Stimulation of ultraviolet-induced apoptosis of human fibroblast UV^r-1 cells by tyrosine kinase inhibitors

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Abstract **Damnacanthal is an anthraquinone compound isolated from the root of *Morinda citrifolia* and was reported to have a potent inhibitory activity towards tyrosine kinases such as Lck, Src, Lyn and EGF receptor. In the present study, we have examined the effects of damnacanthal on ultraviolet ray-induced apoptosis in ultraviolet-resistant human UV^r-1 cells. When the cells were treated with damnacanthal prior to ultraviolet irradiation, DNA fragmentation was more pronounced as compared to the case of ultraviolet irradiation alone. The other tyrosine kinase inhibitors, herbimycin A and genistein, also caused similar effects on ultraviolet-induced apoptosis but to a lesser extent. Serine/threonine kinase inhibitors, K252a, staurosporine and GF109203X, rather suppressed the ultraviolet-induced DNA cleavage. Immunoblot analysis showed that pretreatment with damnacanthal followed by ultraviolet irradiation increased the levels of phosphorylated extracellular signal-regulated kinases and stress-activated protein kinases. However, the other tyrosine kinase inhibitors did not increase the phosphorylation of extracellular signal-regulated kinases but stimulated phosphorylation of stress-activated protein kinases. Consequently, the ultraviolet-induced concurrent increase in both phosphorylated extracellular signal-regulated kinases and stress-activated protein kinases after pretreatment with damnacanthal might be characteristically related to the stimulatory effect of damnacanthal on ultraviolet-induced apoptosis.**

Key words: Ultraviolet; Tyrosine kinase inhibitor; Mitogen-associated protein kinase; Apoptosis

1. Introduction

The induction of apoptosis by ultraviolet (UV) irradiation has been well documented. Finding of the earliest activation of protein kinases after UV irradiation suggested that the receptors for UV can be Src [1], EGF-receptor [2], insulin receptor [3], Syk [4] and ZAP-70 [4]. It was also reported that the activation of these tyrosine kinases is subsequently transduced to Ras, c-Raf and MAP kinases [5]. Three major members of the MAP kinase family, extracellular signal-regulated kinases (ERKs), stress-activated protein kinases (SAPK/JNK) and p38 MAP kinase, are thought to be differentially involved in apoptosis, i.e. activation of SAPK and p38 stimulates apoptosis while activated ERK has a suppressive role [6,7].

Human embryonic RSa cells were established after infection with Rous sarcoma virus and simian virus 40 [8]. UV^r-1 cells were derived from RSa cells mutagenized with ethyl methanesulfonate followed by irradiation with UV [9]. RSa cells are highly sensitive to UV-induced cell-killing while UV^r-1 cells are resistant [9]. Thus, these cell lines are useful for investigation on UV-induced cellular responses [9–11]. However, UV irradiation alone caused severe damage to RSa cells and, therefore, UV^r-1 cells were used in the present investigation on the effects of tyrosine kinase inhibitors on UV-irradiated cells. To elucidate the relevance of tyrosine kinases in UV-induced apoptosis as well as in the activation of the MAP kinase family in UV^r-1 cells, the effects of tyrosine kinase inhibitors such as damnacanthal (Dam), herbimycin A (HMA) and genistein were investigated. Unexpectedly, the results of the present study showed that the UV-induced apoptosis was stimulated by these three inhibitors, especially by Dam. ERKs were synergistically activated by treatment with UV and Dam.

2. Materials and methods

2.1. Materials

Dam was isolated and purified from the root of *Morinda citrifolia* as described previously [12]. Tyrphostin AG1478 was purchased from Sigma. Erbstatin, genistein and K252a were obtained from Seikagaku Kogyo (Tokyo) and staurosporine and GF109203X was from Wako Chemicals (Tokyo). HMA was provided by Yoshimasa Uehara (National Institute for Health, Tokyo).

2.2. Cell culture

The RSa and UV^r-1 cells were cultured in Eagle's MEM supplemented with 10% calf serum as described [9].

