Apoptosis of Human Melanoma Cells by a Combination of Lonidamine and Radiation

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Human melanoma/C32TG cells/Lonidamine/Radiation/Combination treatment/Apoptosis/Caspase/Mitochondria-targeting/Bcl-2/Bad.

Human melanoma is the most aggressive form of human skin cancer, and is notoriously resistant to any current modalities of cancer therapy. Here we show that lonidamine (LND), a mitochondria-targeting non-conventional chemotherapeutic agent, markedly induced apoptosis in radioresistant human malignant melanoma C32TG cells. Either LND or up to 250 µM or X-ray irradiation of up to 15 Gy alone induced only a few percent of the apoptosis when administrated separately. When the two agents were combined, the apoptosis prominently increased to 29.3 %. The apoptotic cells thus induced by the combination treatment showed chromatin condensation, a depletion in \( \Delta \psi_m \), and an activation of caspase-3. A pan-caspase inhibitor Z-Asp-CH\(_2\)DCB completely suppressed the apoptosis. The combination treatment also decreased Bcl-2 and Bad phosphorylation. These results indicate that the mitochondria pathway of apoptosis would devise a new radiotherapy strategy for treating malignant melanoma.

**INTRODUCTION**

Human malignant melanoma is a deadly malady that poorly responds to conventional radiation therapy and chemotherapy.\(^1,2\) Apoptosis is hardly induced by these therapies.\(^3\) Because the p53 status of human malignant melanoma are wild type in general,\(^4\) the resistance of melanoma to these treatments could be due to p53-independent mechanisms.

The anti-apoptotic proteins of the Bcl-2 family protects cancer cells from ant-cancer agents inducing apoptosis.\(^5\) The apoptogenic Bcl-2 family proteins such as Bax and Bad, however, instead promote apoptosis by controlling the release of cytochrome \( c \) from mitochondria.\(^6\) Bad is normally cytosolic in the phosphorylated form, and translocates to mitochondria after dephosphorylation. Dephosphorylated Bad renders cells into a pro-apoptotic state by binding to and inactivating Bcl-2, whereas phosphorylated Bad dissociates from Bcl-2.\(^7\)

Agents designed to target mitochondria have been developed.\(^8,9\) Lonidamine (1-[(2,4-dichlorophenil)methyl]-1H-indazol-3-carboxylic acid) (LND) is a non-conventional chemotherapeutic agent derived from indazol-3-carboxylic acid, and acts on the permeability transition pore of mitochondria to induce apoptosis.\(^10\) LND also enhances the apoptotic response of cancer cells to cisplatin\(^11\) and paclitaxel.\(^12\) The drug is undergoing clinical phase III trial for the treatment of non-small cell lung cancer.\(^13\)

We here investigated and reported that LND remarkably enhanced the radiation-induced-apoptosis of human melanoma cell line C32TG cells with a wild type of p53, and down regulated Bcl-2 and Bad phosphorylation. The results indicate that LND potentiates radiation-induced apoptosis in human melanoma cells.

**MATERIALS AND METHODS**

**Chemicals**

LND was purchased from Sigma, USA. The stock solution of LND was prepared with 100 mM in DMSO and stored at \(-20^\circ C\). Agents including Z-Asp-CH\(_2\)-DCB (pan-caspase inhibitor), Ac-DEVD-MCA (fluorogenic substrates for caspases-3), and 7-amino-4-methylcoumarin (AMC; calibration standard) were purchased from Peptide Institute Inc., Japan.
**Cell line**
C32TG human amelanotic melanoma cells (JCRB0227; Japanese Collection of Research Bioresources) were maintained as a monolayer culture in Ham’s F12 medium (Sigma, USA), supplemented with 10% fetal bovine serum, penicillin G (100 U/ml), and streptomycin sulfate (100 mg/ml). The cells were incubated in a humidified atmosphere with 5% CO₂ at 37°C.

**LND treatment and Irradiation**
The cells (1 × 10⁶) seeded in a T25 tissue culture bottle with 5 ml medium were cultured for 24 h, and followed by a medium change. The cells in 3 ml fresh medium containing various concentrations of LND were incubated at 37°C for a given time. An irradiation to cells at room temperature was given time. An irradiation to cells at room temperature was made with an X-ray machine (Pantak-320S, Shimazu, Japan; 200 kVp, 20 mA, 0.5 mm Cu + 0.5 mm Al filters, focus center distance of 58 mm, 0.9 Gy/min).

