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Improvement of FasL Gene Therapy *In Vitro* by Fusing the FasL to Del1 Protein Domains

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1. Introduction

Gene delivery, transfection, cytotoxicity, and many other factors influence the ability of gene therapy to treat cancer. In addition, as with pharmacologic agents, longer exposure to higher concentrations of gene products should intensify their effects (Wada et al., 2007). Cytotoxic gene products, such as FasL and TRAIL, may remain in tissues after the death of the transfected cells, and they are known to induce apoptosis in both transfected cells and neighbouring cells (Hyer et al., 2003; Kagawa et al., 2001). They have been examined for use in cancer gene therapy and its effects have been examined *in vitro* and *in vivo* (Elojeimy et al., 2006; Griffith et al., 2009). FasL delivered via a viral vector can reduce tumour size and improve prognosis in an explanted tumour model.
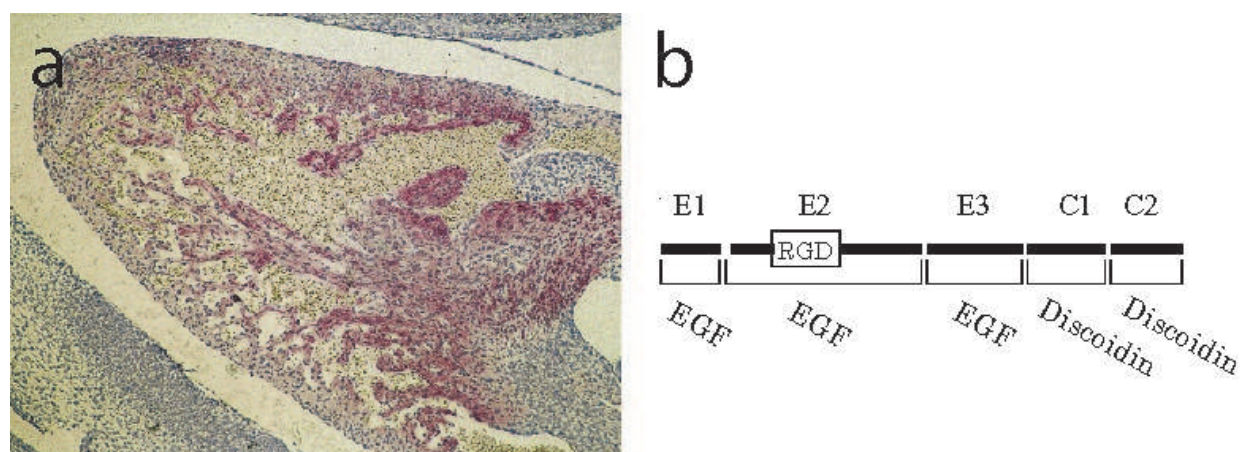
a. Immunohistochemical staining for Del1 in a 14.5 dpc (days post coitum) mouse embryo. Sections were stained with anti-Del1 antibody, followed by an alkaline phosphatase-conjugated secondary antibody and naphthol red as a colorimetric substrate. Immunoreactive protein appears red. Staining for Del1 appeared in the subendocardial region. b. The domain structure of Del1 protein. The RGD sequence in the second EGF motif is indicated.

Fig. 1. Expression and structure of Del1 protein

Currently, multiple injections with a gene therapeutic agent are needed because it is unlikely for a single injection to eliminate a cancer. Therefore, gene therapeutic agents need to be not only safe but inexpensive. Non-viral vectors represent a possible safe and inexpensive way of delivering genes for gene therapy (Lungwitz et al., 2005). However, the

gene transfer efficiency of non-viral vectors remains low. New non-viral vectors that are non-carcinogenic, non-immunogenic, and highly efficient are currently being developed. Here, we discuss the use of non-viral vectors encoding Del1 in cancer gene therapy. Del1 is an extracellular matrix (ECM) protein expressed by embryonic endothelial cells and hypertrophic chondrocytes (Fig. 1a) (Hidai et al., 1998). We examined the biological functions of Del1 domains by generating non-viral vectors encoding fragments of the Del1 gene.

2. Del1 and its application in cancer gene therapy

Del1 is a 480-amino acid protein made up of five regions, including three epidermal growth factor (EGF) repeats (E1-3) at its N-terminus and two discoidin domains (C1 and C2) at its C-terminus (Fig. 1b). The C1 domain is essential for the deposition of Del1 in the ECM, and E3 enhances the ECM deposition mediated by C1 (Hidai et al., 2007). Proteins fused to the E3 and C1 domains are deposited in the ECM, where they accumulate. The E3 domain can also increase the endocytosis of transfected genes, and at high concentrations, E3 induces apoptosis (Kitano et al., 2008, 2010). We therefore examined the possible application of Del1 in cancer gene therapy by transfecting a squamous cell carcinoma line with cDNA encoding a FasL-E3C1 fusion protein. This protein was deposited in ECM, increased apoptosis, and enhanced the efficiency of a following second transfection *in vitro*.

