〈Original Article〉

TNF receptor type 2 transmits caspase-dependent apoptotic signals in fibroblast-like synoviocytes derived from rheumatoid arthritis

Hiroyasu HIRANO[1)], Hideya IGARASHI[1)], Yoshitaka MORITA[2)], Katsuhiko ISHIHARA[1)]

1) Department of Immunology and Molecular Genetics, 2) Department of Rheumatology, Kawasaki Medical School, 577 Matsushima, Kurashiki, 701-0192, Japan

ABSTRACT Signals from tumor necrosis factor α (TNFα) are transduced through two types of receptors, tumor necrosis factor receptor type1 (TNFR1) and type2 (TNFR2), which commonly transduce activation signals for NF-κB, affecting cellular survival, growth, and inflammation. TNFR1 is ubiquitously expressed and mediates caspase-dependent apoptotic signals, whereas TNFR2 is selectively expressed on hematopoietic cells without transducing death signals. We detected TNFR2 transcription in fibroblast-like synoviocytes derived from rheumatoid arthritis (RA-FLS) at various levels, but usually much lower than those of TNFR1. To investigate the function of TNFR2 on RA-FLS in TNFα signaling, we established a stable transfectant overexpressing TNFR2 using the human RA-FLS cell line MH7A and stimulated it with 50 ng/ml TNFα, a concentration that usually induces apoptosis in parent MH7A cells. Since TNFR2 is known to transduce anti-apoptotic signals *via* NF-κB activation, we expected to observe a reduction in apoptotic cells. Contrary to our expectations, the ratio of apoptotic cells in TNFR2 transfectants was higher than that of mock stable transfectants used as a control. This enhanced sensitivity to apoptosis was not inhibited by the addition of either anti-TNFR2 monoclonal antibody (mAb) 80M2, which blocks ligand passing, or antagonistic anti-TNFR1 Ab, indicating that apoptosis was independent of TNFR1 signals. Furthermore, in the presence of antagonistic anti-TNFR1 Ab, the addition of agonistic anti-TNFR2 Ab induced apoptosis with a rapid decrease in TNF receptor-associated factor 2 (TRAF2) and cleavage of caspase-8 and -3. The observed apoptosis was sensitive to an inhibitor of pan-caspase, but not of receptor-interacting protein (RIP) 1. These data clearly indicate the presence of a caspase-dependent, apoptotic signaling pathway downstream of TNFR2.

doi:10.11482/KMJ-E41(2)29 *(Accepted on October 6, 2015)*

Key words: Rheumatoid arthritis, Fibroblast-like synoviocyte, Apoptosis, Caspase

Corresponding author
Katsuhiko Ishihara
Department of Immunology and Molecular Genetics,
Kawasaki Medical School, 577 Matsushima, Kurashiki,
701-0192, Japan

Phone : 81 86 462 1111
Fax : 81 86 464 1187
E-mail: ishihara-im@med.kawasaki-m.ac.jp

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disease that is characterized by hyperplasia of the synovium leading to joint destruction. Inflammatory mediators are produced by macrophages, fibroblasts, and endothelial cells, and induce bone destruction *via* the activation of osteoclasts[1, 2]. The inflammatory cytokine tumor necrosis factor α (TNFα) plays a pivotal role in the pathophysiology of RA, as evidenced by the effectiveness of anti-TNFα therapy in suppressing disease activity and bone destruction[3-5]. However, approximately 30% of RA patients do not respond to anti-TNFα biological reagents[6], and the molecular mechanisms for this unresponsiveness are still unknown.

TNFα binds to two types of receptors, p55 type 1 receptor (TNFR1) and p75 type 2 receptor (TNFR2). TNFR1 expression is ubiquitous, but TNFR2 expression is limited to immune cells[7-9]. TNFR1 has a death domain in the cytoplasmic tail, but TNFR2 does not. Binding of TNFα to TNFR1 recruits TNFR-associated death domain (TRADD) to its death domain, and forms complexes that include TRADD, TNF receptor-associated factor 2 (TRAF2), cellular inhibitor of apoptosis protein-1 (cIAP1), and the serine/threonine kinase, receptor-interacting protein (RIP) 1 (Complex I). TRAF2/cIAP1-mediated ubiquitination of RIP1 activates IκB kinase (IKK). Active IKK phosphorylates IκB, leading to its proteasomal degradation, and then released NF-κB (p65 and p50) translocates to the nucleus and induces transcription of the target genes. RIP1 dissociated from Complex I associates with Fas-associated death domain protein (FADD) and caspase 8 to form Complex IIa, in which caspase 8 is activated to initiate apoptosis[10-12]. A novel type of cell death, necroptosis, is also mediated by TNFR1. In the absence of caspase activity, RIP1 interacts with RIP3 to form Complex IIb, which induces necroptosis. TNFR2 lacks a death domain and stimulation of TNFR2 leads to the activation of NF-κB, but not to the induction of apoptosis.

We inferred that the aberration of TNFR structure or function might contribute to the unresponsiveness of some RA patients to anti-TNFα therapy. We examined TNFR mutations among primary RA-FLSs because mutations in the trans-membrane region of TNFR1 cause continuous inflammation associated with TNF receptor-associated periodic syndrome (TRAPS)/ familial Hibernian fever[13]. We did not detect any mutations, except for a single nucleotide polymorphism, A36G, which has been described previously[14] and is silent, in one case of RA-FLS. Next, we examined the expression levels of both receptors and noticed that the transcription levels of TNFR2 vary among primary RA-FLSs. This prompted us to study the role of TNFR2 in RA-FLS, particular its role in the pathophysiology of RA. However, the properties of TNFR2 are still controversial. For instance, forced expression of TNFR2 in a cervical cancer cell line facilitates apoptosis induced by TNFα stimulation[15]. TNFα induces expression of TNFR2 and promotes cell proliferation in a colorectal cancer cell line[16]. Cellular responses mediated by TNFR2 appear to be cell-type dependent.