2.3. DNA fragmentation analysis

Cells were pretreated with protein kinase inhibitors or the solvent dimethylsulfoxide (DMSO) for 1 h and then mock-irradiated or irradiated with UV (10 J/m^2) as described [9,13]. Cells were further cultured for 40 h in the presence or absence of the inhibitors. Cells were then washed with phosphate-buffered saline for three times and then lysed at 37°C for 3 h in 50 mM Tris-HCl (pH 8.0), 2 mM EDTA, 1% SDS and 100 μg/ml of proteinase K. After the addition of one-tenth volume of 3 M sodium acetate, nucleotides were extracted with phenol/chloroform and then with chloroform. The high-molecular-weight DNA was precipitated by addition of seven-tenth volume of 2-propanol followed by centrifugation at 15 000 rpm for 5 s at room temperature. The low-molecular-weight DNA was recovered in the supernatant and precipitated by incubation at −20°C overnight. After centrifugation, the precipitate was resuspended in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA and 50 μg/ml of DNase-free RNase A and

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Abbreviations: Dam, damnacanthal (3-hydroxy-1-methoxyanthraquinone-2-aldehyde); DMSO, dimethylsulfoxide; ERK, extracellular signal-regulated kinase; HMA, herbimycin A; IC_{50}, half maximum inhibition concentration; SAPK, stress-activated protein kinase; UV, ultraviolet

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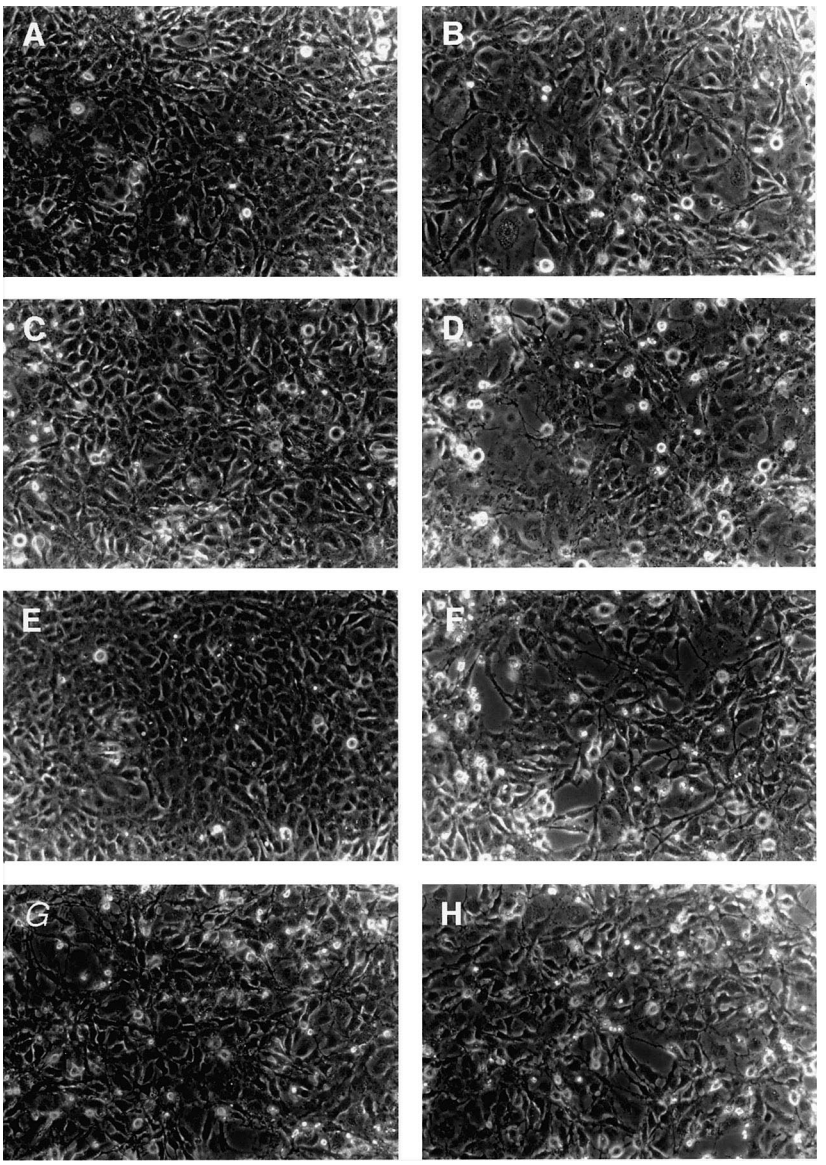

Fig. 1. Phase micrographs of UV^r-1 cells after UV irradiation with or without pretreatment with tyrosine kinase inhibitors. Cells were pretreated with DMSO (0.1%: A and B), HMA (1 μM: C and D), Dam (10 μg/ml: E and F) or genistein (100 μM: G and H) for 1 h and then mock irradiated (A, C, E and G) or irradiated with UV (10 J/m^2) (B, D, F and H). Cells were further cultured for 40 h and phase micrographs were observed. Magnification ×200.

incubated for 3 h at 37°C. The samples were applied to 1.5% agarose gel containing 0.5 μg/ml of 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 [14].

