College of Pharmacy[1], Keimyung University, Daegu; Natural Medicine Research Institute[2], Department of Pharmacy, College of Pharmacy, Mokpo National University, Jeonnam; Department of Oral Pharmacology[3], School of Dentistry and Institute of Oral Bioscience, BK21 plus, Chonbuk National University, Jeonju, Republic of Korea

Heat shock protein 90 inhibitor regulates necroptotic cell death via down-regulation of receptor interacting proteins

Seung Yeon Park[1], Jung-Hyun Shim[2], Jung-Il Chae[3], Young Sik Cho[1]

Received June 10, 2014, accepted July 18, 2014

Young Sik Cho, Ph.D.,College of Pharmacy, Keimyung University, 1000 Sindang-dong Dalseo-gu, Daegu 704-701, South Korea
yscho123@kmu.ac.kr

Pharmazie 70: 193–198 (2015) *doi: 10.1691/ph.2015.4689*

17-(Dimethylaminoethylamino)-17-demethoxygeldanamycin (DMAG) acts as an inhibitor of heat shock protein 90 (HSP 90), which serves as a nodal protein of diverse signaling networks leading to a variety of biological implications. HSP90 plays the role of a chaperone for a variety of client proteins including receptor interacting protein 1 (RIP1). Since RIP1 and RIP3 are, respectively, required for zVAD- and tumor necrosis factor alpha (TNFα)-mediated necrotic cell death, we pursued to address the effects of DMAG on receptor- and nonreceptor-mediated necroptotic cell death. DMAG facilitated the degradation of receptor interacting protein 3 (RIP3) as well as RIP1, a known client protein of HSP90, in L929 cells. Consequently, DMAG rendered cells more sensitive to TNFα stimulation while it rescued cells from necrotic cell death caused by zVAD. From this study, we propose that DMAG-downregulated RIP1 can shift cell death typing from necroptosis to apoptosis. In contrast, the protective effect of DMAG on zVAD-induced cytotoxicity could be partly explained by the fact that zVAD mediates cytotoxicity *via* a RIP1-dependent route. In summary, functional disruption of HSP90 by DMAG destabilized necroptosis proteins RIP1 and RIP3, which in turn regulated zVAD- and TNFα-induced necroptosis. Therefore, pharmacological modulation of necroptotic cell death through HSP90 could be a promising strategy for overcoming cancer drug resistance or protecting ischemic cell death.

1. Introduction

When cells are subjected to environmental and physiological stress, they strive to overcome or adapt to unfavorable situations by primarily inducing heat shock proteins (HSPs) family members such as HSP90, HSP40, HSP70 and HSP27 (Pirkkala et al. 2001). Out of HSP members, HSP90 plays a unique nodal role in cellular homeostasis, supervising cell proliferation and cell-survival mechanisms (Pearl and Prodromou 2006). At the molecular level, it acts as a chaperone by assisting proper folding of its client proteins, thereby regulating stability of various proteins required for tumor growth (Goetz et al. 2003). Geldanamycin (GA) is a specific HSP90 inhibitor that binds to the ATP-binding site of the N-terminal domain of it (residues 1-220) and consequently inhibit ATPase activity of HSP90 (Grenert et al. 1997). In fact, treatment of cells with GA leads to destabilization and further degradation of a variety of client proteins of HSP90. Since HSP90 client proteins play important roles in the regulation of the cell cycle, cell growth, cell survival, apoptosis and oncogenesis, functional inactivation of HSP90 by GA obstructs so effectively the proliferation of cancer cells in experimental animals as to adopt such an approach as the anti-cancer therapy (Miyata 2003, 2005).

When the apoptotic processes are blocked, cells can actively respond to death stimuli by inducing the specialized cell death called programmed necrosis or necroptosis (Declercq et al. 2009). When L929 cells are stimulated with tumor necrosis factor alpha (TNFα), expecially, an alternative cell death pathway to a default cell death apoptosis is activated (Tafani et al. 2000; Thon et al. 2005; Vercammen et al. 1998). Both apoptosis and necroptosis share some regulatory machinery, but exhibit distinct features in many aspects such as morphology, biochemical indications and proteins involved (Cho et al. 2010; Dorn 2013). Particularly, some regulatory proteins including receptor interacting proteins (RIPs) have recently been identified as regulators of TNFα-mediated necroptosis (Hitomi et al. 2008; Moquin et al. 2013; Vanlangenakker et al. 2011; Zhou et al. 2012). Furthermore, RIP1 and RIP3 are proposed to be involved in TNFα- and zVAD-induced necroptosis in L929 cells, respectively (Vanlangenakker et al. 2011; Wu et al. 2011; Zhang et al. 2011). It is interesting that RIP1 is one of client proteins of HSP90, such as Bcr-Abl, Akt and mutated p53 (Neckers and Ivy 2003; Neckers et al. 2007; Zhang et al. 2008). Furthermore, it has been revealed that GA facilitates molecular switch from necroptosis to apoptosis *via* RIP1 down-regulation, when L929 cells are stimulated by TNFα (Vanlangenakker et al. 2011). From those observations and our previous results, we pursued to investigate the modulating effects of 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (DMAG), a water soluble GA analogue, on necroptotic death stimuli in L929 cells. Primarily, we confirmed that DMAG destabilized RIP3 as well as RIP1 in a dose-dependent manner (data not shown). As demonstrated in