2.1 E3 enhances the efficiency of gene transfection

Although viral vectors are highly efficient for gene transfer, they can be carcinogenic and immunogenic (Check, 2002). They also require more time and are more expensive than non-viral vectors. However, non-viral vectors are less efficient for gene transfer. Various methods have been made to improve the efficiency of non-viral vectors. For example, novel chemical transfection reagents have been developed to improve extracellular binding. Also, vectors have been modified with chemicals, growth factor peptides, extracellular matrix proteins, and viral proteins to improve uptake via receptor-mediated endocytosis (Al-Taei et al., 2006; Kikuchi et al., 1996; Nam et al., 2009; Oba et al., 2007).

Although the characteristics of the transfection reagents are important, differences between cell types are a more important determinant of transfection efficiency; some cells are always more easily transfected than others (Von Gersdorff et al., 2006). In this regard, the biological state of a given cell type, such as which endocytic pathways are functional in the cell prior to treatment, may be an important factor in endocytosis-mediated gene transfer. Molecules that initiate and enhance endocytosis are needed to improve endocytosis as a method for delivering exogenous molecules.

Del1 protein is one such a factor that increases the efficiency of transfection *in vitro* and *in vivo* (Kitano et al., 2008). We have found that conditioned medium containing Del1 increases *LacZ* gene transfection using several non-viral gene transfer systems, including lipoplex and polyplex systems. Experiments using deletion mutants and fragments of Del1 have shown that domain E3 of Del1 mediates the enhancement of gene transfer. Incubation of culture cells with as low as 16 pM of recombinant E3 is sufficient to enhance transfection, and 1 nM recombinant E3 enhances the transfection 12-fold. This effect of E3 was observed in every cell type tested. E3 enhanced transfection even when it was administrated to the cell culture medium before transfection. The effects of Del1 on gene transfer are inhibited by both of nystatin, an inhibitor of caveolin-dependent endocytosis, and chlorpromazine, an inhibitor

of clathrin-dependent endocytosis. Furthermore, the addition of E3 to cell culture medium increases phagocytosis *in vitro* (Fig. 2). These results indicate that E3 activates several kinds of endocytosis.
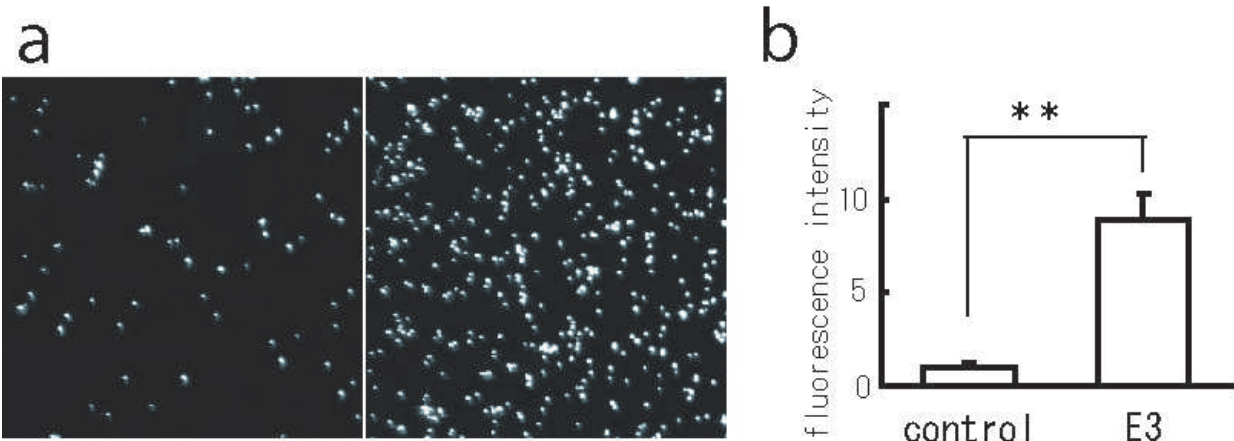
a. Confluent COS-7 cells were cultured with 1-μm diameter fluorescent beads (Invitrogen, Carlsbad, CA) and 1 nM of an alkaline phosphatase (AP)-E3 fusion protein (left side of panel a) for 1 h, washed with PBS, and visualized by fluorescence microscopy. AP protein was used as a negative control (right side of panel a). b. Fluorescence intensity evaluated using image analysis software (AquaCosmos, Hamamatsu photonics, Hamamatsu, Japan). Fluorescence was normalized by the control samples. Results represent means ± SEM (n=6). The results show that recombinant E3 increases phagocytosis.

