Research Article

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UVB-irradiated indole-3-acetic acid induces apoptosis via caspase activation

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Abstract

Objective: Indole-3-acetic acid (IAA) activation has been suggested as a new strategy for cancer therapy. It has been reported that ultraviolet B (UVB) radiation can activate IAA. In the present study, we investigated whether UVB-irradiated IAA (IAA^{UVB}) can induce apoptosis of G361 human melanoma cells and examined the apoptotic pathway involved.
Methods: DNA fragmentation was measured to examine apoptosis. IAA^{UVB}-induced signaling pathways were investigated by Western blot analysis.
Results: Our results show that IAA^{UVB} reduced cell viability of G361 human melanoma cells, and induced DNA fragmentation, a hallmark of apoptosis. We also found that c-Jun NH_2-terminal kinase (JNK) and p38, which are activated by IAA^{UVB}, are not associated with this cell death. We further investigated the IAA^{UVB}-mediated apoptotic pathway after pretreatment with NS398, vitamin C, and N-acetylcysteine (NAC). Although NS398, an inhibitor of cyclooxygenase-2, was not protective, vitamin C and NAC ameliorated IAA^{UVB}-mediated cell death. In addition, when cells were pretreated with a caspase inhibitor, IAA^{UVB}-induced apoptosis was inhibited.
Conclusions: These results suggest that free radicals generated from IAA by UV irradiation may cause apoptosis, and IAA^{UVB} induces apoptosis of G361 human melanoma cells by activating caspases.

Keywords: Indole-3-acetic acid; Ultraviolet B; Melanoma; Apoptosis; Caspase.

Özet

Amaç: İndol-3-asetik asit (IAA) aktivasyonu, kanser tedavisi için yeni bir strateji olarak önerilmiştir. Ultraviole B (UVB) radyasyonunun IAA'yi aktive edebileceği bildirilmiştir. Bu çalışmada, UVB ışınlanmış IAA'nın (IAA^{UVB}) G361 insan melanoma hücrelerinin apoptozunu indükleyip güçsüzleştiremeyeceği ve ilgili apoptotik yol araştırılmıştır.
Metodlar: Apoptozu incelemek için DNA parçalanması ölçülmüştür. IAA^{UVB} kaynaklı sinyal yolakları Western blot analizi izlenmiştir.
Bulgular: IAA^{UVB}'nin G361 insan melanom hücrelerinin hücre yaşamını azalttığını ve apoptozun DNA fragmentasyonunu tetiklediğini göstermektedir. IAA^{UVB} tarafından aktive edilen c-Jun NH2-terminal kinaz (JNK) ve p38'in de bu hücre ölümüyle ilişkili olmadığı bulunmuştur. Daha sonra NS398, vitamin C ve N-asetilsistein (NAC) ile ön tedavi sonrası IAA^{UVB} aracılı apoptotik yol üzerinde araştırmalar yapılmıştır. NS398, siklooksijenaz-2'nin bir inhibitörü olmasına ve koruyucu olmamasına rağmen, C vitamini ve NAC, IAA^{UVB} aracılı hücre ölümünü iyileştirmiştir. Buna ek olarak, hücreler bir kaspaz inhibitörü ile önceden muamele edildiğinde, IAA^{UVB} ile indüklenen apoptoun inhibe edildiği görümüştür.
Sonuç: Bu sonuçlara göre UV radyasyon ile IAA'dan üretilen serbest radikallerin apoptozise neden olabileceği ve IAA^{UVB}'nin kaspazları aktive ederek G361 insan melanom hücrelerinin apoptozunu indüklediğini düşündürmektedir.

Anahtar Kelimeler: Indol-3-asetik asit; Ultraviole B; Melanom; Apoptoz; Kaspaz.

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Introduction

Indole-3-acetic acid (IAA) plays important roles in plant cell division and differentiation [1]. Although IAA is not toxic in and of itself, it is converted into various cytotoxic substances upon interacting with horseradish peroxidase (HRP) [2, 3]. We also reported that the combination of IAA/HRP produces H_2O_2, an active reactive oxygen species (ROS) [4]. Therefore, IAA/HRP combination has been suggested for use as a new cancer therapy [5–7]. However, because it is difficult to deliver HRP to target cancer cells, other methods to activate IAA are needed.