In this study, we revealed that TNFR2 lacking the death domain can transduce caspase-dependent apoptotic signals in the RA-FLS cell line MH7A with forced expression of TNFR2.

MATERIALS AND METHODS

Cells and cell culture

MH7A, an RA-FLS line transformed with SV40TAg, was obtained from the Riken Cell Bank (Ibaragi, Japan). Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum with antibiotics in 10% CO_2 at 37℃ with humidified air. Primary FLSs were described previously[17] and the study protocol was approved

by the Research Ethics Committee of Kawasaki Medical School (#291-2).

Antibodies

FITC-conjugated mouse anti-human TNFR1 monoclonal antibody (mAb) (clone H398, MBL, Nagoya, Japan) and PE-conjugated mouse anti-human TNFR2 mAb (clone 80M2, MBL) were used for the detection of TNFR1 and TNFR2 in FACS analysis. Unlabeled mouse anti-human TNFR2 mAb 80M2 was utilized to stabilize the binding between TNFα and TNFR2 (MBL). Antagonistic mouse anti-human TNFR1 mAb (MAB225, clone 16803, R&D Systems, Minneapolis, MN, USA) was used to neutralize TNFR1. Goat anti-TNFR2 Ab (AB226PB, R&D Systems) was used as agonistic antibody to stimulate TNFR2, as reported by Chan *et al.*[18].

Establishment of a stable transfectant overexpressing TNFR2

cDNA for *Tnfr2* was obtained by RT-PCR with total RNA of MH7A and gene-specific primers for *Tnfr2* (Forward, 5′-GGCCCTCGAGATGGCGCCC GTCGCCGTCTGG-3′; Reverse, 5′-CGGGGATC CTTAACTGGGCTTCATCCCAGC-3′) using high-fidelity DNA polymerase (KOD-Plus-Neo; Toyobo, Osaka, Japan). The PCR product was inserted into the pBluescript II cloning vector (STRATAGENE, La Jolla, CA, USA) and the lack of mutations was confirmed by sequencing with T7 or T3 primers. The vector was replaced with the pEBMulti vector (Wako, Osaka, Japan), and the expression vector was transfected into MH7A using the liposome method (Xtreme Gene; Roche, Basel, Switzerland). After several passages in medium containing hygromycin, the cells were stained with PE-anti-TNFR2 Ab, and cells overexpressing TNFR2 were enriched by cell sorting with FACSAria (BD Biosciences, San Jose, CA, USA). The sorted cells were cultured in the medium to establish stable transfectants.

Quantitative real-time PCR

The total RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Random hexamer-primed cDNAs were prepared using the ReverTra Ace RT Kit (Toyobo). Specific primers for each gene were designed using Universal Primer Design Tools available on the Roche website. Real-time PCR with the SYBR Green 1 dye was carried out using the 7300 Real-Time PCR System (PE Applied Biosystems, Carlsbad, CA, USA). Threshold cycle (Ct) values of the target genes were normalized to those of β-actin.

Cell Proliferation assay

One hundred thousand stable transfectants in 1 ml of culture medium in a 12-well plate were pre-treated with either 2 μg/ml anti-TNFR2 Ab 80M2 or the same concentration of mouse IgG1 for 30 min, then cultured for an additional 72 h in the presence of TNFα at various concentrations (indicated in the Figs. 2A and 3A). At the end of the culture, the cell number was counted using a hemocytometer.

Apoptosis assay

Early and late apoptotic cells were stained with FITC-conjugated Annexin V (BioVision, Inc., Milpitas, CA, USA) and 7AAD, respectively, and were detected simultaneously with the FACSCalibur (BD Biosciences). The early apoptotic cell population was defined as Annexin V^+ $7AAD^-$ cells and the late apoptosis cell population was defined as Annexin V^+ $7AAD^+$ cells in the FACS analysis.

Western blot analysis

Cell lysates were prepared in RIPA lysis buffer with equal cell numbers from each sample. The equivalent whole-cell crude lysates were resuspended in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

sample buffer, boiled for 5 min, resolved using SDS-PAGE, transferred electrically to a PVFD (polyvinylidene fluoride) membrane (Immun-Blot, Bio-Rad, Hercules, CA, USA), and subjected to a western blot analysis with a signal-enhancing kit obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The membrane was probed with rabbit polyclonal antibodies for TRAF2, and anti-Caspase3 or mouse anti-Caspase8 mAb (Cell Signaling Technology, Inc., Danvers, MA, USA). The bound antibodies were detected with HRP-labeled goat anti-rabbit or goat anti-mouse antibodies.

RESULTS

Establishment of the transfectant R2H stably expressing TNFR2

To analyze the properties of TNFR2 in RA-FLS, we established an MH7A- derived stable transfectant, designated R2H, expressing TNFR2 at a high level. RT-PCR analyses revealed that the difference in *tnfr1* expression levels between R2H and parental MH7A (Parent) or mock transfectants (Mock) was only 2-fold. However, the *tnfr2* expression level in R2H was 4,720 times higher than that of mock transfectants. (Fig. 1A). FACS analyses revealed that the mean fluorescence

