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Generation of a sensitive TNFR2-specific murine assays system

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Received October 27, 2015, accepted December 17, 2015

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Pharmazie 71: 235–237 (2016) *doi: 10.1691/ph.2016.5183*

Tumor necrosis factor (TNF)/TNF receptors (TNFR1/TNFR2) are considered to be potential drug targets to treat refractory diseases, including autoimmune diseases and malignant tumors. However, their specific functions, especially in the case of TNFR2, are poorly understood. In this study, we constructed a mouse TNFR2 (mTNFR2)-mediated biological assay system that shows no effects of mouse TNFR1 (mTNFR1) in order to screen mTNFR2-selective stimulating agents. Mouse TNFR1$^{-/-}$R2$^{-/-}$ preadipocytes were transfected with the gene encoding the mTNFR2/mouse Fas (mFas) chimeric receptor in which the extracellular and transmembrane domains of mTNFR2 were fused to the intracellular domain of mFas. Our results demonstrated that this cell line exhibits highly sensitive mTNFR2-mediated cytotoxic effects. We propose that this mTNFR2-mediated biological assay system would be a useful tool to screen for mTNFR2-selective stimulating agents.

1. Introduction

Tumor necrosis factor (TNF) interacts with two receptors, TNF receptor type I (TNFR1) and TNF receptor type II (TNFR2), through which TNF stimulates host immune defense and inflammation (Aggarwal 2003). In recent years, TNF/TNFR interactions have been considered to be potential drug targets for the treatment of refractory diseases, including autoimmune diseases and malignant tumors (Szlosarek and Balkwill 2003). Despite extensive studies on the molecular biology of TNF/TNFR1 signaling, little is known about TNFR2 signaling and biology. The *in vivo* function of TNFR2 could be examined using animals, in particular mouse models. There have been increasing demands for the development of a highly sensitive biological assay system with which to screen mouse TNFR2 (mTNFR2)-selective stimulating agents, as this would lead increased understanding of TNF/TNFR interactions and the development of novel anti-TNF therapies.

Efforts to develop a conventional mTNFR2-mediated biological assay system have been complicated for the following reasons. TNFR1 contains a death domain in its intracellular domain, unlike TNFR2. Death domains can recruit other death domain-containing proteins and induce caspase activation and apoptosis. Therefore, highly TNF-sensitive tumorigenic murine fibroblasts, called L-M cells, are used to evaluate the mTNFR1 mediated bioactivity by measuring the degree of cell cytotoxicity. On the other hand, to evaluate mTNFR2 mediated bioactivity, many researchers have limited their analysis to measuring proliferation of a murine T-cell line (CT6) (Sheehan et al. 1995). Nonetheless, previous studies have demonstrated that human TNF (hTNF), which can bind mTNFR1 but not mTNFR2 (Lewis et al. 1991), induced proliferation of CT6, suggesting that mTNFR1 was also expressed in this cell line (Marr et al. 1999). This complexity has prevented a precise, unambiguous evaluation of mTNFR2 signaling. The development of new strategies to evaluate mTNFR2 without confounding effects of mTNFR1 is therefore highly desirable. The purpose of this study was to construct mTNFR2/mFas preadipocytes (PA) for screening of mTNFR2-selective stimulating agents.

2. Investigations, results and discussion

Fas, also called CD95 or APO-1, belongs to the tumor necrosis factor receptor superfamily and regulates apoptosis signals in response to Fas ligand or stimulatory antibodies (Nagata and Golstein 1995). Because the intracellular (IC) domains of Fas have death domains that mediate apoptotic signals, we designed a mTNFR2/mFas chimeric receptor in which the extracellular (EC) region of mTNFR2 was fused to the IC region of mFas, allowing signaling to induce cell death. In our previous study, we confirmed that the parental TNFR1$^{-/-}$R2$^{-/-}$ PA cell line was resistant to mouse TNF (mTNF)-induced cell death and was sensitive to Fas-induced cell death (Xu and Sethi 1999; Abe et al. 2008), suggesting that this cell line was not influenced by endogenous TNFR1 and that it maintained a Fas-mediated apoptotic signaling cascade. Based on these results, we selected TNFR1$^{-/-}$R2$^{-/-}$ PA to construct an mTNFR2/mFas-expressing cell line. Using the lentivirus vector technique, TNFR1$^{-/-}$R2$^{-/-}$ PA cells were transfected with a gene encoding the mTNFR2/mFas chimeric receptor, in which the EC and transmembrane (TM) regions of mTNFR2 (amino acids 1-293) were fused to the IC region of mFas (amino acids 187-328) (Fig. 1A and B). Stably expressing cells were established by blasticidin (Bsd) resistance gene-mediated selection. To evaluate mTNFR2/mFas chimeric receptor expression levels in these transfectants, flow cytometric analysis using the anti-mTNFR2 monoclonal antibody (mAb) was performed. Specific binding of the anti-mTNFR2 mAb was observed in these transfectants (Fig. 2A), suggesting that expression levels of the mTNFR2/mFas chimeric receptor had increased significantly. We confirmed that the anti-mTNFR2 mAb could not bind to the parental cell line (data not shown). To examine whether mTNFR2-mediated cytotoxic effects could be induced, the viability of mTNFR2/mFas PA cells was evaluated by stimulating the chimeric receptors with mTNF. The mTNFR2/mFas PA cells were killed by mTNF in a concentration-dependent

