UVB-Activated Indole-3-Acetic Acid Induces Apoptosis of PC-3 Prostate Cancer Cells

SU YEON KIM[1], JUNG SU RYU[1], HAILAN LI[1], WOO-JAE PARK[1], HYE-YOUNG YUN[1], KWANG JIN BAEK[1], NYOUN SOO KWON[1], UY DONG SOHN[2] and DONG-SEOK KIM[1,3]

Departments of [1]Biochemistry, Chung-Ang University College of Medicine, and [2]Pharmacology, College of Pharmacy, Chung-Ang University, and [3]Research Institute for Translational System Biomics, Chung-Ang University College of Medicine, Chung-Ang University, 221 Heukseok-dong, Dongjak-gu, Seoul 156-756, Korea

Abstract. *Indole-3-acetic acid (IAA) has recently shown anticancer activity in combination with horseradish peroxidase. The current study demonstrated that IAA irradiated with ultraviolet B (IAA^{UVB}) is able to generate free radicals and induce cell death in a time-dependent fashion in PC-3 prostate cancer cells, while PC-3 cells treated with IAA alone exhibited no toxic responses. It was also found through Western blot analysis that the cytotoxic effect of IAA^{UVB} resulted from apoptosis. Treatment with IAA^{UVB} for 24 hours showed a significant increase in phosphorylated p38 mitogen-activated protein kinase and c-Jun N-terminal kinase, the stress signaling proteins. Furthermore, pro-caspases (-3, -8, and -9) were clearly down-regulated and poly(ADP-ribose) polymerase cleavages were demonstrated in the group treated with IAA^{UVB}. Flow cytometric analysis also demonstrated the induction of apoptosis by IAA^{UVB} in PC-3 cells. In conclusion, this study demonstrated that IAA induced cell death in combination with UVB irradiation by increasing apoptosis in PC-3 cells.*

Indole-3-acetic acid (IAA), the major form of a plant growth hormone in higher plants (auxin), is a heterocyclic compound known to regulate cell division, elongation, and differentiation (1, 2). Since 1940, a number of IAA analogues have been created for use in horticulture and agriculture, and as herbicides for weed control (2). Currently, however, IAA is attracting the interest of medical researchers.

Correspondence to: Dong-Seok Kim, Ph.D., Department of Biochemistry, Chung-Ang University College of Medicine, 221 Heukseok-dong Dongjak-gu, Seoul 156-756, Korea. Tel/Fax: +82 28205768, e-mail: ds_kim@cau.ac.kr

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Reactive oxygen species (ROS), the products of a reaction between IAA and extracellular peroxidase, have been reported to play a role in cell elongation of plants (3). Currently, this ROS-producing reaction of IAA with peroxidase is emerging as a novel anticancer therapy. It has been suggested that IAA has merit as a potential pro-drug for cancer therapy in combination with horseradish peroxidase (HRP) (4). Indeed, there have been many reports describing the possibility of IAA/HRP as a novel anticancer therapy, such as a pro-drug combined with a monoclonal antibody, gene therapy, or polymers (5-7).

It has been previously reported that IAA/HRP had cytotoxic activity against G361 melanoma, while IAA alone did not produce any changes (8), in which the main effector was proposed to be hydrogen peroxide (9, 10). It was also found that IAA can produce free radicals with ultraviolet B (UVB) radiation, as in the IAA/HRP system. The antitumor effect of IAA is sufficiently potent to exert cytotoxic effects on melanoma (11). Furthermore, it was demonstrated that IAA and intense pulse light (IPL) combination therapy significantly slowed the growth of tumors implanted in mice, indicating that IAA might be a promising enhancing agent for photodynamic therapy (PDT) (7, 11, 12).

