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ERK1/2 activation attenuates TRAIL-induced apoptosis through the regulation of mitochondria-dependent pathway

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Abstract

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) functions as an extracellular signal, which triggers apoptosis in tumor cells. In order to characterize the molecular events involved in TRAIL cytotoxic signaling, we attempted to determine the role of extracellular signal-regulated kinase 1/2 (ERK1/2), as well as its downstream targets in TRAIL-treated HeLa cells. Here we demonstrate that TRAIL exposure resulted in the activation of ERK1/2, and the elevation of anti-apoptotic Bcl-2 protein levels. ERK1/2 inhibition with PD98059 promoted cell death via the down-regulation of Bcl-2 protein levels, together with increasing mitochondrial damage, including the collapse of mitochondrial membrane potential, the release of cytochrome *c* from mitochondria to cytoplasm and caspase activity. These results suggest that the ERK1/2 activation is a kind of survival mechanism to struggle against TRAIL-induced stress condition in early stage, via activating cellular defense mechanisms like as the up-regulation of the Bcl-2/Bax ratio, as well as several mitochondrial events.

Keywords: Tumor necrosis factor-related apoptosis-inducing ligand; Extracellular signal-regulated kinase 1/2; Bcl-2/Bax; Cytochrome *c*; Apoptosis

1. Introduction

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a member of the tumor necrosis factor (TNF) family. TRAIL has proven to be capable of inducing apoptosis in several cell lines (Knight et al., 2001; Matsuda et al., 2005). However, its physiological functions remain unknown. TRAIL is widely expressed in normal cells, and is highly homologous with FasL, another cytotoxic member of the TNF family (Knight et al., 2001; Matsuda et al., 2005). Several receptors in human have been determined to bind to TRAIL. These include the death receptors DR4 (TRAIL-R1) and DR5 (TRAIL-R2), the decoy receptors DcR1 (TRAIL-R3) and DcR2 (TRAIL-R4), and osteoprotegerin (Kelley and Ashkenazi, 2004; Yagita et al., 2004). Although some efforts have been made to elucidate the molecular mechanisms underlying TRAIL signaling, the components of the different TRAIL signaling pathways remain largely undefined.

Mitogen-activated protein (MAP) kinase signal transduction pathways in mammalian cells include extracellular signal-regulated kinase1/2 (ERK1/2), c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), and p38 MAP kinase (Schaeffer and Weber, 1999; Chang and Karin, 2001). ERK1/2 is associated principally with

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proliferation and growth factors (Tsukada et al., 2001), while JNK and p38 MAP kinase are induced by stress responses and cytokines, and are able to mediate differentiation and cell death (Nagata and Todokoro, 1999). ERK1/2 has been confidently implicated in the regulation of a variety of cellular processes. However, the precise molecular mechanism of ERK1/2 has still remained controversial. For example, ERK1/2 plays a prominent role in ultraviolet (UV)-evoked p53 phosphorylation (She et al., 2000) and 1-methyl-4-phenylpyridinium (MPP^+)-induced neurotoxicity (Gomez-Santos et al., 2002). By way of contrast, ERK 1/2 activation promotes cell survival in neuronal PC12 cells (She et al., 2000), and ERK1/2 stimulation protects HeLa cells from Fas, TNF, TRAIL receptor-induced apoptosis (Tran et al., 2001). Although ERK1/2 has been definitively demonstrated to be involved in various physiological events, less is understood with regard to its biological roles in TRAIL signaling.

