Systemic Overexpression of TNFα-converting Enzyme Does Not Lead to Enhanced Shedding Activity In Vivo

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Abstract

TNFα-converting enzyme (TACE/ADAM17) is a membrane-bound proteolytic enzyme with a diverse set of target molecules. Most importantly, TACE is indispensable for the release and activation of pro-TNFα and the ligands for epidermal growth factor receptor in vivo. Previous studies suggested that the overproduction of TACE is causally related to the pathogenesis of inflammatory diseases and cancers. To test this hypothesis, we generated a transgenic line in which the transcription of exogenous Tace is driven by a CAG promoter. The Tace-transgenic mice were viable and exhibited no overt defects, and the quantitative RT-PCR and Western blot analyses confirmed that the transgenically introduced Tace gene was highly expressed in all of the tissues examined. The Tace-transgenic mice were further crossed with Tace+/− mice to abrogate the endogenous TACE expression, and the Tace-transgenic mice lacking endogenous Tace gene were also viable without any apparent defects. Furthermore, there was no difference in the serum TNFα levels after lipopolysaccharide injection between the transgenic mice and control littermates. These observations indicate that TACE activity is not necessarily dependent on transcriptional regulation and that excess TACE does not necessarily result in aberrant proteolytic activity in vivo.

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Introduction

Various membrane-bound molecules are subjected to proteolytic cleavage at the cell surface. This proteolytic activity, also referred to as “ectodomain shedding,” plays essential roles in the functional regulation of membrane-bound molecules. Previous studies revealed that the TNFα-converting enzyme (TACE), also known as A Disintegrin and Metalloprotease 17 (ADAM17), is one of the most critical proteolytic enzymes involved in ectodomain shedding in vivo [1–3]. TACE was originally identified as a converting enzyme responsible for the release of membrane-anchored pro-TNFα from cell surface [4,5]. However, subsequent studies found an exceptionally large number of target molecules for TACE, including the ligands for epidermal growth factor receptor (EGFR), TNF receptor (TNFR)-1 and -2, CD62L/L-selectin, and vascular growth factor receptor 2 [6–9]. The importance of the functions of TACE in vivo was further underscored by the observation that mice lacking TACE die perinatally with a highly complex phenotype [6,9]. Importantly, studies in TACE mutant mice revealed that TACE is indispensable for the functional activation of the pro-TNFα and EGFR ligands in vivo [6,9].

There is evidence suggesting that the overexpression of TACE is causally related to the pathogenesis of various disorders [10,11]. The overproduction of TACE has been reported in cancers, including breast cancer, colon carcinoma, lung cancer, and hepatocellular carcinoma [12–16], and inflammatory diseases, such as Sjogren’s syndrome, osteoarthritis, and rheumatoid arthritis [17–20]. In these cases, TACE overexpression was suggested to promote pathogenesis through the excess cleavage of TACE substrates, such as EGFR ligands and TNFα. These observations, in turn, indicate that TACE activity is regulated, at least in part, at the transcriptional level in vivo.

TACE is initially produced as a proteolytically inactive pro-form and is processed by a furin protease in the secretory pathway to produce the mature enzyme [21]. However, the mechanisms underlying the functional activation of TACE remain elusive. TACE can be activated by various stimuli in vitro, including growth factors, phorbol esters, osmotic pressure, ultraviolet irradiation, and cholesterol deprivation, without changing the amount of its mature form [22–25]. Several studies have suggested that the phosphorylation of the cytoplasmic domain is involved in activation of TACE [26–29]; however, other findings have failed to support this hypothesis [22,25]. Furthermore, a recent study showed that tissue inhibitor of metalloprotease 3 (TIMP3), an endogenous inhibitor of MMPs and TACE, directly binds to TACE and thereby regulates its proteolytic activity [29]. However, it has been shown that in cells lacking TIMP3, TACE can still be stimulated by phorbol esters [25], raising questions about the true nature of TACE activation.

To address these issues, we generated a transgenic line of mice that express TACE under the control of a CAG promoter and found that the mutant mice were viable and exhibited no apparent
defects. A higher amount of TACE transcripts and mature protein in the mutant mice tissues were confirmed by quantitative RT-PCR and Western blotting, respectively. The expression levels of the TIMP3 transcripts were not affected by the introduction of the Tace-transgene in any of the tissues examined. This study, therefore, shows that the overproduction of TACE does not necessarily result in the hyperactivation of shedding activity and suggests that the level of TACE activity cannot be explained solely by its level of transcription or the amount of the mature form of the protein.

