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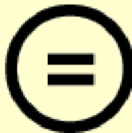

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[UCI]I804:48009-200000108999

Master of Science

Cytotoxic effects of a recombinant immunotoxin CEA(scFv)-PE26 in CEA-expressing cancer cells

CEA 발현 암세포에서 재조합 면역 독소 CEA (scFv) -PE26 의 세포 독성 효과

The Graduate School
of the University of Ulsan

Department of Medicine
Yoo, Jiwon

Cytotoxic effects of a recombinant immunotoxin CEA(scFv)-PE26 in CEA-expressing cancer cells

Supervisor Han Choe

Master's thesis

Submitted to
the Graduated School of the University of Ulsan
in partial Fulfillment of the Requirements
for the Degree of

Master of Science

by

Yoo, Jiwon

Department of Medicine
Ulsan, Korea
August 2018

Cytotoxic effects of a recombinant immunotoxin CEA(scFv)-PE26 in CEA-expressing cancer cells

This certifies that the dissertation
of Yoo, Jiwon is approved

Jang, Yeon-Jin
Committee Vice-chair Dr.

Han Choe
Committee member Dr.

Leem, Chae-Hun
Committee member Dr.

Department of Medicine
Ulsan, Korea
August 2018

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LIST OF ABBREVIATIONS

ABC	Ammonium bicarbonate
ACN	Acetonitrile
BSA	Bovine serum albumin
CV	Column volume
DAPI	4',6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
E. coli	*Escherichia coli*
FACS	Fluorescence activated cell sorter
FBS	Fetal bovine serum
$Furin_{cs}$	Furin cleavage site
GFP	Green fluorescence protein
GPC	Gel Permeation Chromatography
HC	Hill coefficient
CEA	Carcinoembryonic antigen
HPLC	High performance liquid chromatography
IAA	Iodoacetamide
IC_{50}	50% inhibit concentration
IMAC	Immobilized metal ion affinity chromatography
IPTG	Isopropyl 1-thio-β-d-galactopyranoside

LAL	Limulus Amebocyte Lysate
LB medium	Luria-Bertani medium
LC	Liquid chromatography
MBP	Maltose binding protein
MS	Mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
OD	Optical density
PBS	Phosphate buffered saline
PE	*Pseudomonas* exotoxin A
PI	Isoelectric point
PVDF	Polyvinylidene difluoride
RIT	Recombinant immunotoxin
RPMI 1640	Roswell Park Memorial Institute 1640 Medium
RT-PCR	Real-time polymerase chain reaction
scFv	Single chain Fv
SE	Standard Error
SEC	Size exclusion
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TEV	Tobacco etch virus
TEVrs	Tobacco etch virus protease recognition site
TFA	Trifluoroacetic acid

Abstract

Cytotoxic effects of a recombinant immunotoxin CEA(scFv)-PE26 in CEA-expressing cancer cells.

Yoo, Jiwon

The Graduate School

University of Ulsan

College of Medicine

Pseudomonas exotoxin A (PEA) is derived from the gram-negative bacillus *Pseudomonas aeruginosa*. This toxin interferes with protein synthesis via the ribosylation of EF-2 (Elongation factor 2) and thereby causes apoptosis. In the present study, I designed a recombinant immunotoxin (RIT) derived from PEA that would retain specific cytotoxic capabilities but not cause non-specific adverse events such as vascular leak syndrome. This engineered RIT comprised 26 kDa of the full length PEA of 38 kDa. This truncated form lacked the B cell and T cell epitopes present in the native protein so that it would have reduced immunogenicity whilst maintaining its toxicity. Moreover, I generated a more targeted immunotoxin via the addition of an scFv fragment that is specific for carcinoembryonic antigen (CEA), which is overexpressed on the surface of diverse carcinoma types relative to normal cells. To efficiently produce and purify this RIT, a bacterial expression vector was used that contained a hexahistidine-maltose binding protein (His6-MBP) tag. The BL21(DE3) *E.coli* strain was used to produce the protein from which the overexpressed fusion product, his6-MBP-CEA(scFv)-PE26, was purified using affinity chromatography. I achieved a yield of 12.6 mg of CEA(scFv)-PE26 from a 2 L culture. I

tested the growth effects of this RIT in the gastric cancer cell line, MKN45, and colon cancer cell lines, SNU-C4 and HCT-116; and the liver cancer cell line, HepG2 . The viability of the MK45 cell line, which is the only one of the four tested that overexpresses CEA, was significantly reduced to about 20%. In contrast, the SNU-C4 and HCT-116 cells showed a 70% and 90% viability level, respectively, after exposure to CEA(scFv)-PE26 . The IC_{50} value in the MKN 45 cells for this RIT was 1.22 ± 0.56 ng/mL (n=3), which is lower than that reported for other RIT molecules. I also confirmed by flow cytometry analysis that my novel immunotoxin binds specifically to the CEA expressed on the MKN45 cell surface but does not interact with the other cell types that were analyzed. In conclusion, CEA(scFv)-PE26 is a novel RIT that shows potential as a future anticancer drug that can specifically target CEA-expressing cancer cell.

Keywords: Carcinoembryonic antigen (CEA), Pseudomonas exotoxin A, CEA(scFv)-PE26, Recombinant immunotoxin, Soluble overexpression, Purification

Introduction

Pseudomonas exotoxin A (PEA) is a bacterial toxin generated by *P. aeruginosa* that comprises 613 amino acids with AB toxin structure-function properties. The A domain corresponds to the catalytic domain (Ib and III, aa 365-613), and the B domain to the translocation domain (II, aa 253-364). The B domain transfers the A domain into the cytoplasm of the cells and a binding domain is also contained in the toxin (Ia, aa 1-252) that binds to cell surface receptors [1, 2]. PEA binds to its cell surface receptor and enters the cell through receptor-mediated endocytosis [3]. In the endosome, the translocation domain is cleaved by Furin protease and the A domain is then translocated across the endoplasmic reticulum membrane and delivered to the host cytoplasm [4]. The catalytic ADP ribosylation of elongation factor 2 (EF2) then occurs, leading to apoptotic cell death [5]. The potency of PEA has suggested its potential as a possible new anti-cancer therapeutic. However, the native toxin has issues such as a lack of specificity and the promotion of vascular leak syndrome that would prevent its clinical use [6]. Previous studies have shown however that recombinant immunotoxins (RITs) that are designed based on a 38 kDa portion of PE (PE38) are possible new cancer treatment drugs when the cell-binding domain is replaced with an antibody fragment that targets a specific cancer cell [7, 8].

In the current study, I developed a novel RIT comprising a single-chain Fv (scFv) from an anti-carcinoembryonic antigen (CEA) monoclonal antibody fused to a 26 kDa fragment from PEA (PE26). CEA is usually present at very low levels in the blood of healthy adults, but becomes highly expressed in some types of cancer including gastric carcinoma, pancreatic carcinoma, lung carcinoma, breast carcinoma, and most notably in colorectal carcinoma. Labetuzumab govitecan (IMMU-30) was referred in order to obtain the sequence of CEA (scFv). It is designed to kill the cancer specifically by targeting the expressed carcinoembryonic antigen on the tumor surface [9, 10]. The PE26 truncated fragment of PEA was generated by deleting most of domain II (aa 253-364) but retaining the furin processing

site and a portion of domain Ib (aa 365-404), thus removing the B- and T- cell epitopes [11, 12]. To silence the B-cell epitopes , six hydrophilic residues were substituted with alanine, glutamic acid and histidine [13, 14]. The resulting engineered immunotoxin had lower non-specific toxicity and immunogenicity and was resistant to degradation by lysosomal proteases [15-17]. This RIT therefore not only become a more effective inhibitor of cancer cell activity, but also showed about an 8-fold lower toxicity in mice and diminished potency to induce capillary leak syndrome in rats [18].

