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# Increase in ultraviolet sensitivity by overexpression of calpastatin in ultraviolet-resistant UV$^r$-1 cells derived from ultraviolet-sensitive human RSa cells

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## Abstract

**Human RSa cells are highly sensitive to apoptotic-like cell death by ultraviolet irradiation (UV) while UV$^r$-1 cells are their variant with an increased resistance to UV. Three days after UV at 10 J/m$^2$, the viability of RSa cells was approximately 17% while that of UV$^r$-1 cells was 65%. This different survival might reflect apoptotic cell death since apoptosis-specific DNA ladder was more clearly observed in RSa cells than in UV$^r$-1 cells after UV. Addition of ALLN/calpain inhibitor I to the culture medium after UV resulted in similar survival (14 – 18%) between RSa and UV$^r$-1 cells. Immunoblot analysis showed down-regulation of protein kinase C$\theta$, Src, Bax and $\mu$-calpain after UV was more prominent in UV$^r$-1 than in RSa cells. Activated $\mu$-calpain appeared within 1 h post-UV only in UV$^r$-1 cells. The expression of calpastatin, a specific endogenous inhibitor of calpain, was higher in RSa than in UV$^r$-1 cells. To further examine the role of calpain in UV-induced cell death, cDNA of human calpastatin was transfected into UV$^r$-1 cells. The results showed that overexpression of calpastatin suppressed down-regulation of Src, $\mu$-calpain and Bax. Concomitantly, colony survival after UV was reduced in calpastatin-transfected cells as compared to vector control cells. Our results suggest that activation of calpain might account for, at least in part, the lower susceptibility to UV-induced cell death in UV$^r$-1 cells.** *Cell Death and Differentiation* (2000) **7,** 531 – 537.

**Keywords:** ultraviolet; signal transduction; down-regulation; calpain; Bax; apoptosis

**Abbreviations:** ALLN/calpain inhibitor I, *N*-acetyl-Leu-Leu-norleucinal; DMSO, dimethylsulfoxide; MTT, 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide; SAPK, stress-activated protein kinase; UV, ultraviolet irradiation

## Introduction

Irradiation of eukaryotic cells with far-ultraviolet light (UVC, principally 254 nm wavelength) induces cellular DNA damage, which is followed by activation of DNA-repair mechanisms. Defects in the latter result in increased cell death.[1] On the other hand, specific signal transduction pathways including activation of protein kinases and transcription factors are also induced by ultraviolet irradiation (UV).[2,3] Previous studies have shown that one of the earliest post-UV responses is activation of tyrosine kinases such as Src,[4] EGF-receptor,[5] insulin receptor,[6] Syk[7] and ZAP-70.[8] These signals are subsequently transduced by Ras, c-Raf, ERK MAP kinases, stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) and p38 MAP kinase, followed by enhancement of transactivation by AP-1 (c-Jun/ATF-2),[4,9] SRF[10] and NF-$\kappa$B.[9] In another pathway, DNA damage leads to activation of p53, which induces p21$^{Cip1}$, Bax, Mdm2, GADD45 etc.[11 – 13] Caspases play a key role in induction of apoptotic cell death,[14] and it was suggested that the high expression of Bax leads to activation of caspases.[15]

$Ca^{2+}$-activated neutral cysteine proteinase calpain is one of the major cytoplasmic nonlysosomal proteases.[16,17] Three isozyme forms, $\mu$-calpain, m-calpain and muscle-specific calpain 3 (p94), have been reported.[18] The catalytic activity of calpain is specifically inhibited by an endogenous inhibitor calpastatin.[19] The following observations suggested the proapoptotic role of calpain: (1) calpain inhibitors such as acetyl-Leu-Leu-norleucinal (ALLN, calpain inhibitor I), acetyl-Leu-Leu-methioninal (ALLM, calpain inhibitor II) and benzyloxycarbonyl-Leu-Leu-Tyr diazomethylketone suppressed dexamethasone-induced apoptotic cell death in thymocyte,[20,21] serum deprivation-induced apoptosis in muscle satellite cells,[22] apoptosis in rat cerebellar granule neurons exposed to low potassium-containing medium,[23] TGF-$\beta$-induced apoptosis in primary cultures of hepatocytes,[24] NGF-deprivation-induced neuronal cell death,[25] calphostin-induced apoptosis in U937 human promonocytic leukemia,[26] and reovirus-induced apoptosis in murine L929 fibroblast.[27] Calpain inhibitors also suppressed activation of caspase-7 during B cell receptor crosslinking on immature B cells.[28] (2) Potentiation of calpain activity by depleting calpastatin is sufficient to cause apoptosis of neutrophils.[29] Calpastatin was degraded by caspases during apoptosis in staurosporine-treated Jurkat T-cells.[30,31] (3) Degradation of calpain substrates

