 12,000×g, and the resulting supernatants were loaded onto a Ni-NTA column equilibrated with 10 ml of 50 mM Tris-Cl and 1% Triton X-100 (pH 7.4). After adding 10 ml of 50 mM Tris-Cl and 50 mM imidazole (pH 7.4) for washing and 10 ml of 50 mM Tris-Cl and 300 mM imidazole (pH 7.4) for elution, the eluent buffer was exchanged with 1× phosphate-buffered saline (PBS, pH 7.4) containing 10% glycerol using a PD-10 desalting column.

Preparation of reconstituted hET₄₄ nanodiscs

Purified hET₄₄ and MSP-1 were mixed with POPC dissolved in 100 mM sodium cholate at a hET₄₄:MSP-1:POPC molar ratio of 1:30:60. After the addition of 200 mg/ml Bio-BeadsSM-2 (Bio-Rad, Hercules, CA, USA), the resuspended solution was incubated at 4°C with mixing by rotation at 100 rpm and centrifuged at 12,000×g for 5 min to remove detergent. Then, the supernatants were dialyzed in 1× PBS (pH 7.4) and concentrated using Amicon Ultra® spin columns (Merck Millipore; 30 kDa cutoff). The concentrated supernatants were loaded onto a Superdex 200 gel filtration chromatography column (Cytiva, Marlborough, MA, USA) for development in 35 ml of 1× PBS (pH 7.4), and the fractions showing both hET₄₄ and MSP-1 protein bands in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis were recovered.

Construction of a human naive immune scFv library

VH and VL genes of human immunoglobulins, which were prepared from peripheral blood mononuclear cells (PBMCs) of anonymous donors as described in the literature, were PCR amplified using 200 μM dNTPs, 1 μM mixed oligonucleotides (MSJ#07–MSJ#16 for VH and MSJ#17–MSJ#37 for VL), 2.5 units of Phusion High-Fidelity DNA polymerase, and 100 ng of cDNA as a template. Then, the VH and VL genes were assembled by PCR amplification using two primers (MSJ#38/MSJ#39) and a template (pMAZ-IgL-GlycoT®), which were assembled from two light chains of full-length IgG for AG8, each VH and VL gene was PCR amplified using primers (MSJ#01 and MSJ#02) and was subcloned into pET28a(-1) (Novagen, Burlington, MA, USA) at the Ndel/BamHI restriction endonuclease sites to generate pET28-MSP-1. To construct plasmids encoding the heavy and light chains of full-length IgG for AG8, each VH and VL gene was PCR amplified using a phagemid (pEL3X-AG8) isolated from the phage library screen and the primer pairs MSJ#42/MSJ#44 for VH and MSJ#46/MSJ#48 for VL. Then, the DNA fragments encoding the IgG constant region (CH1-CH2-CH3) of trastuzumab, which were prepared by PCR amplification using a primer pair (MSJ#43/MSJ#45) and a template (pMAZ-IgL-GlycoT®), were assembled with the VH DNA fragments using a primer pair (MSJ#42/ MSJ#45). A primer pair (MSJ#46/MSJ#49) was used to assemble the DNA fragments for the VL gene, and the human Cλ DNA fragments were amplified using primers (MSJ#47/MSJ#49) and a template (pMAZ-IgL-GlycoT®). pMAZ-AG8H and pMAZ-AG8L were constructed by ligation of the resulting heavy and light-chain DNA of AG8 IgG, respectively, into the pMAZ-IgL-GlycoT plasmid at the BssHII and XbaI sites.

MATERIALS AND METHODS

Reagents

All oligonucleotide primers and plasmids used in this study are described in Supplementary Tables 1 and 2. Restriction enzymes, Phusion High-Fidelity DNA polymerase, and T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA, USA). Oligonucleotide primers and VCSM13 helper phage stock were obtained from Integrated DNA Technologies (Coralville, IA, USA) and Agilent Technologies (Santa Clara, CA, USA), respectively. 1-Palmitoyl-2-oleoylphosphatidylcholine (POPC), an anti-M13 antibody conjugated to horseradish peroxidase (HRP), and 1-Step Ultra 3,3′,5,5′-tetramethylbenzidine (TMB) substrates were purchased from Avanti Polar Lipids (Alabaster, AL, USA), Bethyl Laboratories (Montgomery, TX, USA), and Thermo Fisher Scientific (Waltham, MA, USA), respectively. All other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise.