Escherichia coli (*E.coli*) is the most popular host system for RIT production as these molecules do not require post-translational modifications such as glycosylation for their bioactivity [19, 20]. A high-yield of these proteins can thus be achieved very cost-effectively [21] but the efficient production of recombinant product in *E.coli* can often lead to insoluble protein forms known as inclusion bodies [21, 22]. Inclusion body proteins lack biological activity and require solubilization and refolding to recover their functions. RITs have been successfully produced from inclusion bodies in *E.coli* [23], but their activities are sometimes not recoverable even after refolding. To overcome some of these issues, I adopted a fusion protein approach by employing a maltose-binding protein (MBP) tag and subsequently optimized the expression conditions and solubility of the fusion product in *E.coli.* I have now purified an MBP-CEA-PE26 fusion protein and observed that the MBP tag raises the solubility of the expressed product and promotes its proper folding. I tested this recombinant molecule in a cytotoxicity assay and found strong activity in CEA-expressing cancer cells and no activity in non-CEA-expressing cells.

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Material and Method

1. Materials

1-thio-β-d-galactopyranoside (IPTG) and Dithiothreitol (DTT) were purchased from Anaspec (Fremont, CA). Tris-HCl and Coomassie brilliant blue R-250 were sourced from Amresco (Solon, OH). Imidazole was obtained from Daejung Chemicals (Siheung, Korea) and ampicillin was purchased from Duchefa Biochemie (Haarlem, Netherlands). NaCl and glycerol were obtained from Samchun Chemical (Pyeongtaek, Korea). The HisTrap^{TM}FF was purchased from GE healthcare (Piscataway, NJ). *E.coli* BL21 (DE3), SHuffle and Origami 2 cells were obtained from Novagen (Madison, WI). For Gateway cloning, integrase, lambda integrase and excisionase were obtained from Elpis Biotech (Daejun, Korea). The dialysis membranes were purchased from Viskase (Darien, IL). Amicon Ultra was sourced from Merck Millipore (Darmstadt, Germany). Triton X-114 and 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). The SNU-C4, MKN-45, HepG2 and HCT-116 cell lines were obtained from the Korea Cell line Bank (Seoul, Korea). RPMI-1640, fetal bovine serum (FBS), and other culture reagents were purchased from Gibco/Thermo Fisher Scientific (Waltham, MA). The enhanced chemiluminescence (ECL) western blotting detection reagent was purchased from Amersham (Buckinghamshire, UK). The CEACAM5 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

2. Construction of the CEA(scFv)-PE26 expression plasmid

The CEA(scFV)-PE26 expression vector was generated using the Gateway cloning system based on BP and LR recombination reactions. The BP reaction is a recombination reaction between attB and attP sites and LR reaction is a reaction between attL and attR sites. The gene of interest is amplified with the help of a tagged primer pair and it is carried out to

transfer the sequence of interest to one or more destination vectors in simultaneous reactions, making the technology high throughput. The entry clone is mixed with the appropriate gateway vector and gateway clonase enzyme. Recombination between these generates two molecules. One molecule contains the DNA segment of interests, the other molecule is a by-product.

Briefly, a codon optimized sequence encoding the 480 amino acids of CEA(scFv)-PE26 was synthesized (GenScript, Piscataway, NJ). The CEA(scFv) portion of this sequence was linked to the furin processing site, RHRQPR/G. Following the furin cleavage site, a GGSG linker was connected to the PE26 sequence (Figure 1A). The tobacco etch virus recognition site (TEVrs), ENLYFQ/G, was placed at the N-terminus of the CEA-PE26 nucleotide. The entire DNA sequence was then subcloned into a pDONOR207 vector via a BP reaction to obtain an entry clone, pENTR-CEA-PE26. Following the BP reaction, an expression vector was created by an LR reaction between pENTR-CEA-PE26 and the destination vector, pDEST-HMGWA. The fidelity of the final vector was confirmed by sequencing (Macrogen, Daejeon, Korea). PE26 and CEA(scFv)-GFP vectors were also generated by Gateway cloning and used as negative controls in the subsequent cytotoxicity assays and FACscan analyses.

3. Fusion protein expression

The expression plasmids were transformed into different *E.coli* cell types i.e. BL21 (DE3), SHuffle and Origami 2. Each culture was incubated in LB broth at 37°C at 200 rpm for 24 hour . One day later, a single colony was picked up and seed-cultured into 4 mL of LB medium containing 50 μg/mL ampicillin. This small culture was then grown at 37°C overnight and then transferred to 2 L of LB medium added ampicillin and tetracycline at 1:100 ratios. This culture was incubated at 37°C with shaking at 200 rpm. To induce the overexpression of the recombinant product, 0.5 mM IPTG was added to the culture batch when the OD_{600} reached 0.62. The temperature condition was then maintained at 37°C for 4 h

and 18°C for 18 h . The bacterial cells were then harvested and analyzed by SDS-PAGE using a 10% tricine gel.

4. Purification of CEA(scFv)-PE26 from *E. Coli*

The *E.coli* were harvested from 2 L cultures by centrifugation at 3000 rpm for 20 min at 4°C. The supernatant was then removed and pellets were stored at -80°C. For cell lysis, the collected pellets were thawed in ice and resuspended in 150 mL immobilized metal ion affinity chromatography (IMAC) binding buffer (20 mM Tris-HCl, 300 mM NaCl, 5% glycerol [v/v], pH 8.0) and sonicated using an ultrasonic cell disruptor JY99-IIDN (Ningbo Scientz Biotechnology, Guangdong, China) until the lysate was completely homogenized. The suspension was then centrifuged at 15000 rpm for 10 min at 4°C and the supernatant collected for purification using a 20 mL HisTrap FF column (GE healthcare, Piscataway, NJ). After equilibration with a 5 column volume (CV) of IMAC binding buffer, the column was fed with the crude sample. Non-specific protein was removed with 5 CV of IMAC washing buffer containing 30 mM imidazole. The MBP-CEA(scFv)-PE26 fusion protein was completely eluted from the column with 5 CV of IMAC elution buffer (20 mM Tris-HCl, 300 mM NaCl, 5% glycerol [v/v], 500 mM imidazole, pH 8.0). To block and negative effects of the NaCl and imidazole on the TEV protease, the elution buffer was changed to NaCl-free IMAC buffer (20 mM Tris-HCl, 5% glycerol [v/v], pH 8.0) using a dialysis membrane (Viskase, Darien, IL). The eluted fusion protein was then digested with TEV protease at a ratio 1:5 (w/w) for 18 h at room temperature (RT). For a second round of IMAC purification, the digested solution was added to 300 mM NaCl and loaded onto 5 mL HisTrap FF column pre-equilibrated with 5 CV of IMAC binding buffer. As expected, the target CEA(scFv)-PE26 protein product did not bind to the Ni resin and was collected from the flow through. This preparation was subsequently concentrated using an Amicon Ultra-0.5 Centrifugal Filter (Millipore ,Massachusetts) The finally purified CEA(scFv)-PE26 was then dialyzed

against PBS buffer at 4°C and, after the removal of endotoxin (see procedure below), was stored at -80°C until use.

5. Purification of PE26 and CEA(scFv)-GFP from *E. coli*

As a negative control recombinant products for use in the cytotoxicity assay, PE26 without CEA(scFv) was prepared and purified. The bacterial cells were harvested from 1 L cultures and purified essentially as described for CEA(scFv)-PE26. CEA(scFv)-GFP was also prepared for use in the cytotoxicity assay and FACscan analysis. Briefly, MBP-CEA(scFv)-GFP was produced from 1 L *E. coli* cultures. Following centrifugation at 3000 rpm for 20 min at 4°C, the supernatant was removed and pellets were stored at -80°C. The pelleted cells were subsequently resuspended in 100 mL IMAC binding buffer (20 mM Tris-HCl, 250 mM NaCl, 5% glycerol [v/v], pH 8.0) and sonicated as described earlier. The resulting suspension was centrifuged at 15000 rpm for 10 min at 4°C and the supernatant was collected for purification on a 5 mL HisTrap FF column.

