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Scythe cleavage during Fas (APO-1)-and staurosporine-mediated apoptosis

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ABSTRACT

Scythe is a nuclear protein that has been implicated in the apoptotic process in *Drosophila melanogaster*; however, its role in apoptosis of mammalian cells is not fully elucidated. Here we show that cleavage of Scythe by caspase-3 occurs after activation of both the extrinsic (i.e. Fas/APO-1-mediated) and the intrinsic (i.e. staurosporine-induced) apoptosis pathway. Moreover, this caspase-dependent cleavage correlates with Scythe translocation from the nucleus to the cytosol. We also show that cytosolic re-localization of Scythe is required for Fas/APO-1-triggered phosphatidylserine (PS) exposure, a signal for macrophage clearance of apoptotic cells. Our data suggest that Scythe cleavage may represent a marker for caspase-3 activation and implicate cytosolic re-localization of Scythe in the pathway of PS exposure.

1. Introduction

Apoptosis is a physiological process responsible for the removal of supernumerary, damaged or harmful cells. This process is critical for embryonic, development, tissue homeostasis and defense against pathogens [1]. Generally, it is induced through two major pathways, an extrinsic pathway and an intrinsic pathway [2]. The initiation of the extrinsic pathway of apoptosis is triggered via death receptors on the cell surface, while initiation of the intrinsic pathway is triggered through activation of mitochondria and release of pro-apoptotic factors. Hence, death signals induce mitochondrial membrane permeabilization and cytochrome c release and activation of the initiator caspase, caspase-9 [3]. Among the numerous proteins implicated in apoptosis is Scythe (also known as BAT3; HLA-B associated transcript; Bag 6). Scythe has been shown to interact with Reaper, a central regulator of developmental apoptosis in *Drosophila melanogaster*, and the interaction between Scythe and Reaper was shown to be required for Reaper-induced apoptosis in *Xenopus* egg extracts [4,5].

A previous study indicated that inactivation of Scythe in the mouse resulted in lethality associated with pronounced developmental defects in the lung, kidney, and brain [6]. Co-incident with organogenesis defects was widespread aberrant apoptosis and proliferation, and resistance of Scythe-deficient fibroblasts to apoptosis.

Scythe also possesses an amino acid sequence that can bind the ATPase domain of HSP70 family. This region of Scythe has been shown to interact with HSP70 suggesting that Scythe may function through regulation of the activity of apoptotic signaling molecules [7]. Further biochemical evidence that Scythe plays a role in apoptosis come from the identification of a caspase-3 cleaved Scythe C-terminal fragment, which shows pro-apoptotic activity after its translocation to the cytosol [8]. Interestingly, among cell stress inducing reagents such as ricin, cycloheximide, thapsigargin, tunicamycin, camptothecin, and etoposide, only ricin was found to cause the cleavage of Scythe and the cytosolic release of the cleaved fragment, suggesting that the interaction between Scythe and ricin might promote this proteolytic process [8]. In another study it was shown that cytosolic Scythe interacts with and regulates the stability and location of apoptosis-inducing factor (AIF) after apoptotic stimulation mediated by endoplasmic reticulum stress [9]. The aim of the present study was to determine whether Scythe cleavage is a common event during apoptosis. Our data show that cleavage of Scythe by caspase-3 occurs both after extrinsic and intrinsic activation of the apoptotic process and that this cleavage is required for Scythe re-localization to the cytosol since pre-treatment with the pan-caspase inhibitor Z-VAD-FMK blocked this translocation. Moreover, cytosolic Scythe seems to contribute to PS exposure since silencing of the expression of this protein results in a significant decrease of the levels of phosphatidylserine (PS) on the surface of the apoptotic cells. We propose that the nuclear cleavage of Scythe may

Abbreviations: AIF, apoptosis-inducing factor; HSP, heat shock protein; PS, phosphatidylserine; NLS, nuclear localization signal; Z-VAD-FMK, *N*-benzyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone

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serve as a marker for apoptosis. Furthermore, the cytosolic re-localization of Scythe appears to play an active role in the pathway leading to PS exposure in Fas-triggered cells.