Construction of plasmids

The mouse ET₄₄ (mET₄₄) gene (NCBI Gene ID: 13617) was synthesized by GenScript (Piscataway, NJ, USA). The pP9-mET₄₄ plasmid was constructed by Gibson assembly of the mET₄₄ DNA fragments amplified by polymerase chain reaction (PCR) using primers (MSJ#01 and MSJ#02) and the pP9 plasmid digested with the SamI restriction enzyme. The gene encoding membrane scaffold protein (MSP-1), derived from the apolipoprotein A-I gene (NCBI Gene ID: 335), was assembled by PCR using primers (MSJ#03–MSJ#06) and was subcloned into pET28a(-1) (Novagen, Burlington, MA, USA) at the Ndel/BamHI restriction endonuclease sites to generate pET28-MSP-1. To construct plasmids encoding the heavy and light chains of full-length IgG for AG8, each VH and VL gene was PCR amplified using a phagemid (pEL3X-AG8) isolated from the phage library screen and the primer pairs MSJ#42/MSJ#44 for VH and MSJ#46/MSJ#48 for VL. Then, the DNA fragments encoding the IgG constant region (CH1-CH2-CH3) of trastuzumab, which were prepared by PCR amplification using a primer pair (MSJ#43/MSJ#45) and a template (pMAZ-IgL-GlycoT®), were assembled with the VH DNA fragments using a primer pair (MSJ#42/ MSJ#45). A primer pair (MSJ#46/MSJ#49) was used to assemble the DNA fragments for the VL gene, and the human Cλ DNA fragments were amplified using primers (MSJ#47/MSJ#49) and a template (pMAZ-IgL-GlycoT®). pMAZ-AG8H and pMAZ-AG8L were constructed by ligation of the resulting heavy and light-chain DNA of AG8 IgG, respectively, into the pMAZ-IgL-GlycoT plasmid at the BssHII and XbaI sites.

of preparing a functional form of a GPCR antigen with a conformation similar to that of the complex seven transmembrane α-helical structure of native GPCRs, and the limited exposure of extracellular regions of GPCRs as a target for antibodies. Due to these hurdles in developing anti-GPCR antibodies, only two therapeutic antibodies against GPCR antigens—erenumab (Aimovig®) and mogamulizumab (Poteligeo®), targeting the calcitonin gene-related peptide receptor and chemokine receptor 4, respectively—have been approved by the US FDA, in contrast to the clinical and marketing successes of a number of therapeutic antibodies targeting other types of antigens.

In this study, we report the successful isolation of a human antibody antagonizing the functions of ETA and the evaluation of its antitumor activity. ETA nanodiscs were prepared by over-expressing ETA in E. coli and reconstituting the detergent-solubilized form with lipids and membrane scaffold proteins (MSPs). Screening of an in-house-constructed human antibody phage display library against ETA nanodiscs enabled us to isolate an antibody that binds specifically to ETA. The resulting human antibody regulating the downstream signaling of human ETA showed potent antitumor effects in both in vitro tests and an in vivo xenograft mouse model. This study demonstrates that this antibody targeting human ETA could be used to elucidate the functions of endothelin receptors and could be developed as a potential therapeutic agent for cancer.

M.-S. Ju et al. Experimental & Molecular Medicine (2021) 53:1437 – 1448
Preparation of phage particles from the scFv library

E. coli ER2738 cells harboring naïve immune scFv library plasmids were inoculated and grown for 1 h in 10 ml of Super Broth (SB) medium (Becton Dickinson Diagnostic Systems, DiFCO™, USA) supplemented with 100 μg/ml carbenicillin. The culture broth was diluted 1:100 in 1 L of SB medium containing the same antibiotic and incubated at 37 °C with shaking at 250 rpm for 4 days. The OD600 of the supernatant was measured to be approximately 0.8–1.0. Then, 1 ml of VCSM13 helper phage (1 × 1012 pfu) and 70 μg/ml kanamycin were added, and the incubation was continued for 16 h at 37 °C with shaking at 250 rpm to induce the production of scFv-displaying phage particles. The culture broth was centrifuged at 10,000xg, and the supernatants were mixed with polyethylene glycol (PEG)/NaCl solution containing 10% (w/v) PEG 8000 and 3% (w/v) NaCl. The supernatants were resuspended in 1× PBS and 3% bovine serum albumin (pH 7.4), and the recovered phage particles were stored at 4 °C prior to use.

Library screening and phasing

In total, 50 μl of 4 μg/ml G3p protein purified as described previously25 was coated onto a 96-well plate (Corning, Corning, NY, USA) at 4 °C for 16 h. After extensive washing of the wells, 50 μl of H2O2 reconstituted nanodiscs (4 μg/ml) was added, and the plate was incubated at room temperature for 2 h. Before screening, the library phage particles were immobilized onto the wells in the plate immobilized with H2T2 nanorods, a negative selection procedure was conducted. The library phage particles were immobilized in wells immobilized with empty nanorods containing only MSP-1 and a lipid that did not contain H2T2. Next, 50 μl of the resulting supernatants were added to the wells preimmobilized with H2T2 nanorods. After the wash was performed with 1× PBS (pH 7.4), bound phage particles were eluted in 100 μl of glycine-HCl buffer (pH 2.2) and neutralized by the addition of 20 μl of 2 M Tris (pH 8.0). Then, 120 μl of the resulting neutralized, recovered phages and 1 ml of VCSM13 helper phage particles were added to infect E. coli ER2738, and the amplified phages were used for the next round of biopanning. The number of washing cycles was increased in each subsequent round of biopanning to enrich high-affinity binders. After five rounds of biopanning, E. coli ER2738 cells were infected with eluted phages, and 400 individual clones were cultured in 1 ml of SB medium at 37 °C with shaking at 250 rpm until the OD600 reached 0.6. Then, 50 μl of VCSM13 helper phages and 70 μg/ml kanamycin were added to the infected E. coli ER2738 cells. After overnight cultivation, the supernatant was used for phage enzyme-linked immunosorbent assay (ELISA).