After equilibration with 5 CV of IMAC binding buffer, the column was fed with the crude sample and non-specific protein was removed with a further 5 CV of IMAC washing buffer containing 100 mM imidazole. The MBP-CEA(scFv)-PE26 fusion protein was completely eluted from the column with 5 CV of IMAC elution buffer (20 mM Tris-HCl, 300 mM NaCl, 5% glycerol [v/v], 500 mM imidazole, pH 8.0). The eluted protein was collected and digested with TEV protease at a ratio of 1:5 and incubated at RT for 18 h. The preparation was then dialyzed against 20 mM Tris, 250 mM NaCl, 5% glycerol (v/v), pH 8.0 using a 10 kDa cut-off membrane. The dialyzed sample was next loaded onto 5 mL Ni column again. Washing buffer containing 50 mM imidazole (10 CV) was passed through the column and the product was eluted with the same washing buffer containing 100 mM imidazole. The target protein preparation was then applied to an MBP Trap HP column (GE Healthcare, Piscataway, NJ). After equilibration with 5 CV of IMAC binding buffer (20 mM

Tris, 250 mM NaCl, 5% glycerol [v/v], pH 8.0), the protein was loaded onto the column and the target protein was eluted in the flow through. The purified CEA(scFv)-GFP was concentrated to about 1 mg/ml then dialyzed against PBS buffer supplemented with 5% glycerol . After removal of endotoxin with Triton X-114, the protein was stored at - 20ºC.

6. Electrophoresis and quantification of protein expression and solubility

Proteins were added to 5 x sample buffer (312.5 mM Tris-HCl, 50% glycerol, 5% SDS, 0.05% bromophenol blue, 100 mM DTT, pH 6.8) before loading onto a 10% tricine SDS-PAGE gel. The protein bands were stained using Coomassie brilliant blue R-250. The expression level, solubility and purity of the proteins was assessed using an ImageJ image analyzer (http://imagej.nih.gov.ij) and quantified as described previously [24-28)].

7. Determination of CEA(scFv)-PE26 purity by HPLC

The recombinant proteins were evaluated using size exclusion chromatography-high performance liquid chromatography (SEC-HPLC) to determine their purity. The binding solution was prepared using PBS since the final product was stored in PBS buffer. For equilibration, more than 10 CVs of PBS buffer at pH 7.4 were applied to a protein-pak 300SW SEC 7.5 × 300 mm column. The protein itself was then applied to the column with a flow rate of 1 mL/min for 25 minutes. The elution peaks were detected at 280 nm. This experiment was performed at room temperature.

8. Endotoxin removal and endotoxin assay

Purified proteins were treated with 1% of the protein volume of Triton X-114 (Sigma-Aldrich, St. Louis, MO) at 4ºC for 30 min and at RT for 10 min. The sample was

then centrifuged at 13000 rpm for 10 min to remove the Triton detergent. The Toxin Sensor Chromogenic LAL Endotoxin Assay Kit (GenScript , NJ) was then used to measure the endotoxin level in accordance with the manufacturer's instructions. Briefly, the purified CEA(scFv)-PE26 was diluted with endotoxin-free LAL water to a 1 μg/mL concentration of which a 100 μL aliquot was placed into an endotoxin-free vial along with endotoxin standards at 1, 0.1, 0.05, 0.025 and 0.01 EU/mL. Reconstituted LAL reagent was then added to each of the vials (100 μL) with gentle mixing, followed by incubation at 37ºC on a heating block for 6 min. A 500 μL aliquot of color-stabilizer #1 was added as a stop solution and mixed thoroughly. This was followed by the sequential addition of 500 μL of color stabilizers #2 and #3 with mixing. A 150 μL aliquot of each final mixture was transferred into a 96-well-plate and the 545 nm absorbance was then measured using an ELISA plate reader. The units of endotoxin present in the sample were calculated using a standard curve obtained from the standard solutions.

9. LC-MS/MS analysis

To confirm the purification of CEA(scFv)-PE26, LC-MS/MS analysis was conducted. Briefly, a CEA(scFv)-PE26 sample was subjected to SDS-PAGE and the recombinant product was excised from the gel. The CEA(scFv)-PE26 gel piece was then destained using 100 mM ammonium bicarbonate (ABC) in 50% acetonitrile (ACN) solution. The gel fragment was next washed in 100 mM ABC and 100% ACN was added to dehydrate the gel at room temperature. The dried gel was then incubated in 50 mM DTT in 50 mM ABC at 50ºC for 1 h, washed again in 50 mM ABC, then added to 55 mM iodoacetamide (IAA) in 50 mM ABC for 1 h at room temperature again in the dark . After this incubation, the gel fragment was further washed in 100% of ACN and digested with 0.1 μg/μL of trypsin in a 20 μL volume with 2 mM $CaCl_2$ for 1 h on ice. After this step, 50 mM ABC was added and the fragment was placed at 37 ºC for 16 h. The supernatant and gel fragment were

washed again with 40% ACN supplemented with 0.1% trifluoroacetic acid (TFA) via smooth vortexing and the new supernatant was combined with the previous one . The supernatant was then dried in a speed vacuum. LC-MS/MS was performed using an Ultimate 3000 liquid chromatography system (Thermo, Waltham. MA) system connected to a Q Exactive plus biopharm spectrometer (Thermo).

10. Western blot analysis

For western blotting, cell lines or tumor cells were lysed for 30 minutes at 4°C using proprep (Intron bio, Seongnam, Korea) containing protease inhibitor cocktail (BP-477; Boston BioProducts, Worcester, MA) and cleared by centrifugation at 13,000 rpm. Protein aliquots of 50 μg were loaded onto each well, electrophoretically separated on 10% SDS-PAGE gels, and transferred onto polyvinylidene difluoride (PVDF) membranes. Blocking of the membrane was done for 30 min using skim milk (Bioworld, Philadelphia, PA) and the blots were incubated with antibodies for CEA (A0116; DAKO, Glostrup, Denmark) and β-actin (A5441; Sigma, St. Louis, MO). Blots were developed using the SuperSignal West Pico Chemiluminescent Substrate (34080; Pierce, Rockford, IL).

11. RNA preparation and real-time quantitative PCR

MKN45, SNU-C4 and HepG2 cells were treated with Trizol (Gibco BRL) to extract RNA. The RNA samples were subsequently reverse-transcribed using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). The resulting cDNA was amplified using specific primers for CEACAM5 which is a gene in the CEA family. Real-time polymerase chain reaction (RT-PCR) was performed using an iCycler iQ system with the iQ SYBR Green Supermix (Bio-Rad Laboratories) and the transcript levels were quantified using the comparative Ct method and normalized to the S18 housekeeping gene. The

following primer sequences were used: CEACAM5, forward 5′-AAG AAA TGA CGC AAG AGC CTA TGA C-3′ and reverse 5′-CCC GAA AGG TAA GAC GAG TCT GAC-3′; S18, forward 5’-TTT GCG AGT ACT CAA CAC CAA CA-3’ and reverse 5’-CCT CTT GGT GAG GTC AAT GTC TG-3’. The Step-One Real-Time PCR System amplification conditions were used (Applied Biosystems, Foster City, CA) as follows: 95°C for 10 minutes, 95°C over 40 cycles for 15 seconds, and 60°C for 1 minute. Melting curve analysis was performed to evaluate the specificity of the PCR product fluorescence. The quantity of each transcript was calculated as described by the protocol for the instrument.