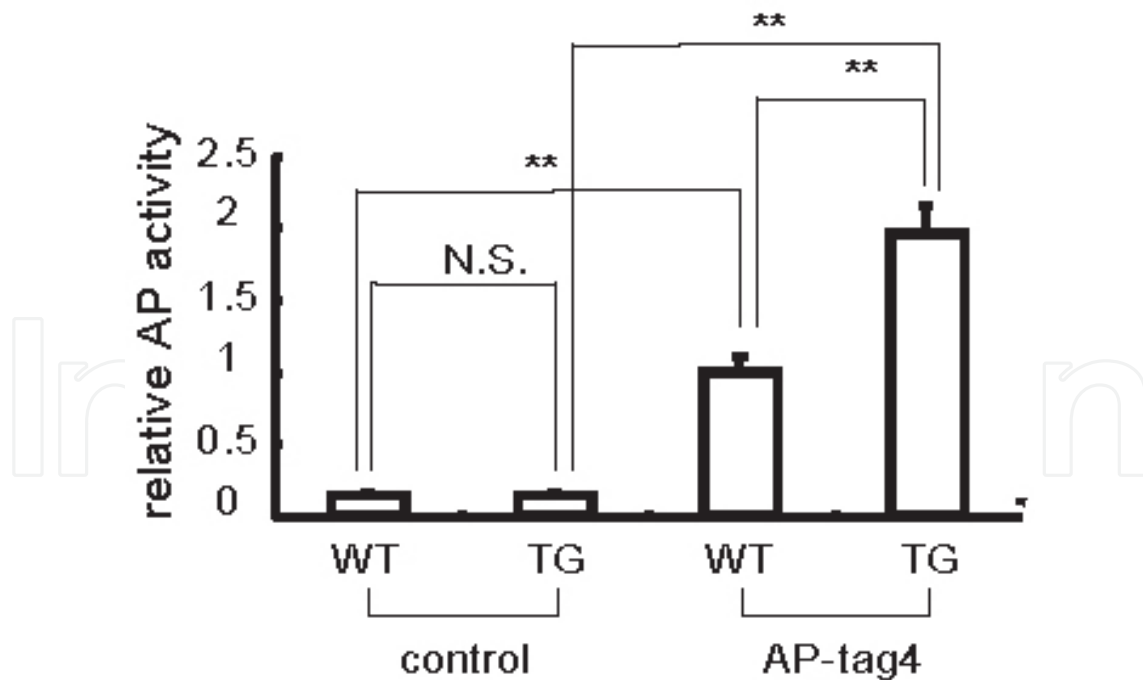Fig. 2. Effect of recombinant E3 on phagocytosis

Fig. 3. Effect of recombinant E3 on gene transfer *in vivo*

The effects of Del1 on gene transfer were observed *in vivo*. We found two-fold higher serum alkaline phosphatase activity following transfection with a heat-stable alkaline phosphatase (AP) cDNA in mice over-expressing Del1 compared wild-type littermates (Fig. 3). These

results suggest that the E3 fragment of Del1 in ECM can be used as a general enhancer of non-viral gene transfer.

Del1 over-expressing mice (TG) and wild-type mice (WT) were intravenously injected with a cDNA encoding AP using jet-PEI (Polyplus-transfection, San Marco, CA, USA). Mice injected with an empty vector were used as negative controls. After 24 h, serum AP activity was measured. AP activities were normalized by the AP activity of wild-type mice. Results represent means ± SEM (n=6). N.S., not significant. These results revealed that Del1 increased the susceptibility to gene transfer *in vivo*.

2.2 E3 induces apoptosis

Del1 is present in branchless cavities, such as the heart and umbilical veins, as well as in avascular tissue with hypertrophic chondrocytes in developing embryos (Hidai et al., 1998). In transgenic mice, constitutive expression of Del1 decreases the total volume of the vascular bed (Hidai et al., 2005). These findings suggest that Del1 is inhibits angiogenesis activity. However, some evidence suggests that Del1 promotes angiogenesis. Zhong et al. reported that Del1 can stimulate angiogenesis in animal models of ischemia (Zhong et al., 2003). Additionally, Del1 can accelerate tumor growth by enhancing vascular formation (Aoka et al., 2002). Del1 has seemed to have ambiguous characteristics.