Phage ELISA

To isolate phage particles displaying specific anti-H2T2 antibodies, 50 μl of 4 μg/ml of purified human H2T2 protein diluted in 0.05 M Na2CO3 (pH 9.6) was added to each well of a 96-well plate (Corning, Corning, NY, USA) and incubated at 4 °C for 16 h. After blocking with 150 μl of 4% skim milk in 1× PBS (pH 7.4) and washing four times with 150 μl of PBS (pH 7.4) containing 0.02% n-dodecyl-β-D-maltoside (DDM), 50 μl of 5 μg/ml H2T2 reconstituted nanodiscs was added to each well of the plate. Then, the plate was incubated at 25 °C for 1 h, washed with 150 μl of 1× PBS (pH 7.4) containing 0.02% DDM, and treated with 50 μl of rescued phage particles displaying scFvs. After incubating at 25 °C for 1 h and washing four times, 50 μl of anti-M13-HRP conjugates diluted 4000-fold in 1× PBS (pH 7.4) containing 0.02% DDM was added to the plate. After incubation for 1 h at 25 °C and four washes in 150 μl of 1× PBS containing 0.02% DDM (pH 7.4), 50 μl of 1-Step™ Ultra TMB was added to each well, and the plate was incubated for 20 min to develop the signal. After quenching the signal by the addition of 50 μl of 4 N H2SO4, the ELISA-binding signal was detected by measuring the absorbance at 450 nm in an Epoch plate reader (BioTek, Winooski, VT, USA).

Luciferase assay

A luciferase assay was performed using a dual-luciferase reporter assay system (Promega, Madison, WA, USA) according to the manufacturer’s instructions. Poly-L-lysine-coated plates were coated onto 96-well plates (Corning, Corning, NY, USA) by incubation at 37 °C for 1 h, and cells were then seeded at a density of 5 × 103 cells/well. The luciferase reporter plasmids were cotransfected with the control plasmid encoding Renilla luciferase into the cells in the plate, and AG8 phage supernatants were added after 24 h. Then, a mixture of dye reagent was added after 48 h, and luciferase activity was measured using a VICTOR Light luminescent (PerkinElmer, Inc. Waltham, MA, USA). The transfection efficiency was evaluated by normalization to Renilla luciferase activity as a control.

Mammalian cell culture

CHO-K1 cells expressing human ETA were maintained as monolayer cultures on 100-mm cell culture dishes in Ham’s F12 medium supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic–antimycotic solution at 37 °C in a humidified atmosphere containing 5% CO2. The established human colorectal cancer cell lines HT-29 and HCT-116 were purchased from the Korean Cell Line Bank (Seoul, Korea) and maintained in HyClone RPMI-1640 medium (Cytiva, Marlborough, MA, USA) supplemented with 10% HyClone FBS (Cytiva, Marlborough, MA, USA), 1% penicillin-streptomycin, and 1% sodium pyruvate at 37 °C in a humidified atmosphere of 5% CO2.

Expression and purification of AG8 IgG

The pMAZ-AG8H and pMAZ-AG8B plasmids, which encode the heavy and light chains of AG8 IgG, respectively, were constructed using an eCube Plasmid DNA Mini Kit (PhireKorea, Seoul, Korea) and transformed into the Expi293 cells using polyethyleneimine, as described in the literature26. After resuspension of the cells in 300 ml of Gibco FreeStyle™ medium (Thermo Fisher Scientific, Waltham, MA, USA), incubation at 37 °C with shaking at 125 rpm under 8% CO2, centrifugation at 4000xg, and the supernatants were mixed with polyethylene glycol (PEG)/NaCl solution containing 4% (w/v) PEG 8000 and 3% (w/v) NaCl. The pellets were resuspended in 1× PBS (pH 7.4) and 3 ml of 100 mM glycine-HCl buffer (pH 2.5) was loaded onto the column for elution. The eluents were immediately neutralized by the addition of 1 ml of Tris-Cl (pH 8.0). After buffer exchange with 1× PBS (pH 7.4) using Amicon Ultra 4 spin columns (Merck Millipore), the concentration and purity of AG8 IgG were analyzed by measuring the absorbance at 280 nm and by 4–15% SDS–PAGE.

