

Preclinical studies for pharmacokinetics and biodistribution of Ad-stTRAIL, an adenovirus delivering secretable trimeric TRAIL for gene therapy

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<http://dx.doi.org/10.3858/emm.2011.43.10.065>

Accepted 21 July 2011

Available Online 8 August 2011

Abbreviations: Ad, adenovirus; AUC, area under the curve; BBB, brain-blood barrier; CSF, cerebrospinal fluid; i.c., intracranial; LOD, limits of detection; qPCR, quantitative polymerase chain reaction; stTRAIL, secretable trimeric TRAIL; TNF, tumor necrosis factor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand

Abstract

Malignant glioma is the most frequent type in brain tumors. The prognosis of this tumor has not been significantly improved for the past decades and the average survival of patients is less than one year. Thus, an effective novel therapy is urgently needed. TNF-related

apoptosis inducing ligand (TRAIL), known to have tumor cell-specific killing activity, has been investigated as a novel therapeutic for cancers. We have developed Ad-stTRAIL, an adenovirus delivering secretable trimeric TRAIL for gene therapy and demonstrated the potential to treat malignant gliomas. Currently, this Ad-stTRAIL gene therapy is under phase I clinical trial for malignant gliomas. Here, we report preclinical studies for Ad-stTRAIL carried out using rats. We delivered Ad-stTRAIL intracranially and determined its pharmacokinetics and biodistribution. Most Ad-stTRAIL remained in the delivered site and the relatively low number of viral genomes was detected in the opposite site of brain and cerebrospinal fluid. Similarly, only small portion of the viral particles injected was found in the blood plasma and major organs and tissues, probably due to the brain-blood barrier. Multiple administrations did not lead to accumulation of Ad-stTRAIL at the injection site and organs. Repeated delivery of Ad-stTRAIL did not show any serious side effects. Our data indicate that intracranially delivered Ad-stTRAIL is a safe approach, demonstrating the potential as a novel therapy for treating gliomas.

Keywords: gene therapy; glioma; pharmacokinetics; TNF-related apoptosis-inducing ligand

Introduction

Malignant glioma is the most frequent type of brain tumors. Despite recent therapeutic advances, the prognosis of this malignancy remains very bad (Maher *et al.*, 2001; Lefranc *et al.*, 2005) and the average survival of patients after conventional chemotherapy and radiotherapy is less than one year. Thus, it is evident that development of an effective novel therapy is urgently required (Weller and Fontana, 1995).

Gene therapy is considered to be one of the newly investigated approaches to treat malignant gliomas. This approach delivers therapeutic genes into the tumor tissues to express the killer factors, or to enhance the systemic immune responses, through which tumor cells are eliminated. Particularly, since drug delivery is limited by the brain-blood barrier (BBB) in brain tumors, gene therapy directly

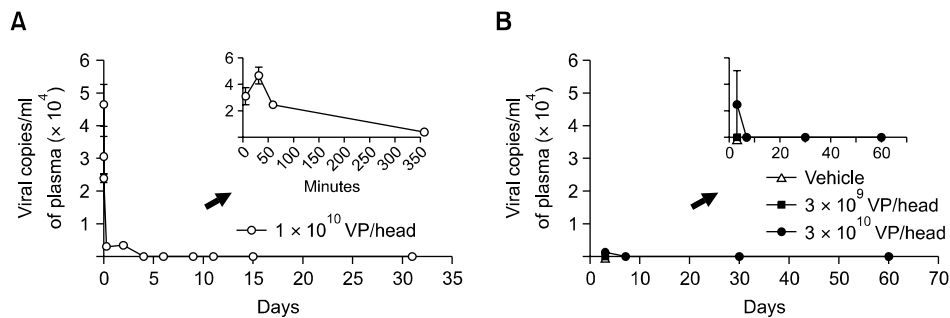


Figure 1. Profile of Ad-stTRAIL distribution in plasma after single or multiple (5 times, 1-week interval) i.c. administration. Ad-stTRAIL concentrations after (A) single administration of 1×10^{10} VP/head of Ad-stTRAIL and (B) multiple administrations of 2 doses of Ad-stTRAIL and vehicle. In the multiple administrations study, Ad-stTRAIL was injected 5 times with a 1-week interval, and sampling was performed on the 3rd, 7th, 30th and 60th day, respectively, after final injection (LOD: 2,500 copies of Ad-stTRAIL/ml of plasma).

delivering therapeutic genes into tumor tissues is an effective option for treating brain tumors.