2. Materials and methods

2.1. Reagents and cell lines

Agonistic anti-Fas monoclonal antibodies (clone CH-11) were purchased from Medical & Biological Laboratories, Ltd. (Nagoya, Japan). Staurosporine, the caspase substrate aspartate-glutamate-valine-aspartate-7-amino-4-methyl-coumarin (DEVD-AMC), and the pan-caspase inhibitor *N*-benzyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone (Z-VAD-FMK), were all purchased from Sigma (St Louis, MO, USA). The Jurkat human T leukemic cell line, the Raji human B lymphoma cell line, the U266 myeloma cell line and the HL-60 promyelocytic leukemia cell line were obtained from the European Collection of Cell Cultures (Salisbury, UK) and cultured in RPMI-1640 medium (Sigma) supplemented with 10–15% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin (Gibco, Paisley, Scotland).

2.2. Caspase-3-like activity

The measurement of DEVD-AMC cleavage was performed in a fluorometric assay as described previously [10]. The cleavage of the fluorogenic peptide substrate was monitored in a TECAN Infinite® 200 plate reader (Labsystems, Stockholm, Sweden) using 355 nm excitation and 460 nm emission wavelengths. Fluorescence units were converted to pmol of AMC cleavage per minute based on a standard curve of free AMC.

2.3. Phosphatidylserine exposure

PS exposure was quantified by dual staining with propidium iodide (PI) (Sigma) and Annexin V (Annexin-V-Fluos staining kit, Roche Diagnostics GmbH, Manheim, Germany) [11]. Cells were analyzed within 30 min after staining on a FACS Calibur (BD Biosciences, San Jose, CA, USA) operating with CellQuest software (BD Biosciences).

2.4. Western blotting

For protein detection, western blotting was performed according to standard procedures. The following primary antibodies were used: GAPDH (Ambion, Austin, TX, USA), Scythe (Abcam, Cambridge, United Kingdom) cleaved caspase-3 (AH Diagnostics, Stockholm, Sweden), PARP (Biomol International, L.P. Plymouth Meeting, PA, USA) and Histone H3 (Santa Cruz Biotechnology Santa Cruz, CA, USA). After washing, the membranes were incubated with a peroxidase-conjugated secondary antibody (Pierce, Rockford, IL, USA) and bound antibody was visualized by enhanced