such as non-erythroid $\alpha$-spectrin and Bax was observed during apoptosis in staurosporine-treated neuroblastoma cells[32] and topoisomerase I inhibitor-treated HL-60 cells.[33]

On the other hand, some reports suggested that calpain activity is necessary for cell survival. Calpain inhibitors produced apoptosis in human HL-60 cells and prostate cancer cells.[34,35] Calpain 3 deficiency is associated with myonuclear apoptosis possibly due to inability to degrade I$\kappa$B$\alpha$.[36] Furthermore, calpain-independent cell death was also observed in thymocyte triggered by heat shock and by valinomycin and in cultured rat cardiomyocytes during metabolic inhibition.[21,37] Thus, the role of calpain in apoptosis is controversial and depends on cell types and treatment.

We have previously reported the mechanisms of UV-induced cell death using UV-hypersensitive human RSa cells as well as its UV-resistant derivative UV$^r$-1 cells.[38,39] Despite the clear difference in UV-resistance, no significant difference was observed in the activity of UV-induced DNA repair synthesis between RSa and UV$^r$-1 cells.[39,40] These suggest that certain alteration(s) in the signal transduction pathways could be the underlying mechanism of the different UV-susceptibility between RSa and UV$^r$-1 cells. By comparing UV-induced molecular changes in these cells, one might be able to identify a causative molecule responsible for UV-induced cell death. In the present study, we found that sensitivity to calpain inhibitors, activation of $\mu$-calpain and degradation of calpain substrates after UV were different between RSa and UV$^r$-1 cells. The possible development of calpain in UV-induce cell death was further examined by transfection of calpastatin cDNA in UV$^r$-1 cells.

## Results

### Comparison of UV-induced cell death between RSa and UV$^r$-1 cells

Human RSa cells were much more sensitive to UV than UV$^r$-1 cells when the survival was measured by colony-forming assay.[39] Similar difference in UV sensitivity was also observed 3 days after UV. The relative viability measured by MTT method was much lower in RSa cells as compared to that in UV$^r$-1 cells after irradiation at 5 and 10 J/m$^2$ (Figure 1). Survival of UV$^r$-1 cells after UV at 15 J/m$^2$ was still higher than that of RSa cells irradiated at 10 J/m$^2$. Addition of ALLN/calpain inhibitor I to the culture medium after UV induced only marginal decrease in survival in RSa cells but marked decrease in the viability of UV$^r$-1 cells. In the presence of ALLN, the viability of both RSa and UV$^r$-1 cells after UV at 10 J/m$^2$ was approximately 15% (Figure 1). Thus, the different UV sensitivity between RSa and UV$^r$-1 cells might attribute to the calpain proteolytic system.

### Comparison of UV-induced DNA fragmentation between RSa and UV$^r$-1 cells

To determine that the difference in UV-induced cell death between RSa and UV$^r$-1 cells is related to apoptosis, we investigated internucleosomal DNA cleavage, a typical event

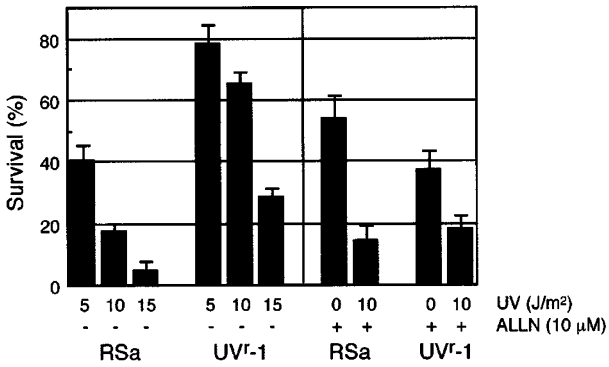


**Figure 1** Comparison of viability after UV between RSa and UV$^r$-1 cells. RSa and UV$^r$-1 cells were mock-irradiated or irradiated with ultraviolet at 5, 10 and 15 J/m$^2$, and then cultured for 3 days in the absence or presence of ALLN. After incubation with MTT, absorbance at 570 nm, reflecting the viable cell number, was measured and expressed as a percentage of that of mock-irradiated cells cultured in the absence of inhibitors. Data are mean of three experiments and error bars represent S.D.