Physicochemical analysis of AG8 IgG

Antibody aggregation was measured with a Waters Alliance 2695 system (Milford, MA, USA) and a Waters BioSuite high-resolution size-exclusion chromatography (SEC) column (7.5 mm × 300 mm, 10-μm particle size). Samples (10 μl, 1 mg/ml) were injected, and separation was conducted using isocratic elution with 0.05 M Na2CO3 (pH 9.6) as the mobile phase was eluted at a flow rate of 1.44 ml/min. The mobile phase was 0.1% trifluoroacetic acid (TFA) in water (Eluent A) and 0.1% TFA in acetonitrile in water (Eluent B) at a flow rate of 1 ml/min, a linear increase from 20 to 80% Eluent B; 18–30 min, washing, and re-equilibration. The injection concentration and volume were the same as those used for SEC. The intact masses of the antibody were determined with RP-HPLC using a Waters Acquity i class UPLC system. Separation was performed on a Thermo MabPac™ RP column (2.1 mm × 50 mm, 4-μm particle size) at a flow rate of 0.2 ml/min. The mobile phase was prepared by mixing 0.1% formic acid in water (Eluent A) and 0.1% formic acid in acetonitrile (Eluent B). After linear gradient elution for 2 min with an increase in the ratio of Eluent B to 25% followed by isocratic elution with 25% Eluent B, the sample was separated by linear gradient elution (25–45% Eluent B). The effluent was analyzed with a Thermo Fisher LTQ Orbitrap mass spectrometer (Thousand Oaks, CA, USA) using Fourier transform (FT) mode. The resolution and mass range of the FT-based mass spectrometer were 120,000 and m/z 400–4000, respectively. The injection concentration and volume were 0.1 mg/ml and 5 μl, respectively. Glycan profiling was performed with a Rapi-Flour labeling kit (Waters, Milford, MA, USA), and all procedures were performed as described previously25,26.

ELISA

For coating, 50 μl of G3p (4 μg/ml, diluted in 0.05 M Na2CO3 (pH 9.6)) was added to a 96-well polystyrene plate, and the plate was incubated at 4 °C for 16 h. After the addition of 150 μl of 4% skim milk in 1× PBS (pH 7.4) and incubation for 2 h for blocking, 50 μl of 4 μg/ml H2T2 reconstituted in 0.5% sarkosyl was added to the plate. Then, the plate was washed four times with 150 μl of 1× PBS containing 0.05% Tween 20 (PBST, pH 7.4), and 50 μl of AG8 IgG serially diluted in 1× PBS (pH 7.4) was added. After the plate was washed with 150 μl of PBST, 50 μl of a goat anti-human IgG (H + L) antibody-HRP conjugate (5000-fold dilution; Thermo Fisher Scientific, Waltham, MA, USA) was added. After the plate was washed...
with 150 µl of PBST, 50 µl of 1-Step™ Ultra TMB was added, the plate was incubated for 20 min, and 50 µl of 4 N H2SO4 was added to the wells to quench the ELISA signal. The absorbance at 450 nm was analyzed in an Epoch plate reader (BioTek, Winooski, VT, USA).

**Calcium flux assay**

Changes in the cytosolic Ca2+ concentration upon hETα binding to the ET-1 ligand were analyzed as described in the literature[2]. After incubation of 1 x 107 hETα-overexpressing CHO-K1 cells or HT-29 colorectal cancer cells with 5 µM Fura-2-acetoxymethyl ester (Fura-2-AM) dye at 25 °C for 1 h, serially diluted scAB AG8 was added. After incubation at 25 °C for 1 h, 10 nM ET-1 was added, and the resulting fluorescence emission at 510 nm, with separate excitation at 380 and 340 nm, was monitored using a FluoroMate FS-2 fluorescence spectrometer (Scinco, Seoul, Korea) to evaluate the Ca2+ concentration changes upon intracellular endothelin signaling.

**Proliferation assay**

Cancer cell proliferation was analyzed using a CyQUANT™ NF cell proliferation kit (Thermo Fisher Scientific, Waltham, MA, USA). Cells were seeded in 96-well plates at a density of 2-3 x 103 cells/well. After 24 h of incubation, ET-1 and the anti-ETα antibody were treated in a 1:1 ratio in 2% FBS medium, and the medium was replaced with RPMI-1640 medium. After 24 h, CyQUANT™ NF dye reagent was added, and the cells were incubated at 37 °C for 30 min. Then, the fluorescence intensity was measured as the ratio of the fluorescence at 530 nm to that at 485 nm using an Infinite M200 Pro microplate reader (TECAN, Männedorf, Switzerland).