2.4. Preparation of cell extract and immunoblot analysis

Cells were pretreated with protein kinase inhibitors and then irradiated with UV (10 J/m^2) as described above. Cells were further cultured for 1 h in the presence or absence of the inhibitors. Cells were then washed with phosphate-buffered saline for 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, 100 μM leupeptin, 100 μM antipain, 100 μM pepstatin A and 100 μM acetyl-Leu-Leu-norleucinal) for 10 min at 4°C. The cell lysate was centrifuged at 13 000×*g* for 10 min and the supernatant was lyophilized and used as 'cytoplasmic extract'. The pellet of the centrifugation was washed once with the lysis buffer, directly dissolved in SDS-sample buffer and used as 'nuclear extract'. Immunoblot analysis was carried out using the ECL system (Amersham) as described elsewhere [15]. The antibodies used were anti-phospho-specific ERK, anti-phospho-specific SAPK, anti-phospho-specific p38 MAP kinase and anti-phospho-specific c-Jun antibodies (New England Biolabs).

3. Results

The inhibitory activities of protein kinase inhibitors on the proliferation of UV^r-1 cells were first examined to determine the half maximum inhibition concentration (IC_{50}) of each compound (data not shown). The concentration of IC_{50} of each inhibitor was used in the following experiments.

UV^r-1 cells were pretreated with HMA [16], Dam [12], genistein [17] or the solvent DMSO for 1 h and then irradiated with UV at 10 J/m^2. The cells were further cultured for 40 h and the morphological change was investigated (Fig. 1). UV irradiation alone caused some delay in the cell growth. However, phase morphology of irradiated cells was similar to that of unirradiated cells (Fig. 1A,B). Treatment with HMA in-

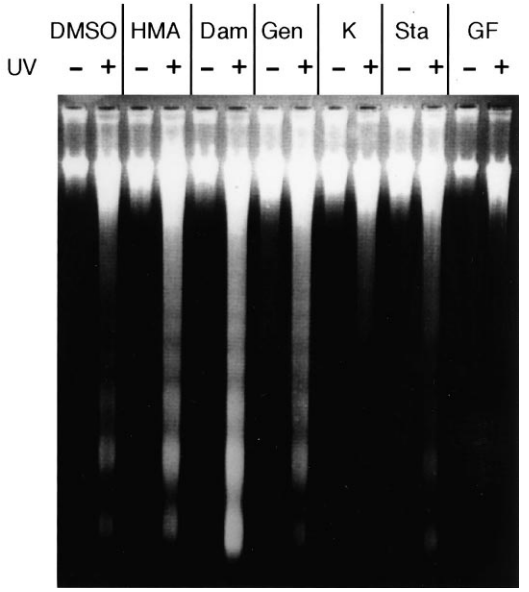

Fig. 2. Induction of DNA fragmentation after UV irradiation and treatment with protein kinase inhibitors. UV^r-1 cells were pretreated with DMSO (0.1%), HMA (1 μM), Dam (10 μg/ml), genistein (100 μM: Gen), K252a (2 μM: K), staurosporine (100 nM: Sta) or GF109203X (2 μg/ml: GF) for 1 h and then mock irradiated (−) or irradiated with UV at 10 J/m^2 (+). 40 h after the irradiation, DNA was isolated and analyzed on a 1.5% agarose/ethidium bromide gel.

duced a marginal change in the morphology, yet it synergistically induced morphological damages with UV irradiation, i.e. the plasma membrane became rough and long processes appeared (Fig. 1C,D). Some of the cells were detached from the culture dishes. Similar synergism was also observed for pretreatment with Dam and UV irradiation. Treatment with Dam alone caused no detectable morphological change in UV^r-1 cells at concentrations lower than 10 μg/ml (approximately 40 μM) (Fig. 1E). However, the cells pretreated with Dam and then irradiated with UV showed a clearly different appearance (Fig. 1F). Cells with long processes and an irregular shape were frequently observed. On the other hand, treatment with genistein alone induced a rough membrane as well as long processes, and UV irradiation further stimulated these changes (Fig. 1G,H).