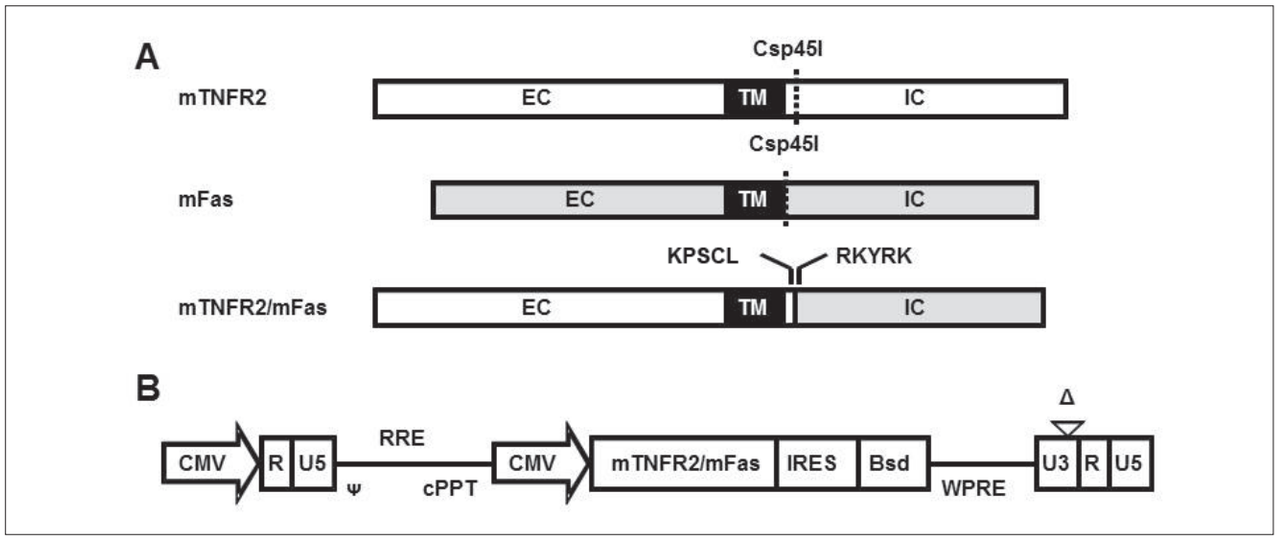

Fig. 1: Construction of mTNFR2/mFas chimeric receptor gene and vector. (A) The cDNA structures of mTNFR2, mFas and fusion genes (mTNFR2/mFas) are shown. EC: extracellular domain, TM: transmembrane domain, IC: Intracellular domain. (B) Schematic representation of self-inactivating (SIN) LV plasmid (CSII-CMV-mTNFR2/mFas-IRES-Bsd). CMV, cytomegalovirus promoter; ψ: packaging signal; RPE, rev responsive element; cPPT, central polypurine tract; IRES, encephalomyocarditis virus internal ribosomal entry site; Bsd, blasticidin; WPRE, wood chuck hepatitis virus posttranscriptional regulatory element. Δ: deleting 133 bp in the U3 region of the 3' long terminal repeat.