**Western blot analysis**

Whole-cell protein lysates were prepared using RIPA buffer (iNTRON Biotechnology, Seongnam, Korea) supplemented with protease inhibitor cocktail (Roche, Basel, Switzerland), and total protein samples were quantified using a BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). After separation of equal amounts of the protein lysates on 10% Bis–Tris protein gels (Thermo Fisher Scientific, Waltham, MA, USA), transfer to PVDF membranes (Merck Millipore, USA), and blocking with 5% skim milk, the membranes were incubated with HRP-conjugated anti-β-actin, anti-phospho-ERK1/2, anti-total-ERK1/2, anti-phospho-AKT (S473), or anti-total-AKT antibodies (Cell Signaling Technology, Danvers, MA, USA). Then, the membranes were washed in 0.05% Tween 20 in Tris-buffered saline and incubated with a 1:5000 dilution of anti-rabbit IgG -HRP conjugate (Bio-Rad, USA) as the secondary antibody. Specific bands were detected using a WEST-ZOL plus Western Blot Detection System (iNTRON Biotechnology, Seongnam, Korea).

**RNA extraction and quantitative real-time PCR (qRT–PCR) analysis**

The total RNA was isolated using an RNeasy Mini Kit (Qiagen, Germantown, MD, USA) following the manufacturer’s protocol. Reverse transcription was conducted using 1 µg of total RNA as a template and SuperScript™ III Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA, USA). qRT–PCR was performed in triplicate in LightCycler 480 system with SYBR Green I Master Mix (Roche, Mannheim, Germany) and the appropriate primers (MSJ#50/MSJ#51), and the target gene expression levels were normalized to the β-actin level. The values from independent experiments were averaged, and are presented as the means ± standard deviations.

**Mouse xenograft model**

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the National Cancer Center Research Institute (NCCRI). The NCCRI is an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International)-accredited facility and abides by the Institute of Laboratory Resources (ILAR) guidelines. Five-week-old female nude mice (BALB/c) was injected as the negative control. After tumor volumes per group) after 7 days. Then, AG8 IgG (1.125 mg/kg) was injected as the negative control. After tumor volumes and body weights were measured prior to antibody injection, the tumors were measured using a caliper, and the volumes were calculated as follows: \( V = \text{Width}^2 \times \text{Length} \times 1/2. \) The mice were sacrificed 27 days after cancer cell injection.

**Statistical analysis**

Statistical analyses were performed with Student’s t test, and \( P < 0.05 \) was considered statistically significant.

**RESULTS**

**Preparation of functional hETα antigens mimicking the structure of native hETα on the cell membrane**

For screening of monoclonal human antibodies against hETα, it is necessary to prepare a sufficient amount of functional antigen structurally similar to native hETα. However, it is well known that the complex structure of GPCRs, with seven transmembrane α-helices, is difficult to express in heterologous hosts[3]. In a previous study, we overexpressed hETα in E. coli by fusion of the P9 sequence of *Pseudomonas* phage Φ6 (PhI6) to the N-terminal region of hETα (Fig. 1a)[22]. As reported in the previous work, both hETα and mETα were successfully overexpressed in E. coli through the fusion of the N-terminal P9 motif, and they were purified from sarkosyl-solubilized cell membrane fractions using Ni-NTA affinity chromatography (Fig. 1b, c). In an ELISA, purified hETα showed binding affinity not only for its ligand ET-1 but also for human Gαq, which is an essential component of GPCR downstream signaling (Fig. 1d, e). To prepare a functional hETα antigen with a native-like structure, we reconstituted purified hETα, MSP-1, and lipids in an optimized ratio, and antigen-embedded nanodiscs were successfully recovered by size-exclusion chromatography (SEC) (Fig. 1f–h).

**Isolation of a human ETα-specific antibody using a constructed human antibody library and immobilized hETα nanodiscs**

To isolate a specific ETα human antibody, we constructed a phage library displaying human scFv antibodies (library size: \( >1 \times 10^{10} \) individual clones, as estimated from the number of transformants) by PCR amplification of VH and VL genes existing in the immune repertoire of human B cells (Fig. 2a). The purified hETα nanodiscs were immobilized on the plate in an orientation-controlled manner through capture by precipitated human Gαq, so that the scFv antibodies could efficiently access the extracellular region of hETα. After five rounds of negative screening of the phage library against empty nanodiscs and biopanning against immobilized hETα nanodiscs with increasing numbers of washing cycles in successive screening rounds (Fig. 2b), we observed that phages displaying a high affinity for hETα nanodiscs were enriched based on the output phage titers (Supplementary Table 3). As determined by phage ELISA, five individual clones exhibited a high signal for binding to the hETα nanodisc, and DNA sequencing of the five clones revealed that all had the same scFv sequence (Fig. 2c), suggesting successful enrichment of a particular human antibody clone via the hETα affinity-based screening system. Next, we aligned the sequences of the variable regions of the scFv antibody (AG8) with the germline sequences of those of human immunoglobulins using IMGT/V-QUEST[23]. The sequence analysis results revealed a sequence identity of 94.44% between the VH region of AG8 and the human immunoglobulin heavy-chain.
Fig. 1 Preparation of the ETₐ antigen for isolation of an anti-hETₐ antibody. a Expression cassette for endothelin receptor type A. b, c SDS–PAGE gel images showing purified human ETₐ (hETₐ) (b) and mouse ETₐ (mETₐ) (c). d, e ELISA results showing the binding of purified hETₐ to its ligands ET-1 (d) and Gαi3 (e). f Overall scheme showing the method for preparing reconstituted ETₐ nanodiscs and empty nanodiscs. g, h SDS–PAGE gel image (g) and gel filtration chromatogram (h) showing the hETₐ nanodisc and empty nanodisc fractions; Lane 1: hETₐ nanodisc fraction; Lane 2: empty nanodisc fraction.
variable region V1–8 genes (IMGT ID: M99637), and the sequence of the AG8 VL light chain was 90.68% identical to that of the human immunoglobulin kappa chain variable region V1–17 genes (IMGT ID: KM455566).