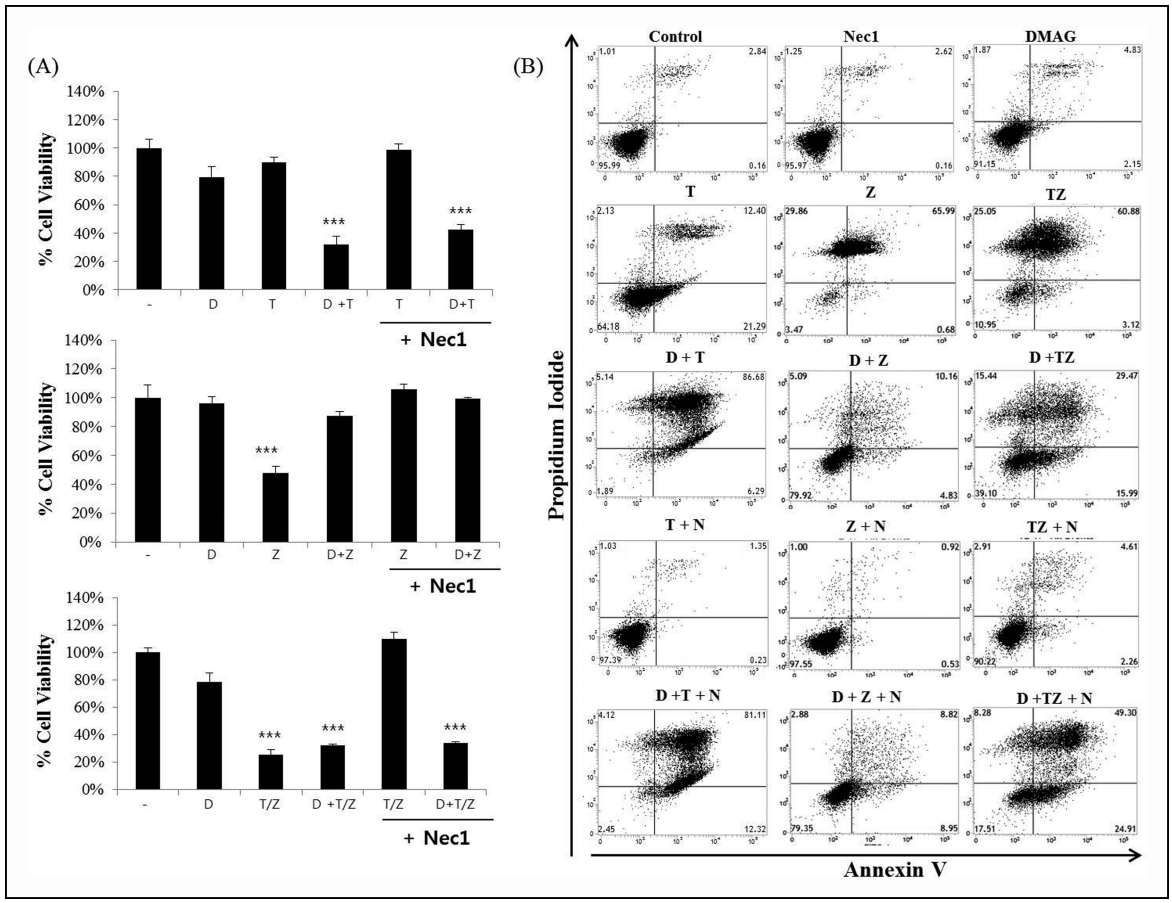

Fig. 1: Effects of DMAG on the cell death caused by death stimuli such as TNFα, zVAD and zVAD/TNFα. (A) L929 cells were pretreated with 1 μM DMAG or vehicle for 15 h. Thereafter, cells were subjected to TNFα (upper panel), zVAD (middle panel) and TNFα/zVAD (lower panel) stimulation for 3 h, 24 h and 3 h, respectively. In addition, Nec-1 at a dose of 10 μM was investigated for its protective effects against necroptotic stimuli in DMAG- or vehicle-pretreated cells. Death stimuli-induced cell death was relatively compared with control group by measuring cell viability through MTS assay. (B) To delineate the cell death profiles, L929 cells were treated under the same conditions as indicated above. Then, harvested cells were labeled with AnxV and PI for subsequent flow cytometry analysis. The two-color fluorescent plots were arranged in the following order from top to bottom. Top: control group, 2nd row: T, Z or T/Z stimulation, 3rd row: DMAG pretreatment, and then T, Z or T/Z stimulation, 4th row: T, Z or T/Z stimulation along with Nec-1, bottom: DMAG pretreatment, and then T, Z or T/Z stimulation with or without Nec-1. N, D, T and Z stand for Nec-1, DMAG, TNFα and zVAD, respectively. *$p < 0.05$ against control; **$p < 0.01$ against control; ***$p < 0.005$ against control.

viability assays and flow cytometric analyses, DMAG sensitized the cytotoxic response to TNFα while it protected substantially cells from zVAD-induced cytotoxicity. Mechanistically, DMAG down-regulated RIP1 through HSP90 inactivation, which in turn shifted cell death modes by inducing caspase activity and delaying IκB degradation. In contrast, it appeared to protect cells from zVAD *via* degradation of RIP1, which is responsible for zVAD-mediated toxicity.