Prostate cancer is the most commonly diagnosed cancer and the second most common cause of death in American men (13). One man in six is diagnosed with prostate cancer in their lifetime and the death rate is up to 3% (14). Although it has been reported that IAA irradiated with ultraviolet B light (IAA^{UVB}) induces apoptosis of human melanoma cells, the effects of IAA^{UVB} on prostate cancer cells have not been studied. The current therapy options for patients with prostate cancer have serious side effects and few salvage options exist when current therapy fails (15). This situation leads to the search for new and safer methods to cure prostate cancer.

The present study determined the effects of IAA in combination with UVB in PC-3 cells, and examined the possible application as a new PDT for prostate cancer.

Materials and Methods

Materials. IAA and Trolox were obtained from Sigma (St. Louis, MO, USA). 2,7-Dichlorofluorescin diacetate (DCFH-DA) was obtained from Calbiochem (San Diego, CA, USA). Antibodies recognizing caspase-8 (sc-7890), caspase-3 (sc-7272), caspase-9 (sc-8355), and actin (I-19) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and anti-PARP antibody were obtained from BD Pharmingen (San Diego, CA, USA). Antibodies against phospho-specific p38 mitogen-activated protein kinase (MAPK; Thr180/Tyr182, #9211S), total p38 MAPK (#9212S), phospho-specific c-Jun *N*-terminal kinase (JNK; Thr 183/Tyr 185, #9251), and total JNK (#9258) were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA).

Cell cultures. PC-3 cells were obtained from ATCC (Rockville, MD, USA). Cells were grown in F12K medium supplemented with 10% FBS and 1% penicillin + streptomycin (10,000 U/ml and 10 mg/ml, respectively) in 5% CO_2 at 37˚C.

Crystal violet assay. Cell viability was measured using crystal violet assays. After treating PC-3 cells for 24 h, culture media were removed and replaced with 1% crystal violet in 10% ethanol for five min at room temperature. The cells were then rinsed four times with distilled water, stained, and crystal violet was extracted with 95% ethanol. Absorbance was determined at 590 nm using an ELISA reader (VERSAMAX; Molecular Devices, Sunnyvale, CA, USA).

Free radical determination. Free radical formation was determined using DCFH-DA, which is oxidized by free radicals to dichlorofluorescein (DCF). To activate DCFH-DA, 350 μl of a 1 mM stock solution of DCFH-DA in ethanol was mixed with 1.75 ml of 0.01 N NaOH and allowed to stand for 20 min before adding 17.9 ml of 25 mM sodium phosphate buffer (pH 7.2). Reaction mixtures contained activated DCFH-DA solution and IAA (500 μM), and were irradiated with UVB (100 mJ/cm^2). Absorbancies were determined at room temperature at 490 nm.

Western blot analysis. PC-3 cells were grown in 60-mm culture dishes. After serum starvation for 24 h, cells were treated for 24 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 inhibitors [Complete™; Roche, Mannheim, Germany], 1 mM Na_3VO_4, 50 mM NaF, and 10 mM EDTA). Twenty micrograms of protein per lane were separated by SDS-polyacrylamide gel electrophoresis and blotted onto PVDF membranes, which were then saturated with 5% fat-free dried milk in Tris-buffered saline containing 0.4% Tween 20. Blots were then incubated with the appropriate primary antibodies at a dilution of 1:1000, and further incubated with horseradish peroxidase-conjugated secondary antibody. Bound antibodies were detected using an enhanced chemiluminescence plus kit (Amersham International, Little Chalfont, UK).

Flow cytometric analysis for apoptosis. After treating PC-3 cells with IAA^{UVB}, IAA alone, or UVB alone for 24 h, culture supernatants were collected for floating dying and apoptotic cells. Adherent cells were then harvested by brief trypsinization. Two cell fractions were then mixed and washed with PBS. Cells were labeled with FITC-conjugated annexin V and propidium iodide (PI) using TACS™ Annexin V-FITC kits (Trevigen, Gaithersburg, MD, USA) and flow cytometric analyses were performed on a FACSCalibur™ (Becton Dickinson, San Jose, CA, USA). The fluorescence of FITC- and PI-labeled cells was then measured.