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), known as a TNF family member (Wiley *et al.*, 1995; Pitti *et al.*, 1996), has a unique feature inducing tumor-specific apoptosis with a limited normal cell death (Ashkenazi *et al.*, 1999; Walczak *et al.*, 1999). This feature draws a great attention to develop TRAIL as an anti-cancer therapy selectively killing tumor cells. TRAIL is mainly expressed as a membrane-bound protein, and only approximately 10% is known to exist as a soluble form of protein through the processing of extracellular region by metalloproteases or cysteine proteases (Degli-Esposti, 1999; Kang *et al.*, 2000).

Previously, we reported an enforced trimerized secretable form of TRAIL, composed of a secretion signal sequence, a isoleucine zipper (ILZ)-based trimerization domain, and TRAIL moiety (amino acids 114-281) (Kim *et al.*, 2006). Using this secretable trimeric TRAIL (stTRAIL), we have developed a stTRAIL-expressing adenovirus, Ad-stTRAIL, and demonstrated that Ad-stTRAIL effectively limits growth of glioma in animal tumor models. When combined with chemotherapeutic agent, tumor suppressor activity of Ad-stTRAIL was significantly promoted, resulting in increased animal survival (Jeong *et al.*, 2009). Most importantly, Ad-stTRAIL did not show any significant adverse effects in animals.

Currently, Ad-stTRAIL is under phase I clinical trial for treating malignant gliomas. Here, we report the preclinical data collected prior to entering the clinical trial, particularly on pharmacokinetics and biodistribution of Ad-stTRAIL after single or multiple administrations into the brain of Sprague-Dawley (SD) rats. We examined distribution of Ad-stTRAIL in various regions of the brain including cerebrospinal fluid (CSF), injection and opposite injection site of brain, in plasma and in various

organs. Most Ad-stTRAIL remained to the injected site rather than diffused to the organs or blood plasma. Repeated delivery of Ad-stTRAIL did not show any serious side effects in animals. Our data suggest that direct delivery of Ad-stTRAIL into the brain is safe and potentially applicable for treating gliomas as a novel therapy.

Results

Pharmacokinetics of Ad-stTRAIL in blood plasma

Generally, the BBB prevents coming in and out of the drugs across brain and adenovirus can cause a dysfunction of organs. Thus, it is important to know whether Ad-stTRAIL delivered into the brain comes out of the brain and freely diffuses throughout the whole body through the blood circulation. To address this question, we administered Ad-stTRAIL into the rat brain and analyzed viral genomes in the blood plasma using the quantitative polymerase chain reaction (qPCR) method. We delivered 1×10^{10} Ad-stTRAIL one time and measured the number of viral genomes in the blood circulation (Figure 1A). Ad-stTRAIL reached the peak (4.6×10^4 viral copies/ml of plasma) 30 min after administration and rapidly decreased. Four days later, Ad-stTRAIL remained below the limits of detection (LOD) (2,500 viral copies/ml of plasma).

Table 1. Pharmacokinetic parameters of Ad-stTRAIL in blood plasma after single i.c. administration (1×10^{10} VP/head, LOD: 2,500 copies of Ad-stTRAIL/ml of plasma)

Parameters	Value
AUC (viral copies*day/ml of plasma)	3.7×10^3
C_{max} (viral copies/ml of plasma)	4.6×10^4
$T_{1/2}$ (hr)	1.5