12. Cytotoxicity assay

MKN 45 ,SNU-C4 and HCT116 cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, and 100 U/mL streptomycin at 37°C in a 5% humidified incubator with a 5% CO_2/95% air atmosphere. Cells were seeded into 96-well plates at a density of 3×10^3 cells/well . CEA(scFv)-PE26 was then added to the cell media at 0.1, 1 or 10 μg/mL. PBS was used as a control and it was processed by duplication. The cells were then incubated for 72 h at 37°C in a 5% humidified incubator with a 5% CO_2/95% air atmosphere. Cell viability was evaluated by measuring the light absorbance at 560 nm. Calculation of the IC_{50} was performed for MKN45 cells only. Briefly, the cells were seeded into 96-well plates at 3,000 cells/well and treated with CEA(scFv)-PE26 at various concentrations (0.001, 0.01, 0.1, 1, 10, 100 ng/mL). At 72 h after treatment with protein, CellTiter-Glo (G7571; Promega, Madison, WI) reagents was added and luminescence was measured using a Victor X3 spectrometer.

Cytotoxicity was calculated using the following equation with Microsoft Excel software:

$$V = top - (top-bottom) / (1 + (IC_{50} / conc.) ^\wedge HC)$$

where V denotes cell viability, top is the highest level of cell viability, bottom is the lowest level of cell viability, conc is the protein concentration and HC is the Hill coefficient of inhibition. The dose-response curve was standardized using non-linear regression analysis.

13. Flow cytometry

For flow cytometry analysis, 10^5 cells were treated with 10 ng/mL CEA(scFv)-GFP and then incubated at 37°C for 1 h. After washing with PBS, the cells were resuspended in 200 μL of PBS and fluorescence was measured using a FACSCalibur flow cytometer with FlowJo software (Ashland, OR).

14. PEGylation of CEA(scFv)-PE26

CEA(scFv)-PE26 was conjugated PEG to the N-terminus. It was used as reductive alkylation method with 20 kDa mPEG aldehyde at low pH. At pH 6.0, CEA(scFv)-PE26 which was purified by Ni column was changed PBS buffer to 0.1 M Na_2HPO_4 by dialysis. At the lower pH, protein became aggregation so pH 6.0 condition was chosen. After then, methoxy PEG-aldehyde was treated to the protein with 1:6 ratio and mixed thoroughly with vortexing. It was incubated 18 h at 4℃ in the dark with inverting condition. To remove the non-PEGylated protein, it was applied to the cation exchange column and gel filtration column but it was hard to seperate. So raised more mPEG treating to obtain the PEGylated protein more.

15. Statistics

Data are presented as mean values ± standard error (SE) of n≥3 samples in 3 independent experiments.

Results

1. Expression and purification of CEA(scFv)-PE26

Expression vectors to produce the recombinant CEA(scFv)-PE26 protein were constructed using the Gateway cloning system (Figure 1B). The protein product contains a His6-MBP tag at its N-terminus and a TEV cleavage site between the tag and the target gene. The expression of the target gene was controlled by a T7 promoter which is inducible with the addition of 0.5 mM IPTG concentration in *E.coli* BL21(DE3) approximately 38-40% for all fusion proteins (Figure 2). Among the three different bacterial strains I tested (BL21(DE3), SHuffle and Origami 2), BL21(DE3) showed the highest expression of the recombinant product and best solubility at 18°C (Table 1). These results indicated that a lower temperature improves recombinant protein solubility in the cytoplasm of *E.coli*. The CEA(scFv)-PE26 was purified with two IMAC steps (Figure 3A). The CEA(scFv)-PE26 product that contains the His6-MBP tag can bind to an Ni column and be subsequently eluted using 500 mM imidazole (Figure 3B). TEV protease can then be used to cleave the tag with an over 90% efficiency at 25°C for 18 h. The resulting tag-free CEA(scFv)-PE26 was purified using a second IMAC round with an Ni column again to remove the cleaved tags and some impurities (Figure 3C). The untagged CEA(scFv)-PE26 was then eluted in the flow through since the tags were trapped by the NiSO4 resin. With a purification yield of 3.6%, 12.6 mg CEA(scFv)-PE26 was obtained from 2 L culture (Table 2). The purity of the final product was almost 97% even though there was no GPC column step. The endotoxin level in the purified CEA(scFv)-PE26 preparation was 0.251 EU/µg which is within the acceptable range (<1 EU/µg) for commercial preparations. The purity of the CEA(scFv)-PE26 was around 97% when it was evaluated by HPLC (Figure 4).

2. Identification of CEA(scFv)-PE26

To verify the CEA(scFv)-PE26 protein, LC-MS/MS analysis was performed using a trypsin digested sample. CEA(scFv)-PE26 was confirmed using this approach with an 81.46% sequence coverage (Figure 5).

3. Expression level of CEA in different cell lines

To investigate the CEA expression level in the MKN45, SNU-C4, HepG2 and HCT-116 lines, the cells in each case were lysed using pro-prep (iNtRON Biotechnology, Seongnam, Korea) The CEACAM5 antibody against CEA was used, which has a size of 180-200 kDa. This band was observed only in MKN45 cells (Figure 6A). Real time PCR using Ct values normalized to the S18 ribosomal protein confirmed this result (Figure 6B).

4. Cytotoxicity assay

Four cell lines were used to test the effects of CEA(scFv)-PE26 against CEA expressing and non-expressing cells. MTT assays indicated that cell viability was decreased to around 20% in the MKN45 gastric cancer cell line after 72 h of treatment with CEA(scFv)-PE26 at 10 μg/mL (Figure 7). For the SNU-C4 and HCT-116 lines, the cell viability was about 70% and 90%, respectively, in this experiment. To measure the IC_{50} value, MKN45 cells were treated with a concentration series of CEA(ScFv)-PE26 for 72 h (0.001, 0.01, 0.1, 1, 10, 100 ng/mL) on MKN45. The IC_{50} value for this RIT in these cells was 1.22 ± 0.56 ng/mL (n=3) with a Hill coefficient of 0.52 ± 0.07 (Figure 8). The CEA(scFv)-GFP negative control had no effects on MKN45 cell viability under the same conditions and PE26 had only effect on MKN45 cell viability 70% at the highest concentration (data not shown).

5. Flow cytometry

Flow cytometry analysis was conducted to determine whether the CEA(ScFv) could bind to the cell surface of the MKN45, SNU-C4, HepG2 and HCT-116 cells. I employed CEA(scFv)-GFP for this analysis and got a positive result only for the MKN45 cells (Figure 9).

6. PEGylation of CEA(scFv)-PE26

To prolong the protein half-life, CEA(scFv)-PE26 was conjugated to the N-terminus of protein using reductive alkylation method with 20 kDa mPEG aldehyde at low pH. At pH 6.0, CEA(scFv)-PE26 was stable without aggregation but at lower than pH 6.0 condition, protein became aggregation. After incubation of CEA(scFv)-PE26 with mPEG-CHO and sodium cyanoborohydride, around 50 % of PEG was conjugated. This PEGylated protein was applied to the cation exchange column and gel filtration column but it was hard to seperate. So raised mPEG amount and incubation time, 18 h to 48 h. Approximately 70-80% of PEG was conjugated to the CEA(scFv)-PE26. It was small volume so increased CEA(scFv)-PE26 amount around 6 mg.

Discussion

The anticancer drugs in current clinical use are typically highly toxic and therefore also affect normal cells and cause side effects such as hair loss and vomiting [29)]. Targeted anticancer drugs that specifically kill cancer cells are therefore highly desirable [30)],[31)]. A knowledge of the various receptors expressed by cancer cells and their binding proteins is crucial for developing such targeted agents. In this regard, antibody-toxin chimeric molecules represent a promising approach as they can bind to specific antigens on the surface of the cancer cells and interfere with their growth [32)]. The purpose of the current study was to investigate this approach further through the use of a recombinant immunotoxin (RIT).