Here, we investigated the effects of DMAG on various necroptotic conditions to understand the molecular mechanisms by which DMAG redirects or protects death modes through regulation of necroptosis-associated proteins. We concluded that DMAG sensitized cells to TNFα stimulation while it protected cells from necrotic cell death caused by zVAD. Accordingly, the comprehensive understanding for pharmacological modulation of cell death will provide insights into clinical control of cell death-associated human diseases.

2. Investigations and results

2.1. Effects of DMAG on responses of cells to zVAD, TNF and zVAD/TNF stimuli

To comprehend the underlying mechanisms by which HSP90 activity could alter cell's responses to the external death stimuli, L929 cells were first treated with DMAG, an HSP90 inhibitor, and then stimulated with TNFα, zVAD or TNFα/zVAD (Fig. 1A). Cell viabilities after treatment with necroptosis inducers were determined by MTS assay. Short-term exposure of cells to TNFα for 3 h did not affect cell viability significantly while zVAD potentiated cytotoxic responses to TNFα by about 60% within only 3 h. Either zVAD or TNFα caused cell death substantially 24 h after treatment. Remarkably, pretreatment of DMAG sensitized cells to TNFα stimulation, lowering cell viability by 50% compared with vehicle alone. In contrast, it successfully protected cells from zVAD, recovering cell viability up to that of the non-stimulated control group. As can be expected, both zVAD- and TNFα/zVAD-mediated cytotoxicity were effectively protected by a RIP1 inhibitor Nec-1. Intriguingly, however, Nec-1 did not rescue DMAG-pretreated cells from TNFα- or TNFα/zVAD-induced cell death at all. To outline cell death modes in detail, flow cytometric analyses were conducted for cells stimulated under the same death contexts as in cell viability assay (Fig. 1B). When the cells were stimulated with TNFα or zVAD for 3 and 24 h, there were apparent increases in cell populations representing early apoptotic, late apoptotic and necrotic death in comparison with non-treated control group. TNFα/zVAD induced more extensively cell death in a short time than did TNFα alone, escalating double-positive staining ($AnxV^+/PI^+$) populations up to around 90 %. As the cells were exposed to TNFα in the presence of DMAG, the accumulation of late apoptotic or necrotic cells was increased more remarkably from 12% to 75% than that in the absence of DMAG. In contrast, DMAG significantly

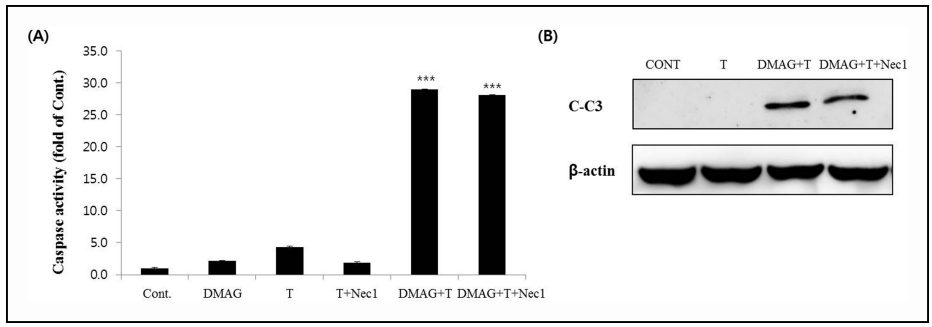

Fig. 2: Measurement of apoptotic caspase 3/7 activity and probing of active caspase 3 in DMAG-pretreated cells that were subjected to TNFα stimulation. (A) L929 cells were pretreated with DMAG for 15 h and then were stimulated with TNFα (10 ng/ml) for 6 h. Apoptotic caspase 3/7 activities from lysed cell were determined by using Triplex assay and then expressed as fold changes relative to control group. (B) To verify caspase 3 activation, vehicle- or DMAG-pretreated cells were stimulated with TNFα for 1 h, and then protein lysates were resolved on SDS-PAGE to have subsequent blots probed with an antibody recognizing specifically a cleaved form of C-3. *p < 0.05 against control;**p < 0.01 against control; ***$P < 0.005$ against control.

protected the cells from zVAD-mediated cytotoxicity, reducing upper right quadrant populations ($AnxV^+/PI^+$) from 65% to 10%. Also, as with TNFα/zVAD, there were few cells alive when cells were stimulated with TNFα/zVAD following DMAG treatment although flow cytometric profiles of AnxV/PI were different from each other. In fact, relative proportions of early apoptotic, late apoptotic/necrotic and late necrotic populations amounted to 5, 91 and 2% in cells stimulated with TNFα/zVAD. When DMAG-pretreated cells were stimulated with TNFα/zVAD, there was an increase (25%) in early apoptotic quadrant with reciprocal reduction (65%) of the upper right quadrant (late apoptotic/necrotic population). Consistent with MTS data, application of Nec-1 rescued effectively cells from undergoing necrosis by TNFα, zVAD or TNFα /zVAD. On the contrary, there were no dramatic protective effects of Nec-1 on TNFα- or TNFα/zVAD-mediated cytotoxicity in DMAG-pretreated cells.