in apoptosis.[41] For this purpose, DNA was isolated from RSa and UV$^r$-1 cells 20 h after UV and analyzed by agarose gel electrophoresis. In RSa cells, a typical DNA ladder was detected after UV at 5 J/m$^2$, which became more evident at 10 to 15 J/m$^2$ (Figure 2). A similar DNA fragmentation pattern was observed between 10 and 48 h after the irradiation (data not shown). In contrast, such DNA ladder pattern was less clear in UV$^r$-1 cells after UV. Some faint discrete bands were observed after irradiation only at 10 J/m$^2$. Since the most prominent DNA fragmentation was noted with UV at 10 J/m$^2$, irradiation at this dose was applied in the remaining experiments.

### Immunoblot analysis following UV

Immunoblot analysis was performed to investigate the cellular alterations in RSa and UV$^r$-1 cells at a molecular level. RSa and UV$^r$-1 cells were treated with UV at 10 J/m$^2$ and further cultured for 1, 2, 4, 8 and 24 h. Cytoplasmic and nuclear cell extracts were prepared as described in Materials and Methods.

Many isoforms of PKC have been reported thus far, and the activities of these PKCs are subtly regulated by $Ca^{2+}$, diacylglycerol and others.[42] The expression level of PKC$\alpha$ was not significantly different between non-irradiated RSa and UV$^r$-1 cells (Figure 3A) and UV did not affect the expression of PKC$\alpha$ in these cells. Likewise, no apparent difference was observed in the expression levels of PKC$\gamma$, PKC$\zeta$, PKC$\lambda$, and PKC$\mu$ between RSa and UV$^r$-1 cells or between these non-irradiated and irradiated cells (data not shown). The expression level of PKC$\theta$ decreased gradually and was hardly detectable at 24 h after UV in UV$^r$-1 cells (Figure 3B). On the other hand, the level of PKC$\theta$ slightly decreased at 4 to 8 h but returned to the original level at 24 h after UV in RSa cells.

Src protein was suggested to be a direct receptor for UV.[4] The expression of Src was lower in non-irradiated UV$^r$-1 cells than in non-irradiated RSa cells (Figure 3C). Furthermore, Src was down-regulated after UV in UV$^r$-1

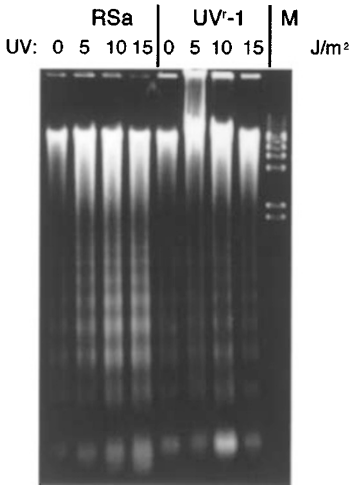


**Figure 2** Induction of DNA fragmentation by UV. RSa and UV$^r$-1 cells were irradiated with ultraviolet at 0, 5, 10 and 15 J/m$^2$ and cultured for 20 h. DNA was isolated and analyzed on 1.5% agarose/ethidium bromide gel. M represents size markers (*λ-Hind*III)