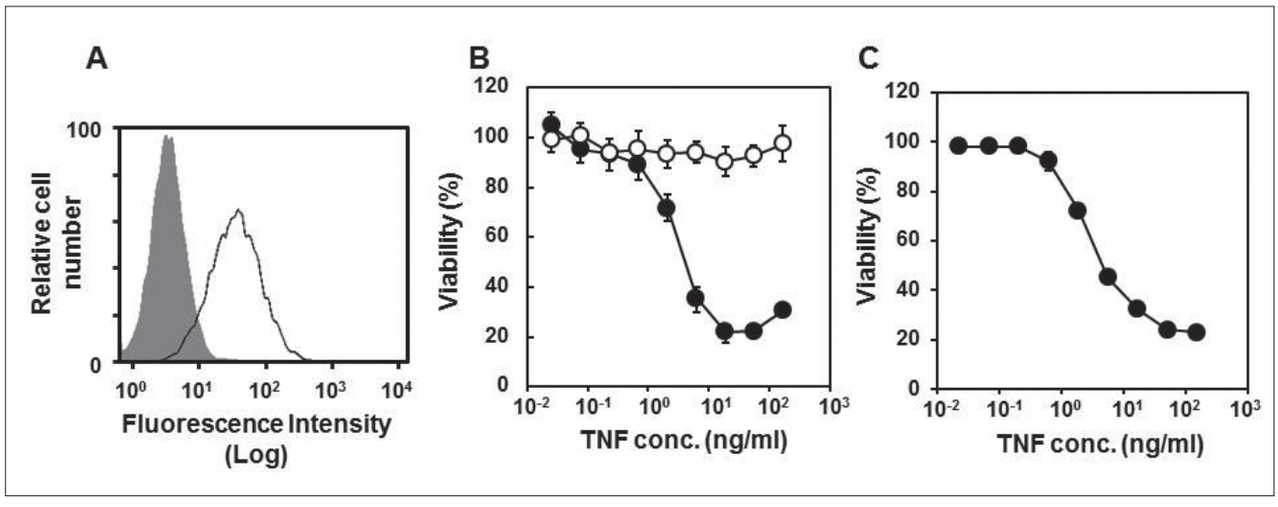

Fig. 2: Simple and highly sensitive assay system for mTNFR2-mediated TNF activity. (A) Expression of the chimeric receptor on mTNFR2/mFas PA was analyzed by flow cytometry using an mTNFR2-specific antibody (open histograms) or an isotype control antibody (shaded histograms). (B) Strong induction of mTNFR2/mFas PA cell death by wtTNF. mTNFR2/mFas PA cells were treated with serial dilutions of mouse TNF (closed circles) or human TNF (open circles) in the presence of CHX (1 μg/ml). After 48 h, the cell viability was measured using the WST-8 assay. Each point represents the mean ± SEM (n= 3 or 5, respectively). (C) TNFR1 mediated bioactivity was measured using the L-M cells. L-M cells were treated with serial dilutions of mouse TNF. After 24 h, the cell viability was measured using the methylene blue assay. Each point represents the mean ± SEM (n=3).

manner, with a median effective concentration (EC50) of 3.01 ng/ml (Fig. 2B). The parental TNFR1$^{-/-}$R2$^{-/-}$ PA cells were resistant to mTNF-induced cell death (Abe et al. 2008), suggesting that these cytotoxic effects were dependent on mTNFR2/mFas activation. The previous CT6 proliferation assays showed that the concentration of mTNF needed to reach 30% of maximal growth induction is 2 ng/ml (Ameloot et al. 2001), suggesting our assay system had the same or higher sensitivity than the conventional method.

In order to examine TNFR1-mediated signaling, we also evaluated the cell viability of mTNFR2/mFas PA cells in the presence of hTNF, which can bind mTNFR1 but not mTNFR2 (Lewis et al. 1991). The hTNF had no effect on the cell viability of mTNFR2/mFas PA cells (Fig. 2B), suggesting that these cells permitted evaluation of mTNFR2 signaling precisely without being affected by mTNFR1. On the other hand, an L-M cytotoxic assay system used to analyze mTNFR1-mediated bioactivity showed that cytotoxic effects were induced by mTNF in a concentration-dependent manner, with an EC50 of 3.37 ng/ml (Fig. 2C). Thus, the constructed mTNFR2/mFas expressing TNFR1$^{-/-}$R2$^{-/-}$ PA cells exerted mTNFR2-mediated cytotoxic effects that were as sensitive as those of the mTNFR1-mediated bioassay system. We are currently applying this assay system and a phage display system (Nomura et al. 2010; Mukai et al. 2006) to generate mTNFR2-selective TNF mutants (unpublished data). We believe that this mTNFR2-mediated biological assay system will be a useful tool to screen for mTNFR2-selective stimulating agents.

