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Cell Biology International 29 (2005) 662–668

Cell Biology International

www.elsevier.com/locate/cellbi

Leupeptin-sensitive proteases involved in cell survival after X-ray irradiation in human RSa cells

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Received 16 March 2004; revised 28 February 2005; accepted 25 March 2005

Abstract

Proteases have received attention as important cellular components responsible for stress response in human cells. However, little is known about the role of proteases in the early steps of cell response after X-ray irradiation. In the present study, we first searched for proteases whose activity levels are changed soon after X-ray irradiation in human RSa cells with a high sensitivity to X-ray cell-killing. RSa cells showed an increased level of fibrinolytic protease activity within 10 min after irradiation with X-ray (up to 3 Gy). The induced protease activity was proved to be inhibited by leupeptin. We next examined whether this protease inducibility is related to the X-ray susceptibility of cells. Treatment of RSa cells with leupeptin prior to X-ray irradiation resulted in lowered colony survival and an increased ratio of G_2/M-arrested cells and apoptotic cells. These results suggest that leupeptin-sensitive proteases are involved in the resistance of human RSa cells to X-ray cell-killing.

Keywords: Human RSa cells; X-ray; Cell-killing; Protease; Leupeptin

1. Introduction

Proteolysis was originally characterized as nonspecific degradation of peptides associated with protein catabolism. However, it is becoming evident that proteolysis provides another important mechanism to control biological processes through the hydrolysis of highly specific peptide bonds (Barrett et al., 1998). We have found that an increase in protease activity promptly after UV (UVC, mainly 254 nm in wavelength) irradiation was associated with hypomutable changes in cultured human RSa cells (Isogai et al., 1998). Protease activity was specifically inhibited by antipain, one of the serine- and cysteine-type protease inhibitors (Isogai et al., 1998; Isogai and Suzuki, 1994). Treatment of cultured human cells with antipain promptly after UV irradiation resulted in an increase in the mutation frequency (Isogai et al., 1998; Isogai and Suzuki, 1994; Suzuki et al., 1997). We therefore suggested that an increase in antipain-sensitive protease activity by UV irradiation is involved in the suppression of cell mutability. Furthermore, we recently found that an increase in protease activity promptly after UV irradiation was associated with changes in the resistance of RSa cells (Sugita et al., 2001; Hiwasa et al., 2000). It is suggested that the increase in protease activity by UV irradiation is involved in the suppression of UV

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doi:10.1016/j.cellbi.2005.03.023

cell-killing. Thus, it is an intriguing method to search for protease activity in human cells under various stress conditions, such as UV and ionizing radiation exposure.

The human cell strain RSa is an embryonic fibroblast, which is transformed by infection with the Rous sarcoma virus and simian virus 40 and characterized with regard to its high UV-sensitivity and hypermutability (Suzuki and Fuse, 1981). RSa cells are also unusually sensitive to X-ray cell-killing (Suzuki and Suzuki, 1990; Kita et al., 2003). As seen in the course of cellular response after UV irradiation, it is conceivable that the increase in protease activity may be involved in the X-ray susceptibility of human cells. So far, several proteases have been reported to be induced by X-ray irradiation, and most of them are involved in X-ray-induced apoptosis. Caspases are a major component of the apoptosis cascade and are induced more than a few hours after X-ray irradiation (Huang et al., 1999; Shinomiya et al., 2000). In some cases, the level of lysosomal protease activity increases several hours after X-ray irradiation in connection with autophagy (Telbisz et al., 2002). Calpain is a calcium-dependent protease with two isozyme forms, *m*-calpain and μ-calpain, and is also thought to be implicated in radiation-induced apoptosis (Watters, 1999). However, there has been no report concerning the protease activity induced promptly after X-ray irradiation. In the present study, we first investigated protease activity within 30 min after X-ray irradiation in RSa cells, and next whether the increase in protease activity is associated with cell susceptibility to X-ray cell-killing.

2. Materials and methods

2.1. Agents

Eagle's minimal essential medium (EMEM) was purchased from Nissui (Tokyo, Japan). Calf serum (CS) was purchased from Thermo Trace Ltd. (Melbourne, Australia). [^{125}I]NaI (3.7 GBq/ml, 629 GBq/mg) was from New England Nuclear (Boston, MA, USA). Octanoyl-*N*-methylglucamide (MEGA-8) and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) were from Dojindo laboratory (Kumamoto, Japan). Aprotinin, chloroquine, fibrinogen, phenylmethylsulfonyl fluoride (PMSF) and thrombin were purchased from Sigma Chemical Co. (St Louis, MO, USA). Pefabloc SC was purchased from Boehringer Ingelheim (GmbH, Germany). The following chemicals were from the Peptide Institute (Osaka, Japan): MG-115, antipain, (L-3-*trans*-carboxyoxirane-2-carbonyl)-L-leucyl-agmatine (E-64) and leupeptin. A mouse monoclonal antibody p53 (DO-1) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Other agents were purchased from Wako Pure Chemical Industries (Osaka, Japan).

2.2. Cells and culture conditions

Cells were cultured with EMEM containing 10% (v/v) CS and antibiotics (60 μg/ml kanamycin) at 37 °C in a humidified atmosphere containing 5% CO_2.

2.3. Preparation of cell lysates

RSa cells at a density of 2×10^6 cells on a 100-mm dish were washed three times with serum-free EMEM preincubated at 37 °C. Then, cells were mock- or X-ray-irradiated. X-ray irradiation was performed as described elsewhere (Kita et al., 2003). Briefly, an X-ray apparatus model MBR-1520R (Hitachi Medical Corporation, Japan) was used at 150 kV and 20 mA with 0.1 mm Cu and 0.5 mm Al filters at a dose of 1.34 Gy/min. After irradiation, cells were cultured with serum-free EMEM for the indicated time. After the culture, serum-free EMEM was removed and then 1 ml of ice-cold lysis buffer [50 mM HEPES–NaOH (pH 7.5), 1 mM EDTA, 0.1% MEGA-8, 5 μM chloroquine, 10 mM 2-mercaptoethanol and 10% glycerol] was dropped on the dish. The lysed cells were scraped off with a piece of silicone rubber. The cell lysates were centrifuged at $10{,}000 \times g$ for 30 min at 4 °C, and the supernatant was used for the estimation of protease activity levels and partial purification of proteases.

2.4. Assay of fibrinolytic protease activity

Fibrinogen was labeled with [^{125}I]NaI by the chloramine-T method (Hunter and Greenwood, 1962). ^{125}I-labeled fibrinogen had a specific radioactivity of 37 MBq/mg protein. The labeled fibrinogen was coated on polystyrene tubes, and then hydrolyzed to fibrin by the addition of thrombin (Sekiya et al., 1985). The cell lysates (0.2 ml) and the partially purified samples were incubated in ^{125}I-fibrin-coated tubes at 37 °C for 20 min in the presence of plasminogen (0.6 μg). The radioactivity (cpm) of released ^{125}I-peptides was counted as described previously (Isogai et al., 1998). The radioactivity increased linearly during incubation for 1 h. Protease activity was estimated principally as cpm/mg protein/min, but as cpm/μl/min in the case of TSK gel G2000SW fractions.

2.5. Purification of proteases

Cell lysates obtained from RSa cells (about 1×10^9 cells) irradiated with and without X-ray (3 Gy) were loaded on 50 ml of a DEAE sepharose CL-6B column equilibrated with lysis buffer. Elution was carried out with lysis buffer containing 1 M NaCl. Then