**Identification of the apoptotic cells**
After being stained with Hoechst33342 (Sigma, USA), the apoptotic cells were defined by the characteristic change of nucleus, as previously described by Choi et al.¹⁴

**Sub-G₁ peak in the DNA profile of FCM**
Fixed with 70% ethanol, washed, and resuspended in 100 µl of PBS(−), the cells were incubated at 37°C for 30 min with the presence of 2 µl of RNase solution (10 mg/ml), and stained for 30 min in the dark with a solution containing propidium iodide (PI) (Sigma. USA) at 1 mg/ml in PBS(−). The DNA content per cell was measured by flowcytometry (FCM). The sub-G₁ peak represents cells with DNA fragmentation.

**Analysis of mitochondrial membrane potential**
ΔΨ was assayed by FCM after the cells were stained with rhodamine 123, as described previously.¹⁵

**Fluorescence staining of mitochondria**
The cells growing in a 60 mm cell culture dish were stained with 500 nM Mito Tracker Deep Red (Molecular Probes Inc.) for 45 min at 37°C. After their staining, the cells were washed in fresh medium, fixed by 3.7% formaldehyde 15 min at 37°C, and rinsed several times in PBS (−). Confocal scanning images of cells were obtained by using a laser-scanning microscope (Olympus, Japan, Tokyo).

**Measurement of caspase activity**
The caspase activity was assayed by a method using caspase fluorescent substrates.¹⁶

**Western blotting analysis**
The cells were lysed with a RIPA buffer containing phosphatase inhibitors (50 mM Tris-HCl (pH 7.2), 150 mM NaCl, 1% NP-40, 1% deoxycholic acid, 0.05% SDS, 100 mM NaF, 2 mM Na₂VO₄, 2 mM DTT, 2 mM PMSF, 1.5% apoprotein) according to the method described by Matsuura et al.¹⁷ The lysates were vortexed and sonicated by a probe-type sonicator 5 times for 1 min each and centrifuged (15,000 rpm × 30 min) at 4°C. The protein concentration of supernatant was determined by the Bio-Rad protein assay kit (Bio-Rad, USA). The equivalent amounts of cell lysates were added with an equal quantity of loading buffer (50 µM Tris-HCl, 0.01% bromophenol blue 15% sucrose, 3% SDS, 10% β-mercaptoethanol), heated at 100°C for 3 min, then electrophoresed in 16% SDS-polyacrylamide gel and transferred onto a PVDF membrane at 60 V for 4 h. The membrane was blocked for 2 h in TBST (137 mM NaCl, 2.68 mM KCl, 25 mM Tris-HCl, 0.1% Tween20) containing 3% Perfect-Block (BIOMOL Research Laboratories Inc., USA and Canada), and incubated with primary antibodies against either Bcl-2 (Oncogene Science Inc., Germany), Bad, or phospho-Bad (BIOMOL Research Laboratories Inc., Germany) and incubated with primary antibodies against either Bcl-2 (Oncogene Science Inc., Germany), Bad, or phospho-Bad (BIOMOL Research Laboratories Inc., Germany). Signals were detected with a Lumi-Light plus (Roche Applied Science Inc., Germany).

**RESULTS**

**LND enhanced X-ray induced apoptosis**
The apoptotic cells were microscopically counted 24 and 48 h after 15 Gy X-ray irradiation, the start of 250 µM LND treatment, or a combination of X-ray irradiation and LND treatment (Fig. 1A). The incidence of apoptosis was 1.0 % for untreated control, and marginally increased to 1.4% by X-ray irradiation. The LND treatment was slightly effective and induced 5.0% apoptosis at 48 h. The combination treatment of 15 Gy X-rays and 250 µM LND synergistically induced apoptosis of the melanoma cells in such a way that 29.3% cells underwent apoptosis at 48 h. The apoptosis induced by the combination treatment of X-rays and LND was further analyzed by changing the radiation dose (Fig. 1B) and LND concentration (Fig. 1C). The apoptotic cells slightly increased to 3.6%, from 1.0%, when radiation dose increased to 20 Gy, from 0 (Fig. 1B). The addition of 250 µM LND resulted in a radiation dose-dependent enhancement so that 43.7% of the cells (p < 0.05) contained apoptosis after 20 Gy X-irradiation. When LND concentration increased to 350 µM from 0 with a fixed radiation dose of 15 Gy, the apoptotic cells increased to 40.6%, from 2%, and the maximum apoptosis induced by LND alone was 5% (Fig. 1C).