3. Experimental

3.1. Cell culture

TNFR1$^{-/-}$R2$^{-/-}$ preadipocytes (PA) and L-M cells were generously provided by Dr. Hotamisligil (Harvard School of Public Health, Boston MA) (Xu et al. 1999) and Mochida Pharmaceutical Co. Ltd. (Tokyo, Japan), respectively. TNFR1$^{-/-}$R2$^{-/-}$ PA, 293T cells and HeLaP4 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, Inc., Tokyo, Japan) with 10% fetal bovine serum (FBS)

and 1% antibiotic cocktail (penicillin 10,000 units/ml, streptomycin 10 mg/ml, and amphotericin B 25 µg/ml; Nacalai Tesque, Kyoto, Japan). L-M cells were cultured in minimum Eagle's medium (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 1% FBS and a 1% antibiotic cocktail.

3.2. Construction of self-inactivating (SIN) lentiviral vector

Vectors were constructed using standard cloning procedures. A DNA fragment encoding the EC and TM domains of mTNFR2 was amplified by polymerase chain reaction (PCR) from mouse spleen cDNA with the following primer pairs: forward primer (5'-GAC CAC CAT GGC GCC CGC CGC CCT CTG GGT CGC GCT GGT CTT TGA AC-3') containing a NcoI site at the 5′-end and a reverse primer (5'-GGC ATC CTT TCG AAG GCA GGA GGG CTT CTT TTT CCT CTG CAC CAG-3') containing a Csp45I site. The resulting amplified fragment was subcloned into the pENTR1A vector (Invitrogen Corp., Carlsbad, CA) to generate pENTR-mTNFR2$^{EC/TM}$. A fragment encoding the IC domain of mFas was amplified by PCR from mouse spleen cDNA with the following primer pair: forward primer (5'-AAT TCC ACT TGT ATT TAT ACT TCG AAA GTA CCG GAA AAG A-3') containing a Csp45I site and a reverse primer (5'-GTC ATC CTT GTA GTC TGC GGC CGC TCA CTC CAG ACA TTG TCC TTC ATT TTC ATT TCC A-3') containing a NotI site at the 5′-end. The mFas DNA fragment was subcloned into pENTR-mTNFR2$^{EC/TM}$ between the Csp45I and NotI sites to combine the EC and TM domains of mTNFR2 to the IC domains of mFas, generating pENTR-mTNFR2/mFas (Fig. 1A). The mTNFR2/mFas DNA fragment was then cloned between the EcoRI and NotI sites of the SIN lentiviral vector construct, which contains the blasticidin (Bsd) resistance gene, generating CSII-CMV-mTNFR2/mFas-IRES2-Bsd (Fig. 1B).

3.3. Preparation of mTNFR2/mFas-expressing cell culture

To prepare the mTNFR2/mFas-expressing cells, TNFR1$^{-/-}$R2$^{-/-}$ PA were infected with a lentiviral vector at a multiplicity of infection (MOI) of 100. The method used to prepare the lentiviral vector has been described previously (Abe et al. 2008; Miyoshi et al. 1999). Stable mTNFR2/mFas-transfectants were selected for growth in culture medium containing 5 µg/ml Bsd (Invitrogen Corp.) for 1 week. Expression of the mTNFR2/mFas chimeric receptor on Bsd-resistant cells was detected by staining with anti-mTNFR2 antibody-PE conjugated or isotype control antibodies (both BD Biosciences, Franklin Lakes, NJ), respectively. Fluorescence was analyzed on a FACS Canto flow cytometer (BD Biosciences), and data were analyzed using FlowJo (Tree Star, Ashland, OR).

3.4. Cytotoxicity assay

mTNFR2/mFas PA were seeded on 96-well plates at a density of 1.5×10^4 cells/well in culture medium. Serial dilutions of mTNF or hTNF were prepared with DMEM containing 1 µg/ml cycloheximide and added to each well. After 48 h incubation, the cell viability was measured using a WST-8 assay kit (Nacalai Tesque) according to the manufacturer's instructions. L-M cells were cultured in 96-well plates in the presence of serially diluted mTNF for 24 h at 3.0×10^4 cells/well. Cytotoxicity was then assessed using a methylene blue assay as described previously (Nomura et al. 2010). Experimental data were analyzed by a logistic regression model to calculate the mean effective concentration (EC50) using GraphPad Prism version 5 (GraphPad Software, San Diego,CA).

Acknowledgements: This study was supported in part by grants-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by Grants-in-Aid for Scientific Research from Japan Society for the Promotion of Science (JSPS). This study also supported in part by by Japan Agency for Medical Research and Development (AMED) and Hoansya Foundation.

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