Effect of RNS on TNFα induced cell death In L929 cells

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ABSTRACT
Reactive nitrogen species (RNS) refer to a group of nitrogen-centered free radicals derived from nitric oxide (NO) capable of exerting nitrosative stress on cellular molecules. In this study, we made an effort to examine the effect of RNS on tumor necrosis factor α (TNFα) induced cell death in L929 cells. First, TNFα-induced apoptosis and necrosis were distinguished based on morphological changes and biochemical markers. Secondly, effect of RNS donor sodium nitroprusside (SNP) on TNFα induced apoptotic and necrotic cell death was measured respectively, which shows that RNS selectively enhance necrotic cell death in L929 cells. Finally, a higher level of C-Jun N-terminal kinases (JNK) activation following exogenous RNS treatment at later stages in necrotic cell death is consistent with previous studies which showed that sustained JNK activation promoted both types of cell death. Data from this study provides some experimental evidence for understanding the complex relationship between RNS and cell death receptor-mediated cell death process.

INTRODUCTION
TNFR1 in the tumor necrosis factor receptor (TNFR) super-family is one of the best understood receptors which are able to induce either apoptotic or non-apoptotic/necrotic cell death upon binding of TNFα. It has been well established that TNFα is able to enhance iNOS expression and thus NO production through the NF-κB pathway. However, the functional impact of such enhancement on TNFα-induced cell death is still largely unknown. In this study, we have made an effort to identify the effect of RNS on apoptosis and necrosis induced in L929 murine fibrosarcoma cell lines with the following objectives: 1) To show TNFα-induced apoptosis and necrosis in L929 cells. 2) To examine the effect of RNS on the two modes of cell death mediated by TNF receptor. 3) To investigate the molecular mechanisms underlying the effect of RNS on the two types of cell death by focusing on C-Jun N-terminal kinases (JNK), whose activation is considered to be an important cellular response to RNS exposure.

MATERIALS AND METHODS
Detection of cell death through Sytox-Hoechst staining method was used for better identification of apoptotic and necrotic cells. Confocal microscopy was used to analyze the cellular locations of FITC-labeled anti-HMGB-1 antibodies. The integrity of the plasma membrane was assessed by determining the ability of cells to exclude propidium iodide (PI) using flow cytometry. Western blotting was used to examine the cellular protein JNK.

RESULTS
1. TNFα induces both apoptosis and necrosis in L929 cells.

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Figure 1 TNFα induce both apoptosis and necrosis in L929 cells. (a) Morphological features of typical apoptosis and necrosis in L929 cells. L929 cells were treated with CHX (10ng/ml) + TNFα (10ng/ml) or zVAD (50uM) + TNFα (10ng/ml) for 3h. Representative images were taken by a phase-contrast microscope. (b) TNFα induce cell death evaluated using Sytox-Hoechst-staining. L929 cells were treated with CHX (10ng/ml) + TNFα (10ng/ml) or zVAD (50uM) + TNFα (10ng/ml) or 3h.

Figure 2 HMGB-1 release occurs during necrosis but not apoptosis. (a) L929 cells were treated with CHX (10ng/ml) + TNFα (10ng/ml) for 3h. Cellular location of nuclear protein HMGB-1 and PI following typical apoptosis and necrosis were observed and recorded. Representative cells were selected and photographed, transmission photos were aimed to show the morphology of the cells. (b) L929 cells were treated with zVAD (50uM) + TNFα (10ng/ml) for 3h. Cellular location of HMGB-1 and PI were examined and recorded.

2. SNP sensitizes TNFα induced both apoptotic and necrotic cell death but with a more significant impact on necrosis.
Figure 3 (a) Flow cytometry analysis of effect of SNP on apoptotic and necrotic cell death. L929 cells were treated with zVAD (50uM) + TNFα (10ng/ml) + SNP (500uM) for 3h (ii) or CHX (10ng/ml) + TNFα (10ng/ml) + SNP (500uM) for 3h (iii). L929 cells treated with H2O2 100uM for 1h was used as a necrotic control (i). FSC scatter was used to examine the cell size, while PI fluorescence intensity was adopted to test membrane integrity. The quantification analysis of the result was shown in (iv) and (v).

3. SNP prolongs JNK activation mainly in TNFα induced cell death.

a.

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<th>Western Blot–L929 Total cell lysis</th>
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Figure 4 SNP prolongs JNK activation in both types of TNFα induced cell death. (a) After treated with CHX (10ng/ml) + TNFα (10ng/ml) + SNP (500uM) or zVAD (50uM) + TNFα (10ng/ml) + SNP (500uM) for the designated time, L929 cells were collected and lysed in M2 buffer and the proteins were subjected to immunoblotting using phosphor-JNK antibodies. zVAD (50uM) and SNP (500uM) alone were used as individual controls and were treated for 3h. Tubulin level was examined as a loading control.

DISCUSSION
1. The differential effect of RNS on TNFα induced apoptotic cell death observed in other cell lines
RNS are known to promote apoptotic cell death in many types of cells, such as macrophages,
thymocytes and tumor cells. Currently, the pro-apoptotic role of RNS was believed to be executed through their potent inhibitory effect on NF-κB activation, the main anti-apoptotic pathway elicited by the activation of the TNF receptor. On the other hand, there is equally strong evidence for the anti-apoptotic function of RNS-mediated protein nitrosylation related to TNFα-induced apoptosis. For example, increased iNOS expression and increased RNS production in cultured hepatocytes following TNFα treatment has been shown to inhibit cell death.

2. **JNK may be a key mediator in the sensitization effect of RNS on TNFα induced both types of cell death**

It is widely accepted that high levels of oxidative stress in the form of both ROS and RNS are contributing factors to cell death processes induced by TNFα. Compared to the understanding of the involvement of ROS on TNFα induced cell death pathway, the signaling pathway mediating RNS-induced JNK activation is relatively less studied at present. Ongoing studies in our lab shows that RNS may elicit its effect on cell death pathway through nitrosylation of components of TNFR1 complex I including TNFR1, TRADD, RIP and TRAF2, which further contribute to the activation of JNK pathway. Thus, there is no doubt that JNK activation plays a critical role in apoptotic cell death mediated by either exogenously administered or endogenously produced RNS.

3. **Further studies possible to enhance our understanding of RNS signaling pathway in TNFα induced cell death**

It is known that NF-kB can down-regulate JNK activation. And this transient JNK activation may help to promote cell survival mediated by NF-kB transcribed genes. Therefore, we can assess involvement of the NF-κB signaling pathway in the cell death process induced by RNS and death receptors through by (a) luciferase assay for examining the transcriptional activity, (b) electrophoretic mobility shift assay (EMSA) for analyzing DNA binding activity, (c) *in vitro* IKK kinase assay for measuring the change of IKK kinase activity.

Moreover, the activation level of JNK can be observed at closely-spaced earlier time points. Different from sustained JNK activation, it is generally believed that transient activation of JNK at early stages mainly plays a protective role in cell death pathway

**REFERENCE**