IAA can also be activated by ultraviolet B (UVB) radiation, thereby producing free radicals [8]. To confirm that free radicals are involved in IAA-induced cell death, we used vitamin C and N-acetylcysteine (NAC) as free radical scavengers. Vitamin C is a strong electron donor, such that it is an effective water-soluble antioxidant against oxidative stress [9]. NAC is another antioxidant and has been used to treat paracetamol poisoning, because it increases glutathione, a biological antioxidant [10]. Furthermore, we entertained the possibility that UVB-treated IAA (IAA^{UVB}) could also produce H_2O_2. IAA^{UVB} did not produce H_2O_2, but did produce other free radicals, which induced apoptosis in G361 human melanoma cells [8]. Therefore, IAA could be used as a new photosensitizer for photodynamic therapy, because IAA alone is not cytotoxic. These results suggest that the combination of IAA and light could serve as a novel candidate for the treatment of malignant melanoma using photodynamic therapy.

c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) are major mediators of various stress signals. Furthermore, JNK and/or p38 MAPK activation is required for cellular apoptosis [8, 11]. Therefore, we sought to determine whether IAA^{UVB} has an influence on JNK and/or p38 MAPK activation, and if so, whether this activation could lead to apoptosis.

Cyclooxygenase-2 (COX-2) is induced by inflammatory stimuli such as lipopolysaccharides [12, 13]. Recent research indicates that ROS induce COX-2 expression in synovial fibroblasts [14]. Furthermore, it has been reported that COX-2 activation is deeply involved in endothelial cell apoptosis [15]. Therefore, it is possible that IAA^{UVB}-induced free radicals can lead to apoptosis via COX-2 activation.

Caspases, a family of cysteine-dependent aspartate-specific proteases, are key agents in the regulation of apoptotic processes [16]. Previously, we reported that IAA^{UVB} activates caspase-8, which results in caspase-3 activation [8]. Therefore, in the present study, we examined whether a pan-caspase inhibitor could block IAA^{UVB}-induced apoptosis of melanoma cells.

Materials and methods

Materials

Indole-3-acetic acid (IAA), vitamin C, and NAC were obtained from Sigma (St. Louis, MO, USA). Antibodies that recognize phospho-JNK (CST-9251) and phospho-p38 (CST-9211) were obtained from Cell Signaling Technology (Danvers, MA, USA). COX2 (sc-34285) and actin (I-19) antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). A pan-caspase inhibitor, benzyloxycarbonyl-Val-Ala-Asp-(O-methyl)-fluoromethylketone (z-VAD-FMK) was purchased from R & D Systems (Minneapolis, MN, USA).

Inhibitors

SB203580, SP600125, and NS398 were purchased from Calbiochem (San Diego, CA, USA). SB203580 is a specific inhibitor of the p38 MAPK pathway, whereas SP600125 is a reversible inhibitor of the JNK pathway. NS-398 is a selective inhibitor of COX-2.

Cell culture

A lightly pigmented human melanoma cell line, G361 (ATCC, Rockville, MD, USA), was grown in RPMI supplemented with 10% fetal bovine serum and penicillin-streptomycin (100 U/mL and 100 μg/mL, respectively) in 5% CO_2 at 37°C.

UVB irradiation

An IAA stock solution (100 mM) was irradiated once with a UVB source (BLE-1T158, Spectronics Corp., Westbury, NY, USA). The energy administered was measured using a Waldmann UV meter (model no. 585100; Waldmann Co., VS-Schwenningen, Germany). To reach a UVB dose of 100 mJ/cm^2, IAA stock solution was irradiated with a UVB lamp for 3 min 16 s. Immediately after UV irradiation, IAA^{UVB} was added to 24-well plates containing G361 cells.