Statistical analysis. The data are expressed as the mean±SD. The statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Dunnett's test. Differences were considered significant at a $p<0.05$.

Results

The UVB-activated IAA (IAA^{UVB}) increases the formation of free radicals. It has been reported that UVB activates IAA to produce free radicals (11). Accordingly, the formation of free radicals was measured at 500 μM of IAA in the presence of 100 mJ/cm^2 of UVB, which resulted in the production of free radicals (Figure 1). As previous work revealed that IAA^{UVB} generated free radicals in a dose-dependent fashion (11), in this study it was confirmed that IAA at 500 μM produced free radicals when irradiated by 100 mJ/cm^2 of UVB.

UVB-activated IAA (IAA^{UVB}) induces cell death of PC-3 cells. The viability of PC-3 cells treated with IAA and IAA^{UVB} was determined. As shown in Figure 2a, crystal violet assay showed treating PC-3 cells with IAA^{UVB} (500 μM and 100 mJ/cm^2) led to a decrease in cell viability. It was observed that the highest dose of IAA (1000 μM) did not kill cells without UVB. However, the cytotoxic actions of IAA were greatly increased in a dose-dependent fashion when co-treated with 100 mJ/cm^2 of UVB. At 200 μM IAA showed >40% cell cytotoxicity in combination with UVB. The doses of IAA and UVB were determined by a free radical forming assay. Therefore, IAA (500 μM) and UVB (100 mJ/cm^2) was used as an experimental dose for further experiments. It was also confirmed that IAA^{UVB} exhibited cytotoxic effects in a time-dependent manner (Figure 2b). After 24 h, >70% of cells failed to survive. This cytotoxic action of IAA^{UVB} was blocked by 40 μg/ml of Trolox, a water-soluble vitamin E derivative, demonstrating that free radicals from IAA^{UVB} are responsible for the observed decrease in cell viability of PC-3 cells (Figure 2c).

Effects of IAA^{UVB} on the stress signaling pathways. It has been noted previously that IAA-derived free radicals caused activation of JNK and p38 MAPK by phosphorylation in G361 human melanoma cells (8). Therefore, this study also examined the effects of IAA^{UVB} on the phosphorylation of JNK and p38 MAPK in PC-3 cells. As shown in Figure 3, it was found that only IAA^{UVB} induced JNK and p38 MAPK phosphorylation, whereas IAA or UVB alone-treated groups failed to activate JNK and p38 MAPK.

Effects of IAA^{UVB} on the apoptotic cell signaling pathways. Based on the above results, this study aimed to determine

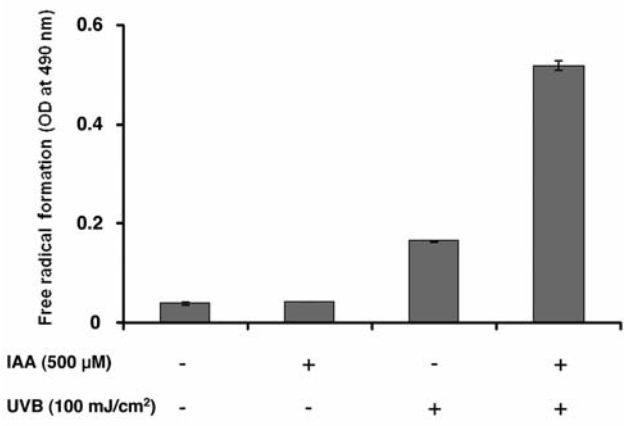

Figure 1. *Free radical production by IAA^{UVB}. The formation of free radicals was determined by DCFH-DA, which is oxidized by free radicals to DCF. IAA (500 μM) in the presence of 100 mJ/cm^2 of UVB resulted in the production of free radicals. Absorbance (OD: optical density) was determined at 490 nm. The values are expressed as the mean±SD.*