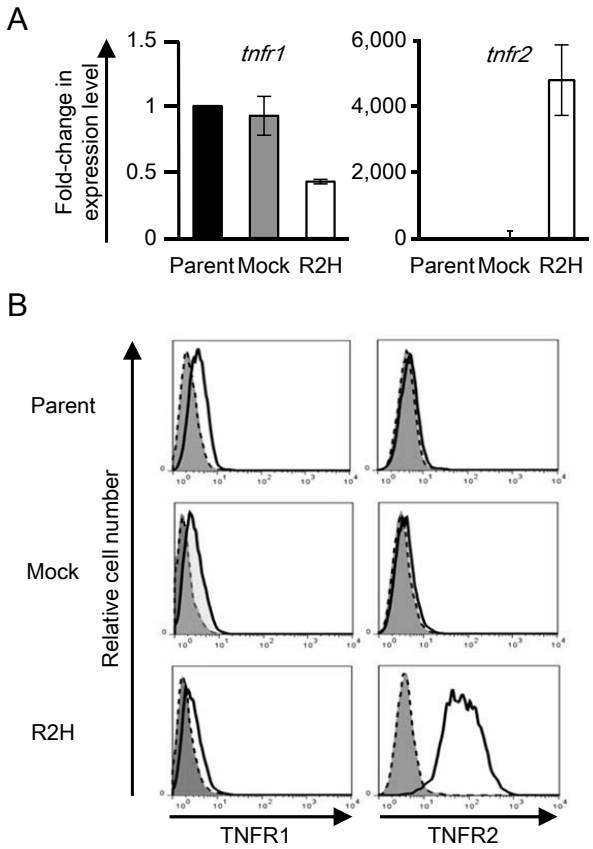

Fig. 1. The expression level of TNFR2 in stable transfectant R2H cells.
A. The transcription of TNFR2 in R2H cells was evaluated by real-time PCR. Parent MH7A cells (Parent) and stable transfectants introduced with the expression vector only (Mock) were used as controls. Fold-changes in the expression level of either *tnfr1* or *tnfr2* were calculated by the ΔΔCT method, setting the parent value as "1."
B. Surface expression of either TNFR1 or TNFR2 was analyzed on FACS with FITC-anti-TNFR1 and PE-anti-TNFR2 Abs, respectively.

intensity of R2H stained with anti-TNFR2 mAb increased, whereas that of mock transfectants showed no increase compared with the unstained control (Fig. 1B). The amount of surface expression of TNFR1 on R2H was similar to the levels on the mock transfectant and parental MH7A cells. We expected that the biological responses of RA-FLS to TNFα would be modified by the forced expression of TNFR2 in R2H.

R2H is highly sensitive to TNFα induced apoptosis

TNFR2-mediated NF-κB activity was expected to have an anti-apoptotic effect; accordingly, we attempted to confirm this activity using R2H with TNFα treatment. Unexpectedly, the R2H cell number was dramatically reduced during a 24-h culture with a high dose of TNFα in comparison with mock cells (Fig. 2A). We examined whether this reduction in living cells was due to the induction of apoptosis by staining with Annexin V. The fraction of early apoptosis R2H cells was markedly increased after stimulation with TNFα (61.2% ± 13.6) in comparison with that in the Mock cells (16.5% ± 1.3) (Fig. 2B and 2C). Of note, even in the absence of TNFα, R2H showed an increased frequency of Annexin V-positive cells (13.8% ± 0.9) compared with the Mock cells (7.5% ± 1.9).

Enhanced apoptosis of R2H by TNFα stimulation was not due to "ligand passing" from TNFR2 to TNFR1

In the ligand passing theory proposed by Goeddel[18], i) TNFα binds to TNFR2 first owing to its higher affinity to TNFα than TNFR1, ii) captured TNFα immediately dissociates from TNFR2 due to its high dissociation constant, and iii) released TNFα binds to proximal TNFR1. Thus, increased expression of TNFR2 may result in an increased amount of TNFα passing from TNFR2 to TNFR1, leading to an enhancement of the TNFR1-mediated apoptotic signal.

The sensitivity of R2H to TNFα was examined in the presence of anti-TNFR2 Ab (80M2), which stabilizes the binding between TNFα and TNFR2, to inhibit the passing of TNFα to TNFR1. However, the addition of 80M2 did not influence the number of living cells (Fig. 3A) or cells in apoptosis (Fig. 3B and 3C) after treatment with TNFα, suggesting that ligand passing is not involved in augmented TNFα -induced apoptosis of R2H and the apoptotic signal was transmitted *via* TNFR2.

Stimulating anti-TNFR2 Ab can induce apoptosis of R2H

To further confirm the possibility that TNFR2 conducts apoptotic signals, the combination of blocking Ab for TNFR1 (MAB225) and stimulating Ab for TNFR2 (AB226PB) was adopted. The inhibitory effect of MAB225 on NF-κB activation was evidenced by the reduction in the phosphorylation status of p65, an indicator of the signal transduction of TNFα *via* TNFR1 (Fig. 4A).

MAB225 alone failed to reduce the TNFα -induced apoptosis of R2H, indicating that TNFR1 signaling was not involved in this apoptotic pathway (Fig. 4B and 4C). The adaptor protein TRAF2 plays a crucial role for TNFR signaling[19]. Of note, the ubiquitin-dependent degradation of TRAF2 by cIAP1 occurs *via* the binding of TNFα to TNFR2[20]. This rapid degradation of TRAF2 was observed when R2H was treated with the combination of MAB225 and AB226PB, indicating this treatment actually stimulates TNFR2 (Fig. 4D). Finally, this combination of Abs more strongly induced Annexin V^+ apoptotic cells for R2H (44.4% ± 20.6) than mock cells (11.3% ± 3.8), revealing the existence of novel TNFR2-mediated apoptotic signals (Fig. 4E and 4F).