Downstream targets of ERK1/2 include ribosomal S6 kinase (RSK) (Merienne et al., 2000), ETS domain transcription factor Elk-1 (Tsai et al., 2000), and the anti-apoptotic Bcl-2 protein family (Desire et al., 2000). The ERK1/2 signaling pathway regulates the expression of Bcl-2 and Bcl-X_L, and promotes the survival of human pancreatic tumor cells (Boucher et al., 2000). Furthermore, Bcl-2/Bcl-X_L overexpression in cells exerts a protective effect against a host of agents, including UV irradiation (Domen et al., 1998), cytotoxic drugs (Srivastava et al., 1999; Takahashi et al., 1999), and p53 (Park et al., 2001). In addition, a great deal of evidence suggests that a variety of apoptotic stimuli affect the formation of mitochondrial permeability transition pores (MPTP), and induce the release of pro-apoptotic molecules, such as cytochrome *c*, from the mitochondria (Jiang and Wang, 2000). When present in the cytoplasm, cytochrome *c* interacts with apoptotic activator factor-1 (Apaf-1) and caspase 9 (Purring-Koch and McLendon, 2000). This complex then, either directly or indirectly, induces apoptotic cell death via caspase 3 activation (Cain, 2003). Recent studies have also reported that Bcl-2 protein overexpression can perform an anti-apoptotic function, by inhibiting the release of cytochrome *c* during TRAIL-induced apoptosis (Sun et al., 2001). However, another study demonstrated that TRAIL-induced cytochrome *c* release is not regulated by the Bcl-2 protein (Keogh et al., 2000). Thus, the relationship between Bcl-2 protein elevated by ERK1/2 and mitochondrial events in TRAIL-induced apoptosis remains a matter of some controversy.

In this study, we have evaluated the possibility that ERK1/2 activation, Bcl-2 protein family, and mitochondrial events are all involved in TRAIL cytotoxic signaling. Therefore, we attempted to more clearly elucidate the relationship between these factors with regard to TRAIL-induced HeLa cell death. Our results suggest that ERK1/2 activation plays a protective role, as a cellular defense mechanism to survive, via the regulation of the Bcl-2/Bax ratio and several mitochondrial events during TRAIL-induced apoptosis.

2. Materials and methods

2.1. Cell culture

Human adenocarcinoma HeLa cells were cultured at 37 °C in Dulbecco's Modified Eagle's Medium (DMEM; Gibco BRL, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco BRL) in a humidified incubator at an atmosphere of 95% air, and 5% CO_2. The cells were transferred to low serum media (1% FBS), 2 h before treatment with recombinant human TRAIL (BIOMOL, Plymouth, PA, USA).

2.2. Cell viability assay

Cells were plated onto 96-well plates (Corning, NY, USA) at a density of 5×10^4 cells/well, in 100 μl of 10% FBS/DMEM without phenol red, then incubated for 24 h. Two hours prior to cell stimulation, the cell media was supplanted with 90 μl of 1% FBS/DMEM without phenol red. TRAIL (4 ng/ml) was added to the plates, which had been pretreated with either vehicle or PD98059 (Calbiochem, San Diego, CA, USA) 2 h prior to TRAIL treatment. Two hours prior to the end of the TRAIL treatment, 10 μl of 10% Triton X-100 was added, followed by the addition of 11 μl of 3-(4,5-dimethyl-thiozol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT; Sigma, St Louis, MO, USA) 10× solution (10 mg/ml). After 4 additional hours of incubation, all remaining media and MTT solution was suctioned off, and the cells and crystallized dyes were dissolved via the addition of 100 μl of 100% DMSO, and 20 min of shaking. Absorbance at 570 nm was measured with an ELISA Reader (Molecular Devices, Sunnyvale, CA, USA). Assay values obtained immediately prior to vehicle treatment were set as 100%, and complete inhibition of MTT reduction (0%) was defined as the value obtained following the addition of 10% Triton X-100.

2.3. Western blotting

Stimulated cells were washed with phosphate-buffered saline (PBS) and lysed in 300 μl of cold RIPA buffer (20 mM Tris–HCl, pH 7.5 containing 1% Triton X-100, 100 mM NaCl, 1 mM Na_3VO_4, 40 mM NaF, 5 mM EGTA, 0.2% SDS, 0.2 mM PMSF, and 100 μM Leupeptin). Cell lysates were then centrifuged for 10 min at 15,800*g*, and 4 °C. The supernatants were harvested and analyzed with regard to protein concentration, using a protein assay kit (Bio-Rad, CA, USA). For electrophoresis, 80 μg of proteins were dissolved in sample buffer (0.1 M Tris, pH 6.8 containing 5% β-mercaptoethanol, 15% glycerol, 3% SDS, and 0.1% bromophenol blue), boiled for 5 min, then separated on 10% SDS gel under reducing conditions. The separated proteins were then transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham Pharmacia Biotech, UK), using a semidry trans-blot system (Schleicher & Schuell, Germany). The blots were blocked for 1 h with