I generated a purpose-built RIT by attaching a truncated form of *Pseudomonas* exotoxin A (PEA) to a scFv antibody fragment that is specific for carcinoembryonic antigen (CEA), a known biomarker expressed on the surface of various cancer cell types. The resulting recombinant product CEA(scFv) -PE26, was then tested for its effects on both CEA-expressing and non-expressing cells. It is known that CEA is expressed during the fetal period but not in normal adult cells and is re-expressed in certain cancer cells [33)]. It is for example known to be expressed in colon, liver, and gastrointestinal cancers [34)]. Although CEA is a biomarker such as colorectal cancer and liver cancer, it is expressed only in a specific cell line because the cell line is cultured continuously and it becomes genetically unstable. And it is presumed that even though the same cancer, CEA expression level is differ from patient to patient [35, 36)].

The expression of CEA was examined in the colorectal cancer cell lines SNU-C4 and HCT-116, hepatoma cell line HepG2, and gastric cancer cell line MKN45. Real time PCR and western blot analysis indicated CEA expression only in MKN45 cells (Figure 6). Consistently, following treatment of MKN45, SNU-C4 and HCT-116 cells for 72 h with CEA (scFv) -PE26, the cell viability was lowest for the MKN45 cells (reduced to 20%) at the 10 μg/mL dose (Figure 8). In contrast, the SNU-C4 and HCT-116 cultures were still

significantly viable with 70-90% of the cells surviving the treatment. Notably also, the CEA(scFv)-PE26 molecule showed a higher potency than that reported previously for PE A-truncated toxin [37)]. In addition, the IC_{50} of CEA (scFv) -PE26 was measured at 1.22 ± 0.56 ng/mL (n=3) with a Hill coefficient of 0.52 ± 0.07 (Figure 9). This is far lower than the 12 ng / mL value reported for a previously constructed RIT in which an anti-CEA monoclonal antibody was linked to a truncated PEA [38)].

In my comparison and control experiments, the viability of CEA(scFv)-GFP treated MKN45 cells was almost unchanged and that of PE26 exposed cells was about 50%. CEA(scFv)-PE26 thus showed a greater cytotoxic potency than PE26 alone. I further examined whether the CEA(scFv) fragment does in fact attach to the surface of a CEA-expressing cancer cell. I used the CEA(scFv)-GFP molecule in flow cytometry analysis with the four cancer cell lines being tested. The GFP wavelength increased only in the MKN45 cell line that shows high CEA expression (Figure 7). Previous flow cytometry studies have shown that the wavelength shift increases and the axis of the graph shifts to the right when normal cells are labeled with GFP, with no such change in unlabeled cells [39)]. In addition, no graph axis shift has been found previously in FACS analysis of HepG2 as a CEA-negative cell line used in other studies [40)]. From these results, it can be concluded that CEA(scFv) binds to the CEA expressed on the surface of MKN45 cells [41)].

However, several RIT studies suggest that there is a limit on RIT, its efficacy is decrease because of the short half-life [31, 42, 43)]. To complement this problem, modifying protein with polyethylene glycol (PEG) is well known as most useful way [44)]. PEG modifies the protein increasing the molecular size and steric hindrance so that can increase the blood-residency of proteins. Moreover, it can increase the proteolytic-stability, plasma half-lives and decrease the immunogenicity and hepatic uptake [42)]. So CEA(scFv)-PE26 was modified with PEG to improve the half-life but it was hard to obtain PEGylated-protein. PEGylation will be continued.

In conclusion, I have constructed, produced, and purified an anticancer drug that can target a molecule, CEA, that is specifically expressed on certain cancer cells. This CEA(scFv)-PE26 molecule only shows specific cytotoxic effects against cancer cells that strongly express CEA and represents a promising new approach to the development of future cancer drugs.

Table 1. Expression and solubility of CEA(scFv)-PE26 in different *E.coli* strains grown at different temperatures

Strain	Expression level (%)		Solubility (%)	
	37°C	**18°C**	**37°C**	**18°C**
BL21(DE3)	38.3	39.9	39.5	47.5
SHuffle	33.5	36.9	27.9	42.3
Origami 2	17.9	18.7	40.5	44.1

Table 2. Prokaryotic purification of CEA(scFv)-PE26

Purification steps	CEA(scFv)-PE26 (from 2 L culture)			
	Total protein (mg)	Purity (%)	CEA(scFv)-PE26 (mg)	Yield (%)
Cell weight	5000 (pellet)	-	-	-
Supernatant	1080	58.6	339.7	100
1st chromatography	45	81.6	19.7	5.8
Final product	12.6	97.03	12.2	3.6

*The yield was obtained from a 2 L culture of the BL21(DE3) *E.coli* strain.

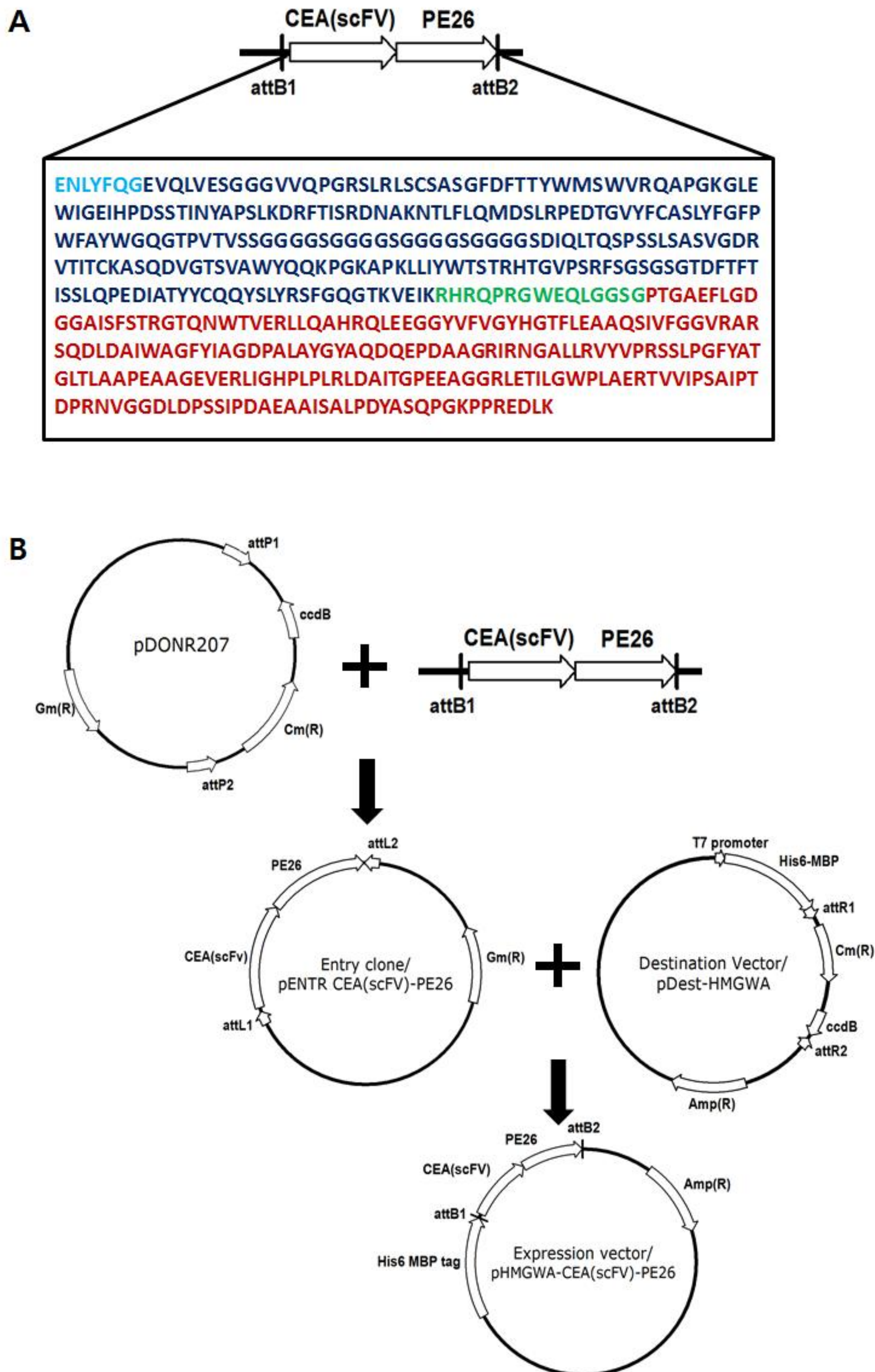

Figure 1. Construction of CEA(scFv)-PE26 (A) CEA(scFv)-PE26 amino acid sequence. (B) Vector map of His6-MBP-CEA(scFv)-PE26 using the Gateway cloning system. The prokaryotic expression of the vector is driven by an IPTG-inducible T7 promoter. Ampicillin is used to select the cells.