AG8 exerts antagonistic effects on ET-1-induced signaling of hET_A

For analysis of the antagonistic effects of the isolated antibody on ET-1-induced hET_A signaling, we employed a β-arrestin Tango assay that enabled monitoring of β-arrestin recruitment through luciferase gene expression. In cells that expressed both hET_A and hET_B, luciferase expression was not activated in the control group treated with bombesin, a ligand unrelated to both hET_A and hET_B; however, the presence of ET-1, a native ligand for both hET_A and hET_B, activated luciferase expression (Fig. 2d). As expected, the addition of phage particles displaying the isolated AG8 scFv inhibited luciferase expression by up to 72% in hET_A-expressing cells. In sharp contrast, cells expressing hET_B, which shares the
To investigate whether purified AG8 can regulate the function of hETA, we expressed the isolated antibody in E. coli as a single-chain antibody (scAb) that contained a human kappa light-chain (HuC) domain and purified it via affinity chromatography using KappaSelect resin (Cytiva, Marlborough, MA, USA) (Fig. 3a). Then, the antagonistic effect of AG8 on hETA was analyzed using fura-2-acetoxyethyl ester (fura-2 AM), a ratiometric calcium indicator, to analyze ET-1-binding-triggered hETA activation, which can be monitored by measuring the increase in the intracellular Ca\(^2+\) level mediated through the inositol trisphosphate (IP3) pathway. In both hETA-expressing CHO-K1 cells treated with 10 nM ET-1 and HT-29 colorectal cancer cells treated with the same concentration of ET-1, the scAb AG8 inhibited the ET-1-induced increase in the intracellular Ca\(^2+\) level, as evidenced by the IC\(_{50}\) values (56 nM in CHO-K1 cells and 51 nM in HT-29 cells). These results clearly demonstrate that AG8 exerted an antagonistic effect on ET-1 ligand-binding-mediated hETA signaling (Fig. 3b, c).

### Physicochemical properties of AG8 IgG

The physicochemical properties of AG8 IgG were characterized by four methods, as shown in Fig. 4. The percentages of the monomeric and aggregated forms of AG8 IgG were 95.23% and 4.77%, respectively, and no other impurities were detected in RP-HPLC analysis. The molecular weight of AG8 IgG was measured both with and without PNGase F treatment, and the accuracies were <1 Da. The glycan profile of AG8 IgG was slightly different from that of the IgG standard, but the result was similar to those shown in other reports. No analyzed physicochemical properties created an issue for subsequent in vitro and in vivo assays.

### In vitro effects of AG8 IgG on cancer cells

Human ET\(_A\) is an important target for cancer treatment because it is highly involved in several signaling pathways that promote cell proliferation, metastasis, and neovascularization. In particular, a high correlation between hETA overexpression and the progression of colorectal cancer has been reported. In two colorectal cancer cell lines, HT-29 and HCT-116, AG8 IgG reduced the proliferation of cells by up to 40% (Figs. 5a and 5b). To investigate how AG8 IgG inhibited the proliferation of these cells, we performed western blot analyses to measure the phosphorylation levels of downstream signaling pathway components. It has been well established that ET-1 binding to hETA promotes the phosphorylation of ERK and AKT in colorectal cancer cells. We found that the addition of AG8 IgG significantly reduced ET-1-induced phosphorylation of both ERK and AKT in colorectal cancer cells. Furthermore, transcription of inhibin A (INHBA), which is highly involved in several signaling pathways that promote cell proliferation, metastasis, and neovascularization, was activated by ET-1 binding to hETA, was decreased upon treatment with AG8 IgG (Fig. 5d). Taken together, these results indicate that the specific binding of AG8 IgG to hETA blocks downstream hETA signaling and inhibits colorectal cancer cell proliferation.