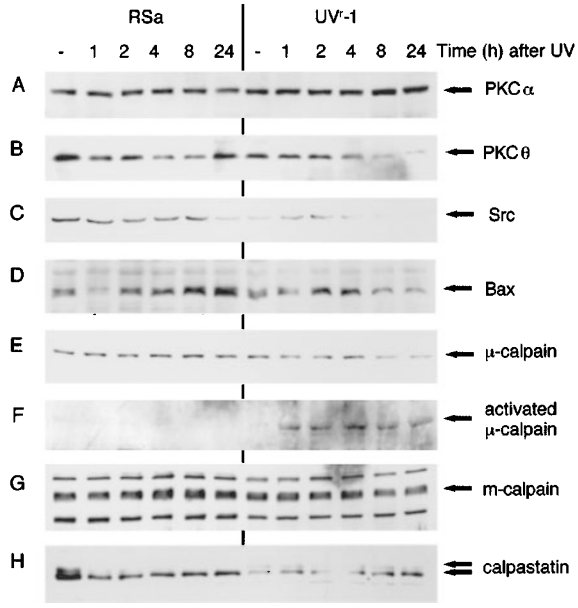


**Figure 3** Effects of UV on the expression levels of signaling molecules in RSa and UV$^r$-1cells. Cells were mock-irradiated or irradiated with ultraviolet at 10 J/m$^2$ and cultured for 1, 2, 4, 8 and 24 h. Cytoplasmic cell extracts were analyzed by immunoblot using anti-PKC$\alpha$ (**A**), anti-PKC$\theta$ (**B**), anti-Src (**C**), anti-Bax (**D**), anti-$\mu$-calpain (**E**), anti-activated $\mu$-calpain (**F**), anti-m-calpain (**G**), and anti-calpastatin (**H**) antibodies

cells while only a slight decrease in Src was observed in RSa cells even at 24 h after UV.

It has been well documented that p53 and Bax play important roles in apoptosis.[43,44] Nuclear accumulation of p53 after UV was reported in other cells previously[44] but was not significantly different between RSa and UV$^r$-1 cells (data not shown). The level of expression of Bax gradually increased in RSa cells at least up to 24 h after irradiation (Figure 3D) possibly in a p53-dependent manner as previously suggested.[12] On the other hand, Bax increased slightly at 2–4 h but returned to the baseline level at 8 h post-irradiation in UV$^r$-1 cells.

The results of Figure 3B–D indicated differences in the down-regulation of certain signaling molecules such as PKC$\theta$, Src and Bax between RSa and UV$^r$-1 cells. One of the proteases that might be involved in the degradation of these molecules is calpain.[33,45,46] Among the calpain family, m- and $\mu$-calpains are widely distributed in most cell types.[47] The expression level of $\mu$-calpain was almost similar in non-irradiated RSa and UV$^r$-1 cells (Figure 3E). However, $\mu$-calpain decreased gradually after UV in UV$^r$-1 cells but not in RSa cells. It is possible that this decrease in $\mu$-calpain was caused by activation and subsequent autolysis of the protease. Activation of $\mu$-calpain is accompanied by autolytic truncation of the amino-terminus of calpain and production of a 76-kDa degradation intermediate.[48] This 76-kDa calpain can be specifically detected by anti-activated $\mu$-calpain antibody which was raised against amino-terminus of the truncated $\mu$-calpain.[48] Immunoblots using this antibody showed that activated $\mu$-calpain appeared within 1 h and was detectable for at least 24 h after UV in UV$^r$-1 cells (Figure 3F). On the other hand, the activated $\mu$-calpain could not be detected in RSa cells irrespective of irradiation. m-calpain was expressed abundantly in both RSa and UV$^r$-1 cells and was not affected by UV (Figure 3G).

Differences in the activation of $\mu$-calpain between RSa and UV$^r$-1 cells were consistent with the expression level of calpastatin, a calpain-specific endogenous inhibitor[49] (Figure 3H). Without irradiation, RSa cells expressed higher levels of calpastatin than UV$^r$-1 cells. Following irradiation, calpastatin was rapidly down-regulated in RSa cells but its level was still higher than that in UV$^r$-1 cells.

## Effects of overexpression of calpastatin

The above results suggest that differences between RSa and UV$^r$-1 cells in down-regulation of signaling molecules as well as in activation of $\mu$-calpain can, at least in part, be explained by the different level of calpain activation. Accordingly, we examined the effect of overexpression of calpastatin in UV$^r$-1 cells by transfection of human calpastatin cDNA. The expression of calpastatin in G418-selected clones was examined by immunoblot. The transfected clones, UCST-4, 10, 11 and 12, expressed elevated levels of calpastatin constitutively compared with the vector-transfected control clones, UCMV-1 and 4, which expressed a low amount of endogenous calpastatin (Figure 4A). Despite overexpression of calpastatin, the phase morphology of calpastatin-transfected clones was indistinguishable from that of control cells (data not shown).

