Suppression of okadaic acid-induced apoptosis by overexpression of calpastatin in human UV^r-1 cells

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Abstract Proteolytic systems have various involvements in apoptotic pathways. To understand the role of calpain in apoptosis, calpastatin, a specific inhibitor of calpain, was overexpressed in human UV^r-1 fibroblasts by transfection of its cDNA. The elevated expression of calpastatin resulted in decreased survival in the presence of okadaic acid (OA) but in no apparent alteration in the sensitivity toward other drugs such as 5-fluorouracil, mitomycin C and methotrexate. After treatment with OA, a typical apoptotic DNA ladder was observed in control vector-transfected cells but not in calpastatin-transfected cells. This indicates that OA-induced apoptosis was suppressed by overexpression of calpastatin. Further immunoblot analysis showed that the OA-induced hyperphosphorylation of c-Jun was inhibited in calpastatin-transfected cells. This might be involved in the resistance to OA-induced cell death in calpastatin-overproducing cells.

Key words: Apoptosis; Okadaic acid; Calpastatin; c-Jun

1. Introduction

Apoptotic cell death is regulated by a variety of molecules. Anti-apoptotic activity was reported for Bcl-2, Akt and ERK mitogen-activated protein kinases (MAP kinases), whereas pro-apoptotic effects were observed for p53, Bax, Bad, stress-activated protein kinase/Jun N-terminal kinase (SAPK/JNK) and p38 MAP kinase (for review see [1]). The apoptotic pathway is also regulated by proteases such as caspases, calpains and proteasome [2]. Calpains are of great interest since they have been reported to cleave signaling molecules including protein kinase C, Src, epidermal growth factor receptor, c-Jun, c-Fos, p53, etc. [3–8]. Two major calpains, μ-calpain and m-calpain, are ubiquitously expressed, and the activity of both calpains is specifically inhibited by calpastatin [9]. A synthetic calpain inhibitor, acetyl-Leu-Leu-norleucinal (ALLN), has frequently been used for investigating the roles of calpains [10]. ALLN induced apoptosis in human prostate adenocarcinoma cells [11] and human HL-60 promyelocytic leukemia cells [12], whereas it showed anti-apoptotic activity on transforming growth factor β-induced apoptosis in rat hepatocytes [13] and *N*-methyl-D-aspartate-induced apoptotic cell death in human CHP100 neuroblastoma cells [14]. Thus, the role of calpain in apoptosis is controversial.

Okadaic acid (OA) is a potent inhibitor of protein phosphatase 1 and protein phosphatase 2A [15], and is a useful probe for the study of cellular phosphorylation regulation [16]. Treatment with OA resulted in apoptotic cell death which was accompanied by hyperphosphorylation of c-Jun [17], induction of p21^{Cip1} and Bax [18] and modulation of Raf-1, protein kinase C and MAP kinases [19]. It remains to be clarified which of these signaling molecules has a key role in OA-induced apoptosis.

In this study, to examine the role of calpains in OA-induced apoptosis, calpastatin cDNA was transfected into human fibroblast UV^r-1 cells. The overexpression of calpastatin resulted in a marked suppression of apoptosis induced by treatment with OA. Possible involvement of Bad and MAP kinases is discussed.

2. Materials and methods

2.1. Materials

Okadaic acid and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA).

2.2. Cell culture

The human cell strain RSa is an embryonic fibroblastic strain transformed by infection with Rous sarcoma virus and simian virus 40 [20]. UV^r-1 cells were derived from RSa cells mutated with ethyl methanesulfonate, then treated with UV followed by selection of surviving cells [21]. These cells were cultured in a medium containing Eagle's MEM supplemented with 10% calf serum.

2.3. Transfection of calpastatin

Human calpastatin cDNA [22] was inserted into the *Xba*I site of pRc/CMV eukaryotic expression vectors (Invitrogen) and transfected into UV^r-1 cells using LipofectAMINE reagent (Life Technologies). Transfected cells were selected in the presence of G418 (400 μg/ml) for 2 weeks.

2.4. Methods for assessing cell survival

Cells in the logarithmic growing phase (5×10^3) were plated in each well of 96-well plates containing various concentrations of OA, 5-fluorouracil (5-FU), mitomycin C (MMC) and methotrexate (MTX), and cultured for 3 days according to the method of Mosmann [23] as described previously [24]. The activity of mitochondrial succinic dehydrogenase was measured by incubation for 4 h in the presence of MTT (0.5 mg/ml) followed by measurement of absorbance at 570 nm with a reference wavelength of 655 nm. Absorbance reflects the viable cell number, and the relative viability was expressed as a percentage of control cells cultured in the absence of test drugs.