2.2. Induction of caspase activity when L929 cells were stimulated with TNFα in the presence of DMAG

To better study switching of TNFα-driven necroptosis into apoptosis in DMAG-treated L929, Triplex assay designed to assess apoptosis and necrosis was employed. It is shown in Fig. 2A that TNFα itself did not noticeably induce caspase activity of L929 cells. However, pretreatment of cells with DMAG elevated caspase activity 30-fold compared with the vehicle-pretreated group upon TNFα stimulation. Consistent with the results shown in the viability assay, Nec-1 did not suppress the increased caspase activity in cells treated with DMAG plus TNFα. To specify apoptosis-associated caspase at a molecular level, caspase 3 (C-3) was monitored for its cleavage by immunoblotting with an specific antibody against it (Fig. 2B). The active C-3 form was not observed in both non-stimulated and TNFα-stimulated group. DMAG treatment facilitated the cleavage of C-3 in response to TNFα, triggering apoptosis, but its C-3 activation was not affect by even Nec-1 treatment, which can block necroptosis effectively.

2.3. Effects of DMAG on downstream signaling molecules (RIP1 and RIP3) following TNFα or TNFα/zVAD stimulation

To gain some clues as to how DMAG may be involved in switching of TNFα-mediated cell death from necroptosis to apoptosis, kinetics of RIP1/3 degradation and IκB degradation/phosphorylation were primarily investigated when cells were exposed to death stimuli with or without DMAG (Fig. 3). DMAG pretreatment induced markedly expression of HSP70 in L929 cells whereas it down-regulated not only RIP3, but also

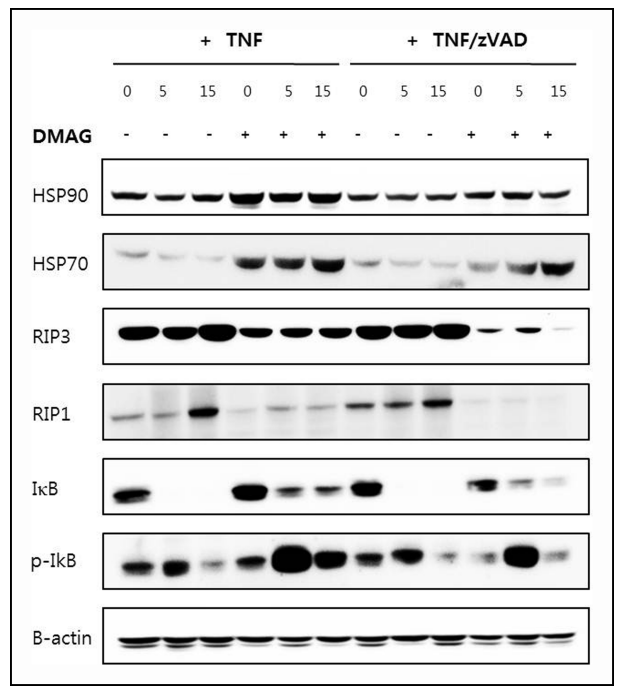

Fig. 3: . Effects of DMAG on downstream signaling molecules triggered from TNFα or TNFα/zVAD stimulation. To investigate effects of HSP90 inhibition on its downstream molecules after TNFα or TNFα/zVAD stimulation, L929 cells were pretreated with DMAG for 15 h, and then stimulated with TNFα or TNFα/zVAD for the indicated times. The extracted total proteins were then subjected to SDS-PAGE, followed by western blotting with the specific antibodies against RIPs or IκB. HSP70 upregulation was presented as a signature of HSP90 inactivation.

RIP1. Largely, down-regulating effects of DMAG on RIP proteins were more manifested in TNFα/zVAD-treated cells than in cells left treated with TNFα alone. Furthermore, IκB phosphorylation and degradation were investigated with times in response to TNFα and TNFα/zVAD since RIP1 plays a key role in NFκB activation as well as cell death. As cells were stimulated with TNFα or TNFα/zVAD, IκB was rapidly degraded within 5 min, accompanied by the immediate increase of phosphor-IκB levels. In contrast, when DMAG-treated cells were stimulated with TNFα, IκB degradation was delayed up to 15 min, and IκB was highly phosphorylated 5 min after TNFα stimulation.

3. Discussion

It has been well documented that both zVAD and TNFα induce programmed necrosis in L929 cells, in which caspases are not activated in response to TNFα (Thon et al. 2005; Vanlangenakker et al. 2011). To examine the modulatory effects of HSP90