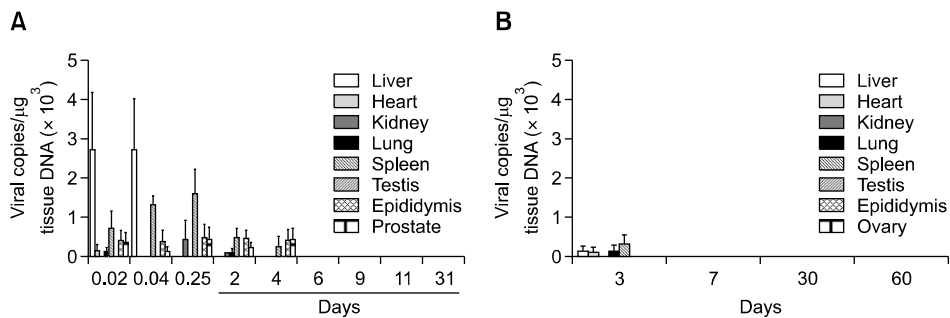


Figure 2. Biodistribution of Ad-stTRAIL in various tissues after single or multiple i.c. administration of Ad-stTRAIL. Ad-stTRAIL concentrations after (A) single administration of 1×10^{10} VP/head and (B) multiple administrations of 3×10^{10} VP/head. In the multiple administrations study, Ad-stTRAIL was injected 5 times with a 1-week interval, and sampling was performed on the 3rd, 7th, 30th and 60th day, respectively, after final injection (LOD: 500 copies of Ad-stTRAIL/ μ g total tissue DNA).

From these data, the PK parameters obtained were 3.7×10^4 viral copies \times day/ml of plasma as AUC, 4.6×10^4 viral copies/ml of plasma as C_{max} , and 1.5 h as $T_{1/2}$ (Table 1).

When Ad-stTRAIL is applied to human patients as an anti-cancer therapy, Ad-stTRAIL is expected to be repeatedly delivered to patient's brain. Thus, also to check the changes of Ad-stTRAIL in the blood circulation when repeatedly delivered, we administered different doses of Ad-stTRAIL (3×10^9 , or 3×10^{10} VP/head) for five times at a 1-week interval and then measured Ad-stTRAIL in the blood plasma (Figure 1B). For the group administered with 3×10^9 viral particles (VP), Ad-stTRAIL reached a level below LOD three days after final administration in all the animals. Similarly, when administered with 3×10^{10} VP, Ad-stTRAIL also reached a level below LOD three days after final administration in eight out of ten animals. Regardless of the delivered viral doses, the viral levels in every individual animal reached a level below LOD from day 7 to day 60 following administration. Our data indicate that only a small portion of Ad-stTRAIL comes out of the brain due to the BBB and rapidly eliminated from the blood circulation. Furthermore, repeated delivery does not lead to an accumulation of Ad-stTRAIL in the blood plasma.

Biodistribution of Ad-stTRAIL in organs

We then next analyzed distribution of Ad-stTRAIL in major organs following administration to the brain. When 1×10^{10} Ad-stTRAIL was administered once, Ad-stTRAIL was detectable only in a few organs including liver and spleen (Figure 2A). Nevertheless, the levels of Ad-stTRAIL in the organs were significantly low (less than 2.7×10^3 viral copies/ μ g total DNA), when compared to the levels observed in the injected site of the brain (1.5

$\times 10^8$ viral copies/ μ g total DNA) at 30 min after administration. Ad-stTRAIL also rapidly disappeared from these organs and reached a level below LOD six days after administration. The peak amount of Ad-stTRAIL detected in the organs did not exceed 0.0019% of that of the injected site.

When Ad-stTRAIL was repeatedly administered at a dose of 3×10^9 , or 3×10^{10} VP/head for five times at a 1-week interval, it was also detectable in several major organs on 3 days after final administration (Figure 2B). The peak amount of Ad-stTRAIL at this time point was as low as 0.0003% of that of the injected site. Ad-stTRAIL decreased to a level below LOD seven days after the final administration. Our data indicate that Ad-stTRAIL escaping from the brain reaches organs in a limited fashion and quickly disappears as time passes even after repeated administrations, suggesting that repeated administration of Ad-stTRAIL as a treatment regimen may be safe.

Distribution of Ad-stTRAIL in brain after a single administration

In addition to examining the systemic distribution (Figures 1 and 2), we also analyzed the local distribution of Ad-stTRAIL at the sections of brain, such as the injection site, the opposite injection site and CSF following delivery of Ad-stTRAIL into the brain. The opposite injection site refers to the brain tissues located at the diagonally opposite side of the injection site on the axis of the central brain. We administered 3×10^9 , 1×10^{10} , or 3×10^{10} Ad-stTRAIL per head once for each dose and measured Ad-stTRAIL at each target site. At the injection site (Figure 3A), the levels of Ad-stTRAIL were sustained high for relatively longer period of time, although gradually decreased. For the group administered with 1×10^{10} VP, the levels of Ad-stTRAIL at the injection site were 1.5×10^8 viral