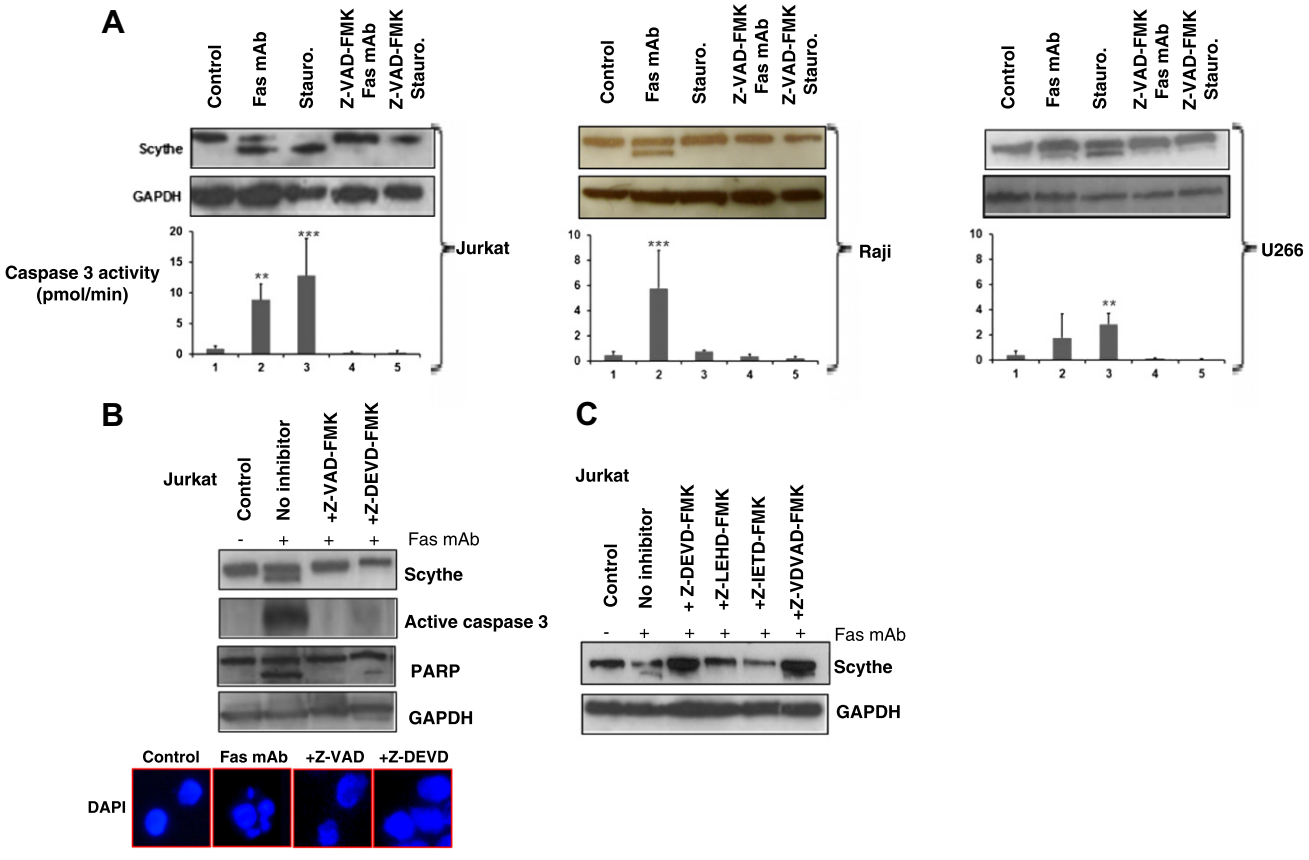

Fig. 1. Caspase-3-dependent cleavage of Scythe occurs after extrinsic and intrinsic activation of apoptosis. (A) Scythe is cleaved in a caspase-dependent manner in Jurkat, Raji and U266 cells. Cells were left untreated or treated with Fas mAb (250 ng/ml) or staurosporine (2 μM) in the presence or absence of the pan-caspase inhibitor Z-VAD-FMK (20 μM). After 3 h cells were collected and lysed for western blotting. GAPDH was used as loading control. The fluorogenic substrate DEVD-AMC was used for assessment of caspase-3-like enzyme activity. Data are presented as the mean ± S.D derived from two independent experiments performed in triplicate. $^{**}P < 0.01$ $^{***}P < 0.001$ (one-way ANOVA). (B) Cleavage of Scythe is mediated by caspase-3 and occurs in the presence of other signs of apoptosis. Jurkat cells were left untreated or treated with Fas mAb (250 ng/ml) in the presence or not of the pan-caspase inhibitor Z-VAD-FMK (20 μM) and the specific caspase-3 inhibitor Z-DEVD-FMK (20 μM). After 3 h cells were collected and lysed for Western blotting (Scythe, active caspase-3, cleaved PARP and GAPDH as loading control) and for immunofluorescence analysis (nuclei are stained in blue by DAPI). (C) Jurkat cells were left untreated or treated with Fas mAb in presence of specific inhibitors of caspase 9, 8, 3, and 2 and Scythe cleavage was analysed by western blotting. Membranes were re-probed for GAPDH as a control for equal loading of protein.