TNFR2-mediated apoptotic pathway is caspase-dependent

Apoptotic signals are initiated by the binding of

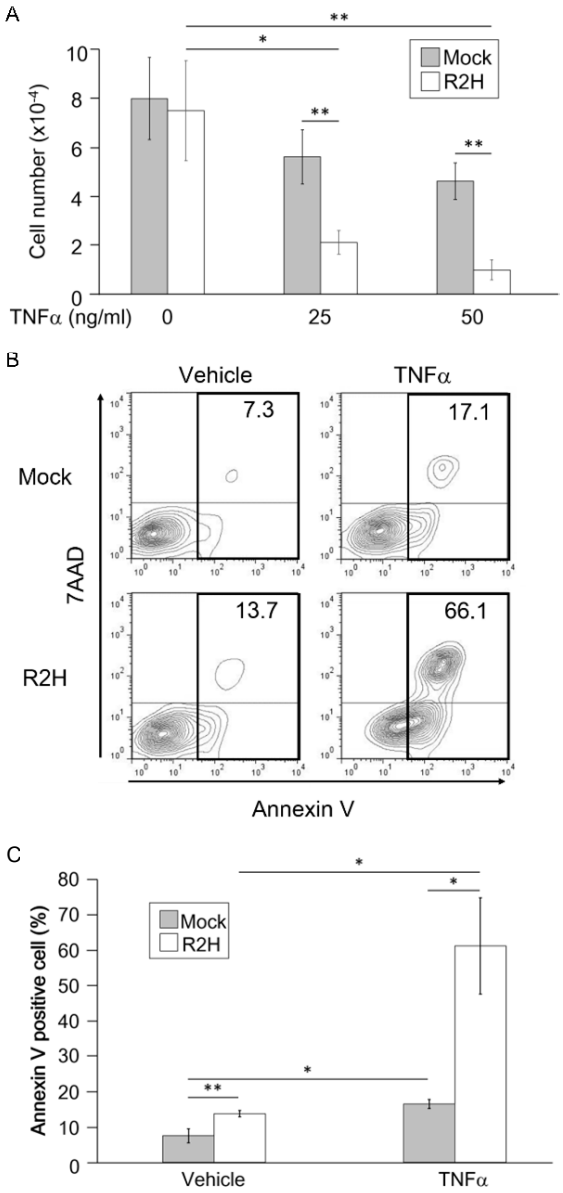

Fig. 2. Growth retardation and enhanced cell death of R2H.

A. Mock and R2H cells were plated at a density 1×10^5/well in a 12-well plate. The cells were cultured for 24 h in the presence or absence of TNFα at various concentrations indicated in the figure. Then, the cells were harvested and counted using a hemocytometer. Gray bars and white bars indicate Mock and R2H cells, respectively. A representative result is shown from three independent experiments. Results are presented as means ± S.D. from the triplicate experiments. Differences between mock and R2H cells treated with TNFα marked with * and ** were statistically significant (*$p < 0.05$, **$p < 0.01$).

B. Mock or R2H cells were cultured for 24 h in the absence or presence of TNFα at a concentration of 50 ng/ml, and were stained with FITC-conjugated Annexin V and 7AAD. Apoptotic cells were defined as Annexin V^+ cells in the FACS analysis. The numbers in the representative FACS profile indicate the sum of the frequencies of Annexin V^+ $7AAD^-$ and Annexin V^+ $7AAD^+$ populations.

C. The percentage of Annexin V positive cells was shown as bar graphs. Gray bars and white bars indicate Mock and R2H cells, respectively. Results are presented as means ± S.D. from three independent experiments including one indicated in B. Differences between mock and R2H cells treated with TNFα marked with * and ** were statistically significant (*$p < 0.05$, **$p < 0.01$).

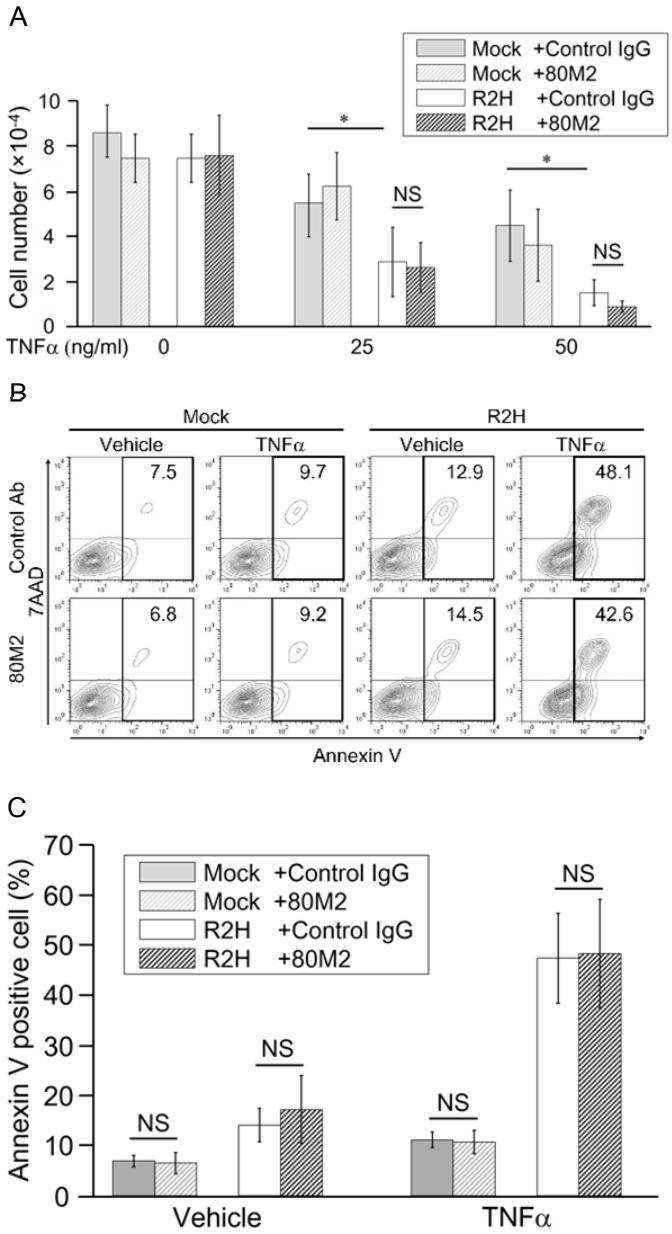

Fig. 3. No effect of anti-TNFR2 Ab (80M2), which stabilizes the binding between TNFα and TNFR2, on TNFα-induced apoptosis of R2H.