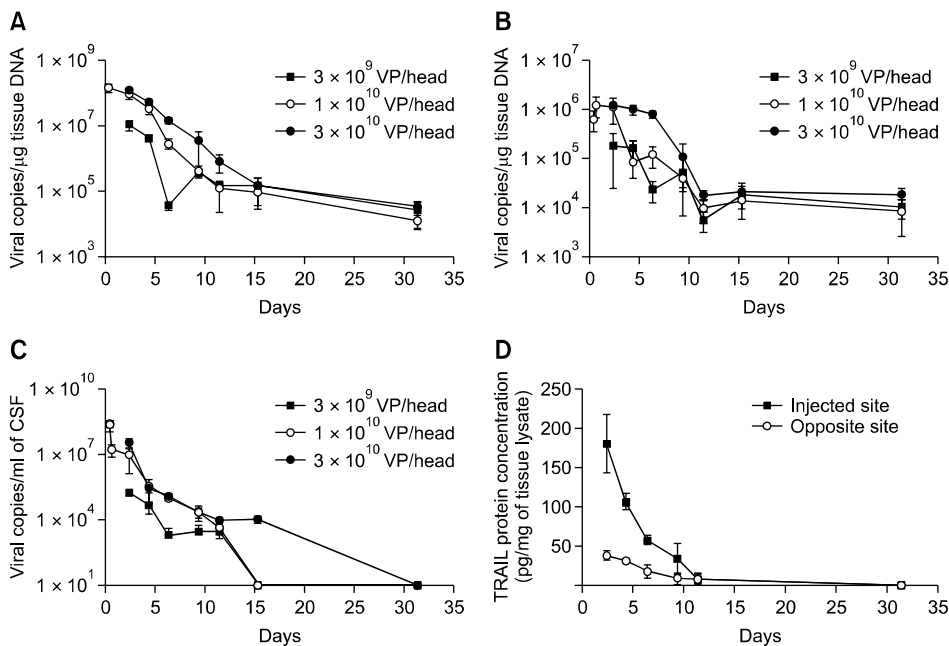


Figure 3. Ad-stTRAIL distribution in brain after single i.c. administration of Ad-stTRAIL. Ad-stTRAIL concentration in injected site (A), opposite site (B), and CSF (C). Profile of expressed stTRAIL proteins in brain after single administration of 1×10^{10} VP/head dose of Ad-stTRAIL (D). (LOD: 500 copies of Ad-stTRAIL/ μg total tissue DNA, 10,000 copies/ml of CSF, 25 pg of stTRAIL/mg tissue lysate)

copies/ μg total DNA at 30 min after administration. In this group, the levels of Ad-stTRAIL at day 31 were 1.2×10^4 viral copies/ μg total DNA (Figure 3A) which is less than 0.01% of the peak. Based on the data collected, the PK parameters obtained for 1×10^{10} VP delivered were 4.2×10^8 viral copies \times day/ μg of tissue DNA as AUC, 1.5×10^8 viral copies/ μg of tissue DNA as C_{max} , and 2.2 days as $T_{1/2}$ (Table 2). The half-life of 2.2 days at the injection site is much longer than that of 1.5 h in the blood circulation.

Analysis of the viral levels at the opposite injection site of brain revealed that the levels of Ad-stTRAIL started to increase 30 min after administration, reached the peak level (1.2×10^6 viral copies/ μg total DNA) after 6 h, and then gradually decreased (Figure 3B). When administered with 1×10^{10} VP, the peak level of Ad-stTRAIL at the opposite injection site of brain was 0.83% of the level measured at the first time following administration. Our data suggest that although diffused to the opposite injection site in a certain degree, most Ad-stTRAIL is restricted to the injection site in brain.

We also examined whether Ad-stTRAIL is detectable in the cerebrospinal fluid (CSF) through which Ad-stTRAIL can spread over the brain tissues. When administered with 1×10^{10} VP, the levels of Ad-stTRAIL in the CSF were highest (2.7×10^8 viral copies/ml of CSF) 30 min after administration (Figure 3C). Six h later, the levels decreased to 1.8×10^7 viral copies/ml of CSF which corresponds to 6.63% of the peak level. After 15 days, the levels of Ad-stTRAIL reached to 1×10^4

Table 2. Pharmacokinetic parameters of Ad-stTRAIL at the injection site of brain after single i.c. administration (1×10^{10} VP/head, 500 copies of Ad-stTRAIL/ μg total tissue DNA)

Parameters	Value
AUC (viral copies \times day/ μg of tissue DNA)	4.2×10^8
C_{max} (viral copies/ μg of tissue DNA)	1.5×10^8
$T_{1/2}$ (day)	2.2

viral copies/ml of CSF, which is lower than LOD. Our data suggest that most Ad-stTRAIL exists at the injection site of brain and only a small portion of virus spreads over the brain tissues through the CSF.