We have found that Del1 induces cell death *in vitro* (Fig. 4) (Kitano et al., 2010). Treatment of cells with Del1 results in chromatin condensation and DNA laddering, suggesting apoptosis. Experiments using TUNEL and annexin V staining also suggest that Del1 induces apoptosis. The apoptosis-inducing activity of Del1 is localized in E3. As little as 25 pM recombinant E3 is sufficient to induce apoptosis, and we have found that E3 induces apoptosis in all adhesive cell types examined. Because of this ability to induce cell death, the E3 domain of Del1 might be useful for cancer gene therapy.
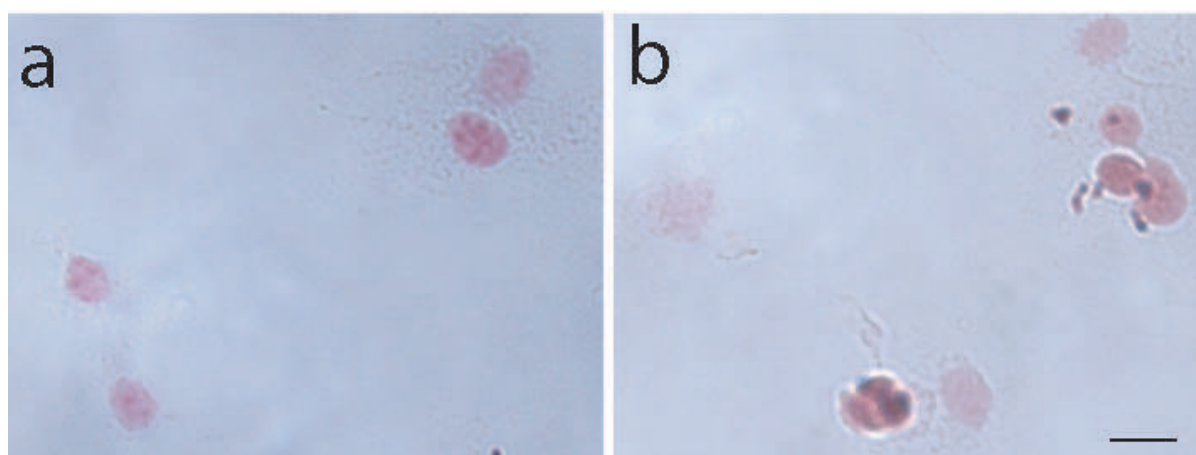
COS-7 cells were transfected with Del1 cDNA (a) or an empty vector. (b). After 24 h, fragmented DNA was detected with a TACS2 TdT-blue label *in situ* apoptosis detection kit (Trevigen, Gaithersburg, MD, USA). Cells were counterstained with Nuclear Fast Red. Bar , 20 μm.

Fig. 4. *In situ* detection of DNA fragmentation induced by a recombinant E3

The C1 domain of Del1 also mediates apoptosis. Hanayama et al. reported that the C1 domain binds to phosphatidylserine (PS), a component of the plasma membrane (Hanayama et al., 2004). In healthy cells, PS is maintained on the inner leaflet of the plasma membrane lipid bilayer. However, in apoptotic cells, PS is present in the outer leaflet. Del1

may link phagocytes with apoptotic cells via the integrin-binding RGD sequence in the E2 domain and the PS-binding C1 domain, which can bind to apoptotic cells. Thus, Del1 can both initiate apoptosis and enhance the elimination of apoptotic cells.

2.3 C1 as a deposition domain

The ECM is a critical factor in morphogenesis (Fujiwara et al., 2011; Sakai et al., 2003). Because the organization of the ECM directly influences the structure of tissues and organs, determining how ECM organization is regulated can help clarify the process of morphogenesis. We therefore investigated how Del1 is assembled in the ECM using an AP-Del1 fusion protein. We found that the fusion protein is secreted from cells and deposited in the ECM (Fig. 5).


Fig. 5. Del1 deposition domain can immobilize AP protein

CHO cells were cultured and transfected with cDNA of an AP-Del1 fusion protein. Cells were removed from tissue culture plates with EDTA and the ECM remaining was collected with a cell scraper, followed by centrifugation. Collected ECM pellets were incubated with p-nitrophenyl phosphate in microcentrifuge tubes. The ECM pellet on the left was collected from wild-type cells, the pellet in the middle from cells transfected with a cDNA encoding AP, and the pellet on the right from cells transfected with cDNA encoding the AP-Del1 fusion protein. The pellet on the right was stained purple indicating that the AP-Del1 fusion protein was present in the ECM.

Using various Del1 deletion mutants, we have found that the C-terminus of the C1 domain, which contains a lectin-like structure, mediates ECM deposition (Hidai et al., 2007). The efficiency of deposition is influences by the presence of other domains in Del1. A fragment containing E3 and C1 (E3C1) had the highest level of ECM deposition, with approximately 70% of the secreted AP fusion protein deposited in the ECM. In contrast, fragments containing C2, which is highly homologous to C1, were present at much lower levels in the ECM. The E3C1 fragment was deposited in the ECM by all cell types examined, although the efficiency varied. Digestion of the ECM with bovine testis hyaluronidase released Del1 from ECM, suggesting that glycosaminoglycans are involved in the deposition of Del1.

In vivo gene transfer experiments in mice showed that the deposition domain of Del1 dramatically alters the distribution of exogenous proteins. The AP activity in liver was 8 times