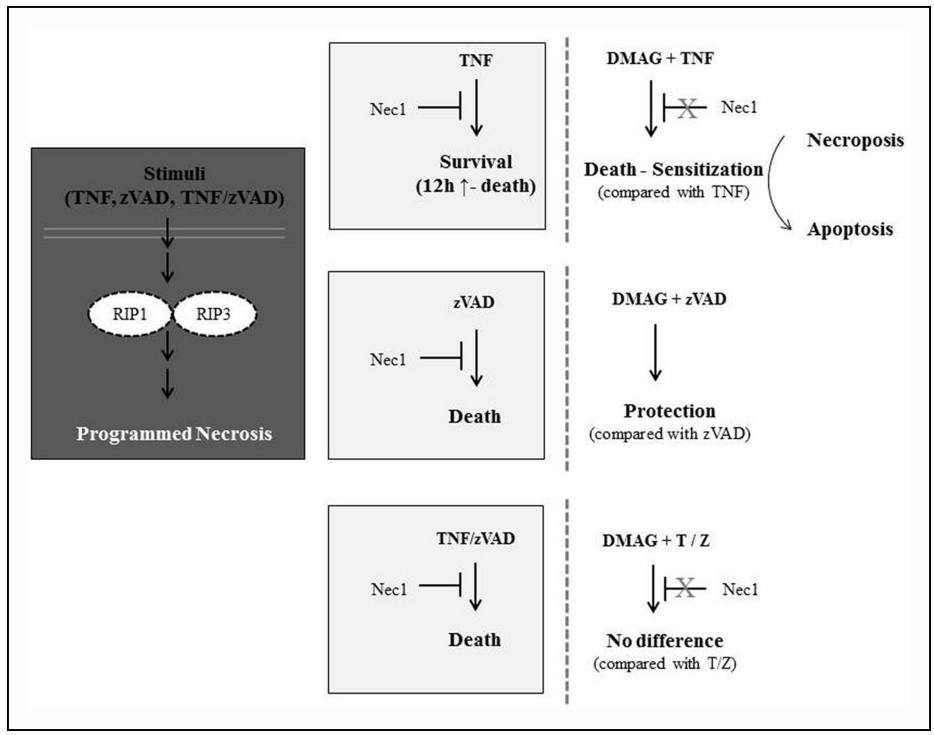

Fig. 4: The schematic summary demonstrating the way how DMAG exerts differential effects on necroptotic stimuli. TNFα, zVAD and zVAD/TNFα caused cell death of L929 in a necroptotic manner. The treatment of cells with Nec-1, a RIP1 inhibitor, could effectively suppress zVAD/TNFα-mediated cytotoxicity as well as zVAD and TNFα alone. Pretreatment of cells with DMAG accelerated not only TNFα-mediated cell death as rapidly as can be completed within 3 h, but also switched TNFα-driven cell death modes from necroptosis to apoptosis, which would not be protected by Nec-1. zVAD also exerted necrotic cell death *via* a route distinct from TNFα, but its cytotoxic activity was successfully reduced by DMAG pretreatment. Moreover, a combined treatment of cells with zVAD/TNFα caused cell death much more radically than that of zVAD or TNFα alone. As observed in cells stimulated with TNFα following DMAG, DMAG treatment diverted TNFα/zVAD-executed necroptosis to apoptosis, consequently nullifying the protective effect of Nec-1.

on necroptotic stimuli, we primarily carried out cell viability assays and FACS analyses for DMAG-pretreated cells that were subjected to necroptotic death stimuli. FACS data revealed that exposure of cells to zVAD for 24 h caused most populations to appear in a double positive quadrant of AnxV/PI staining. In addition, zVAD potentiated cytotoxic response of cells to TNFα, killing more than 90% of cells acquired for analyses. zVAD has already been proposed to augment TNFα-induced cell death through the inhibition of caspases and unidentified another pathways although its underlying mechanism remains elusive (Huang et al. 2005; Wu et al. 2011). Some populations of cells stimulated with TNFα for a short time (3 h) were positively stained with AnxV alone, a labeling marker of apoptosis. Caspase-independent primary necrotic cells can unexpectedly exhibit AnxV-positive/PI-negative staining before proceeding into PI-positive population (Sawai and Domae 2011). Therefore, it is required to use Nec-1 in order to discriminate between primary necrotic and apoptotic AnxV-positive/PI-negative cells. In this study, Nec-1 rescued completely TNFα-induced cell toxicity, implying that AnxV-positive cells undergo necroptosis but not apoptosis. As demonstrated in a previous study, zVAD and TNFα induced necrotic cell death *via* RIP1 and RIP3, respectively, in L929 cells (Park et al. 2014). In fact, RIP1-knockdown cells are readily susceptible to TNFα treatment, but substantially protect cells from zVAD exposure. Moreover, it has been highlighted that RIP1 knockdown overrides the protective effects of RIP3 silencing on TNFα-mediated cytotoxicity. Here, we showed that treatment of HSP90 inhibitor DMAG could degrade RIP1 and RIP3 proteins considerably. Treatment of cells with DMAG destabilized readily RIP1 to remain at a low level within 15 h. It is not surprising that RIP1 is subject to degradation upon DMAG treatment, since RIP1 is one of various client proteins of HSP90. Strikingly, cellular RIP3 was also downregulated by DMAG pretreatment. It could not be ruled out that RIP3, like RIP1, might be a new client protein of HSP90, although there have been no reports for such perception yet. As described above, both RIP1 and RIP3 are key regulators of programmed necrosis that are triggered following TNFα-TNFR binding (Cho et al. 2009; He et al. 2009; Zhang et al. 2009). Since the identification of RIP1 and RIP3, a few proteins have been so far revealed as signaling molecules of programmed necrosis through RNA interferences screening and a wide array of genomic analysis (Hitomi et al. 2008). When it comes to signaling pathways leading to programmed necrosis, it has been established that RIP1-RIP3 necrotic complex functions as a core to recruit a mixed lineage kinase domain-like (MLKL) and a mitochondrial phosphatase phosphoglycerate mutase family member 5 (PGAM5), which in turn leads to mitochondrial malfunction and necrotic cell death (Kanamaru et al. 2012; Sun et al. 2012; Wang et al. 2012). In addition, the deacetylase activity of sirtuin 2 (SIRT2) has been proposed to regulate RIP1-RIP3 necrotic complex formation and its resulting programmed necrotic cell death (Narayan et al. 2012). Therefore, we postulated that alterations in cellular RIP1 and RIP3 levels showed differential responses to TNFα, zVAD and TNFα/zVAD. It has been already acknowledged that TNFα-mediated cell death is potentiated by RIP1 silencing. More clearly, RIP1 depletion in L929 cells redirects from TNFR-associated death domain (TRADD)-independent necroptosis to TRADD-dependent apoptosis in response to TNFα stimulation (Vanlangenakker et al. 2011). This notion was corroborated by our present results that DMAG-downregulated RIP1 was closely correlated with altered responses to TNFα, demonstrating TNFα-induced caspase activity and Nec-1's failure to lessen cytotoxicity of TNFα in DMAG-pretreated cells. Cellular caspase-3 zymogen was cleaved into an active form by DMAG pretreatment with subsequent TNFα stimulation, prompting that it executes apoptotic cell death through activation of other caspases as well as rele-