Comparison of adenoviral distribution in brain between single and multiple administrations

When Ad-stTRAIL is applied to patients as an anti-cancer therapy, multiple administrations are expected. Thus, it is important to know the changes in distribution and pharmacokinetics of Ad-stTRAIL in brain following multiple administrations. To address these questions, we administered 3×10^9 or 3×10^{10} of Ad-stTRAIL to the brain of rats for one time or five times with a 1-week interval and analyzed distribution and pharmacokinetics of Ad-stTRAIL at the injection site, opposite injection site and CSF. Comparison results revealed that tissue distribution and pharmacokinetics of Ad-stTRAIL in brain were similar, regardless of delivery times (Table 3). Interestingly, multiple administrations at 1-week in-

Table 3. Comparison of Ad-stTRAIL distribution in brain following single and multiple administrations of Ad-stTRAIL (3×10^{10} VP/head group)

Group	Single administration group			Multiple administrations group			
	Day	2	6	31	3	7	30
Injected site of brain		1.2×10^8	1.4×10^7	3.4×10^4	1.3×10^8	6.1×10^6	1.1×10^4
Opposite site of brain		1.2×10^6	7.9×10^5	1.9×10^4	5.0×10^5	1.2×10^5	4.2×10^3
CSF		3.5×10^7	1.2×10^5	1.0×10^1	4.2×10^6	2.0×10^4	0

Unit: viral copies/ μ g total DNA for brain tissue/Viral copies/ml of CSF for CSF, LOD: 500 viral copies/ μ g of total DNA/10,000 viral copies/ml of CSF.

terval did not show any additive effects in viral levels. As observed early, the viral levels were higher at the injection site than the opposite injection site or CSF, although decreased gradually. Our data indicate that multiple administrations of Ad-stTRAIL at 1-week interval do not lead to viral accumulation at the injected site and adjacent brain tissues.

Profile of expressed stTRAIL protein in brain after single administration of Ad-stTRAIL

Ad-stTRAIL expresses stTRAIL protein with an activity of selectively inducing apoptosis in tumor cells. Since the levels of stTRAIL expressed are correlated with therapeutic efficacy of Ad-stTRAIL, pharmacokinetics of stTRAIL at the administered site is important to be analyzed. We administered 1×10^{10} VP of Ad-stTRAIL into the brain of rats and measured the levels of stTRAIL protein at the injection site and the opposite injection site as well (Figure 3D). The levels of stTRAIL protein at the injection site and the opposite injection site were 181.1 pg/mg tissue lysate and 38.7 pg/mg tissue lysate, respectively, two days after administration, and then gradually decreased to less than 25 pg/mg tissue lysate at day 31, which is a level below LOD. It took 3.2 days that the levels of stTRAIL decreased to 50% of its initial amount following administration of Ad-stTRAIL. Our data indicate that expression of stTRAIL is restricted to the administered brain tissues where most of the Ad-stTRAIL delivered exists. Pharmacokinetics of stTRAIL was similar to that of Ad-stTRAIL, although there was a time delay required for expressing protein from the stTRAIL expression cassette of Ad-stTRAIL.

Discussion

In this study, we analyzed the distribution of an adenoviral gene therapy vector, Ad-stTRAIL, in the plasma, brain and major organs after single or multiple intracranial administrations in SD rats. When a single dose of Ad-stTRAIL was administered to the

brain, Ad-stTRAIL reached maximum concentration in plasma at 0.5 h after administration (Figure 1A). Taking into account the typical body weight and volume of plasma of rats (average weight = 200 g; blood volume = 7% of weight; plasma = 55% of blood volume), we estimate that the total amount of Ad-stTRAIL in plasma at its peak was approximately 3.2×10^5 viral copies. This amount corresponds to 0.003% of the total dose administered (1×10^{10} viral particles/head). Thus, our data indicate that only a small portion of the intracranially administered Ad-stTRAIL reached the systemic blood circulation.