The aforementioned down-regulation of Src after UV was also observed in UCMV-1 whereas the amount of Src did not diminish after irradiation of UCST-4 cells (Figure 4B). Likewise, the decrease in $\mu$-calpain expression after UV was suppressed in UCST-4 but not in control UCMV-1 cells (Figure 4C). The amount of Bax increased transiently and then decreased after UV in UCMV-1 cells (Figure 4D) as

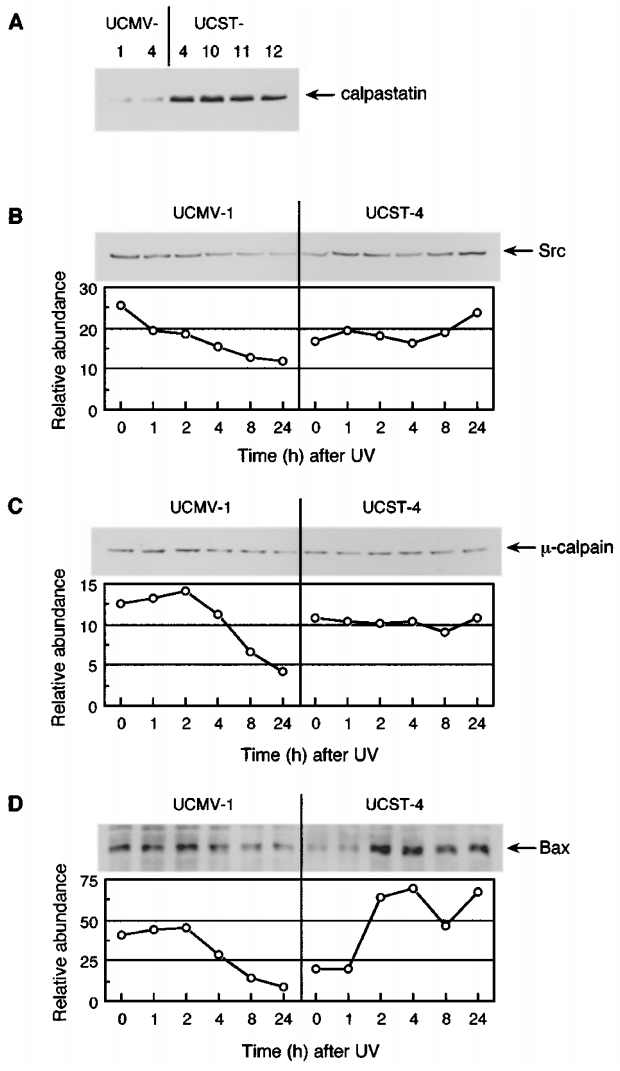


**Figure 4** Expression of calpastatin, Src, $\mu$-calpain and Bax in transfected clones. (**A**) The expression of calpastatin in vector-transfected control clones, UCMV-1 and 4, and calpastatin-transfected clones, UCST-4, 10, 11 and 12, was investigated by immunoblotting using anti-calpastatin antibody. (**B**–**D**) Expression of Src (**B**), $\mu$-calpain (**C**) and Bax (**D**) in UCMV-1 and UCST-4 cells following UV for the indicated periods was analyzed by immunoblotting using anti-Src, anti-$\mu$-calpain and anti-Bax antibodies, respectively. Quantitation of the data shown in the upper panels was performed by scanning the X-ray films with a densitometer (Hoefer Scientific Instruments, GS300), and the relative abundance (arbitrary unit) is shown in the lower panels

observed in parent UV$^r$-1 cells (Figure 3D). However, the high expression level of Bax was maintained up to 24 h after irradiation of UCST-4 cells. This implies that the expression level of Bax can be regulated by the calpain-calpastatin system.

The sensitivity of these transfected clones to UV-induced cell death was then examined by colony-forming assay. The results showed that calpastatin-transfected clones, UCST-4 and 10, were more sensitive to UV than vector-transfected UCMV-1 cells (Figure 5). The survival of UCST-4 and 10 after treatment with UV at 13.5 and 18 J/m$^2$ was significantly lower than in UCMV-1 ($P<0.05$ and $P<0.01$,

