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Role of Caspases and Reactive Oxygen Species in Rose Bengal-Induced Toxicity in Melanoma Cells

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Abstract

Objective
We have previously shown that Rose Bengal (RB) alone, not as a photosensitiser, could induce apoptotic- and non-apoptotic cell death in different melanoma cell lines. To clarify RB-induced toxicity mechanisms, role of caspases and reactive oxygen species (ROS) were studied in melanoma cells.
Materials and Methods
Human melanoma cell lines, Me 4405 and Sk-Mel-28 were cultured in DMEM medium. Cell viability was quantitated by MTT assay. Apoptotic cells were determined using PI staining of DNA fragmentation by flow cytometry (sub-G1 peak). Role of caspase were studied using the pan-caspase inhibitor z-VAD-fmk. ROS was measured using DCF-DA by flow cytometry analysis.
Results
This study showed that while z-VAD-fmk completely inhibited apoptosis of melanoma induced by tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), it only partially blocked RB-induced apoptosis in Me4405 and Sk-Mel-28 melanoma cell lines. RB also increased ROS production in melanoma cells but pretreatment with antioxidant γ-glutamylcysteinylglycine (GSH) could not decrease RB-induced toxicity.
Conclusion
Both caspase-dependent and -independent pathways were induced by RB in melanoma cells. RB-induced generation of ROS does not play a significant role in RB-induced toxicity and it is independent of ROS production in melanoma cells.

Keywords: Caspases, Melanoma, Rose Bengal, ROS.

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Introduction

Melanoma is among the top six cancers to cause death and morbidity in the world. However, there has been little progress in the medical treatment of metastatic melanoma because of the absence of effective systemic therapies such as chemotherapy, irradiation and immunotherapy. This is believed to be primarily due to resistance of melanoma cells to apoptosis induced by therapeutic agents. In view of this, there is increasing interest in identification of agents for treatment of melanoma (1, 2).

Rose bengal (4, 5, 6, 7-tetrachloro-2', 4', 5', 7'-tetraiodofluoresceindisodium), has been used as a systemic diagnostic for hepatic function, ophthalmologic disorders and as a photosensitiser in photodynamic treatment. We have previously reported that RB itself and not as a photosensitiser (100- 200 μM) could induce pronounced cell death in melanoma cell lines. This toxicity was predominantly due to non-apoptotic cell death but in some melanoma cell lines, RB could also induce apoptosis (3). In an attempt, it was sought to study the mechanisms of RB-induced toxicity in melanoma cells. Therefore, role of caspases activation and ROS production were studied in melanoma cells.

Materials and Methods

Reagents

RB was supplied by Sigma. Recombinant human tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) was supplied by Immunex (Seattle, WA). The preparation was supplied as a leucine zipper fusion protein, which required no further cross-linking for maximal activity. The general caspase inhibitor, Z-Val-Ala-Asp (Ome)-CH2F (z-VAD-fmk), were purchased from Calbiochem (La Jolla, CA). The antioxidant γ-glutamylcysteinylglycine (GSH) and fluorescent probe 2', 7'-dichlorofluorescein diacetate (DCF-DA) were purchased from Sigma Chemical Co (St. Louis, MO). Propidium iodide (PI), sodium citrate and Triton X-100 were purchased from Sigma. DMEM and FCS were purchased from Commonwealth Serum Laboratories (Melbourne, Australia). MTT Cell Proliferation Assay Kit was purchased from Molecular Probes (Eugene, UR).

Cell culture

Human melanoma cell lines, Me4405 and Sk-Mel-28 (Dr. Hersey' laboratory, University of Newcastle, Australia) have been described previously (4).

The cell lines were cultured in DMEM containing 5% FCS. Cells were seeded overnight, and then incubated with RB in the dark.

Cell viability

Cell viability was measured by MTT cell proliferation assay (3) using Vybrant MTT Cell Proliferation Assay Kit. Briefly, cells were seeded at 10000/well onto flat-bottomed 96-well culture plates and allowed to grow for 24 h followed by treatment with RB (100 and 200 μM) in dark. After removing the medium, each well was washed completely with PBS to remove the red color of RB. Cells were then labelled with MTT and resulting formazan was solubilized with DMSO. Absorbance was read in a microplate reader (Bio-Rad) at 540 nm.

Apoptosis

Apoptotic cells were determined by staining using the PI method described elsewhere (3, 4). In brief, melanoma cells were cultured overnight in a 24-well plate and treated with RB or TRAIL for 24 h in the dark. Floating and adherent cells were then harvested and incubated overnight at 4°C in the dark with 750 μl of a hypotonic buffer (50 μg/mL PI in 0.1% sodium citrate plus 0.1% Triton X-100) before flow cytometric analysis using a FACScan flow cytometer (Becton

Dickinson). Me 4405 and SK 28 cells were treated with z-VAD-fmk (20 μM) 1 h before adding TRAIL (200 ng/ml) or RB (200 μM) for another 24 h.