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Abbreviations: ALLN, acetyl-Leu-Leu-norleucinal; DMSO, dimethylsulfoxide; JNK, Jun N-terminal kinase; IC_{50}, half-maximum inhibition concentration; MAP kinase, mitogen-activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OA, okadaic acid; SAPK, stress-activated protein kinase

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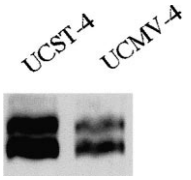

Fig. 1. Expression of calpastatin in calpastatin-transfected clones. Cytoplasmic cell extracts of a calpastatin-transfected clone, UCST-4, and a vector-transfected clone, UCMV-4, were analyzed by immunoblot using anti-calpastatin antibody.

2.5. DNA fragmentation analysis

Cells were cultured for 48 h in the presence of OA at concentrations of 0, 5, 10, 15 and 20 nM, and then lysed for 3 h at 37°C in a solution containing 50 mM Tris-HCl (pH 8.0), 2 mM EDTA, 1% SDS and 100 μg/ml proteinase K. Following high-molecular-weight DNA precipitation by the addition of 7/10 volume 2-propanol and centrifugation at 15 000 rpm for 5 s at room temperature, the low-molecular-weight DNA was recovered from the supernatant and precipitated by incubation overnight at −20°C. After centrifugation, the precipitate was re-suspended and treated with DNase-free RNase A for 3 h at 37°C. The samples were electrophoresed on 1.5% agarose gel containing 0.5 μg/ml ethidium bromide [25].

2.6. Preparation of cell extract and immunoblot analysis

Cells were treated with OA at a concentration of 10 nM for 24 h. Control cells were without treatment. Cells were then washed with phosphate-buffered saline three times and incubated in lysis buffer (0.5% Nonidet P-40, 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 50 μM leupeptin, 50 μM antipain, 50 μM pepstatin A and 50 μM ALLN) for 10 min at 4°C. The cell lysate was centrifuged at 13 000×*g* for 10 min and the supernatant was analyzed by immunoblot using anti-calpastatin (Takara, Kyoto, Japan), anti-phospho-specific SAPK and anti-phospho-specific c-Jun antibodies (New England Biolabs, Beverly, MA, USA). The reaction was detected using the ECL system (Amersham) as described previously [26,27].

3. Results

3.1. Transfection of calpastatin cDNA

Human calpastatin cDNA was inserted into the constitutive expression vector pRc/CMV and transfected into UV^r-1 cells. The expression of calpastatin in G418-selected clones was examined by immunoblot. Two bands were observed, possibly due to the different phosphorylation levels of calpastatin (Fig. 1) [28]. One of the transfected clones, UCST-4, expressed an elevated level of calpastatin relative to an empty vector-transfected control clone, UCMV-4. Thus, these two clones were used in the following experiments.

3.2. Different chemosensitivity to OA between control and calpastatin-transfected cells

To investigate the chemosensitivity to cytotoxic drugs of control and calpastatin-transfected cells, the relative number of viable cells after culture for 3 days in the presence of varying concentrations of the drugs was measured by the MTT method. OA suppressed the proliferation of UCMV-4 cells more effectively than UCST-4 cells (Fig. 2A). Half-maximum inhibition concentrations (IC_{50}) of OA were 6 and 10 nM in UCMV-4 and UCST-4 cells, respectively. On the other hand, chemosensitivity toward 5-FU, MMC and MTX was not significantly different between UCMV-4 and UCST-4 cells (Fig. 2B–D). Thus, the reduced sensitivity of UCST-4 cells was specific to OA.

3.3. Comparison of OA-induced DNA fragmentation between control and calpastatin-transfected cells

To investigate further the different sensitivities to OA between UCMV-4 and UCST-4 cells, we investigated internucleosomal DNA cleavage, a typical event in apoptosis [29].