vant targets. Interestingly, DMAG showed a protective effect on zVAD-induced cytotoxicity as well. This could be partly explained by RIP1 down-regulation, as deduced by our previous report that RIP1 silencing but not RIP3 protects cells from zVAD (Park et al. 2014). This notion is further strengthened by the finding that the protective effect of GA against oxygen-glucose deprivation/zVAD-induced neuronal injury is associated with RIP1 protein instability (Chen et al. 2012). Besides RIP1, possibly, other client proteins of HSP90 might be involved in the modulation of cell death caused by TNFα or zVAD. It has been reported that another RIP family protein RIP3 conducts a crucial role for TNFα-mediated necroptosis by forming a complex with RIP1 (Vanlangenakker et al. 2011; Zhang et al. 2011). Thus, it was at first predicted that DMAG would alleviate cytotoxic responses of cells to TNFα since it could keep RIP3 at low levels. However, DMAG rather directed cells' response to TNFα toward apoptotic cell death but not survival. It seemed likely that the sensitizing effect of RIP1 down-regulation could surpass the protective effect of lowered RIP3 levels on TNFα. Remarkably, cells that express both RIP1 and RIP3 at low levels through RNA interferences show different responses to TNFα or TNFα/zVAD stimulation from those pretreated with DMAG (Park et al. 2014). Both RIP1 and RIP3 knockdown can successfully protect cells from TNFα and TNFα/zVAD, as well as zVAD, thereby inferring that a variety of proteins regulated by DMAG may intricately affect cell death machinery in the context of TNFα signaling. Conclusively, DMAG destabilized RIP3 proteins as well as RIP1, an HSP90 client protein. We revealed that fluctuation in RIP3 and RIP1 levels had differential effects on necrotic cell death stimuli such as zVAD, TNFα and zVAD plus TNFα. Consequently, our study can be recapitulated as the following scheme from the results achieved so far (Fig. 4). Based on our findings that RIP1 and RIP3 were, respectively, required for zVAD- and TNFα-mediated cell death, and that DMAG could degrade both RIP1 and RIP3, the modulating effects of DMAG on death stimuli delineated. Kinetic responses of cells to TNFα alone indicated that necrotic cell death was initiated 12 h after treatment (data not shown). However, treatment of cells with DMAG not only accelerates TNFα-caused cell death, but also directs TNFα-driven necroptosis into apoptosis, which is not be rescued by Nec-1. Another necroptotic agent zVAD also exerts necrotic cell death *via* a route distinct from TNFα, but its cytotoxic potency is successfully diminished by DMAG pretreatment. Moreover, a combined treatment of cells with zVAD and TNFα causes cell death much more radically than that of zVAD or TNFα alone, and furthermore, zVAD/TNFα-mediated cytotoxicity can be effectively controlled by Nec-1, as with antagonizing effects of Nec-1 on zVAD or TNFα exposure. Similar to reprogramming effects of DMAG on TNFα-mediated cell death, DMAG switches TNFα/zVAD-induced necroptosis into apoptosis so that Nec-1 fails to protect TNFα toxicity any more.

Taken together, the molecular understanding of mechanisms by which cell demise can be elaborately regulated will provide insights into harnessing cell death/survival so as to be suited for therapeutic uses such as cancer fighting and treatment of ischemic cell death.

4. Experimental

4.1. Cell line and cell culture

L929 cells (ATCC CCL-1) were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were grown in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (FBS), and allowed to incubate at 37 °C in a 5% CO_2 incubator. Cells were maintained under optimal conditions by trypsin digestion and subculture of cells at a low density.

4.2. Reagents

Mouse TNFα was obtained from eBioscience (San Diego, CA, USA). The cell proliferation assay kit (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium, MTS reagent) and ApoTox-Glo^{TM} Triplex assay kit were bought from Promega (Madison, WI, USA). zVAD and propidium iodide (PI) were purchased from Sigma (St. Louis, MO, USA). DMAG was kindly provided by the Ulsan National Institute of Science and Technology (UNIST, Ulsan, South Korea). Anti-RIP1 and anti-RIP3 were from BD Pharmingen (San Diego, CA, USA) and ProSci (Poway, CA, USA), respectively. The antibody that can specifically recognize a cleaved form of caspase-3 was from Cell Signaling Technology (Danvers, MA). FITC-annexin V (AnxV) detection kit was bought from BD Biosciences (Franklin Lakes, NJ, USA). Other chemical reagents used were of analytical grade.