In accordance with the low levels of Ad-stTRAIL observed in the blood, Ad-stTRAIL was also observed only at low concentrations in various organs. Ad-stTRAIL concentrations were highest in the liver and spleen, which is similar to observations made in previous studies on the PK/BD of adenoviral vectors (Hiltunen *et al.*, 2000; Ni *et al.*, 2005; Sharma *et al.*, 2009). However, even at its peak level, Ad-stTRAIL concentrations in the liver and the spleen were significantly lower than in the brain and below detection limit by 6 days after administration. Considering the limited biodistribution observed in this study, we expect a benign toxicity profile of Ad-stTRAIL after intracranial administration. In a separate toxicology study using the same dose and route of administration, no histopathological abnormalities were observed in various organs including the liver (unpublished results).

The distribution of Ad-stTRAIL within the brain after intracranial administration of Ad-stTRAIL was also analyzed. While most of the Ad-stTRAIL was retained at the injection site, low levels of Ad-stTRAIL were detectable in other regions of the brain. Two days after intracranial injection, the concentration of Ad-stTRAIL at the injection site was more than 80-fold higher than at the opposite side of the brain. In contrast, TRAIL protein expressed from the Ad-stTRAIL vector showed a less pronounced localization, with the concentration of TRAIL at the injection site being approximately 5-fold higher than at the opposite side of the brain. The discrepancy between the Ad-stTRAIL dis-

tribution and the TRAIL protein distribution may be attributed to the fact that the Ad-stTRAIL vector is designed to express TRAIL as a secretable protein, which is much smaller in size than the adenoviral vector (MW of TRAIL approximately 67 kDa as compared to 170,000 kDa for Ad-stTRAIL) and therefore spreads more easily within the tissue.

Comparison between single and multiple administrations of the Ad-stTRAIL showed that the distribution and elimination profile of Ad-stTRAIL after multiple administrations was very similar to the single administration profile, and no accumulation of Ad-stTRAIL was observed after multiple administrations. The limitation in transfer of Ad-stTRAIL from the injection site to the blood circulation and organs after intracranial injection and the rapid clearance of the Ad-stTRAIL may have desirable consequences in terms of safety and efficacy, such as a lack of induction of unintended immune reactions directed against the adenoviral vector. Ad-specific immune reactions are generally expected after systemic or repeated intramuscular administration of adenoviral vectors and may negatively impact the safety and efficacy of adenoviral gene therapy. However, our results suggest that Ad-directed immunity may be weak or absent after intracranial administration due to the very limited systemic exposure following this route of administration. In accordance with this assumption, we did not detect production of neutralizing antibodies against Ad-stTRAIL at 60 days after multiple intracranial administrations (data not shown).

In conclusion, we investigated the biodistribution of Ad-stTRAIL after single and multiple intracranial administrations in rats. Most of the Ad-stTRAIL was retained at the injection site, and systemic exposure was observed to be very low. Also, drug accumulation was not observed after multiple administrations. Based on these results, we expect a favorable safety profile of Ad-stTRAIL when used in a clinical setting for the treatment of glioma. The safety and efficacy of the Ad-stTRAIL gene therapy vector is currently being investigated in a phase I clinical trial in patients with recurrent malignant gliomas.

Methods

Preparation of recombinant adenovirus Ad-stTRAIL

An E1/E3-deleted replication-incompetent adenoviral vector was constructed through Cre-lox recombination (Hardy *et al.*, 1997). Ad-stTRAIL amplified in 293F cells (Invitrogen, Carlsbad, CA) was purified using an anion exchange chromatography and an immobilized metal affinity chromatography.

Purified virus was dialyzed using formulation buffer (10 mM Tris [pH 7.5], 75 mM NaCl, 5% [w/v] sucrose, 0.02% [v/v] Tween-80, 1 mM MgCl₂, 100 μM EDTA, 0.5% [v/v] Ethanol and 10 mM L-Histidine) and kept at -70°C until used.

Animal and intracranial delivery of Ad-stTRAIL

5-week-old male and female Sprague-Dawley (SD) rats were used for all the experiments. For single administration study, animals received formulation buffer or three doses of Ad-stTRAIL (3×10^9 , 1×10^{10} or 3×10^{10} viral particles/30 μl/head) for one time intra-cranially. Injection site was putamen region of brain, which was exactly localized at 3.1 mm right, 0.4 mm front and 6.0 mm deep from the bregma. For the study of multiple administrations, animals received intra-cranially formulation buffer or two doses of Ad-stTRAIL (3×10^9 or 3×10^{10} viral particles/30 μl/head) once a week for five times. The injection procedure for multiple administrations was the same as that performed in the study of single administration.