Materials and Methods

Generation of Tace-transgenic mice

A mouse Tace cDNA with an HA-epitope (YPYDVPDYA) sequence added to the 3′-terminus was generated using PCR-based methods and subcloned into the pCAGGS vector. HA-epitope tagged-TACE has been shown to exhibit comparable shedding activity towards its substrates to non-tagged wild-type TACE [22,30]. A transgene fragment containing the CAG promoter, Tace cDNA, HA epitope, and polyadenylation signal sequence (Fig. 1A)
was injected into fertilized zygotes obtained from superovulated donor mice. Several founder mice were obtained, and two independent lines were selected for further analysis based on the expression levels of the transgene. The phenotypes of these two transgenic lines (line-004 and -005) were nearly identical, and the data obtained using the line-004 are presented in this study. A Southern blot was performed using genomic DNA collected from the tail to confirm a single integration site of the transgene and to determine the copy number of the integration. All of the animal experiments in this study were approved by the Institutional Animal Care and Use Committee of the Keio University, School of Medicine (Permit Number: 09101).

### Histology

The tissues were fixed in 4% paraformaldehyde/PBS, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. The sections were photographed using a DXM1200 camera (Nikon, Tokyo, Japan) and a BX50 microscope (Olympus, Tokyo, Japan).

### Quantitative RT-PCR

RNA was extracted from the tissues or cultured cells using Sepasol RNA I Super G (Nacalai Tesque, Kyoto, Japan) and reverse-transcribed using ReverTra Ace (Toyobo, Osaka, Japan). The PCR amplification and quantification were performed using SYBR premix ExTaqII (Takara Bio, Shiga, Japan) and LightCyclerII (Roche). The relative mRNA expression levels were normalized to the expression level of the $\beta$-Actin transcripts. The sequences of the oligonucleotides used in this study will be provided upon request.

### Western blotting

The tissues and cells were lysed in lysis buffer (1% Triton X-100, 150 mM NaCl, 0.5 mM EDTA, 10 mM Tris-HCl (pH 7.4), 1 mM 1,10-phenanthroline, and protein inhibitor cocktail (Sigma-Aldrich)), and the lysed samples were separated on SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The anti-sera against the cytoplasmic domain of mouse TACE was produced as previously described [31]. The anti-HA-epitope antibody (clone; 3F10), anti-ADAM10, and anti-$\beta$-Actin antibody...
were purchased from Roche, Calbiochem, and Sigma-Aldrich, respectively.

Shedding assay

Mouse embryonic fibroblasts (mEFs) collected from E13.5 Tace-Tg embryos were immortalized with the SV40 large T antigen, as previously described [32]. The cells were transfected with alkaline phosphatase (AP)-tagged TGFα using Fugene HD (Roche), as previously described [22]. Fresh Opti-MEM (Invitrogen) medium with or without the indicated reagents (PMA and/or GM6001) was added 24 h after the transfection and incubated for an hour. The AP activity was measured by colorimetry, as previously described [33,34].

Cell surface labeling

The cells were washed twice in ice-cold PBS, and the cell surface molecules were labeled using Pierce Cell Surface Protein Isolation Kit (Thermo) according to the manufacturer’s instructions, with some modifications. The labeled samples were affinity precipitated with neutravidin beads overnight at 4°C, and the affinity precipitated material was separated by SDS-PAGE and analyzed by Western blotting.

Septic shock

Septic shock was induced by the intraperitoneal injection of lipopolysaccharide (LPS, 5 µg) and D-galactosamine (20 mg). The mice were closely monitored until the mortality was 100%. For the analysis of the serum cytokines, the sera were collected at 3 h after the intraperitoneal injection of LPS and D-galactosamine (n = 18). The sera were collected under unchallenged conditions (−) or 3 h after the intraperitoneal injection of LPS and D-galactosamine (n = 18). The sera were collected under unchallenged conditions (−) or 3 h after the intraperitoneal injection of LPS and D-galactosamine (n = 18). (C) Quantitative RT-PCR of Timp3 in the liver, lung, skin, spleen, bone marrow cells (BM), and thymus collected from the control (Ctrl) and Tace-Tg mice. Bars, S.D. ns, not significant.

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Statistical analysis

The Student’s t-test for two samples, assuming equal variances, was used to calculate the p values. The statistical analyses were performed using Prism 5 (GraphPad software), and p values smaller than 0.05 were considered statistically significant. All of the experiments were conducted in triplicate.