4.3. Cell viability

L929 cells grown on the 96-well plate were primarily stimulated with zVAD or TNFα or TNFα /zVAD. In brief, trypsinized cells were plated in 96-well plates at a density of 1×10^4 cells per well. On the following day, cells were subjected to either TNFα (10 ng/ml), zVAD (10 μM) or TNFα (10 ng/ml) plus zVAD (10 μM) stimulation and allowed to incubate for 3 h, 24 h or 3 h, respectively. To see the effects of DMAG on necroptotic stimuli, cells were pretreated with 1 μM DMAG for 15 h, and then exposed to death stimuli described above. Also, Nec-1 at a dose of 10 μM was tested to address whether it could affect responses of vehicle- or DMAG-pretreated cells to death stimuli. To determine cell viability, MTS reagents were directly added to the culture medium. The plates were further incubated for color development at 37 °C, and then read at a wavelength of 490 nm with the plate reader.

4.4. Flow cytometry

To delineate cell death profiles that were caused by TNFα, zVAD and TNFα/zVAD, cells stained with annexinV (AnxV)/propidium iodide (PI) were analyzed by flow cytometry. L929 cells were seeded into each well of 12-well plates at a density of 2×10^5 per well. On the following day, vehicle- or DMAG-pretreated cells were subjected to death stimuli with or without Nec-1 under the same conditions as indicated in viability assay. After stimulation for the indicated times, the resulting trypsinized cells were transferred to new tube, and stained with AnxV/PI according to the manufacturer's protocol. The staining profiles of cell populations were analyzed with FACS machine (BD FACSVersa).

4.5. Determination of cellular caspase (3/7) activities and detection of caspase 3 (C-3) activation

L929 cells, which were pretreated with vehicle or 1 μM DMAG, were stimulated with TNFα for 6 h. The apoptotic caspase 3/7 activities in cell lysates were measured by Triplex assay. For further detection of an active caspase 3 resulting from caspase 3 cleavage, proteins from cell lysates were resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and its transferred blot was probed with an antibody against the active caspase 3 (cleaved form). The specific protein was visualized by incubation of the membrane with a horseradish peroxidase (HRP)-conjugated secondary antibody and development of chemiluminescent substrates.

4.6. Immunoblotting analysis

Cell lysates were resolved on SDS-PAGE, and then proteins of interest were detected on the transferred membrane as a single band using specific antibodies. In brief, total proteins in lysates were subjected to SDS-PAGE and subsequently transferred to nitrocellulose membranes. The transferred membranes were blocked with 5% skim milk and then probed with the indicated antibodies (1:1,000) and their matching horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5,000). The specific bands were visualized using a chemiluminescence detection kit according to the manufacturer's protocol (GE healthcare).

4.7. Statistics

The results obtained from each experiment were expressed as mean ± standard deviation from at least three independent experiments. The significance was set at $p < 0.05$ for each analysis using student's t-test.

Acknowledgements: This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2013R1A1A2010212 to YS Cho) and Golden Seed Project (2130051 to JI Chae), Ministry of Agriculture, Food and Rural Affairs. Also, we appreciated Dr. ByoungHeon Kang

(UNIST, Ulsan, South Korea) for his kind donation of DMAG and helpful comments on this manuscript.

References

Chen WW, Yu H, Fan HB, Zhang CC, Zhang M, Zhang C, Cheng Y, Kong J, Liu CF, Geng D, Xu X (2012) RIP1 mediates the protection of geldanamycin on neuronal injury induced by oxygen-glucose deprivation combined with zVAD in primary cortical neurons. J Neurochem 120: 70–77.

Cho YS, Challa S, Moquin D, Genga R, Ray TD, Guildford M, Chan FK (2009) Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. Cell 137: 1112–1123.

Cho YS, Park SY, Shin HS, Chan FK (2010) Physiological consequences of programmed necrosis, an alternative form of cell demise. Mol Cells 29: 327–332.

Declercq W, Vanden Berghe T, Vandenabeele P (2009) RIP kinases at the crossroads of cell death and survival. Cell 138: 229–232.

Dorn GW, 2nd (2013) Molecular mechanisms that differentiate apoptosis from programmed necrosis. Toxicol Pathol 41: 227–234.

Goetz MP, Toft DO, Ames MM, Erlichman C (2003) The Hsp90 chaperone complex as a novel target for cancer therapy. Ann Oncol 14: 1169–1176.

Grenert JP, Sullivan WP, Fadden P, Haystead TA, Clark J, Mimnaugh E, Krutzsch H, Ochel HJ, Schulte TW, Sausville E, Neckers LM, Toft DO (1997) The amino-terminal domain of heat shock protein 90 (hsp90) that binds geldanamycin is an ATP/ADP switch domain that regulates hsp90 conformation. J Biol Chem 272: 23843–23850.

He S, Wang L, Miao L, Wang T, Du F, Zhao L, Wang X (2009) Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-alpha. Cell 137: 1100–1111.

Hitomi J, Christofferson DE, Ng A, Yao J, Degterev A, Xavier RJ, Yuan J (2008) Identification of a molecular signaling network that regulates a cellular necrotic cell death pathway. Cell 135: 1311–1323.