Cell viability determination by crystal violet assay

Cell viability was measured using a crystal violet assay [17]. After incubating G361 cells with IAA (1 mM)

or IAA^{UVB} (1 mM/100 mJ/cm^2) for the indicated times (0–8 h), culture medium was removed and replaced with 0.1% crystal violet in 10% ethanol for 5 min at room temperature. The cells were then rinsed four times with distillated water, and adherent crystal violet was extracted with 95% ethanol. Absorbance was determined at 590 nm using an ELISA reader. Data represent the mean ± SD of triplicate assays expressed as percentages of the control. Each experiment was repeated at least twice independently, and representative results are shown.

Detection of DNA fragmentation

After serum starvation for 24 h, G361 cells were treated with IAA^{UVB} (1 mM/100 mJ/cm^2). Cells were further cultured for 3–24 h, then harvested. Genomic DNA was isolated using genomic DNA purification kits according to the manufacturer's recommendations (Promega, Madison, WI, USA). Ten micrograms of DNA from each sample were separated by 1.9% agarose gel electrophoresis and visualized by ethidium bromide staining.

Western blot analysis

G361 cells were grown in 60-mm culture dishes, starved of serum for 24 h, treated with IAA^{UVB} (1 mM/100 mJ/cm^2) for 1–4 h, and lysed in cell lysis buffer [62.5 mM Tris-HCl (pH 6.8), 2% SDS, 5% β-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride, protease inhibitor cocktail (Roche, Mannheim, Germany), 1 mM Na_3VO_4, 50 mM NaF, and 10 mM EDTA]. Ten micrograms of protein per lane was separated by SDS-polyacrylamide gel electrophoresis and blotted onto PVDF membranes, which were then blocked with 5% fat-free dried milk in Tris-buffered saline containing 0.4% Tween 20. Blots were incubated with the appropriate primary antibodies at a dilution of 1 : 1000, and then further incubated with HRP-conjugated secondary antibody. Bound antibodies were detected using enhanced chemiluminescence plus kits (Amersham International, Little Chalfont, UK).

Statistics

Differences between results were assessed for significance using the Student's t-test.

Results

IAA^{UVB}-induced apoptosis of human melanoma cells

We examined the effect of IAA^{UVB} on the viability of G361 human melanoma cells. IAA was irradiated with UVB (100 mJ/cm^2), then administered to G361 cells. When G361 cells were incubated with 1 mM IAA^{UVB}, cell viability decreased in a time-dependent manner (Figure 1A). By 8 h after IAA^{UVB} addition, cell viability had decreased by more than 50%. When IAA was not irradiated with UVB, it was not effective at all (Figure 1B). We next examined DNA fragmentation after treating cells with IAA^{UVB} (1 mM) for varying time periods. Increasing the treatment time period resulted in more extensive DNA fragmentation (Figure 1C). These results show that IAA^{UVB} induces apoptosis of human melanoma cells.

Possible pathways governing the apoptosis induced by IAA^{UVB}

We examined possible pathways involved in IAA^{UVB}-induced apoptosis. G361 human melanoma cells were treated with IAA^{UVB} for 0–4 h, and the levels of several proteins involved in apoptosis were measured. We determined the levels of cyclooxygenase-2, phospho-JNK, and phospho-p38. All of these proteins were increased upon treatment with IAA^{UVB} (Figure 2A). Among these, phospho-p38 was the most prominent. It was present at a very low level in non-treated control cells, and was markedly increased by IAA^{UVB} treatment. The level was sustained for 4 h. However, the level of phospho-JNK was briefly increased at 1 h, and returned to normal at 4 h. The level of cyclooxygenase-2 was increased by IAA^{UVB} treatment. These results suggested that increased expression of COX2 and activation of JNK and p38 may be involved in IAA^{UVB}-induced apoptosis of human melanoma cells.

We then tested the effects of specific inhibitors for these possible apoptosis mediators along with vitamin C and NAC. Although neither SB203580, SP600125, nor NS398 was protective, vitamin C and NAC protected G361 cells from IAA^{UVB}-induced cytotoxicity (Figure 2B and C). These results suggested that neither COX2, JNK, nor p38 mediate IAA^{UVB}-dependent cytotoxicity, but instead that an oxidative process initiated by IAA^{UVB} may underlie its cytotoxic effect.