The combination treatment of radiation and LND induced the characteristic changes of apoptosis such as cell shrinkage, plasma membrane blebbing, chromatin condensation, and segregation (data not shown). Furthermore, we analyzed sub-G₁ cells that represented cells with DNA fragmentation. The sub-G₁ cells were not observed in non-treated cells (Fig. 2A), J. Radiat. Res., Vol. 45, No. 2 (2004); http://jrr.jstage.jst.go.jp
LND and Radiation Induces Apoptosis

Fig. 1. Effects of LND and X-ray irradiation on the apoptosis of C32TG cells. A: Time-dependent increases in apoptosis. Time of 15 Gy radiation and starting time of LND treatment are set at 0 h. B: Radiation dose-dependence of apoptosis. The cells were treated with a fixed concentration of 250 µM LND, received varying radiation doses at 0 h, and were incubated for 48 h. C: The LND concentration-dependence of apoptosis. The cells were treated with a fixed radiation dose of 15 Gy and a varying concentration of LND. The cells were incubated for 48 h. The bars represent mean ± SEM (n = 3).

Fig. 2. Flowcytometric analysis of DNA contents. The cells were either not treated (A), irradiated with 15 Gy X rays (B), treated with 250 µM LND (C), or treated with the combination treatment (D) for 48 h. PI fluorescence intensity is shown as a horizontal scale, but the number of cells is shown as abscissa.

Fig. 3. Mitochondrial membrane potential (ΔΨ) after radiation, LND treatment or the combination treatment. The cells were either not treated (A), irradiated with 15 Gy radiation (B), treated with 250 µM LND, (C) or treated with the combination treatment (D) for 48 h. Rhodamine123 fluorescence intensity is shown as a horizontal scale (indicating ΔΨ), but the number of cells is shown as abscissa.

LND 250 µM treated cells (Fig. 2B), or X-ray irradiated-cells (Fig. 2C) 48 h after treatment. The combination treatment of 15 Gy X-rays and 250 µM LND, however, clearly induced a sub-G1 peak (Fig. 2D).

These results demonstrate that the combination of X-ray and LND synergistically induce a typical apoptosis of C32TG melanoma cells.

LND treatment accompanied the loss of ΔΨ and morphological change in mitochondria.

We analyzed the mitochondrial membrane potential 48 h after the treatment with 15 Gy X-rays, 250 µM LND, or the combination treatment of 15 Gy X-rays and 250 µM LND. A single peak was found for non-treated cells (Fig. 3A) and also for 15 Gy irradiated-cells (Fig. 3B). However, the treatment of cells with LND alone for 48 h produced two peaks, indicating low ΔΨ and high ΔΨ cells (Fig. 3C). The two
peaks were also observed for cells treated with a combination of 15 Gy radiation and 250 µM LND (Fig. 3D). A slight increase of \( \Delta\psi \) observed for X-ray irradiated cells (Fig. 3A vs. 3B) could be due to increased G2 cell population (Fig. 2A).

The treatment of cells with 250 µM LND for 48 h condensed mitochondria (Fig. 4C). The mitochondria condensation was not observed for cells receiving 15 Gy radiation (Fig. 4B). The combination treatment with 15 Gy radiation and 250 µM LND not only condensed mitochondria but also induced chromatin condensation and the segregation of nuclei (Fig. 4D).

Role of caspase in apoptosis induction by the combination treatment

We measured caspase-3 activity by using caspase-3/7 fluorogenic substrate Ac-DEVD-MCA (Fig. 5A). Cells treated with 250 µM LND showed a slight but insignificant increase (1.4-fold, \( p = 0.21 \)) in caspase-3 activity, but no increase in caspase-3 activity was detected after 15 and 30 Gy radiation. Cells treated with the combination of 15 Gy radiation and 250 µM LND showed a much higher caspase-3 activity; thus an increment of about 2.1-fold (\( p < 0.05 \)) was observed.