higher and the serum activity 30 times lower in mice injected intravenously with a cDNA encoding an AP-E3C1 fusion protein than in mice injected with a cDNA encoding AP alone. In addition to AP protein, the E3C1 sequence of Del1 can immobilize several proteins, including yellow fluorescent protein (YFP) (Fig. 6). Therefore, the E3C1 sequence should be a powerful tool for targeting therapeutic proteins to target tissues and thereby increasing the efficacy and decreasing side-effects.
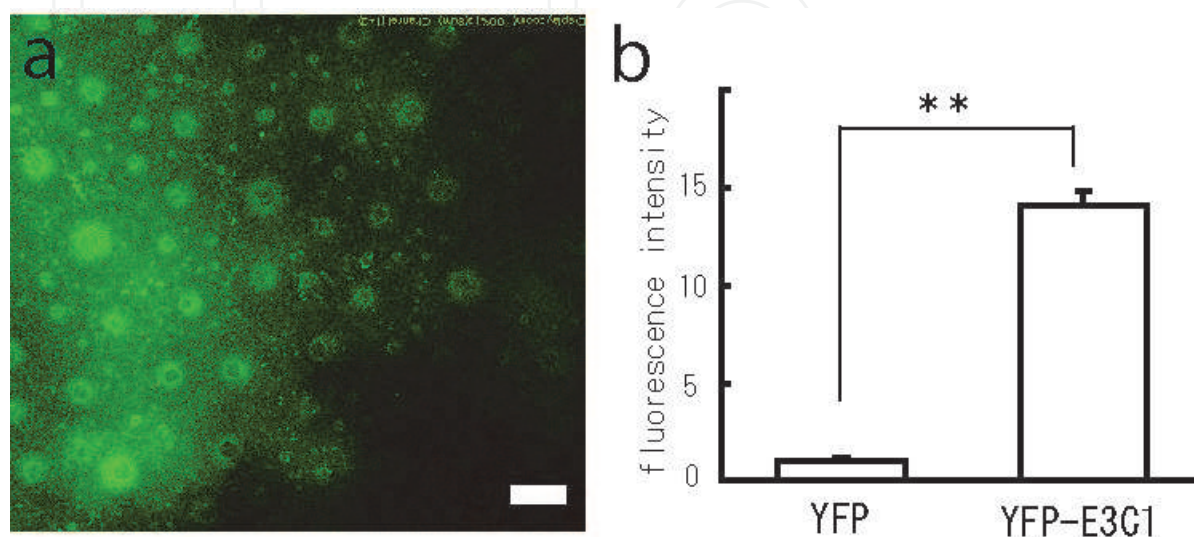
a COS-7 cells were transfected with cDNA encoding YFP fused with E3C1. After 48 h, cells were removed with EDTA and observed by fluorescence microscope. Bar, 10 μm. Specific staining of the ECM was observed. b. Fluorescence intensity in the ECM. Values were corrected by the fluorescence from unconditioned ECM. Values were normalized to the fluorescence from cells expressing cDNA encoding YFP alone. Results represent mean ± SEM (n=6). The data indicate that E3C1 can immobilize YFP protein in ECM.

Fig. 6. YFP-E3C1 fusion stains the ECM

To examine whether the E3C1 domain interferes with the function of fused enzymes, we fused it to 3α-hydorxysteroid dehydrogenase and expressed it in the prostatic cancer-derived, androgen-dependent cell line LNCap (Hidai et al., 2009). The 3α-hydorxysteroid dehydrogenase-E3C1 fusion protein was localized in the ECM and metabolized dihydrotestosterone in the medium, inhibiting cell growth. Thus, the E3C1 domain can target proteins to the ECM without interfering with their function.

2.4 Potential of a FasL-E3C1 fusion protein in cancer gene therapy
We next considered fusing the E3C1 domain to FasL, a cytotoxic protein that has been studied for cancer gene therapy. A FasL-E3C1 fusion protein was deposited and concentrated in the ECM and enhanced the efficiency of subsequent transfections with the same plasmid (Fig. 7).

SCCKN oral squamous cell carcinoma cells (Urade et al., 1992) were transfected with a non-viral vector encoding a FasL-E3C1 fusion protein or encoding FasL alone (control). Three days later, the cells were transfected with a cDNA encoding AP. E3C1 was expected to target FasL to the ECM, increase cytotoxicity, and increase the efficiency of the secondary transfection with AP.
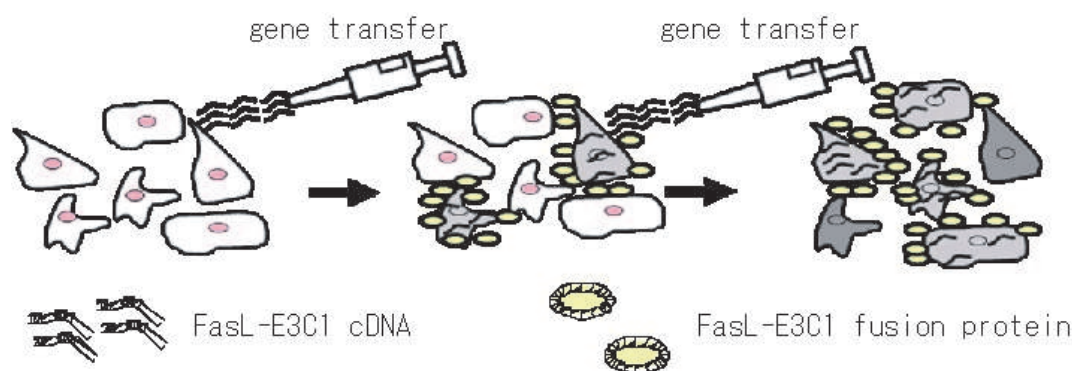
Fig. 7. Scheme describing repetitive gene therapy using a FasL-E3C1 fusion protein.