We also examined the protective effect of a pan-caspase inhibitor, z-VAD-FMK, on IAA^{UVB}-dependent cytotoxicity.

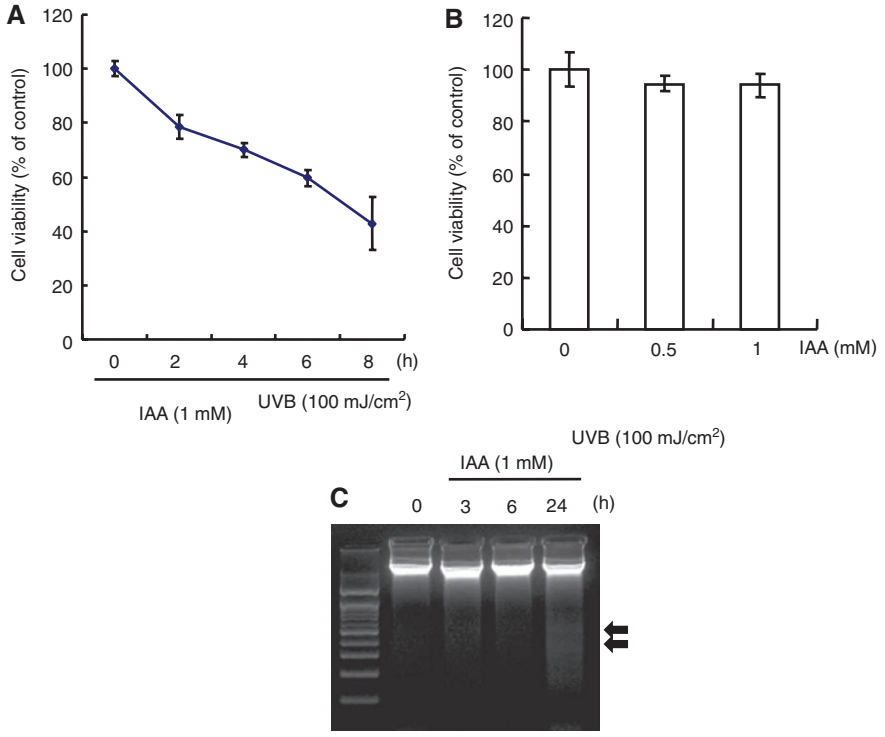

Figure 1: Effects of IAA^{UVB} on apoptosis of G361 human melanoma cells.
(A) G361 cells were treated with IAA (1 mM) which was irradiated with UVB (100 mJ/cm^2). After treatment for 0–8 h, cell viabilities were measured using a crystal violet assay. (B) G361 cells were treated with IAA (0.5 or 1 mM). After treatment for 24 h, cell viability was measured using crystal violet assay. Data represent the means ± SD (n = 3). (C) G361 cells were treated with IAA^{UVB} for 0–24 h. Ten micrograms of genomic DNA was extracted from cells and separated by 1.9% agarose gel electrophoresis. After staining with ethidium bromide, gels were photographed under UV.

When G361 human melanoma cells were treated with 1 mM IAA^{UVB} for 6 h, cell viability was decreased by ~40% (Figure 3). The cell viability was increased by pre-treating cells with the pan-caspase inhibitor. These results show that caspase activation is a cause of IAA^{UVB}-induced death of human melanoma cells.

Discussion

Since IAA alone has no cytotoxic effect on mammalian cells, IAA is considered a prodrug that can be activated by HRP [2, 7]. We also used IAA as a prodrug and potential cancer therapy. However, we activated IAA using light instead of HRP [8]. That study indicated that UVB stimulated IAA and produced free radicals, which could cause human melanoma cell apoptosis. IAA also reportedly promotes the efficacy of photodynamic cancer therapy using phenothiazinium dyes [18]. Therefore, in the present study, we sought to determine the signal transduction pathway by which IAA^{UVB} causes apoptosis.