whether or not IAA^{UVB} induces cell death through activation of the apoptotic pathways. It is well-known that caspase-3, -8, and -9 are involved in the apoptotic process and poly(ADP-ribose) polymerase (PARP) cleavage is the most apparent phenomenon when cells undergo apoptosis. PARP, a 116 kDa protein, is cleaved into 85-kDa fragments during apoptotic cell death. To verify activation of the apoptotic pathways, anti-caspase-8, -9, and -3 antibodies directed against the precursor forms of the respective caspases were used. By Western blot analysis, although IAA or UVB had little effect on the activation of all caspases, it was shown in this study that the expression of precursors of caspase-3, -8, and -9 were decreased and PARP was definitely cleaved into two fragments by 24 h treatment with IAA^{UVB} (Figure 4). These results suggest that IAA^{UVB} combination therapy may have a stronger capability of inducing apoptosis in PC-3 cells.

IAA^{UVB} induced apoptosis in PC-3 cells. Despite the identification of apoptotic changes in the levels of caspases and PARP cleavage, the apoptotic process in IAA^{UVB}-treated PC-3 cells was further investigated using flow cytometry. Figure 5 shows various cellular states according to annexin V binding and propidium iodide (PI) uptake. Cells were categorized into the following four populations after 24 h of IAA^{UVB} treatment: vital cells (annexin V$^-$/PI$^-$); early apoptotic cells (annexin V$^+$/PI$^-$); late apoptotic cells (annexin V$^+$/PI$^+$); and necrotic cells (annexin V$^-$/PI$^+$). As shown in Figure 5, the fractions of apoptotic annexin V$^+$/PI$^+$ cells only increased in the IAA^{UVB} group, confirming that IAA^{UVB} kills PC-3 cells *via* apoptosis.