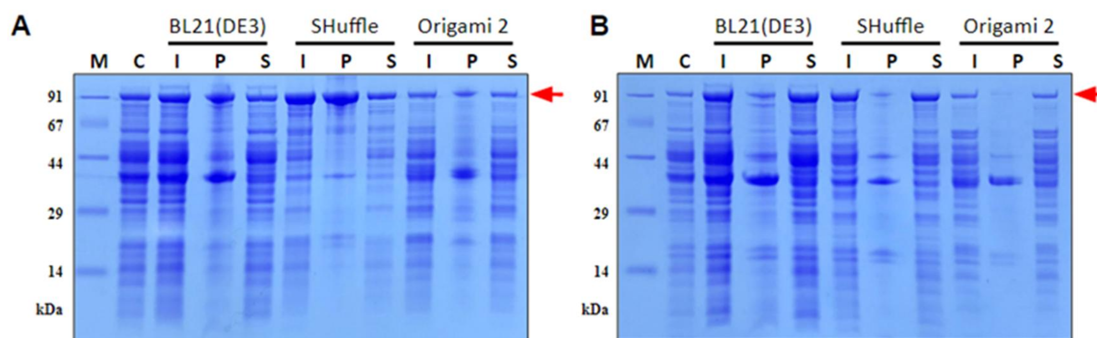

Figure 2. Expression and solubility analysis of CEA(scFV)-PE26 fusion proteins in different *E.coli* strains Expression of the recombinant fusion proteins was induced by 0.5 mM IPTG . Recombinant expression was tested at an induction temperature of 37ºC (A) or 18ºC (B). The arrows indicate the migrated CEA(scFv)-PE26 product fused with an His6-MBP tag. M, molecular weight markers; C, total cell protein prior to IPTG induction as a negative control; I, total cell protein after IPTG induction; P, pellet fraction after cell sonication; S, soluble supernatant after cell sonication.

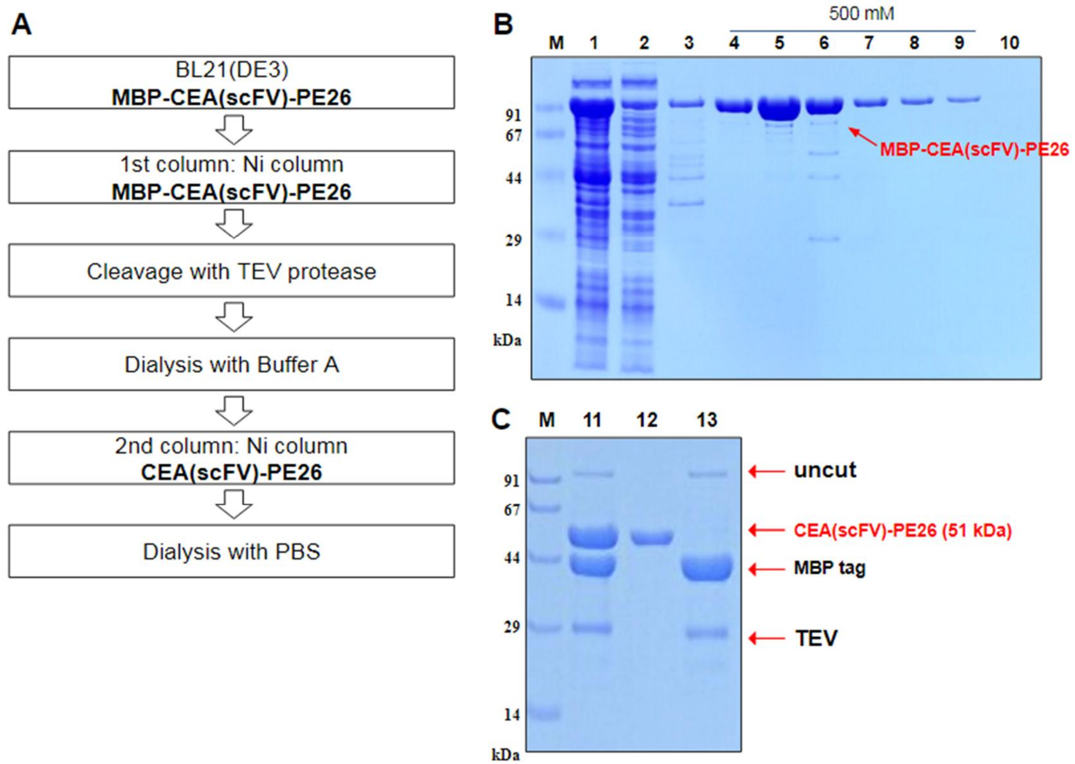

Figure 3. Purification of CEA-PE26 (A) Flow chart of the purification steps for the recombinant immunotoxin. (B) First column step. M, molecular weight markers; lane 1, total cell protein after IPTG induction; lane 2, flow through when the total cell protein was bound to the Ni column; lane 3, washing with 30 mM imidazole; lanes 4-9, elution of the fusion protein with 500 mM imidazole; lane 10, washing with 1 M imidazole. (C) Second column step. M, molecular weight markers; lane 11, pooling and TEV protease treatment of the proteins from lanes 4-9; lane 12, CEA(scFv)-PE26 was eluted with the flow through; lane 13, TEV-uncut protein and His6-MBP tag elution with 1 M imidazole.

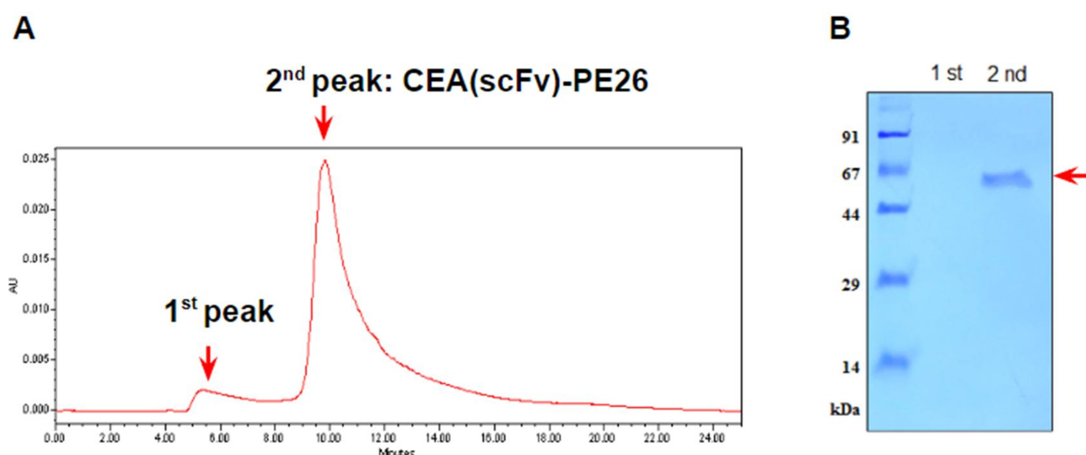

Figure 4. SEC-HPLC analysis of CEA(scFv)-PE26 (A) CEA(scFv)-PE26 was analyzed by HPLC using a protein-pak 300SW SEC 7.5 × 300 mm column to evaluate the purity. The x-axis indicates the retention time (min) and the y-axis shows the absorbance at 280 nm (arbitrary units, AU). The first peak appeared at 5.33 min and the second, which is the main peak for CEA(scFv)-PE26, appeared at 9.831 min. (B) fractions from (A) were analyzed using SDS-PAGE. The first and second peak fractions were concentrated at the same volume and loaded at equivalent amounts. The 51 kDa protein present in the second peak fraction only is CEA(scFv)-PE26.