We examined the effect of a pan-caspase inhibitor (Z-Asp-CH2DCB) on the appearance of apoptotic cells (Fig. 5B). The apoptotic cells marginally increased after 5 Gy radiation (0.8%), 15 Gy radiation (1.3%), or 250 µM LND treatment (2.4%) after 48h, compared with non-treated cells (1.0%). Combination treatments of either 250 µM LND or 5 or 15 Gy radiation produced 20% and 30% apoptotic cells, respectively, the values being prominently higher than a single treatment produced. When we added the inhibitor to the medium shortly before and during the combination treatments, apoptosis was significantly (\( p < 0.001 \)) reduced to 1%, a level similar to that induced by radiation alone.
Bcl-2 expression and Bad phosphorylation after treatment with LND and X-rays

We conducted a Western blot analysis to study Bcl-2 expression and Bad phosphorylation after radiation with or without LND treatment (Fig. 6). The cells treated with 250 µM LND alone did not change Bcl-2 expression. A radiation of 15 Gy and 30 Gy also slightly increased the Bcl-2 expression. The combination treatment with 15 Gy radiation and 250 µM LND30 resulted in a decrease of Bcl-2 expression. Bad phosphorylation was not affected by LND treatment or by radiation alone (Fig. 6). Also, the Bad expression was not affected by LND treatment or radiation alone. However, the cells treated with the combination of radiation and LND decreased not only the Bad expression but also the Bad phosphorylation.

DISCUSSION

This study showed that the combined treatment of X-ray radiation and LND efficiently induced apoptosis (Fig. 1). Radiation stress is known to induce apoptotic signaling via mitochondrial pathway involving caspase-9 and -3 activation.18,19) Because the combination treatment of 15 Gy radiation and 250 µM LND significantly increased caspase-3 activity (Fig. 5), and because the pan-caspase inhibitor completely blocked apoptosis induction, the caspase-3 pathway should at least be involved in the apoptosis induced by the combination treatment.

Mitochondrial permeability transition (MPT) is one of major pathways involved in stress-inducing apoptosis, and mitochondrial permeability transition is a process before caspase cascade to execute apoptosis.20) In this study, LND but not X-rays reduced MPT or induced mitochondrial membrane potential (Fig. 3). Because LND did not induce apoptosis (Fig. 1C), the C32TG cells could be deficient in any downstream pathway of apoptosis. Apaf-1, which is required for a caspase-activating pathway, is inactivated in human malignant melanoma cells.20) If radiation had activated Apaf-1 of C32TG cells in the present study, the apoptosis signals of MPT could go through a caspase-activating pathway.

We showed here that the combination treatment downregulated Bcl-2 expression (Fig. 6), suggesting that a Bcl-2 down-regulation mechanism was involved in the combination treatment-induced apoptosis. Bcl-2, like protease, is cleaved by caspase during apoptosis in human malignant melanoma cells.21) In this study, caspase like protease was not activated by LND treatment, whereas the combination treatment showed caspase dependent apoptosis (Fig. 5A, B). Bcl-2 family proteins have been implicated in the regulation of the mitochondrial pathology, such as the electrochemical gradient across the inner mitochondrial membrane.22,23) Bcl-2 and Bcl-Xl exert an anti-apoptotic effect, whereas other Bcl-2-related proteins, such as Bak and Bax, antagonize Bcl-2 and Bcl-Xl, thus inducing apoptosis.24) Bcl-2 downregulation might be important to induce apoptosis in human malignant melanoma cells.

Downregulation by the combination treatment was also observed for Bad, and more prominently for phosphorylated Bad (Fig. 6). This follows that Bad and/or dephosphorylated Bad was relatively more abundant than phosphorylated Bad in cells that received the combination treatment. The Bad rich status is active in sending anti-apoptotic signals through binding Bcl-2.25) We do not exclude the possibility, however, that ATP depression could be involved in the apoptosis induced by the combination treatment.

These findings suggest that LND has a potential for a new radiosensitizing agent to treat human melanoma.

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