A. Transfectant cells were plated at 1×10^5 /well and pretreated with 2 μg/ml 80M2 for 30 min. The cells were cultured for an additional 24 h in the presence or absence of TNFα at various concentrations indicated in the figure. Then the cells were harvested and counted using a hemocytometer. Asterisks (*) indicate statistically significant differences (*$p < 0.05$), and NS means not significant.

B. Mock and R2H cells were cultured for 24 h in the absence or presence of TNFα at a concentration of 50 ng/ml, and were stained with FITC-conjugated Annexin V and 7AAD. Apoptotic cells were defined as Annexin V^+ cells in the FACS analysis. The numbers in the representative FACS profile indicate the sum of Annexin V^+ $7AAD^-$ and Annexin V^+ $7AAD^+$ populations.

C. The percentage of Annexin V positive cells was shown as bar graphs. Results are presented as means ± S.D. of four independent experiments. NS means not significant.

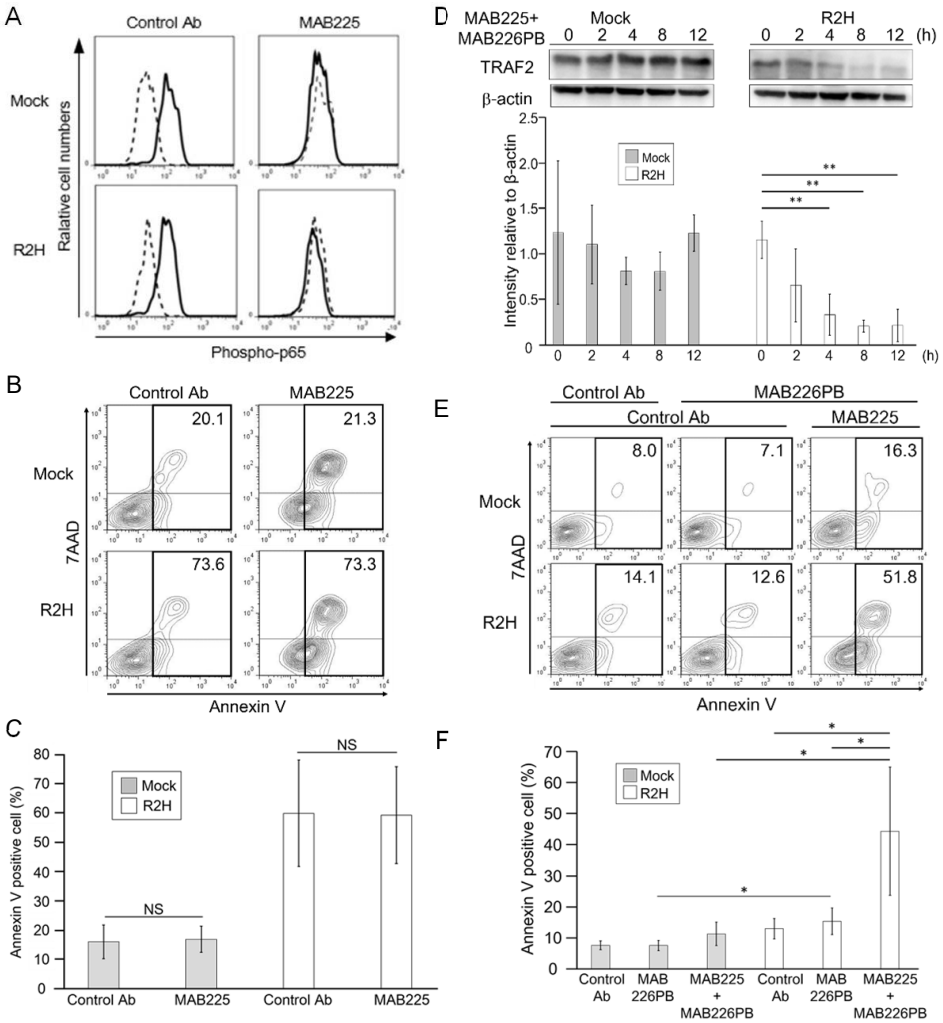

Fig. 4. No contribution of signals from TNFR1 to TNFα-induced apoptosis of R2H.

A. The activation status of NF-κB was measured by the intensity of PE-conjugated anti-phosphorylated p65 (RelA) on FACS. Dashed line: unstimulated, solid line: stimulated with TNFα at a concentration of 50 ng/ml for 5 min.

B. Mock and R2H cells, pretreated with 3 μg/ml neutralizing anti-TNFR1 Ab (MAB225) or isotype-matched control Ab for 30 min, cultured for an additional 24 h in the presence of TNFα at a concentration of 50 ng/ml, and stained with FITC-conjugated Annexin V and 7AAD. Apoptotic cells were defined as Annexin V^+ cells in the FACS analysis. The numbers in the representative FACS profile indicate the sum of the frequencies of Annexin V^+ $7AAD^-$ and Annexin V^+ $7AAD^+$ populations.