Measurement of reactive oxygen species generation

ROS was monitored by measurement of hydrogen peroxide generation. As described elsewhere (5), cells that were seeded in 24-well plates overnight with or without treatment with RB, were incubated with the fluorescent probe 2', 7'-dichlorofluorescein diacetate (DCF-DA) for 30 min. The medium was removed to a 75-mm Falcon polystyrene tube and the adherent cells were trypsinized and collected into the same tube.

After washing twice with PBS, the intensity of DCF-DA fluorescence was determined by using a FACScan flow cytometer (Becton Dickinson, Sunnyvale, CA), with an excitation wavelength of 480 nm and an emission wavelength of 530 nm.

Statistical analysis

All results were expressed as mean ± SEM. The significance of difference was evaluated with ANOVA and Bonfrroni's test. A probability level of $p<0.05$ was considered statistically significant.

Results

RB induces apoptosis of melanoma through caspase-dependent and independent pathways

To determine the role of caspase activation in RB-induced apoptosis, SK 28 and Me 4405 melanoma cell line were treated with the pan-caspase inhibitor, z-VAD-fmk, 1 h before adding RB at 200 μM for a further 24 h. Figure 1 shows that while z-VAD-fmk completely inhibited apoptosis of melanoma induced by tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), a member of the TNF family that is known to induce apoptosis in the melanoma cell lines (4), it only partially blocked RB-induced apoptosis. This suggests that both caspase-dependent and -independent pathways were induced by RB in melanoma cells.

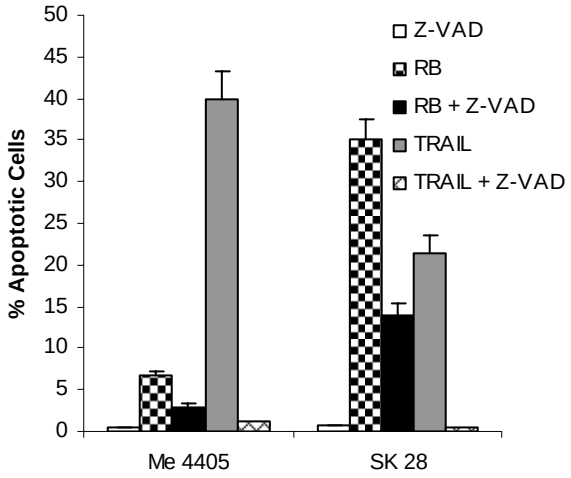

Figure 1. Effects of inhibition of caspases on RB-induced apoptosis of melanoma. Apoptosis induced by RB is partially caspase-dependent. Me 4405 and SK 28 cells were treated with z-VAD-fmk (20 μM) 1 h before adding TRAIL (200 ng/ml) or RB (200 μM) for another 24 h. Apoptosis was measured by propidium iodide method using flow cytometry. *Columns,* mean of three individual experiments; *bars,* SEM.

RB-Induced toxicity of melanoma is independent of the generation of ROS

We examined whether RB may mediate its effects by generation of ROS, by using DCF-DA in flow cytometry analysis. To avoid any interaction between absorbance of RB and DCF-DA florescence, we measured ROS production in 100 μM of RB. At this concentration, RB did not decrease the florescence intensity of DCF-DA and did not produce florescence itself in FITC area (data was not shown).

As shown in Figure 2A, the levels of DCF-DA fluorescence in Me 4405 and SK 28 cells treated with RB were markedly increased, which could be clearly detected at 30 min, and remained increased until 6 h after exposure to RB. To study whether increased production of ROS may play a role in RB-

induced toxicity of melanoma, we treated Me 4405 and SK 28 cells with the antioxidant, GSH (5 mM), 2 hrs before adding RB for a further 24 hrs. Figure 2B, shows that pretreatment with GSH had no effects on RB-induced toxicity, while it markedly decreased RB-induced production of ROS (Figure 2C). Pretreatment with GSH did not decrease RB-induced apoptosis in the Sk-Mel-28 cell line at 200 µM concentration (data was not shown). This suggests that the RB-induced generation of ROS does not play a significant role in RB-induced toxicity of melanoma.