Results and Discussion

Mice systemically overexpressing TACE exhibit no overt defects

To understand how the enhanced expression of Tace transcripts would affect the TACE activity in vivo, we generated transgenic mice that expressed an HA-tagged TACE driven by a CAG promoter (Fig. 1A). The HA-epitope was added to the C-terminus of the cytoplasmic domain of TACE to facilitate the detection of the protein; an HA-tagged TACE expression vector used in previous studies showed no interference on the activity of TACE in vitro [22]. The TACE-transgenic mice (henceforth referred to as Tace-Tg mice) were viable and fertile and did not show any overt developmental defects (Fig. 1B). Matings between the wild-type control mice and Tace-Tg mice yielded offspring in the Mendelian ratio (wild-type mice, 186; Tace-Tg mice, 201; $X^2 = 0.446$).

We first evaluated the expression levels of the TACE transcripts in the Tace-Tg mice by quantitative RT-PCR. As shown in Figure 1C, a significant increase in the transcript levels of TACE was observed in all the tissues examined, including the liver, lung, skin, spleen, bone marrow (BM), and thymus. The relative increase was lower (approx. 4-6 times) in the immune organs (spleen, BM, and thymus) and higher (approx. 30-300 times) in the other tissues, indicating that the basal levels of TACE transcripts and/or CAG promoter activity differ across these tissues. The increased expression of the TACE protein in these tissues was also confirmed.
using an anti-HA antibody (which specifically binds to the transgene-
ically introduced HA-tagged TACE) and an anti-TACE
antibody (which recognizes both endogenous and transgene-
ically introduced TACE) (Fig. 1D). Necrotomy of 8-week-old Tace-Tg
and control littermate mice showed no apparent defects at either
the macroscopic or histological level (Fig. 1E). These observations
indicate that a highly enhanced expression level of TACE does not
have a profound impact on normal development or homeostasis.
To confirm that the transgenically introduced TACE was fully
functional in vivo, we mated the Tace-Tg mice with Tace+/– mice
[32] to generate Tace-Tg/Tace−/− mice. The Tace-Tg/Tace−/−
mice expressed no endogenous TACE protein, only expressing the
transgenic HA-tagged TACE under the control of the CAG
promoter. These mutant mice were also viable and revealed no
apparent defects at either the macroscopic or histological level
(Fig. 1E and data not shown). We, therefore, concluded that
transgenic HA-tagged TACE was fully functional in vivo and that
the transcriptional regulation of Tace by its endogenous promoter
is not necessarily essential for the functional regulation of TACE
during development and postnatal growth.

Unaltered shedding activity in Tace-Tg-derived mEFs

To confirm that the introduction of transgenic HA-tagged
TACE resulted in an increase in the total amount of the mature
protein, we examined the expression level of mature TACE by
labeling the proteins expressed on the cell surface with membrane-
permeable biotin. The TACE protein is initially produced as a
pro-form and is cleaved by a furin protease before it is expressed as
a proteolytically active mature form on the cell surface [21].
Therefore, only the mature form could be labeled with a
membrane-permeable biotin. We first confirmed the increase in
the Tace transcript levels in the mEFs derived from the Tace-Tg
mice (Fig. 2A). As shown in Figure 2B (left panels), a Western blot
of the cell lysates using an anti-TACE antibody revealed an
increase in the amount of both the pro-form (black arrowhead)
and mature form (white arrowheads) in the Tace-Tg mEFs. The
expression and maturation of the HA-tagged TACE were also
confirmed by Western blotting using an anti-HA antibody (Fig. 2B,
left panels). The lysates were affinity precipitated using neutravi-
din-conjugated beads and detected with anti-TACE and anti-HA
antibodies. Consistent with the results of the Western blot of the
cell lysates, there was a sharp increase in the amount of the biotin-
labeled mature form in the Tace-Tg mEFs (Fig. 2B, right panels).
These observations show that the Tace-Tg mEFs express more
mature TACE than the control mEFs, and that the maturation
and trafficking of the exogenous HA-tagged TACE to cell surface
are not hampered in the Tace-Tg mice. The increase in the
amount of mature TACE was also confirmed in the Tace-Tg
splenocytes (Fig. 2C).