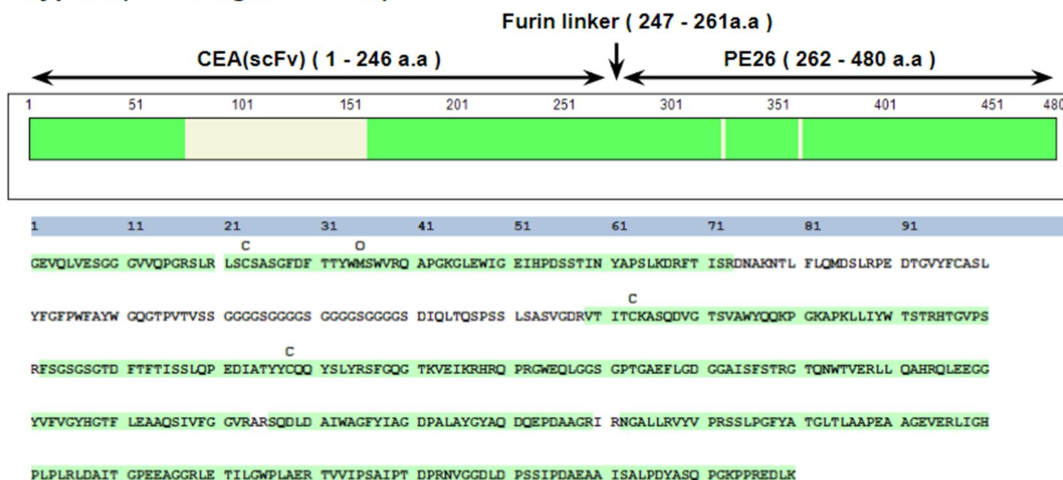

Figure 5. Identification of the CEA(scFv)-PE26 analized by LC-MS/MS performed by trypsin digested sample. The percent coverage for protein identification is represented by the identified peptides in the total protein sequence. The protein was digested by trypsin and the coverage was 81.46%.

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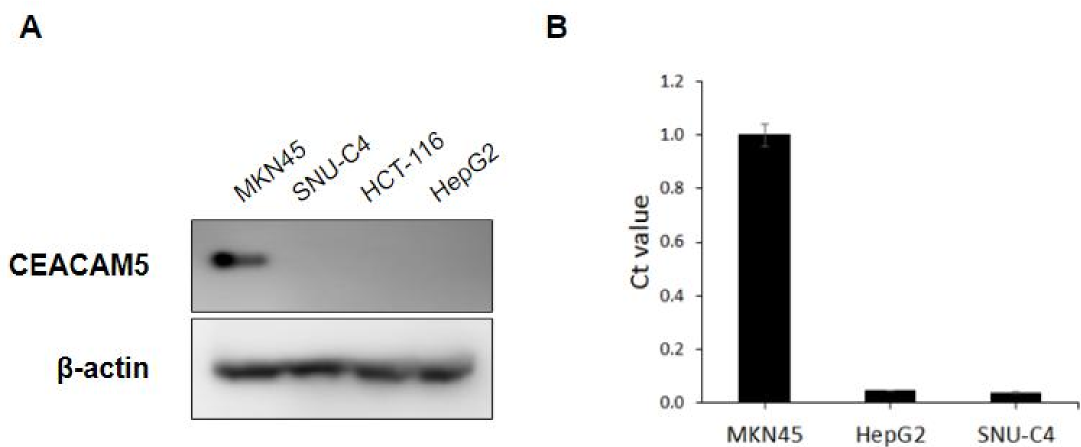

Figure 6. Expression of CEA in different cancer cell types (A) MKN45, SNU-C4, HCT-116 and HepG2 cells were analyzed by western blotting using a CEA antibody (CEACAM5) with β-actin used as a loading control. CEA was detected only in the MKN45 cell line. (B) Real time PCR confirmed the higher CEA expression in MKN45 cells.

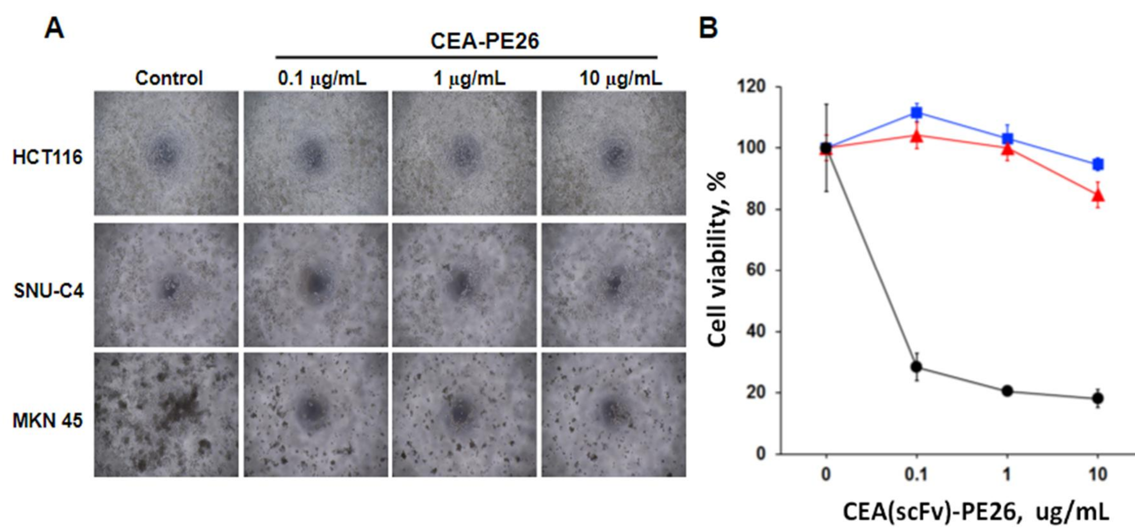

Figure 7. MTT assay (A) Morphology changes in the indicated cells after treatment with CEA(scFv)-PE26. (B) Effects of the purified CEA(scFv)-PE26. ■ : HCT-116, ▲ : SNU-C4, ● : MKN-45. At the 0.1 μg/mL concentration of recombinant toxin, the viability of MKN45 cells was decreased to about 20% with no effect evident in the other cell types.