Previously, we showed that IAA^{UVB} did not generate ROS, but rather produced other kinds of free radicals [8]. Oxidized IAA can generate many other free radicals aside from ROS, such as indolyl, skatolyl and peroxyl radicals [19–21]. Thus, it is difficult to identify the free radicals responsible for IAA^{UVB}-induced apoptosis. Nevertheless, IAA^{UVB} clearly induced lipid peroxidation [8]. Although further studies are needed, lipid peroxidation was also initiated by indolyl radical cations in a previous study [22]. Therefore, it is possible that these free radicals could activate JNK and p38 MAPK. In the present study, we clearly showed that IAA^{UVB} induces the phosphorylation of JNK and p38 MAPK. Since JNK and/or p38 MAPK activation is known to cause apoptosis [8, 11], we pretreated cells with SP600125 (a JNK pathway inhibitor) or SB203580 (a p38 MAPK pathway inhibitor) before IAA^{UVB} treatment. However, neither SP600125 nor SB203580 blocked IAA^{UVB}-induced cell death. These results indicate that JNK and/or p38 MAPK activation did not induce apoptosis of G361 melanoma cells.

In a previous report, we suggested that IAA^{UVB} might produce peroxyl radicals [8]. Moreover, it has been reported that 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH, a peroxyl radical generator) induces COX-2 expression in human skin keratinocytes [23].