The apparent lack of developmental defects in the Tace-Tg mice
indicates that the increase in the Tace transcript levels does not
significantly affect the shedding of its substrates. To test this
hypothesis, we performed an in vitro shedding assay using AP-
tagged TGFα, as previously described [22]. We introduced an AP-
tagged TGFα expression vector into immortalized mEFs and indirectly
evaluated the shedding activity by measuring the AP activity
released in the supernatant using colorimetry [34]. TGFα is a well-
established TACE substrate, and the cleavage of the membrane-
bound pro-TGFα can be significantly stimulated in vitro with
phorbol esters, such as phorbol 12-myristate 13-acetate (PMA). As
shown in Figure 2D, we found no significant difference in the
shedding profile of AP-TGFα between the mEFs derived from the
control and Tace-Tg embryos. The shedding activity was similarly
enhanced upon PMA stimulation and suppressed by a broad-

range metalloprotease inhibitor, GM6001. These observations
indicate that the excess TACE protein in the Tace-Tg-derived
mEFs does not significantly affect the overall shedding activity, at
least under the present experimental conditions.

LPS-induced production of soluble TNFα is comparable
between control and Tace-Tg mice

Several studies have shown a causal relationship between the
enhanced expression of TACE and cancer progression and
inflammatory disease [10,11,35], and these studies have indicated
that the upregulation of TACE leads to an enhanced shedding of
its substrates and, consequently, a more aggressive phenotype.
Conversely, these observations also suggest that the effect of the
increased expression of TACE could be manifested under pathological conditions. To test this hypothesis, we next evaluated
the production of soluble TNFα in serum using a murine model of
endotoxin shock. The control and Tace-Tg mice were intraperi-
toneally injected with LPS to induce TNFα production; LPS is a
major component of the cell membrane of Gram-negative bacteria, and it elicits a strong immune response in mammalian
immune cells. Because LPS can stimulate the production of TNFα
in immune cells, we hypothesized that the amount of soluble
TNFα released into the supernatant would show a positive correlation with the amount of TACE expressed in the immune
cells. However, contrary to our expectation, there was no
difference in the serum levels of TNFα or any of the TACE
substrates examined (TNFα receptor 1, TNFα receptor 2, and
CD61(2L)) between the control and Tace-Tg mice under unchall-
eged conditions or after LPS treatment (Fig. 3A). Furthermore,
we also found that the Tace-Tg mice were not more susceptible to
LPS-induced septic shock than the control animals; in fact, they
were slightly more resistant (Fig. 3B).

Given these observations, we next examined whether there was
a change in the expression levels of TIMP3 in the Tace-Tg mice-
derived cells and tissues. TIMP3 is a critical regulator of TACE,
and Timp3−/− mice were shown to exhibit an overt immune
response due to the overproduction of soluble TNFα [36,37].
Furthermore, it has recently been shown that TIMP3 suppresses
TACE activity by directly binding to TACE dimers and that MAP
kinase activation stimulates TACE activity by suppressing the
dimerization of TACE and the binding of TIMP3 to TACE [29].
Therefore, in theory, overt TACE activity can be offset if the
expression of TIMP3 correlates with that of TACE. However, we
did not find any difference between the control and Tace-Tg mice
with regard to the transcription levels of Timp3 in any of the organs
examined (Fig. 3C). These observations indicate that even though
TIMP3 is certainly a critical regulator of TACE, the activity of
TACE cannot be simply deduced from the ratio between the
TIMP3 and TACE expression levels. In fact, although Timp3−/−
mice have been shown to develop hepatic inflammation due to
increased TACE-TNFα activity [36], we did not observe any
defects in the Tace-Tg liver, even though approximately 300 times
more TACE transcript was expressed in the Tace-Tg versus the control
liver (Fig. 1C).

The mechanisms underlying the activation of TACE remain
controversial. It is clear that the cleavage of the prodomain is
necessary for TACE maturation, and recent studies have revealed
that immune cells lacking iRhom2 (RHBDF2), a proteolytically
inactive member of the rhomboid protease family, are defective for
this process and incapable of releasing soluble TNFα [38,39]. The
present study indicates that although the conversion of TACE from
the pro- to mature form is critical for TACE to become
functional, the amount of mature TACE does not directly
correlate with the overall shedding activity. Therefore, it may be
that cells require only a certain amount of TACE and that any excess TACE above the threshold level does not significantly contribute to the overall shedding activity in a given cell or tissue. In agreement with this hypothesis, the present study also suggests that the transcription of Tace does not have to be rigorously regulated, and Nature reviews Cancer 8: 929-941. During the revision of the manuscript, an independent study [40] presented data in agreement with our results that systemic overexpression of TACE in vivo does not lead to overt defects.

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