2.4.1 The E3C1 sequence of Del1 targets FasL to the ECM

2.4.1.1 Materials and methods

Cell culture

CHO cells were purchased from ATCC and grown in α-minimum essential medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen). The human oral squamous cell carcinoma cell line, SCCKN, a gift from Dr. Hayashido, Hiroshima university, was grown in RD medium (45% Dulbecco's modified Eagle's medium [Invitrogen], 45% RPMI 1640 medium (Invitrogen), and 10% fetal bovine serum. Cells were cultured in 5% CO_2 at 37°C.

DNA constructs

Mouse FasL cDNA was a gift of Dr. Tagawa, Chiba cancer center. Mouse Del1 cDNA in pcDNA3 (Invitrogen, Carlsbad, CA) was a gift from Dr. Quertermous, Stanford university. First, a cDNA fragment encoding the mouse FasL gene was amplified by reverse transcriptase-PCR using the forward primer 5′-TACCGAGCTCGGATCCATGCAGCAGCCCATGAATTAC and the reverse primer 5′-GGCACTGTGCTGGATATCAAGCTTATACAAGCCGAA and then cloned into pcDNA3D (Invitrogen), resulting in pFasL. Next, a fragment encoding the E3 and D1 sequence (E3D1, amino acids 122–316 of mouse Del1) was amplified with the forward primer 5′-TGTGAAGCTGAGCCTTGCAGAATGGCCGGA and the reverse primer 5′-ACAGCCTGAGAGCTCACAGCCAAGAAGTT and cloned into the 3′-end of the FasL gene in pFasL, resulting in pFasL-E3D1. The recombinant proteins expressed by these constructs also had a V5 epitope tag at their C-terminal ends.

Immunoblotting

Immunoblotting was used to determine whether E3C1 can target FasL protein to the ECM. CHO cells were used for the experiment because they are more efficiently transfected than SCCKN cells. CHO cells was cultured in 60-mm tissue culture plates and transfected with pFasL-E3D1, pFasL, or a empty vector using jet-PEI (PolyPlus-transfection; San Marcos, CA). Six hours later, the medium was replaced with 3 ml of fresh medium. After 72 h, cells were harvested by incubation with 10 mM EDTA and then solubilized with SDS-sample buffer. Next, the remaining ECM was fixed with 1 ml of 10% trichloroacetic acid in PBS (Wako, Osaka, Japan) and harvested with a cell scraper. One-fourth of the protein from the samples was analyzed by SDS-polyacrylamide gel electrophoresis, after which protein was

transferred to a poluvinylidene difluoride membrane (ATTO, Tokyo, Japan). After blocking and incubation with anti-V5 antibody (Invitrogen), anti-laminin antibody (Sigma, Saint Louis, MO), or anti-tubulin antibody (Oncogene, San Diego, CA), and HRP conjugated secondary antibody (Cell signaling technology, Denvers, MA). an ECL advance western blotting detection kit (Amersham, Piscataway, NJ) was used to detect immunoreactive protein.

2.4.1.2 Results

Immunoblotting of cell lysate showed that the recombinant FasL and FasL-E3C1 fusion proteins had the expected sizes (Fig. 8a). Immunoblotting of conditioned ECM remaining after cell removal showed that FasL-E3C1 fusion protein but not FasL was present in the ECM (Fig. 8b).
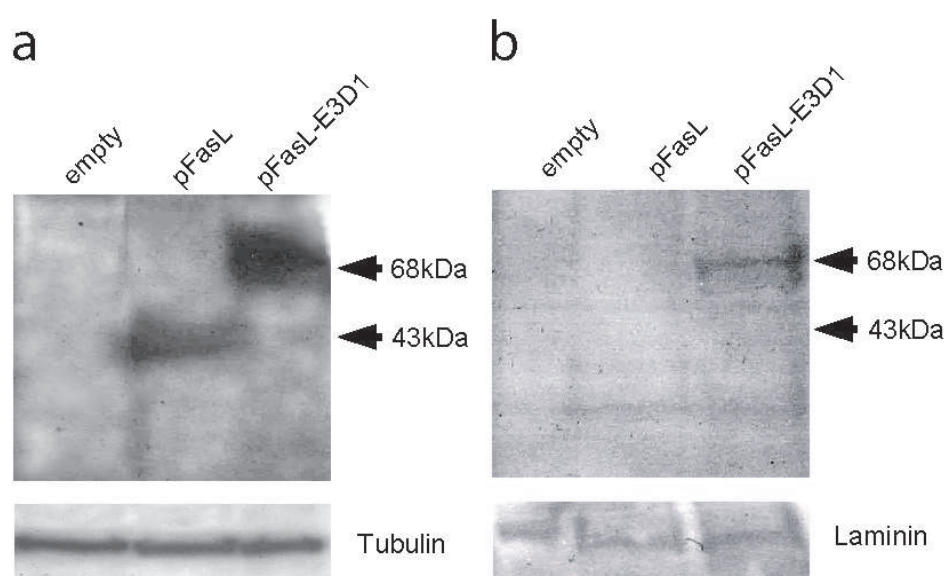

Fig. 8. Immunoblotting

Immunoblotting of cell lysate (a) and ECM (b) from CHO cells transfected with empty vector, pFasL, or pFasL-E3C1. Immunoblotting for tubulin and laminin was used to confirm equal loading.