Huang J, Wu L, Tashiro S, Onodera S, Ikejima T (2005) The augmentation of TNFalpha-induced cell death in murine L929 fibrosarcoma by the pan-caspase inhibitor Z-VAD-fmk through pre-mitochondrial and MAPK-dependent pathways. Acta Med Okayama 59: 253–260.

Kanamaru Y, Sekine S, Ichijo H, Takeda K (2012) The phosphorylation-dependent regulation of mitochondrial proteins in stress responses. J Signal Transduct 2012: 931215.

Miyata Y (2003) Molecular chaperone HSP90 as a novel target for cancer chemotherapy. Nihon Yakurigaku Zasshi 121: 33–42.

Miyata Y (2005) Hsp90 inhibitor geldanamycin and its derivatives as novel cancer chemotherapeutic agents. Curr Pharm Des 11: 1131–1138.

Moquin DM, McQuade T, Chan FK (2013) CYLD deubiquitinates RIP1 in the TNFalpha-induced necrosome to facilitate kinase activation and programmed necrosis. PLoS One 8: e76841.

Narayan N, Lee IH, Borenstein R, Sun J, Wong R, Tong G, Fergusson MM, Liu J, Rovira, II, Cheng HL, Wang G, Gucek M, Lombard D, Alt FW, Sack MN, Murphy E, Cao L, Finkel T (2012) The NAD-dependent deacetylase SIRT2 is required for programmed necrosis. Nature 492: 199–204.

Neckers L, Ivy SP (2003) Heat shock protein 90. Curr Opin Oncol 15: 419–424.

Neckers L, Kern A, Tsutsumi S (2007) Hsp90 inhibitors disrupt mitochondrial homeostasis in cancer cells. Chem Biol 14: 1204–1206.

Park SY, Shim JH, Cho YS (2014) Distinctive roles of receptor-interacting protein kinases 1 and 3 in caspase-independent cell death of L929. Cell Biochem Funct 32: 62–69.

Pearl LH, Prodromou C (2006) Structure and mechanism of the Hsp90 molecular chaperone machinery. Annu Rev Biochem 75: 271–294.

Pirkkala L, Nykanen P, Sistonen L (2001) Roles of the heat shock transcription factors in regulation of the heat shock response and beyond. Faseb J 15: 1118–1131.

Sawai H, Domae N (2011) Discrimination between primary necrosis and apoptosis by necrostatin-1 in Annexin V-positive/propidium iodide-negative cells. Biochem Biophys Res Commun 411: 569–573.

Sun L, Wang H, Wang Z, He S, Chen S, Liao D, Wang L, Yan J, Liu W, Lei X, Wang X (2012) Mixed lineage kinase domain-like protein mediates necrosis signaling downstream of RIP3 kinase. Cell 148: 213–227.

Tafani M, Schneider TG, Pastorino JG, Farber JL (2000) Cytochrome c-dependent activation of caspase-3 by tumor necrosis factor requires induction of the mitochondrial permeability transition. Am J Pathol 156: 2111–2121.

Thon L, Mohlig H, Mathieu S, Lange A, Bulanova E, Winoto-Morbach S, Schutze S, Bulfone-Paus S, Adam D (2005) Ceramide mediates caspase-independent programmed cell death. Faseb J 19: 1945–1956.

Vanlangenakker N, Bertrand MJ, Bogaert P, Vandenabeele P, Vanden Berghe T (2011) TNF-induced necroptosis in L929 cells is tightly regulated by multiple TNFR1 complex I and II members. Cell Death Dis 2: e230.

Vercammen D, Beyaert R, Denecker G, Goossens V, Van Loo G, Declercq W, Grooten J, Fiers W, Vandenabeele P (1998) Inhibition of caspases increases the sensitivity of L929 cells to necrosis mediated by tumor necrosis factor. J Exp Med 187: 1477–1485.

Wang Z, Jiang H, Chen S, Du F, Wang X (2012) The mitochondrial phosphatase PGAM5 functions at the convergence point of multiple necrotic death pathways. Cell 148: 228–243.

Wu YT, Tan HL, Huang Q, Sun XJ, Zhu X, Shen HM (2011) zVAD-induced necroptosis in L929 cells depends on autocrine production of TNFalpha mediated by the PKC-MAPKs-AP-1 pathway. Cell Death Differ 18: 26–37.

Zhang DW, Shao J, Lin J, Zhang N, Lu BJ, Lin SC, Dong MQ, Han J (2009) RIP3, an energy metabolism regulator that switches TNF-induced cell death from apoptosis to necrosis. Science 325: 332–336.

Zhang DW, Zheng M, Zhao J, Li YY, Huang Z, Li Z, Han J (2011) Multiple death pathways in TNF-treated fibroblasts: RIP3- and RIP1-dependent and independent routes. Cell Res 21: 368–371.

Zhang T, Hamza A, Cao X, Wang B, Yu S, Zhan CG, Sun D (2008) A novel Hsp90 inhibitor to disrupt Hsp90/Cdc37 complex against pancreatic cancer cells. Mol Cancer Ther 7: 162–170.

Zhou Z, Han V, Han J (2012) New components of the necroptotic pathway. Protein Cell 3: 811–817.