C. The percentage of Annexin V positive cells was shown as bar graphs. Gray bars and white bars indicate Mock and R2H cells, respectively. Results are presented as means ± S.D. from five independent experiments. NS means not significant.

D. The kinetics of the amount of TRAF2 after selective stimulation through TNFR2. Mock and R2H cells were treated with 3 μg/ml agonistic anti-TNFR2 Ab (AB226PB) for 30 min in the presence of neutralizing anti-TNFR1 Ab (MAB225) and evaluated at the indicated time points by a western blotting analysis (upper panel). The graph shows the relative amounts of TRAF2 protein quantified on a densitometer normalized with those of β-actin as an internal control (lower panel). Gray bars and white bars indicate Mock and R2H cells, respectively. Asterisks (**) indicates statistically significant difference (**$p < 0.01$).

E. Apoptosis assay was performed using 3 μg/ml agonistic anti-TNFR2 Ab (AB226PB) or isotype-matched control Ab instead of TNFα in B. The number in the representative FACS profile indicates the sum of the frequencies of Annexin V^+ $7AAD^-$ and Annexin V^+ $7AAD^+$ populations.

F. The percentage of Annexin V positive cells was shown as bar graphs. Gray bars and white bars indicate Mock and R2H cells, respectively. Results are presented as means ± S.D. from six independent experiments. Asterisks (*) indicate statistically significant differences (*$p < 0.05$).

TRADD-FADD complex to the death domain within the cytoplasmic portion of TNFR1. Subsequently the complex activates caspase-3 and -8, the direct effecter molecules for apoptosis. Since TNFR2 lacks the death domain, it seemed unlikely that the TNFR2-mediated pathway utilizes caspases. Contrary to the expectation, cleaved-product of caspase 3 (17/19 kDa) was clearly detected in R2H at 2 h after stimulation with the combination of MAB225 and AB226PB, as shown in Fig. 5A. Cleaved-product of Caspase8 (43 kDa) was also detected under the same conditions (Fig. 5A). These data indicate that caspase-3 and -8 are involved in the TNFR2-mediatied apoptosis.

Recent studies have demonstrated that not only caspase-dependent apoptosis pathways, but also RIP1/RIP3-dependent necroptosis pathways are mediated by TNFR1[21]. The pan-caspase inhibitor zVAD-FMK (zVAD) significantly reduced the Annexin V^+ cells of R2H induced by the combination of MAB225 and AB226PB (from 43.3% ± 16.3 to 16.0% ± 5.3). However, the RIP1 inhibitor necrostatin-1 (Nec-1)[21, 22] had no apparent effect on Annexin V^+/7AAD$^+$ dead cells of R2H (from 43.3% ± 16.3 to 40.4% ± 14.5), indicating caspase activation is required for the TNFR2-mediated apoptosis pathway (Fig. 5B and 5C).

DISCUSSION

TNFα is thought to play a pivotal role in inducing hyperplasia of the RA synovium through NF-κB activation[23]. The importance of NF-κB in inhibiting TNFR1-mediated apoptosis in human primary FLS is also evidenced by the dual knock-down of p65 and p50 subunits of NF-κB[24]. TNFR2 lacks a death domain and stimulation of TNFR2 leads to activation of the RIP/IKK complex and NF-κB without involvement of the pro-apoptotic pathway. Thus, it is reasonable to think that TNFR2 signals have a cytoprotective effect[25, 26]. However, the cellular responses mediated by TNFR2 appear to be cell-type dependent. For instance, forced expression of TNFR2 in the cervical cancer cell line HeLa results in enhanced apoptosis by TNFα stimulation, although the molecular mechanism for this effect has not been elucidated. In the present study, we showed that TNFR2 signals have a cytocidal effect in the RA-FLS cell line. Furthermore, we clearly demonstrated that this cytocidal signal from TNFR2 is caspase-dependent.

Thus, TRAF2 seems to inhibit caspase-8 activation by recruiting cIAP1 to the TNFR1 complex following TNFα stimulation[20]. One mechanism that may account for TNFR2-mediated apoptosis signals is the rapid downregulation of TRAF2 found in R2H treated with stimulating Ab to TNFR2. In TNFR1-mediated apoptosis signaling, TNFα binding to TNFR1 recruits FADD to the TNFR1 signaling complex, which promotes the recruitment of pro-casapase-8 to FADD *via* the death domain. The oligomerized pro-casapase-8 on FADD is activated by auto-cleavage, and mature caspase-8 successively cleaves both pro-casapase-3 and pro-Bid, leading to their activation as pro-apoptotic molecules. Therefore, FADD is thought to be an indispensable platform for casapase-8 activation. Thus, in TNFR2-mediated apoptosis, future studies are necessary to determine how caspase-8 is activated independent on FADD. If the molecules connecting TNFR2 to caspase-8 are identified, they are potential targets for manipulations to artificially induce apoptosis of proliferative RA-FLS.

Ban *et al.* revealed that agonistic stimulation of TNFR2 induces the apoptosis of auto-reactive T cells from diabetes patients[27]. Future developments of the methods to activate cell surface proteins on specific target cells will facilitate the use of this strategy to treat auto-immune diseases, and might have applications for the elimination of pro-inflammatory FLS in RA, as shown in this study.