To elucidate whether these morphological changes reflect apoptosis, DNA fragmentation analysis was carried out. Irradiation with UV induced a ladder of fragmented DNA which is typical for apoptosis (Fig. 2). None of the tyrosine kinase inhibitors including HMA, Dam and genistein, did not produce DNA fragmentation per se. However, cells pretreated with these inhibitors and then irradiated with UV showed a remarkable increase in the fragmented DNA. Especially, Dam showed the most potent effect among the inhibitors examined. On the other hand, serine/threonine protein kinase inhibitors such as K252a, staurosporine and GF109203X suppressed UV-induced DNA cleavage (Fig. 2).

It has been reported that UV irradiation induces signal transduction including phosphorylation of ERKs, SAPKs, p38 MAP kinase and c-Jun [5]. These phosphorylation can be specifically detected by an immunoblot using phospho-specific antibodies. Activated ERKs can be detected by the phospho-specific antibody which recognizes phosphorylation at both threonine-202 and tyrosine-204 in ERKs [18]. Phosphorylated ERK1/2 increased slightly at 1 h after UV irradiation (Fig. 3A). Treatment with Dam alone enhanced the phosphorylation of ERK1/2. Pretreatment with Dam followed by UV irradiation resulted in a further increase in the level of phosphorylated ERK1/2. On the other hand, treatment with AG1478, HMA, erbstatin or genistein did not increase the phosphorylated ERKs in UV-irradiated cells. Genistein rather decreased the level of phosphorylated ERKs. Serine/threonine kinase inhibitors, K252a, staurosporine and GF109203X, were without effect on the expression level of phosphorylated ERKs.

Activated SAPK is also specifically detected by using a specific antibody that recognizes phosphorylation at threonine-183 and tyrosine-185 [19]. UV irradiation slightly increased the phosphorylation of SAPK. Pretreatment with Dam, AG1478, erbstatin or genistein enhanced the phosphorylation of SAPK in UV-irradiated cells while that with K252a and staurosporine reduced it (Fig. 3B). Phosphorylated p38 MAP kinase was not detectable by using the phospho-specific antibody irrespective of the treatment with inhibitors or UV irradiation (data not shown).

The transcriptional activity of c-Jun is regulated by phosphorylation at Ser-63 and Ser-73 which is induced by activated SAPK [20]. Phosphorylated c-Jun can be specifically detected by using the phospho-specific anti-c-Jun antibody.

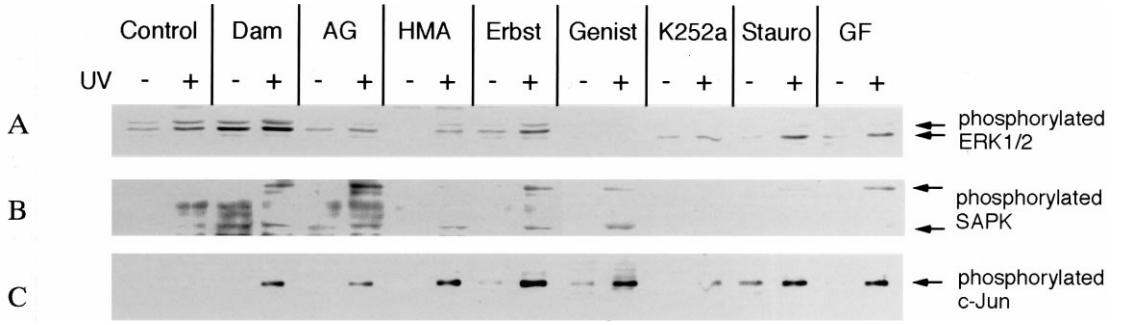

Fig. 3. Effects of protein kinase inhibitors and UV irradiation on signalling molecules in UV^r-1 cells. Cells were pretreated with DMSO (0.1%: Control), Dam (10 μg/ml), Tyrphostin AG1478 (100 nM: AG), HMA (1 μM), erbstatin (5 μg/ml: Erbst), genistein (100 μM: Genist), K252a (2 μM: K), staurosporine (100 nM: Stauro) or GF109203X (2 μg/ml: GF) for 1 h and then mock irradiated (−) or irradiated with UV at 10 J/m^2 (+). After culturing for 1 h, cell extracts were prepared and analyzed by immunoblot using anti-phospho-specific ERK (A), anti-phospho-specific SAPK (B) and anti-phospho-specific c-Jun (C) antibodies. Cytoplasmic extracts were examined in A and B, while nuclear extracts were analyzed in C.