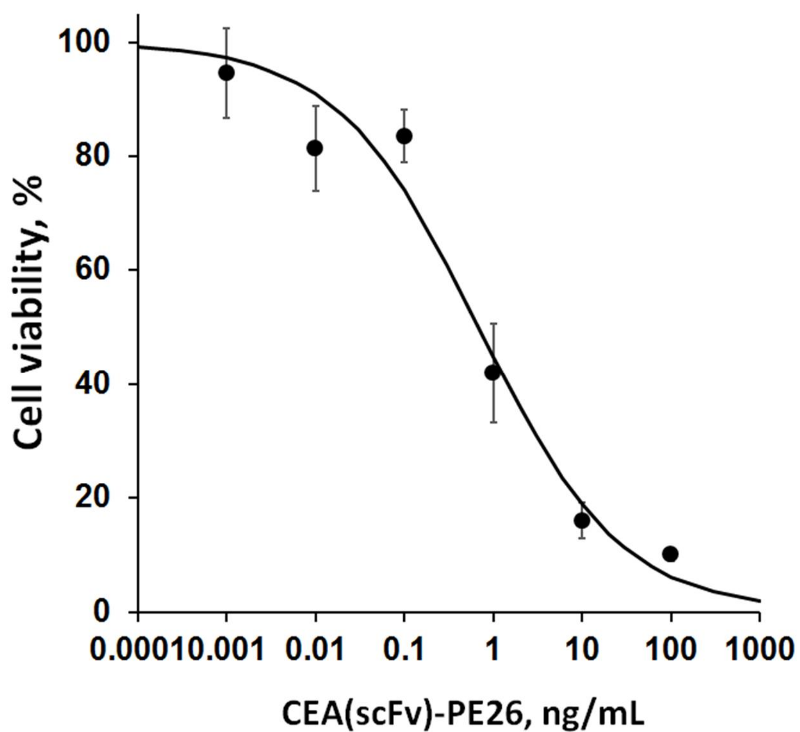

Figure 8. IC_{50} assay for CEA(scFv)-PE26 in MKN45 cells The IC_{50} of CEA(scFv)-PE26 in MKN45 cells was calculated at 1.22 ± 0.56 ng/mL (n=3) with a Hill coefficient of 0.52 ± 0.07.

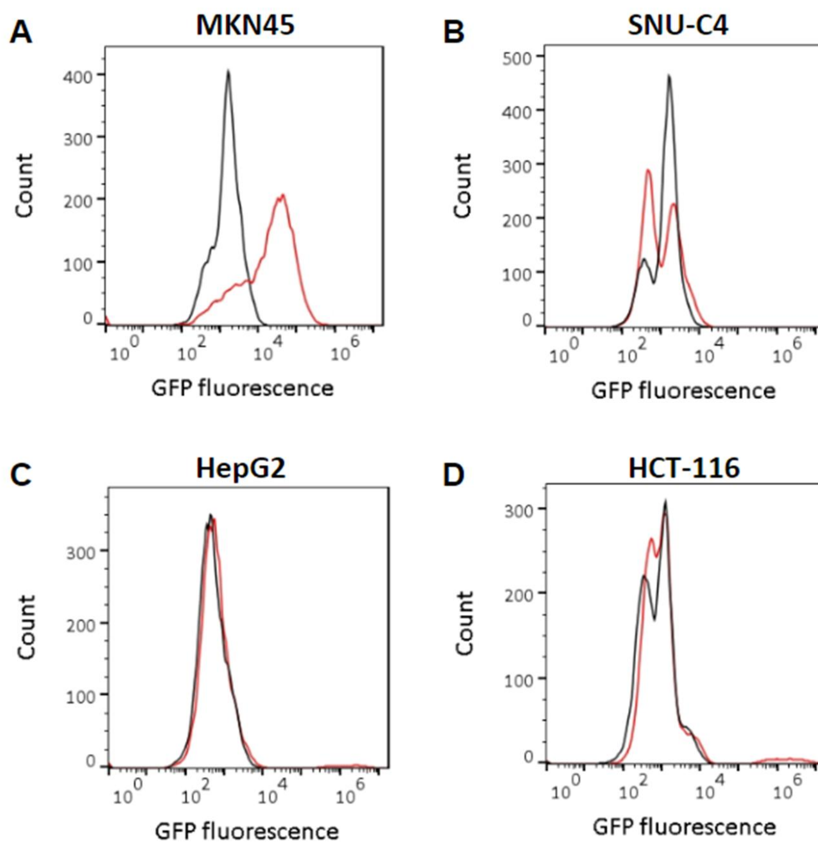

Figure 9. Flow cytometry analysis of CEA(scFv)-GFP binding to cancer cells The black line denotes un-treated cells and the red line indicates CEA(scFv)-GFP treated cells. After a one hour incubation with CEA(scFv)-GFP, a shift in the graph was only evident in the MKN45 cells.

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국문요약

CEA 발현 암세포에서 재조합 면역 독소
CEA (scFv) -PE26 의 세포 독성 효과

유지원
울산대학교 대학원
의학과

Pseudomonas exotoxin A (PEA)는 녹농균이라는 그람-음성 간균에서 기원한 독소이며, 세포 내 단백질 합성에 관여하는 신장인자 2(elongation factor 2)를 리보실화함으로써 단백질 합성을 방해하여 세포사멸을 일으키는 것으로 알려져 있다. 본 연구에서는 기존에 38 kDa 으로 잘라 사용했던 PEA 를 26 kDa 으로 더 작게 만들어 혈관누출증후군을 좀 더 보완할 뿐만 아니라, B cell 과 T cell 의 항원 결정부위를 제거하여 자체 면역원성을 줄이되 그 독성을 유지할 수 있도록 디자인 하였으며 이러한 독소를 암 특이적으로 발현 하는 발암배아성 항원 (Carcinoembryonic antigen, CEA)와 scFv 를 연결하여 표적화된 면역독소를 디자인 하였다. 이를 효율적으로 생산하기 위하여 헥사히스티딘 말토스 결합 단백질 (HIS6-MBP)을 포함한 발현벡터를 제작하였고 BL21(DE3), SHuffle 그리고 Origam2 와 같은 대장균에서 His6-MBP-CEA(scFv)-PE26 의 과발현을 유도하였으며 그 중 가장 발현률이 높은 BL21(DE3)를 선택하였다. 과발현된 벡터를 가지고 친화 크로마토그래피를 사용하여 단백질을 정제하였고 최종적으로 2 L 의 배양액에서 약 12.6 mg 의 CEA(scFv)-PE26 가 얻어졌다. 이렇게 정제된 면역독소를 사용하여, CEA 발현에 따른 세포독성 비교를 진행하였다. 세포독성

평가를 진행하기 전에, 여러 암세포주 (위암 세포주; MKN45, 대장암 세포주; SNU-C4, HCT116 및 간암세포주; HepG2)에서 CEA 단백 발현율을 Western Blot 과 Real time PCR 을 이용하여 조사한 결과 MKN45 세포주에서만 CEA 발현율을 보였으며, 다른 세포주에서는 CEA 발현이 매우 작아 검출되지 않았다. 또한 CEA 발현이 다양한 암세포들에 CEA(scFv)-PE26 를 72 시간 동안 처리했을 경우의 세포생존율을 조사하기 위하여 MTT assay 를 진행하였다, 그 결과 동일 농도 10 µg/mL 에서 MKN45 세포주의 세포 생존율이 약 20% 까지 유의성 있게 감소되었으며 이와는 반대로 SNU-C4 는 70%, HCT-116 은 90% 가량의 세포생존율을 보였다. MKN45 의 IC_{50} 수치는 1.62 ± 0.85 ng/mL 로 기존의 다른 재조합면역독소가 갖는 IC_{50} 수치보다 낮아 저농도로도 치료효과를 볼 수 있음을 시사한다. 더 나아가 면역독소가 CEA 발현 특이적으로 결합하는지를 확인하기 위하여 MKN45, SNU-C4, HCT-116 및 HepG2 에 CEA(scFv)-GFP 를 처리하여 FACscan 을 진행한 결과, CEA 발현율이 높았던 MKN45 에서만 green fluorescence 파장의 증가를 볼 수 있었으며 다른 세포주에서는 파장의 증가를 의미하는 그래프 축의 이동을 볼 수 없었다. 이러한 결과를 종합해 볼 때, 재조합 면역독소인 CEA(scFv)-PE26 는 CEA 에 특이적으로 발현되는 암을 표적화하는 표적항암제로서의 가치를 기대해 볼 수 있다.

중심단어: 발암배아성항원, 녹농균, CEA(scFv)-PE26, 재조합면역독소, 수용성 과발현, 정제