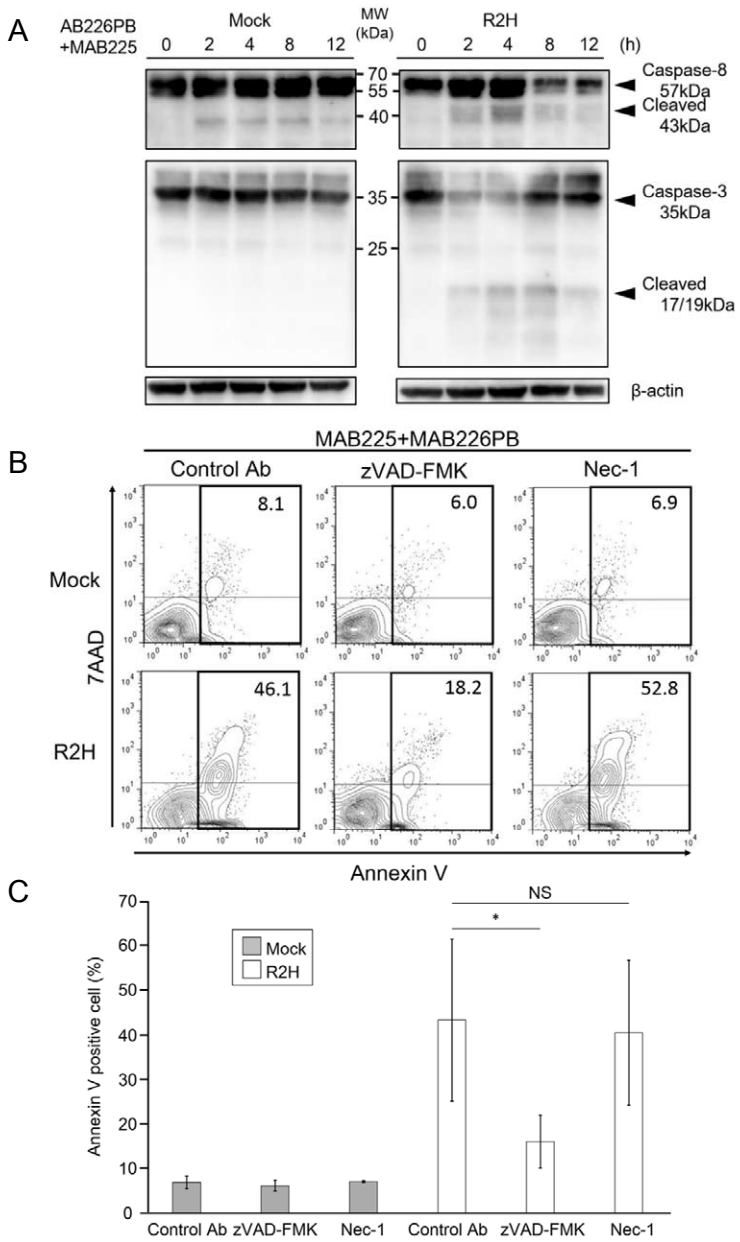

Fig. 5. Activation of Caspase-8 and -3 in R2H cells stimulated with agonistic anti-TNFR2 Ab.

A. Cleavages of Caspase-8 and -3 after treatment of Mock and R2H cells with 3 μg/ml of agonistic TNFR2 Ab (AB226PB) for 30 min in the presence of neutralizing TNFR1 Ab (MAB225) were evaluated at the indicated time points by a western blotting analysis. Representative data from three independent experiments are shown.

B. Mock and R2H cells, pretreated with 50 μM zVAD or 100 μM Nec-1 for 60 min, were subsequently treated with 3 μg/ml neutralizing anti-TNFR1 Ab (MAB225) for 30 min. Those cells were cultured for an additional 24 h in the presence of 3 μg/ml agonistic anti-TNFR2 Ab (AB225PB), and stained with FITC-conjugated Annexin V and 7AAD. Apoptotic cells were defined as Annexin V^+ cells in the FACS analysis. The numbers in the representative FACS profile indicate the sum of the frequencies of Annexin V^+ $7AAD^-$ and Annexin V^+ $7AAD^+$ populations.

C. The percentage of Annexin V positive cells was shown as bar graphs. Gray bars and white bars indicate Mock and R2H cells, respectively. Results are presented as means ± S.D. from five independent experiments. Asterisks (*) indicate statistically significant differences ($*p < 0.05$), and NS means not significant.

CONFRICT OF INTEREST

Y.M. has received research supports and/or honoraria from Abbvie, Astellas, Bristol-Myers, Chugai, Eisai, Eli lilly, Mitsubishi-Tanabe, Pfizer, and Takeda.

ACKNOWLEDGEMENTS

This work was supported by Research Project Grants (26-G2 to H.H, and 25-B68 to H.I.) from Kawasaki Medical School and Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, Culture and Technology of Japan (25462390 to H.I.). We would also like to thank Mss. Kenmotsu and Funatsu for their technical assistance in this study.

REFERENCES

1） Bottini N, Firestein GS: Duality of fibroblast-like synoviocytes in RA: passive responders and imprinted aggressors. Nat Rev Rheumatol 9: 24-33, 2013

2） Firestein GS: Evolving concepts of rheumatoid arthritis. Nature 423: 356-361, 2003

3） Lipsky PE, van der Heijde DM, St Clair EW, *et al.*: Infliximab and methotrexate in the treatment of rheumatoid arthritis. Anti-Tumor Necrosis Factor Trial in Rheumatoid Arthritis with Concomitant Therapy Study Group. N Engl J Med 343: 1594-1602, 2000

4） van der Heijde D, Klareskog L, Rodriguez-Valverde V, *et al.*: Comparison of etanercept and methotrexate, alone and combined, in the treatment of rheumatoid arthritis: two-year clinical and radiographic results from the TEMPO study, a double-blind, randomized trial. Arthritis Rheum 54: 1063-1074, 2006

5） Breedveld FC, Weisman MH, Kavanaugh AF, Cohen SB, Pavelka K, van Vollenhoven R, Sharp J, Perez JL, Spencer-Green GT: The PREMIER study: A multicenter, randomized, double-blind clinical trial of combination therapy with adalimumab plus methotrexate versus methotrexate alone or adalimumab alone in patients with early, aggressive rheumatoid arthritis who had not had previous methotrexate treatment. Arthritis Rheum 54: 26-37, 2006