### Inhibition of tumor growth by AG8 IgG in BALB/c nude mice

We next confirmed the anticancer effects of AG8 IgG in vivo. A xenograft mouse model was established by subcutaneous injection of colorectal cancer cells into the flanks of BALB/c nude mice. We found that the addition of AG8 IgG significantly reduced tumor growth in BALB/c nude mice.
mice, and AG8 IgG was administered at 2-day intervals by intratumoral injection (1.125 mg/kg per injection). After 27 days, tumor growth in the AG8-treated mice was decreased 40% relative to that in PBS-treated mice (Fig. 5e, f), clearly showing that AG8 IgG exerted significant antitumor effects in mice bearing colorectal cancer xenografts.
In silico modeling of the structure of AG8

Comparison of the sequences of the hETα (UniProtKB ID: P25101) and hETβ (UniProtKB ID: P24530) proteins determined their sequence identity and similarity to be 53.9% and 71.8%, respectively. To infer the structure of hETα, for which no crystal structure is available, two crystal structures (PDB codes: 5GLI and 5GLH for ligand-free hETβ and ET-1-bound ETβ, respectively) were used for in silico analysis. Models of both ligand-free and ligand-bound hETα were constructed using the sequence of hETα and the two crystal structures of hETβ (5GLI and 5GLH). Superimposition of the resulting two hETα models showed that the root-mean-square deviation between the two models was 2.694 Å (Fig. 6a). The in silico analysis showed that the endothelin-binding site in hETα was located in the region inside the 7 transmembrane helices, as in the ET-1-bound ETβ structure38, and that the conformations of two transmembrane helices (TM6 and TM7) were changed more significantly than those of the other transmembrane helices upon binding to ET-1. To analyze the AG8 binding site in hETα, a structural model of AG8 was generated using Discovery Studio 2019, and the potential binding sites were listed in order of stabilization energy using the docking function of the software. The results revealed that the extracellular loop 3 (ECL3) region connected to the 6th and 7th transmembrane helices of hETα showed the most stable binding (Fig. 6b). Interestingly, this region exhibited the highest degree of conformational change upon binding to ET-1.

**DISCUSSION**

In this study, we overexpressed a type of GPCR with an intrinsically complex structure using a bacterial expression system and prepared a protein in the form of a nanodisc to maintain a GPCR structure similar to that of native GPCRs expressed in the cell membrane environment. This antigen preparation strategy enabled us to isolate a human anti-GPCR antibody with high target antigen selectivity and the capability to regulate intrinsic
and overexpression of hET\(_A\) have an important effect on the human antibody. The regulation of downstream hETA signaling. Aberrant activation of the transmembrane domain of hETA is predicted by protein docking analysis is represented by a dotted circle. The numbers 1 through 7 in the yellow circles indicate the positions of structural modifications during chemical conjugation or genetic fusion of a part of a GPCR antigen with a carrier protein.

Our group has also fused carrier proteins such as keyhole limpet hemocyanin and ovalbumin with synthetic peptides encoding the N-terminus, extracellular loop 1 (ECL1), extracellular loop 2 (ECL2), or extracellular loop 3 (ECL3) of hET\(_A\) for isolation of anti-GPCR antibodies. However, our antibody screening trial performed through animal immunization using the prepared antigen consisting of a synthetic GPCR peptide subunit fused with a carrier protein was not successful. To overcome these limitations, we prepared a GPCR antigen in nanodisc form. Nanodiscs reconstituted with a protein such as a GPCR, phospholipids, and MSPs have been used in various studies on membrane proteins. Cai et al. solubilized the human glucagon-like peptide-1 receptor (GLP-1R) with detergent and successfully produced a nanodisc using MSP and phospholipids, and they confirmed binding activity with its ligand GLP-1 and with the G\(_S\) protein. In a similar way, the self-assembly of detergent-solubilized hET\(_A\) with POPC and MSP enabled us to produce hET\(_A\) nanodiscs leading to successful isolation of an hET\(_A\)-specific human antibody.

Through a β-arrestin recruitment Tango assay, we confirmed that the isolated antibody AG8 selectively bound to hET\(_A\), enabling the regulation of downstream hET\(_A\) signaling. Aberrant activation and overexpression of hET\(_A\) have an important effect on the survival of patients with a variety of cancers, such as breast, cervical, colorectal, ovarian, prostate, and head and neck cancers. Currently, the main antagonists targeting endothelin receptors approved for clinical trials include sitaxentan, bosentan, macitentan, and ambrisentan, all of which are small-molecule compounds. A clinical trial for sitaxentan was withdrawn, and bosentan and macitentan are dual ET\(_A\) and ET\(_B\) antagonists, whereas ambrisentan is the only antagonist known to selectively bind to ET\(_A\). Kappes et al. used ambrisentan in a preclinical murine model of metastatic breast cancer and confirmed that it inhibited cancer cell migration, invasion, and metastasis by selectively binding to ET\(_A\) without interfering with the physiological vasodilator function controlled by ET\(_B\). This suggests that selective binding of antagonists to a specific type of endothelin receptor is likely to be beneficial for cancer therapy. AG8 IgG, with high ET\(_A\) selectivity, could be a candidate therapeutic agent for cancers in which patient survival prognosis is affected by dysfunction or overexpression of ET\(_A\).