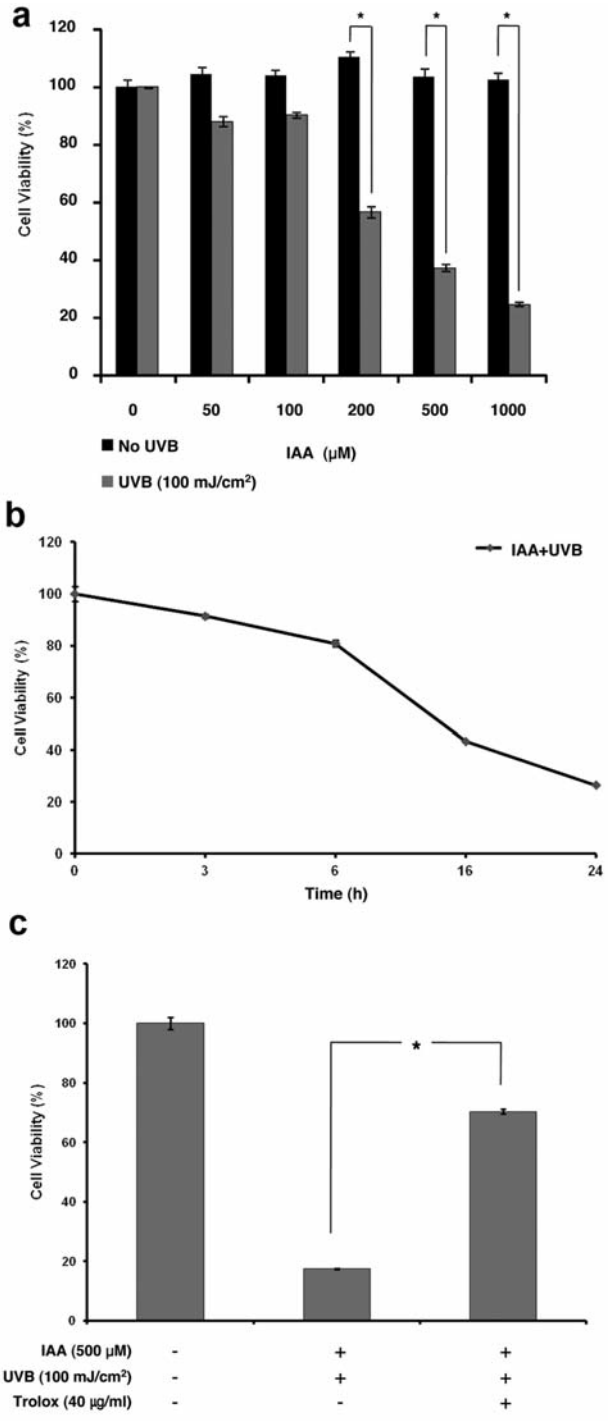

Figure 2. *Cytotoxic effects of IAA^{UVB} on PC-3 prostate cancer cells. A: Dose-dependent increase in cytotoxicty of IAA^{UVB}. Serum-starved cells were treated with varying concentrations (50-1000 μM) in the absence or in presence of UVB (100 mJ/cm^2). After treatment for 24 h, cell viability was measured. B: Time course cytotoxic effect of IAA^{UVB}. After treatment with IAA (500 μM) and UVB (100 mJ/cm^2), cell viability was checked for 24 h. C: The blockade of IAA^{UVB}-induced cytotoxicity by Trolox. Cells were pre-treated with Trolox (40 μg/ml), followed by IAA^{UVB} treatment (500 μM and 100 mJ/cm^2). All cell viabilities were measured by the crystal violet assay. All values are expressed as the mean±SD (*:p<0.05).*

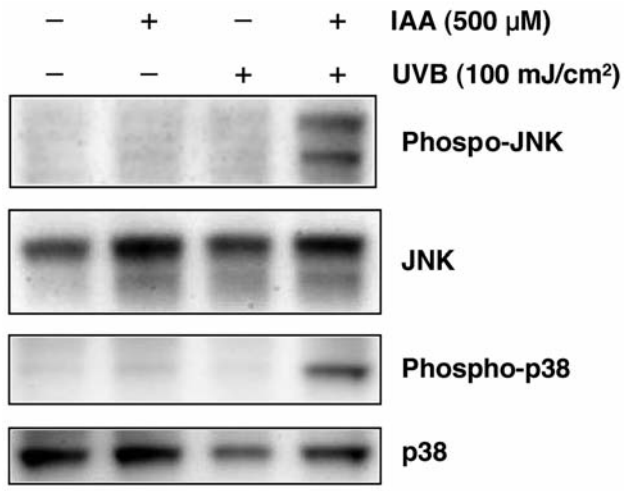

Figure 3. *Phosphorylation of the JNK and the p38 MAP kinase by IAA^{UVB} in PC-3 cells. Serum-starved cells were incubated with IAA (500 μM) or UVB (100 mJ/cm²) or IAA+UVB for 24 h. Cell lysates were then analyzed by Western blot analysis with antibody against phospho-specific JNK or p38 MAPK. Active forms of JNK and p38 MAPK were only detected in the IAA^{UVB}-treated group.*

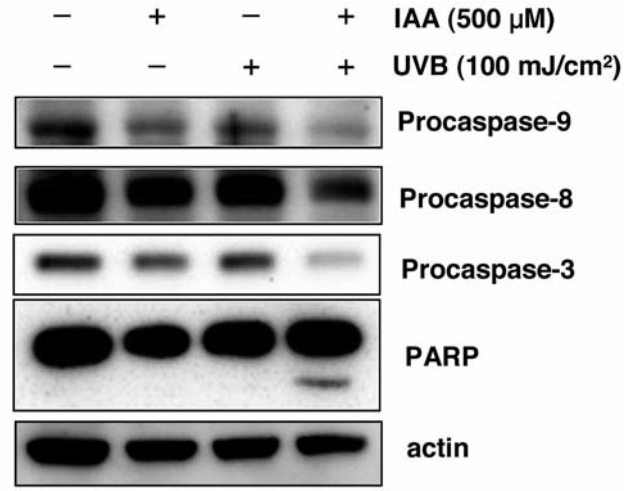

Figure 4. *Activation of caspases and induction of PARP cleavage by IAA^{UVB}. After treatment with IAA (500 μM), UVB (100 mJ/cm²), or IAA+UVB for 24 h, the activation of caspases (-3, -8, and -9) and PARP cleavage were investigated by Western blot analysis. Antibody against pro-caspases (-3, -8, and -9) were used for caspase activation. Strongly down-regulated pro-caspases and cleaved PARP were only detected in the IAA^{UVB} group.*