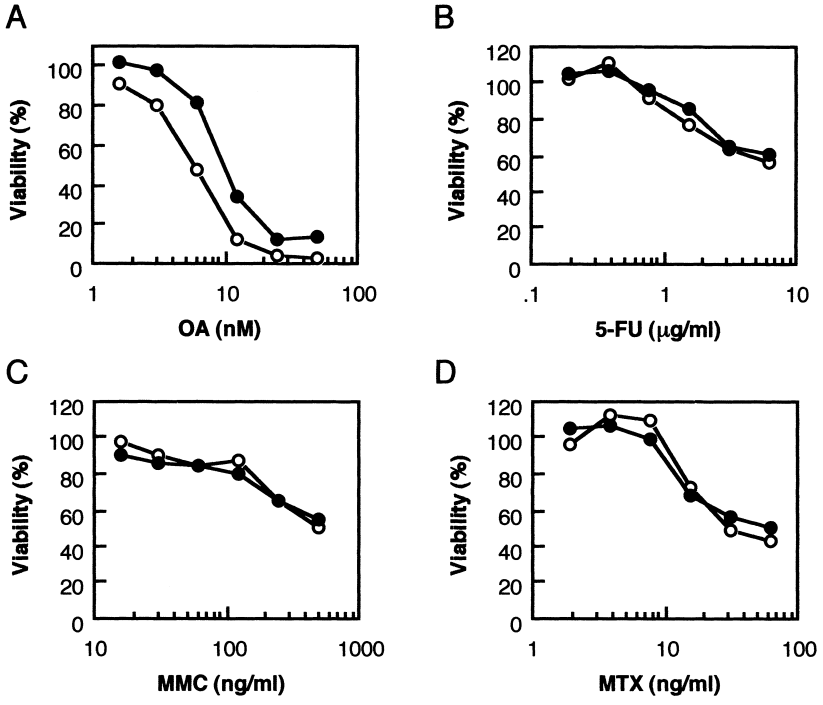

Fig. 2. Effects of OA on the proliferation of UCMV-4 and UCST-4 cells. UCMV-4 (open circles) and UCST-4 cells (closed circles) were cultured for 3 days in the presence of varying concentrations of OA (A), 5-FU (B), MMC (C) and MTX (D). After incubation with MTT, absorbance at 570 nm, reflecting the viable cell number, was measured and expressed as a percentage of that in the absence of inhibitors. Data are means of two experiments.

DNA was isolated from cells 48 h after addition of OA and analyzed by agarose gel electrophoresis. Treatment with OA at concentrations of 10, 15 and 20 nM resulted in the appearance of a typical DNA ladder in UCMV-4 cells (Fig. 3). However, no obvious discrete DNA band was observed in UCST-4 cells treated with OA at any concentration. Thus, OA was unable to induce apoptosis in calpastatin-transfected cells. Some decrease in survival of UCST-4 cells at OA concentrations higher than 10 nM (Fig. 2A) might reflect necrotic cell death.

3.4. Immunoblot analysis of signaling molecules after treatment with OA

Immunoblot analysis was performed to investigate the expression levels of signaling molecules. UCMV-4 and UCST-4 cells were treated with OA at 10 nM for 24 h. Cells extracts were prepared as described in Section 2. Activated c-Jun can be detected by the antibody which specifically recognizes phosphorylation at serine-63 of c-Jun. Although the level of phosphorylated c-Jun was similar between untreated UCMV-4 and UCST-4 cells, it increased in UCMV-4 cells 24 h after addition of OA but not in UCST-4 cells (Fig. 4A). The hyperphosphorylation of c-Jun was clearly observed as shifted bands in OA-treated UCMV-4 cells.

It has been reported that serine-63 of c-Jun is phosphorylated by active SAPK [30]. Activated SAPK was observed by a specific antibody that recognized phosphorylation at threonine-183 and tyrosine-185 [30] (Fig. 4B). The level of activated SAPK was higher in UCMV-4 cells than in UCST-4 cells without OA treatment. The activated SAPK increased after addition of OA in both cells but the level in UCMV-4 was still higher than that in UCST-4 cells.

No apparent change in the expression levels of p53, Bax, Bad and activated ERK1 in the nuclear and cytoplasmic fractions was observed after treatment with OA in both UCMV-4 and UCST-4 cells (data not shown). The expressions of $p21^{Cip1}$ and Bcl-2 were under the detectable levels in both cells irrespective of OA treatment (data not shown).