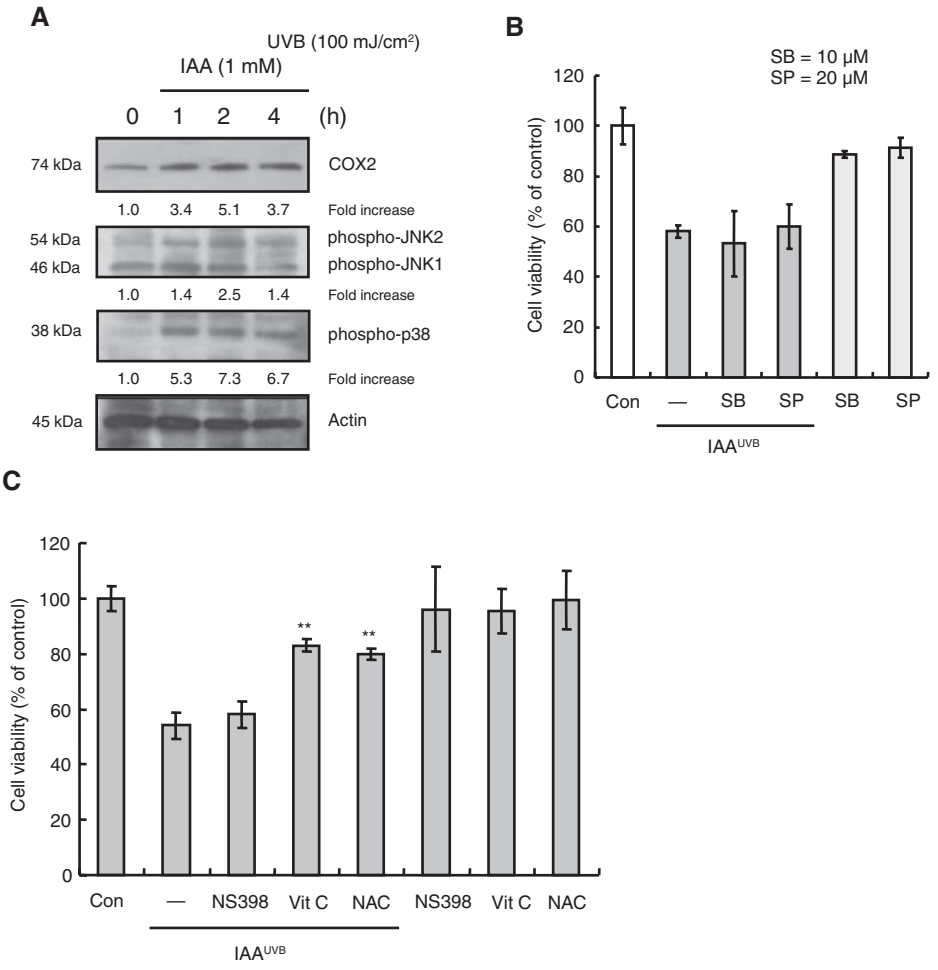

Figure 2: Effects of IAA^{UVB} on signal transduction pathways associated with apoptosis.
(A) After 24 h of serum starvation, G361 cells were treated with IAA^{UVB} (1 mM) for 4 h. COX2, phospho-JNK, and phospho-p38 levels were measured by Western blot analysis, and equal protein loading was confirmed using an anti-actin antibody. Fold increases over the level of the control were determined by densitometric analysis and are shown below each lane. (B) G361 cells were pretreated with 10 μM SB203580 (SB) or 20 μM SP600125 (SP), then treated or not with IAA^{UVB} (1 mM) for 6 h. Cell viabilities were measured using a crystal violet assay. (C) G361 cells were pretreated with 5 μM NS398, 1 mM vitamin C, or 10 mM NAC, then treated with IAA^{UVB} (1 mM) for 6 h or left untreated, after which cell viability was measured. Data represent mean ± SD (n = 3).

Therefore, we also checked whether IAA^{UVB} could induce COX-2 expression. Our results showed that IAA^{UVB} did increase COX-2 protein levels (Figure 2A). It has also been reported previously that COX-2 activation can cause apoptosis [15]. Thus, we used NS398, a selective COX-2 inhibitor, before IAA^{UVB} treatment. However, NS398 did not block IAA^{UVB}-induced cell death. These results suggest that COX-2 expression induced by IAA^{UVB} is not responsible for apoptotic cell death.

Although JNK and p38 MAPK activation and COX-2 expression, are not directly related to IAA^{UVB}-induced apoptosis, free radicals are deeply involved in apoptosis. We clearly showed that antioxidants such as vitamin C and NAC increase protection of G361 cells from these apoptotic stimuli (Figure 2C). Therefore, we propose that IAA^{UVB}-induced free radicals are responsible for its apoptotic effects.

It has been reported that AAPH induces apoptosis of human hepatic HepG2 cells [24]. That study indicated that AAPH activates caspase-9 and -3, which leads to apoptotic cell death. We also reported that IAA^{UVB} resulted in caspase-8 and -3 activation [8], indicating that IAA^{UVB}-induced free radicals activate caspases. Therefore, in the present study, we pretreated cells with a pan-caspase inhibitor, z-VAD-FMK, before administering IAA^{UVB}. The results showed that the pan-caspase inhibitor significantly inhibited IAA^{UVB}-induced apoptosis (Figure 3).

In conclusion, this study showed that IAA^{UVB} causes apoptosis via the activation of caspases in human

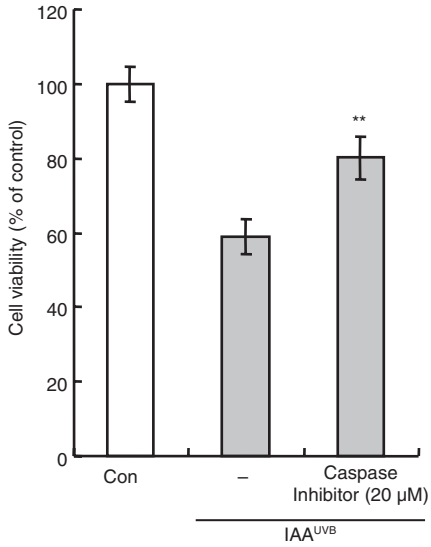

Figure 3: Effects of IAA^{UVB} on the caspase pathway.
The pan-caspase inhibitor, z-VAD-FMK, was added to the culture medium of G361 cells. Cells were then treated with IAA^{UVB} (1 mM) for 6 h, and cell viability was measured. Data represent mean ± SD (n = 3). **, Significantly statistically different, with $p < 0.01$

melanoma cells. These results may help to develop a new photodynamic therapy for melanoma.

Conflict of interest: The authors have no conflicts of interest.

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