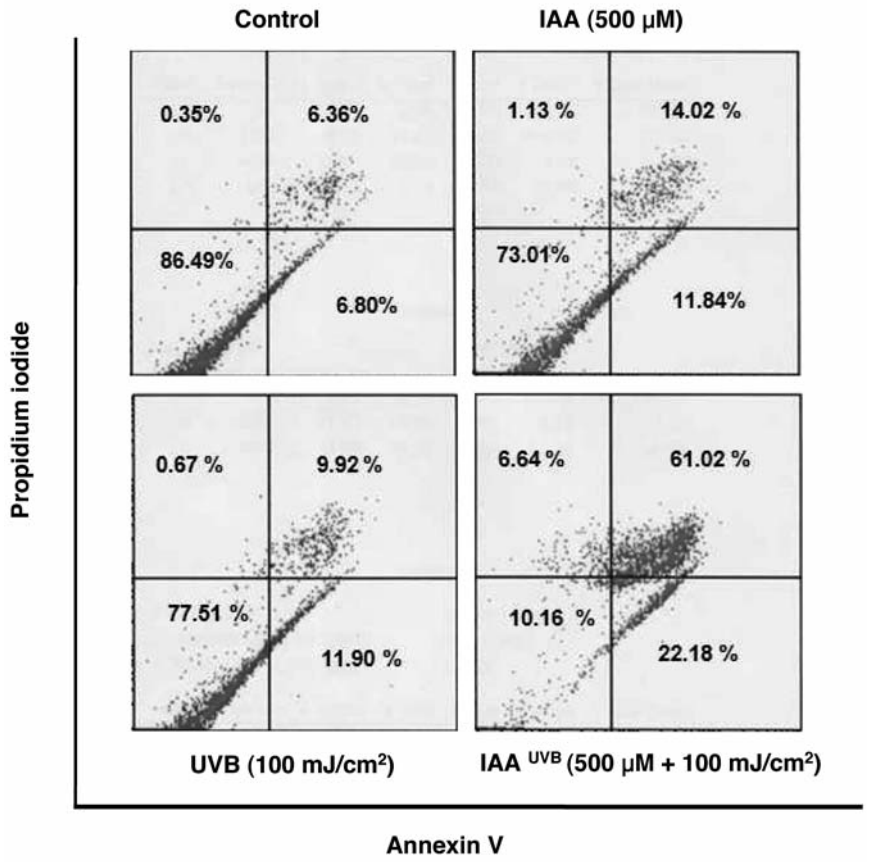

Figure 5. *Apoptosis in IAA^{UVB}-treated PC-3 by flow cytometric analysis. Cells treated with IAA (500 μM), UVB (100 mJ/cm²), or IAA+UVB for 24 h were collected and stained with annexin V and PI for analyzing apoptotic cells by flow cytometry. The highest proportion of apoptotic cells (annexin V+/PI+) was recorded in the IAA^{UVB} group (61.02%).*

Discussion

IAA/HRP combination therapy has long been suggested as a novel anti-cancer therapy (4, 16). The strength of the IAA/HRP system is that IAA alone does not induce any toxic effects on cells. IAA produces ROS in the reaction with HRP, which can effectively inhibit proliferation and induce apoptosis of cancer cells. Application of the IAA/HRP system to cancer therapy is suggested in various ways, such as antibody-directed enzyme/pro-drug therapy, gene-directed enzyme/pro-drug therapy, or polymer-directed enzyme/pro-drug therapy (4, 6, 11).