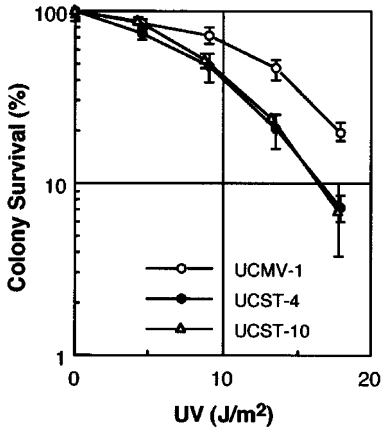


**Figure 5** Cloning efficiency in transfected clones irradiated with ultraviolet. The colony-forming ability of control, UCMV-1 (○), calpastatin-transfected UCST-4 (●) and UCST-10 cells (△), was examined as described in Materials and Methods. Data represent the average percentage of colony numbers relative to that of mock-irradiated control cells. Data are mean ± S.D. of three results

respectively). It is thus conceivable that the proteolytic activity of calpain is necessary for cell survival following UV.

## Discussion

Human RSa cells are highly sensitive to UV-induced cell death while their derivative UV$^r$-1 cells are resistant to it (Figure 1).[39] The present results showed that UV-induced death of these cells reflects, at least in part, apoptosis, as shown in Figure 2. It should be noted that the survival after UV was not significantly different between UV$^r$-1 and RSa cells in the presence of ALLN/calpain inhibitor I (Figure 1). We compared the expression levels of signaling molecules such as PKC, Src, c-Raf, A-Raf, MEK, ERK, SAPK, p38 MAP kinase, phosphatidylinositol 3-kinase, Akt, phospholipase C$\gamma$, c-Jun, p53 and Bax between UV$^r$-1 and RSa cells after UV (Figure 3, data not shown). Among these molecules, UV-induced down-regulation of PKC$\theta$, Src and Bax was observed more clearly in UV$^r$-1 cells than in RSa cells (Figure 3B–D).

Down-regulation of the signaling molecules, a form of feedback regulation, suppresses the expansion and duration of the signal. In many cases, down-regulation was caused by proteolytic degradation of the signaling proteins rather than a decrease in their *de novo* synthesis. Three types of cytoplasmic proteases, calpain, proteasome and caspases, have been frequently suggested to be involved in the down-regulation of signaling molecules. Calpain has been reported to cleave PKC, Src, EGF receptor, c-Jun, c-Fos, p53, Bax, etc.[33,45,46,50–52] On the other hand, proteasome is thought to degrade cyclins, c-Jun, c-Fos, p53, etc.[53–56] PKC$\delta$, MEKK1, Ras GTPase-activating protein and p21$^{Waf1/Cip1}$ can be cleaved by caspases.[57–60] Since all of PKC$\theta$, Src and Bax can be degraded by calpain, we examined the levels of $\mu$- and m-calpains as well as calpastatin. As compared to RSa cells, UV$^r$-1 cells contained lower amounts of $\mu$-calpain and

calpastatin and a much higher amount of activated $\mu$-calpain after UV (Figure 3E,F,H). These suggested that calpain activity can explain the different down-regulation of PKC$\theta$, Src and Bax and possibly the different UV sensitivity between UV$^r$-1 and RSa cells.

Then, we transfected human calpastatin cDNA in UV$^r$-1 cells. As expected, overexpression of calpastatin in UV$^r$-1 cells resulted in the suppression of down-regulation of Src, $\mu$-calpain and Bax (Figure 4B–D). Furthermore, colony survival assay in the present study showed that the susceptibility of calpastatin-transfected clones to UV-induced cell death was significantly higher than that of vector-control cells (Figure 5), suggesting that the catalytic activity of calpain is necessary for the high survival of UV$^r$-1 cells after UV. Thus, failure of calpain activation in RSa cells after UV might explain, at least in part, their high susceptibility to UV-induced cell death. The present study also implies that down-regulation of Src and/or Bax by calpain may be involved in UV-resistance of UV$^r$-1 cells.

In contrast to $\mu$-calpain, the level of m-calpain was hardly affected by UV (Figure 3G). Although m-calpain requires unphysiological high concentrations of $Ca^{2+}$ for activation,[16] a part of m-calpain might be activated by pre-activated $\mu$-calpain in a calpain cascade as suggested by Tompa *et al*.[61] It is possible that this secondary activated m-calpain has a main role in down-regulation of signaling molecules. This idea might account for the delay of decrease in PKCf$\theta$, Src and Bax levels after the appearance of activated $\mu$-calpain in UV$^r$-1 cells (Figure 3B–D and F).