Tissue harvest and DNA isolation

For the study of single administration, animals were sacrificed 5 and 30 min, 1 h, 6 h and 2, 4, 6, 9, 11, 15 and 31 days after administration of Ad-stTRAIL. Blood plasma was sampled at every time point. Brain tissues (from the injection site and opposite injection site), CSF and major organs tissues (from lung, heart, liver, kidney, spleen, testis, epididymis, and ovary) were isolated at the planned time points. For the study of multiple administrations, animals were sacrificed 3, 7, 30 and 60 days after final administration. Blood plasma, brain tissues, CSF and major organ tissues were sampled at every indicated time point. All the samples were stored at -70°C until analysis.

Total DNA was extracted from 25 mg of tissue samples (in case of spleen, 10 mg), 200 μl of plasma or 50 μl of CSF using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) after 3 h of tissue lysis according to the manufacturer's instructions. DNAs extracted were kept at -20°C until analysis.

Quantitative real-time PCR analysis

The primers and probe were designed to target the junction region between TRAIL gene and viral backbone sequence in order to distinguish Ad-stTRAIL from wild-type adenovirus type 5. The primer sequences were 5'-AGC CGCTCCGACACC-3' and 5'-GGCTATGAACTAATGACCC CGTAATTG-3', and the probe sequence was FAM-5'-CGA GTGTTGACATTGATTATTGACTAGTTA-3'-TAMRA. Quantitative PCR (qPCR) was performed using the ABI Prism 7900 HT (Applied Biosystems, Carlsbad, CA). The total DNA isolated from specimens was quantified by UV spectrophotometer, and 100 ng of DNA for each reaction was analyzed by qPCR. The limit of detection was 50 copies of Ad-stTRAIL/100 ng total DNA. For plasma, qPCR was performed using 5 μl of eluate DNA and the limit of detection was 2,500 copies of Ad-stTRAIL/ml of plasma. For CSF, qPCR was performed on 5 μl of eluate DNA and the limit of detection was 10,000 copies/ml of CSF. The qPCR

reaction was performed for 40 cycles with a 15 second denaturing step at 95°C and a 1 min annealing/extension step at 60°C. The levels of Ad-stTRAIL were determined by analyzing a standard DNA curve, ranging from 10⁸ to 10² viral copies, and expressed as the number of viral copies per microgram of total tissue DNA, per milliliter of blood plasma or CSF. Viral genomic DNA extracted from Ad-stTRAIL was used as standard materials, and Easydilution buffer (TAKARA, Shiga, Japan) containing 100 ng of normal SD rat's brain tissue DNA was used as a diluent. Calculation of pharmacokinetic parameters was performed using the WinNonLin S/W (Pharsight, Mountain View, CA).

stTRAIL ELISA

ELISA analysis for stTRAIL was conducted as described before (Kim *et al.*, 2006). Briefly, the brain tissues from the virus injection site were lysed using lysis buffer (25 mM Tris [pH 7.4], 50 mM NaCl, 0.5% Na-Deoxycholate, 2% NP-40, 0.2% SDS, 10% [v/v] protease inhibitor cocktail [Sigma, St. Louis, MO]). After measuring protein concentrations, lysates were diluted to 2.5 mg/ml concentration and 100 µl of diluted lysates were subjected to ELISA. The plates were coated with anti-human TRAIL antibody (Peprotech, Rocky Hill, NJ) and blocked with a blocking solution (2% BSA in PBS), followed by incubation with lysates or rhTRAIL (R&D Systems, Minneapolis, MN) as a standard. After incubation, the plates were treated with biotinylated anti-human TRAIL antibody (Peprotech), and followed by treatment with avidin-HRP (1/1,000 dilution). ABTS solution (Sigma) was treated for color development, and which was measured by reading absorbance at 405 nm.

Acknowledgements

This work was supported by a grant (#1007404) from the Korean Ministry of Knowledge Economy and by a grant (2007-04347 to D.W.S.) from the National Research Foundation of Korea.

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