Ultraviolet radiation is known to excite various biological molecules generating ROS, such as superoxide ion or singlet oxygen (17, 18). ROS formation results from excitation energy transfer through a photoreaction process (19, 20). It was previously reported that IAA can generate free radicals in combination with UVB. The cytotoxic actions on several cancer cells, such as hepatomas and melanomas were also demonstrated (7, 11, 12). Moreover, in order to avoid the adverse effects of UVB, visible light was tested and the anti-cancer activity of the IAA/IPL combination demonstrated (7). However, although it was reported that IAA produces H_2O_2 when oxidized by HRP (9), in a previous study it was found that IAA^{UVB} does not produce H_2O_2 (11), and it thus remains important to resolve the differences between the IAA/HRP and IAA^{UVB} systems. Currently, we are trying to identify the responsible entities.

This study was the first to investigate the effect of the IAA^{UVB} combination on a prostate cancer cell line (PC-3). As in previous studies, it was shown that IAA^{UVB} can produce free radicals through the DCFH-DA assay. DCFH-DA has been used to detect ROS for a number of years in many studies, but free radicals from the IAA^{UVB} combination were shown to be different entities from ROS (11).

Similar to previous work (7, 11, 12), the present results from the cell viability test with PC-3 cells showed that IAA alone was non-toxic to PC-3 cells in comparison with the non-treated group, while IAA^{UVB} treatment produced a significant decrease in cell viability (Figure 2a). Cytotoxicity was effectively reversed by addition of the water-soluble vitamin E derivative, Trolox, which indicates IAA^{UVB}-mediated cell death results from free radicals (Figure 2c).

Although the mechanisms of an IAA combination system (IAA/HRP and IAA^{UVB}) on cellular effects have been previously reported (9, 11), there have been no studies on prostate cancer. Accordingly this study verified the apopotogenic activity and related signaling pathways, including caspase enzymes and MAPKs, to investigate the background of cell death in PC-3 cells. Figure 3 shows that the levels of active forms of JNK and p38 increased in PC-3 cells when treated with IAA^{UVB}. As JNK and p38 MAPK are important mediators of stress signals that ultimately lead to apoptosis, it was assumed that IAA^{UVB} could kill PC-3 cells *via* apoptotic pathways. This finding was supported by examination of caspase enzymes (-3, -8, and -9) and the PARP cleavage assay (Figure 4). Caspases can be thought of as central executioners of the apoptotic pathway (21). Most caspases are activated by autocatalytic activation and induce apoptosis through the caspase cascade, which finally activates downstream effector caspase-3, -6, and -7 (21, 22). One of the characteristic phenomena of apoptosis includes PARP cleavage. PARP cleavage by caspase-3 has been suggested to occur in late stages of apoptosis in order to prevent depletion of cellular energy (23, 24). In this study, it was observed that IAA^{UVB}-treated PC-3 cells present with decreased pro-caspase levels (-3, -8, and -9), which indicated up-regulation of the activated forms of the respective caspase proteases. Also, PARP cleavage by caspase-3 was clearly observed in IAA^{UVB}-treated cells, which explains the ongoing apoptotic process in PC-3 cells. In view of the fact that caspase-9 is the representative effector of the mitochondrial apoptosis pathway, while caspase-8 is a member of the death receptor pathway, the present data suggest that IAA^{UVB} controls both apoptosis pathways like H_2O_2 from IAA/HRP (8).

In addition, it was demonstrated IAA^{UVB}-induced apoptosis by flow cytometric analysis. When cells stained with annexin V and PI were examined by FACS, the apoptosis was significantly enhanced in IAA^{UVB}-treated PC-3 cells, but not in the IAA or UVB group (Figure 5). This data confirmed that IAA^{UVB} effectively induces apoptosis in PC-3 cells, while the same doses of IAA or UVB alone do not harm cells.

Taken together, the data of this study demonstrated that non-cytotoxic IAA induced cell death in combination with UVB irradiation by increasing apoptosis in PC-3 cells.

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