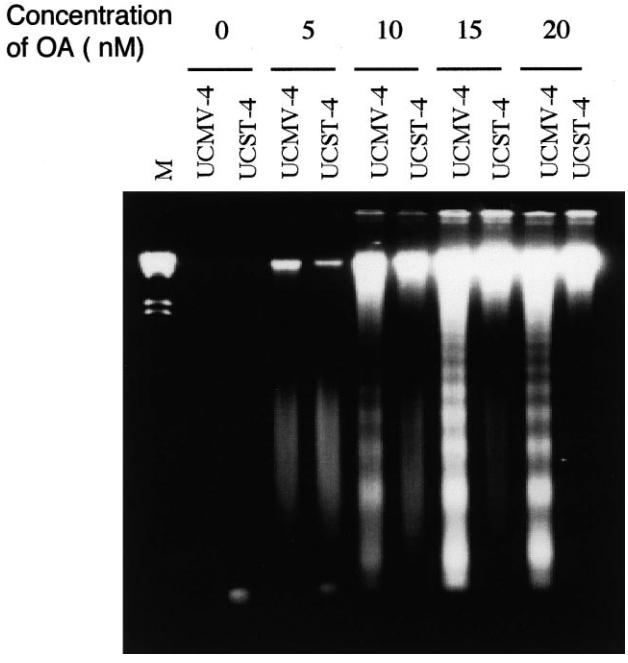

Fig. 3. Induction of DNA fragmentation by OA. UCMV-4 and UCST-4 cells were treated with OA at 0, 5, 10, 15 and 20 nM for 48 h. DNA was isolated and analyzed on 1.5% agarose/ethidium bromide gel. M represents DNA size marker (λ/*Hin*dIII).

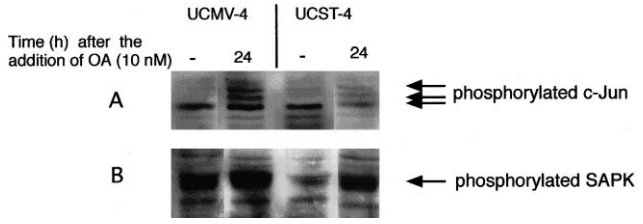

Fig. 4. Effects of OA on the expression of signaling molecules. UCMV-4 and UCST-4 cells were treated with OA at a concentration of 10 nM for 24 h and the cell extracts were analyzed by immunoblot using phospho-specific anti-c-Jun (A) and phospho-specific anti-SAPK (B) antibodies. The results without OA treatment are also shown (−).

4. Discussion

Sheikh et al. reported that treatment with OA resulted in nuclear accumulation of p53, $p21^{Cip1}$ and Bax which might be related to the subsequent apoptotic cell death of human breast carcinoma cells [18]. Dimerization of Bax was reported to be sufficient for induction of apoptosis [31]. However, our present study showed that none of these molecules increased or was translocated into the nuclei in UV^r-1 cells after treatment with OA (data not shown). Among pro-apoptotic molecules thus far examined, activated SAPK increased 24 h after OA treatment in UCMV-4 cells (Fig. 4B). Although p53 was reported to be phosphorylated by SAPK [32], no significant difference in the level of nuclear or cytoplasmic p53 was observed (data not shown). Furthermore, the levels of $p21^{Cip1}$ and Bax, which can be induced by p53, were not altered by treatment with OA (data not shown). These data suggest that the signaling pathway via p53 was not induced by OA in this cell. However, consistent with the previous report of Peng et al. [17], c-Jun was hyperphosphorylated 24 h after addition of OA in UCMV-4 cells, but not in UCST-4 cells (Fig. 4A). Therefore, it is possible that hyperphosphorylation of c-Jun accounts, in part, for the induction of apoptosis in UCMV-4 cells. It should be noted that SAPK/JNK activation was followed by induction of Fas ligand in PC12 cells [33]. Thus, SAPK-to-c-Jun-to-Fas ligand could be a major pathway for OA-induced apoptosis. Overexpression of calpastatin might interrupt this signaling pathway.

Calpains are involved in proteolytic down-regulation of activated protein kinases such as protein kinase C, c-Src and epidermal growth factor receptor [3–5]. It is thus reasonable to speculate that suppression of calpain activity by calpastatin might enhance signal transduction. However, activation of c-Jun was reduced in calpastatin-transfected cells despite some increase in the level of activated SAPK (Fig. 4A,B). This raises the possibility that calpain is not only a suppressive molecule, but can potentiate a signaling pathway through SAPK. Activation of the substrates by calpains was also suggested by Carafoli and Molinari based on the fact that calpain cleaved the substrates into a small number of well defined fragments [8]. Further investigation will elucidate the direct role of calpain in the apoptotic signaling pathway.

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