6） Firestein GS: Biomedicine. Every joint has a silver lining. Science 315: 952-953, 2007

7） Blüml S, Scheinecker C, Smolen JS, Redlich K: Targeting TNF receptors in rheumatoid arthritis. Int Immunol 24: 275-281, 2012

8） Aggarwal BB: Balancing tumor necrosis factor receptor I and tumor necrosis factor receptor II jointly for joint inflammation. Arthritis Rheumatol. 66: 2657-2660, 2014

9） Walczak H: TNF and ubiquitin at the crossroads of gene activation, cell death, inflammation, and cancer. Immunol Rev 244 : 9-28, 2011

10） Micheau O, Tschopp J: Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. Cell 114: 181-190, 2003

11） Schneider-Brachert W, Tchikov V, Neumeyer J, *et al.*: Compartmentalization of TNF receptor 1 signaling: internalized TNF receptosomes as death signaling vesicles. Immunity 21: 415-428, 2004

12） Ofengeim D, Yuan J: Regulation of RIP1 kinase signalling at the crossroads of inflammation and cell death. Nat Rev Mol Cell Biol 14: 727-736, 2013

13） Bachetti T, Ceccherini I: Tumor necrosis factor receptor-associated periodic syndrome as a model linking autophagy and inflammation in protein aggregation diseases. J Mol Med (Berl) 92: 583-594, 2014

14） Barton A, John S, Ollier WE, Silman A, Worthington J: Association between rheumatoid arthritis and polymorphism of tumor necrosis factor receptor Ⅱ, but not tumor necrosis factor receptor Ⅰ, in Caucasians. Arthritis Rheum 44: 61-65, 2001

15） Haridas V, Darnay BG, Natarajan K, Heller R, Aggarwal BB: Overexpression of the p80 TNF receptor leads to TNF-dependent apoptosis, nuclear factor-kappa B activation, and c-Jun kinase activation. J Immunol 160: 3152-3162, 1998

16） Hamilton KE, Simmons JG, Ding S, Van Landeghem L, Lund PK: Cytokine induction of tumor necrosis factor receptor 2 is mediated by STAT3 in colon cancer cells. Mol Cancer Res 9: 1718-1731, 2011

17） Igarashi H, Hashimoto J, Tomita T, Yoshikawa H, Ishihara K: TP53 mutations coincide with the ectopic expression of activation-induced cytidine deaminase in the fibroblast-like synoviocytes derived from a fraction of patients with rheumatoid arthritis. Clin Exp Immunol 161: 71-80, 2010

18） Chan FK, Shisler J, Bixby JG, Felices M, Zheng L, Appel M, Orenstein J, Moss B, Lenardo MJ: A role for tumor necrosis factor receptor-2 and receptor-interacting

protein in programmed necrosis and antiviral responses. J Biol Chem 278: 51613-51621, 2003

19) Tartaglia LA, Pennica D, Goeddel DV: Ligand passing: the 75-kDa tumor necrosis factor (TNF) receptor recruits TNF for signaling by the 55-kDa TNF receptor. J Biol Chem 268: 18542-18548, 1993

20) Naudé PJ, den Boer JA, Luiten PG, Eisel UL: Tumor necrosis factor receptor cross-talk. FEBS J 278: 888-898, 2011

21) Csomos RA, Brady GF, Duckett CS: Enhanced cytoprotective effects of the inhibitor of apoptosis protein cellular IAP1 through stabilization with TRAF2. J Biol Chem 284: 20531-20539, 2009

22) Yuan J, Kroemer G: Alternative cell death mechanisms in development and beyond. Genes Dev 24: 2592-2602, 2010

23) Festjens N, Vanden Berghe T, Cornelis S, Vandenabeele P: RIP1, a kinase on the crossroads of a cell's decision to live or die. Cell Death Differ 14: 400-410, 2007

24) Youn J, Kim HY, Park JH, Hwang SH, Lee SY, Cho CS, Lee SK: Regulation of TNF-alpha-mediated hyperplasia through TNF receptors, TRAFs, and NF-kappaB in synoviocytes obtained from patients with rheumatoid arthritis. Immunol Lett 83: 85-93, 2002

25) Lee UJ, Choung SR, Prakash KV, Lee EJ, Lee MY, Kim YJ, Han CW, Choi YC: Dual knockdown of p65 and p50 subunits of NF-kappaB by siRNA inhibits the induction of inflammatory cytokines and significantly enhance apoptosis in human primary synoviocytes treated with tumor necrosis factor-alpha. Mol Biol Rep 35: 291-298, 2008

26) Zhang L, Blackwell K, Thomas GS, Sun S, Yeh WC, Habelhah H: TRAF2 suppresses basal IKK activity in resting cells and TNFalpha can activate IKK in TRAF2 and TRAF5 double knockout cells. J Mol Biol 389: 495-510, 2009

27) Faustman D, Davis M: TNF receptor 2 pathway: drug target for autoimmune diseases. Nat Rev Drug Discov 9: 482-493, 2010

28) Ban L, Zhang J, Wang L, Kuhtreiber W, Burger D, Faustman DL: Selective death of autoreactive T cells in human diabetes by TNF or TNF receptor 2 agonism. Proc Natl Acad Sci U S A 105: 13644-13649, 2008

【Erratum】

Fig. 4B in the manuscript (doi：10.11482/KMJ-E41（2）29; Kawasaki Medical Journal 41(2) 29-40, TNF receptor type 2 transmits caspase-dependent apoptotic signals in fibroblast-like synoviocytes derived from rheumatoid arthritis. Hirano H, et al) had been changed.