2.4.2 The E3C1 fragment of Del1 improved the effects of FasL

2.4.2.1 Materials and methods

Induction of apoptosis by FasL fusion proteins

SCCKN cells were plated on a 24-weel plate at 30% confluency. After 1 or 4 days, cells were transfected with an empty vector, pFasL, or pFasL-E3C1. Cells were co-transfected with pAP-tag4 (GenHunter, Nashville, TN) as a control for transfection efficiency. After 48 h, cell death was analyzed by measuring lactate dehydrogenase (LDH) in the cell medium using a LDH cytotoxicity detection kit (Takara) according to the manufacturer's protocol (Decker and Lohmann-Matthes, 1988; Legrand et al., 1992). Percent cell death was calculated as 100% x [(LDH activity for the test condition – LDH activity for the negative control)/LDH activity in cells treated with 1% Triton X-100 (positive control)]. LDH activity was normalized to the heat stable AP activity, which was measured as follows. Conditioned medium (20 μl/well) was added to a 96-well plate, heated at 65ºC for 30 min to inactivate endogenous AP

activity, and mixed with 200 µl/well of 1 mg/ml p-nitrophenyl phosphate (Sigma, St Louis, MO) in 1 mM MgCl₂ and 1 M diethanolamine, pH 9.8. The absorbance at 405 nm was measured after 30 to 60 min.

2.4.2.2 Results

In a first set of experiments, cells were transfected with plasmids 1 day after plating (Fig. 9a). In this experiment, expression of the FasL-E3C1 fusion protein was as cytotoxic as FasL. Because the deposition activity of Del1 varies between cells, possibly because of the composition and amount of ECM, we repeated the experiment with transfection 4 days after plating to allow them to produce sufficient ECM (Fig. 9b). Under these conditions, transfection with pFasL-E3C1 was twice as effective as transfection with pFasL at inducing cell death.
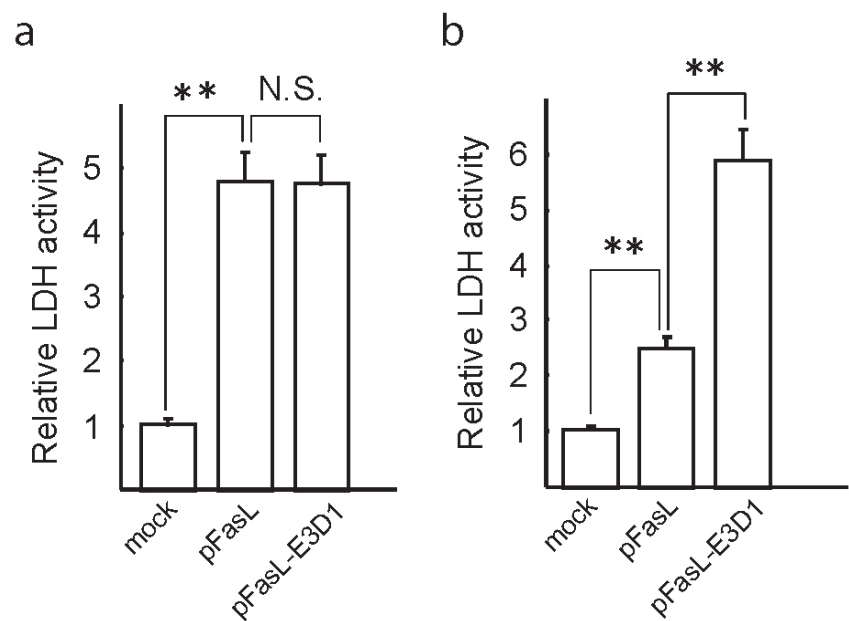

Fig. 9. Effect of E3C1 on the cytotoxicity of FasL

KN cells were transfected 1 (a) or 4 (b) days after plating with an empty vector, pFasL, or pFasL-E3C1. Results represent means ± SEM (n=8). N.S., not significant. Asterisk, P<0.01.