A recent report showed that active Src is degraded by proteasome.[62] PKC$\delta$ and PKC$\zeta$ can be cleaved by caspase-3.[63,64] Thus, we cannot rule out the possibility that other proteolytic systems such as proteasome and caspases might also affect the UV susceptibility of RSa and UV$^r$-1 cells. The activity of signaling molecules can be regulated by various proteolytic enzymes. Further investigation of this issue will identify the key molecule(s) involved in the recovery from UV-induced damage.

## Materials and Methods

### Materials

Ac-Leu-Leu-norleucinal (ALLN)[65] was purchased from Peptide Institute Inc. (Osaka, Japan). MTT (3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide) was purchased from Sigma (St. Louis, MO, USA).

### Cell culture

The human cell strain RSa is an embryonic fibroblastic strain transformed by infection with Rous sarcoma virus and simian virus 40.[38] RSa is highly sensitive to cell death caused by UV.[38,39] UV$^r$-1 cells were derived from RSa cells mutated with ethyl methanesulfonate, then treated with UV followed by a selection of surviving cells.[39] These cells were cultured in a medium containing Eagle's MEM supplemented with 10% calf serum.

### Methods for assessing viability

Cells in logarithmic growth phase were treated with UV[66] and then cultured for 10 min. A total of $5 \times 10^3$ cells were plated in each well of 96-well plates in the absence or presence of 20 $\mu$M ALLN, and cultured for 3 days. 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 according to the method of Mosmann[67] as described previously.[68] Absorbance reflects the viable cell number, and was expressed as a percentage of that of unirradiated cells cultured in the absence of ALLN.

### DNA fragmentation analysis

Irradiated and mock-irradiated cells were cultured for 20 h, and then lysed for 3 h at 37°C in solution containing 50 mM Tris-HCl (pH 8.0), 2 mM EDTA, 1% SDS and 100 $\mu$g/ml proteinase K. Following the addition of one-tenth volume of 3 M sodium acetate, the nucleotides were extracted with phenol/chloroform and then with chloroform. The high-molecular weight DNA was precipitated by the addition of seven-tenth volume 2-propanol followed by centrifugation at 15 000 r.p.m. 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 in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA and 50 $\mu$g/ml of DNase-free RNase A and incubated for 3 h at 37°C. The samples were applied to 1.5% agarose gel containing 0.5 $\mu$g/ml ethidium bromide, and electrophoresed in 90 mM Tris-borate (pH 8.0) and 2 mM EDTA at 100 V for 3 h. DNA was visualized by UV illumination as described previously.[69]

### Preparation of cell extract and immunoblot analysis

Cells were treated with UV (10 J/m$^2$) as described previously[66] and further cultured for 1, 2, 4, 8 and 24 h. Mock-irradiated cells were used as control. Cells were then washed with phosphate-buffered saline (PBS) four times and incubated in lysis buffer [0.5% Nonidet P-40, 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 50 $\mu$M leupeptin, 50 $\mu$M antipain, 50 $\mu$M pepstatin A and 50 $\mu$M ALLN] for 10 min at 4°C. The cell lysate was centrifuged at 13 000 $\times$ *g* for 10 min and the supernatant was lyophilized and used as 'cytoplasmic fraction'. The pellet of the centrifugation was washed once with the lysis buffer, directly dissolved in SDS-sample buffer and used as 'nuclear fraction'.[70] Immunoblot analysis was carried out using ECL system (Amersham) as described previously.[71] The antibodies used were anti-Src (Oncogene Science), anti-PKC$\alpha$, PKC$\gamma$, PKC$\zeta$, PKC$\lambda$, PKC$\mu$ and PKC$\theta$, anti-p53 (Transduction Laboratories), anti-Bax (Santa Cruz Biotechnology), anti-$\mu$-calpain and anti-m-calpain (Chemicon International, Temecula, CA, USA), anti-calpastatin (Takara, Kyoto, Japan) and anti-activated $\mu$-calpain antibodies.[48]

### Transfection of calpastatin

Human calpastatin cDNA[72] 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 $\mu$g/ml) for 2 weeks.

### Colony-formation assay

$UV^r$-1 cells ($1 \times 10^3$) were plated in 100-mm dishes and incubated for 20 h to allow the cells to attach. Cells were then treated with UV and cultured for 2 weeks, then stained with 0.2% methylene blue in 30% methanol. Colonies with a minimal diameter of 2 mm were scored.

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