Immunoblot analysis of the nuclear extract revealed that the level of phosphorylated c-Jun did not significantly increase by UV irradiation or inhibitor treatment alone. However, a noticeable increase in the phosphorylation was observed when cells were pretreated with tyrosine kinase inhibitors and then irradiated with UV (Fig. 3C). The most prominent increase was observed for erbstatin while AG1478 showed less effect. A similar increase in phosphorylated c-Jun was also observed after pretreatment with serine/threonine kinase inhibitors such as staurosporine and GF109203X followed by UV irradiation.

4. Discussion

It has been well documented that irradiation with UV resulted in the early activation of tyrosine kinases [1–4]. This suggests that the induction of apoptotic cell death by UV irradiation was mediated by tyrosine kinases. Thus, it was expected that inhibition of tyrosine kinases resulted in suppression of apoptosis. However, the present study revealed that tyrosine kinase inhibitors rather stimulated UV-induced apoptosis (Fig. 2). Moreover, any of these inhibitors tested did not suppress but potentiated the activation of SAPK (Fig. 3B). Since the activation of SAPK was frequently observed in apoptotic cells [6], this effect of tyrosine kinase inhibitors might be related to their stimulatory effect on UV-induced apoptosis.

Activated SAPK can phosphorylate and activate c-Jun [20]. The level of phosphorylated c-Jun also increased after treatment with tyrosine kinase inhibitors followed by UV irradiation (Fig. 3C). However, the extent of c-Jun phosphorylation was not necessarily correlated to the level of apoptosis, e.g. HMA and genistein potently increased phosphorylated c-Jun but showed less stimulatory effects on UV-induced apoptosis as compared to Dam (Figs. 2 and 3C). Furthermore, c-Jun was also synergistically activated by treatment with serine/threonine kinase inhibitors and UV irradiation despite that these inhibitors rather suppressed UV-induced apoptosis (Figs. 2 and 3C). Therefore, activation of c-Jun by itself may not be sufficient to determine the fate to apoptosis.

The signalling pathway of the activation of SAPK and c-Jun by treatment with tyrosine kinase inhibitors is not fully understood. AG1478 and HMA are an EGF-receptor kinase inhibitor and a Src kinase inhibitor, respectively [21,16]. Hence, these tyrosine kinases seem to be unnecessary for the activation of SAPK as well as for UV-induced apoptosis. This is in good agreement with the recent report of Iordanov et al. that activation of SAPK but not of ERKs in response to UV might be triggered by damage to ribosomal RNA [22]. This pathway was termed the ribotoxic stress response. It is possible that suppression of a tyrosine kinase-mediated pathway evokes the other signalling pathways such as the ribotoxic stress response.

Dam is an anthraquinone compound isolated from the root of *Morinda citrifolia* [12]. Dam has been reported to inhibit specifically tyrosine kinases such as Lck, Src, Fyn and ErbB-2 with half maximum inhibition concentrations between 17 and 700 nM [23]. It is also known to reverse the phenotype of Ki-*ras*-transformed NRK cells [12]. Dam showed a unique activity to induce the activation of ERKs (Fig. 3A) and the most potent stimulatory effect on UV-induced apoptosis among the kinase inhibitors examined (Fig. 2). This might be due to the specific tyrosine kinase inhibitory activity of Dam. Alternatively, Dam might possess an additional activity other than the inhibition of protein kinases. Our recent study revealed that Dam has an inhibitory activity towards serine and cysteine proteinases such as thrombin and calpain [24]. It is possible that this protease inhibitory activity of Dam is also involved in the biological activity of the compound. Further study will elucidate whether the ERK activation in relation to apoptosis is subtly regulated by a serine or a cysteine proteinase.

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