2.4.3 The E3C1 fragment of Del1 enhances the efficiency of a following transfection

2.4.3.1 Materials and methods

Evaluation of the effects of FasL-E3C1 on the efficiency of a second gene transfer

SCCKN cells were cultured at 30% confluency in 96-well plates for 4 days. The cells were transfected with 1 µg of pFasL, pFasL-E3C1, or empty vector (negative control) using jet-PEI. After the cells were cultured for 72 h, the cells that survived the first transfection were transfected with pAPtag-4. Next, the cells were cultured for 24 h before analysis. The AP activity in medium was measured as described above. To count the number of cells, cells were harvested with trypsin EDTA, stained with Trypan blue, and counted using a counting chamber. The AP activity was calculated as the total AP activity in medium/cell numbers.

2.4.3.2 Results

The efficiency of the second transfection was evaluated by measuring the secreted AP activity per cells (Fig. 10). Efficiency increased in the following order: empty vector < pFasL

< pFasL-E3C1. Because cells transfected with empty vector during the first transfection were cultured for 8 days without significant cell death, they may have been too dense to allow for an efficient second transfection, which could explain the higher efficiency of secondary transfection in the pFasL-transfected cells than in the control cells.
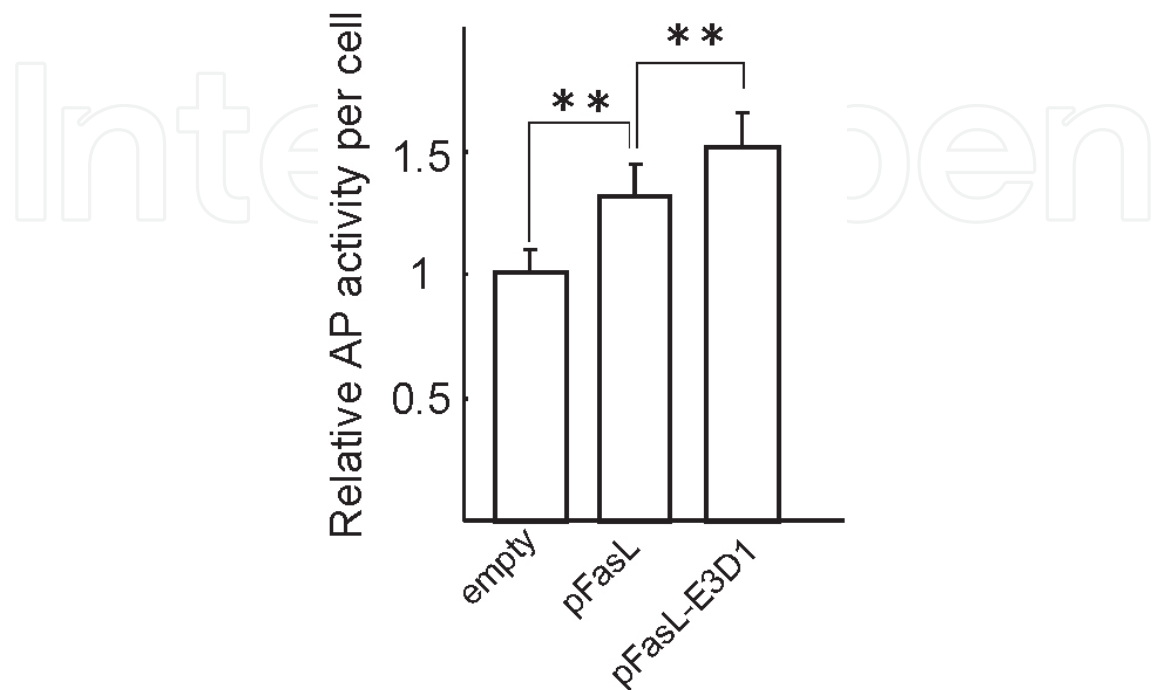

Fig. 10. Effect of E3C1 on secondary transfection with AP

Results represent mean ± SEM (n=8). N.S., not significant. Asterisk, P<0.01.

2.5 Discussion

Expression of Del1 is cell- and tissue-specific. As some reports suggest that Del1 promotes angiogenesis, whereas other reports suggest that it inhibits it, Del1 may be bifunctional. Furthermore, the E3 and C1 domains of Del1 have distinct functions and can act on a variety of cells, and they might be useful as fusion partners to enhance gene therapy using FasL or other proteins.

Neck and esophageal cancer can cause obstruction. Although they can be treated using radiation therapy, its use is limited by the total dosage. As an alternative treatment, we designed a fusion protein of FasL and E3C1 to allow multiple rounds of cancer gene therapy with non-viral vectors. Further study of this fusion protein in the treatment of cancer needs is warranted and should be explored *in vivo* using an explanted tumor model.

3. Conclusion

The E3C1 fragment of Del1 can substantially improve the efficiency of cancer gene therapy using FasL.

4. Acknowledgment

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This book aims at providing an up-to-date report to cover key aspects of existing problems in the emerging field of targets in gene therapy. With the contributions in various disciplines of gene therapy, the book brings together major approaches: Target Strategy in Gene Therapy, Gene Therapy of Cancer and Gene Therapy of Other Diseases. This source enables clinicians and researchers to select and effectively utilize new translational approaches in gene therapy and analyze the developments in target strategy in gene therapy.

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