The protein sequence of hET\(_A\) is 94% identical to that of its mouse homolog. As expected, AG8 IgG showed cross-reactivity with both human and mouse ETA. In the development of anticancer therapeutic antibodies, it is necessary to evaluate antitumor effects using small animal models such as mouse models prior to assessing efficacy in primates and humans. If the antibody binds to the human antigen but not to the corresponding antigen expressed in the animal model, a surrogate antibody with characteristics and binding properties similar to those of the counterpart antigen in the model animal should be produced. Alternatively, a knock-in animal model expressing the human target antigen should be used. As mentioned above, ET\(_A\) has high sequence identity between humans and mice. In addition, the sequences of its ligands ET-1 and ET-3 are identical between the two species, and another endothelin ligand, ET-2, exhibits substantial similarity (95.2%) between humans and mice, suggesting that it is reasonable to evaluate the antitumor effects of AG8 IgG in a non-transgenic mouse xenograft model.

Representative therapeutic antibodies used for colorectal cancer treatment are bevacizumab and cetuximab. These two drugs have been administered in combination with small-molecule drugs such as irinotecan, oxaliplatin, and fluoropyrimidines in treatment regimens. However, long-term treatment with bevacizumab usually increases the expression of soluble VEGF receptor 1 (sVEGFR1) and results in resistance to the drug. In addition, cetuximab shows a general loss of therapeutic efficacy in patients with K-RAS mutations. Therefore, there is an urgent unmet clinical need for the development of improved therapeutic agents for colorectal cancer. ET\(_A\) is activated by both the paracrine and autocrine systems; thus, it affects cancer progression and metastasis in a variety of ways. In this study, we conducted in silico analysis, and the results showed that the ET\(_A\) mRNA expression level in colorectal cancer was higher than that in other cancers (breast, cervical, ovarian, prostate, and head and neck cancers) (Supplementary Fig. 1). Currently, small-molecule-based ET\(_A\) antagonists with FDA approval for treating hypertension, kidney diseases, and hypertension, kidney diseases, and
heart failure have been reported to inhibit tumor progression in a variety of cancer.\textsuperscript{55–56} The IgG antibody AG8 isolated in this study inhibited cell growth by specifically binding to eHTA in colorectal cancer cells, increasing the cytosolic Ca\textsuperscript{2+} level and blocking the activation of ET\textsubscript{a} downstream signaling. In addition, the antitumor efficacy of AG8 IgG was confirmed in a colorectal cancer xenograft model. The tumor growth inhibition observed here was superior to deuterium exchange (HDX), surface plasmon resonance analysis, AG8 IgG, mass spectrometry (MS) analysis using hydrogen- T4 lysozyme to facilitate crystallization by stabilizing the conformation of AG8 IgG, we plan to replace the third intracellular loop (ICL3) with phage display technique. The resulting AG8 IgG showed potent successfully isolated from a human antibody library using a functional coupling of G proteins to endothelin receptors. The druggable human GPCRome. J. Cardiovasc. Dev. Dis. 68, 105 (2021).

In this study, a highly challenging GPCR antigen was prepared, and a human antibody that selectively binds to eHTA was successfully isolated from a human antibody library using a phage display technique. The resulting AG8 IgG showed potent antitumor effects against colorectal cancer. To develop an antitumor therapeutic antibody with enhanced efficacy, we have engineered frameworks in three different regions of AG8 IgG and isolated new antibodies exhibiting higher binding affinity than the parental AG8 IgG for eHTA. Further, we will validate the antitumor efficacy and pharmacokinetics of the antibody by introducing an engineered Fc variant with a prolonged circulating half-life or enhanced effector functions. In addition, the search for additional applicable therapeutic indications is in progress, and the results will be reported in our future publications.

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Experimental & Molecular Medicine (2021) 53:1437 – 1448

M.-S. Ju et al.
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S.-G. Han, Y.G. Yu, W.-K. Lee, Y.-J. Kim, and S.T. Jung conceived the idea. M.-S. Ju, H.-M. Ahn, S.-G. Han, S. Ko, J.-H. Na, M. Jo, C.S. Lim, B.J. Ko, and W.-K. Lee conducted the experiments and performed the data analysis. M.-S. Ju, H.-M. Ahn, W.-K. Lee, Y.-J. Kim, and S.T. Jung wrote the manuscript. All authors read and approved the final manuscript.

COMPETING INTERESTS
The authors declare no competing interests.

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