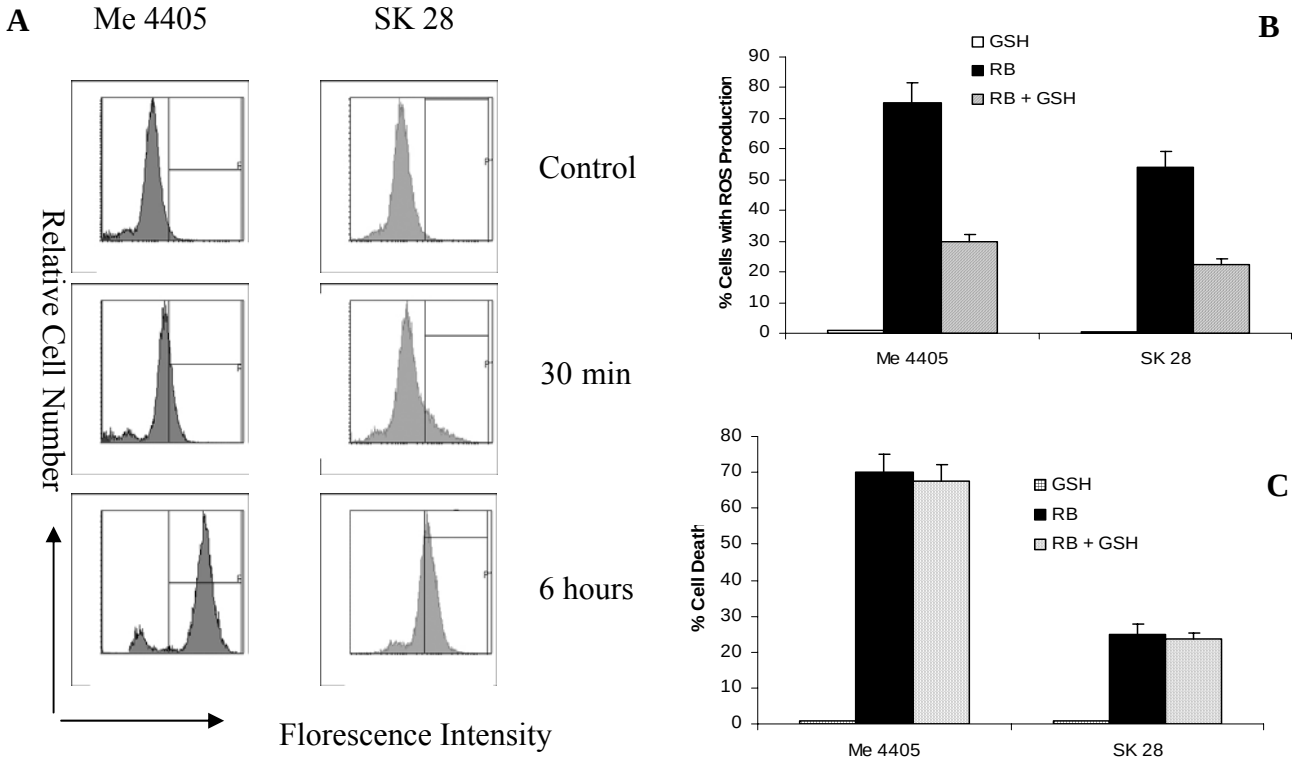

Figure 2. RB-induced toxicity of melanoma is independent of the generation of ROS. **A**, representative flow cytometry histograms of assays of ROS production. Me 4405 and SK 28 cells with or without treatment with RB (100 µM) for indicated time periods. DCF-DA (10 µM) was added for the last 30 min of incubation. The data shown are the mean ± SEM of three individual experiments. **B**, the antioxidant GSH did not inhibit RB-induced toxicity. Me 4405 and SK 28 cells were treated with GSH for 2 hrs before adding RB (100 µM) for another 24 h. Toxicity was measured by the MTT assay. *Columns,* mean of three individual experiments; *bars,* SEM. **C**, the antioxidant GSH inhibited the generation of ROS induced by RB. Me 4405 and SK 28 cells were treated with GSH (5 mM) for 2 h before adding RB (100 µM) for another 24 hrs. ROS production was measured as described in A. *Columns,* mean of three individual experiments; *bars,* SEM.

Discussion

We have previously shown that RB in concentration of 100 and 200 µM could induce both apoptotic and non-apoptotic cell death in different melanoma cell lines (3).

Apoptosis is a gene regulated phenomenon, which is important in both physiological and pathological conditions and characterized by distinct morphological features including:

chromatin condensation, cell and nuclear shrinkage, membrane blebbing and oligonucleosomal DNA fragmentation. Three major apoptotic pathways originating from separate subcellular compartments have been identified - the death receptor, mitochondrial and the endoplasmic reticulum pathway (6-8). Although each pathway is initially mediated by different mechanisms, they share a common final phase of apoptosis, consisting of activation of the executioner caspases and dismantling of substrates critical for cell survival (9, 10).

In this study it is shown that both caspase-dependent and -independent pathways were involved in RB-induced apoptosis. It is indicating some factors other than caspases such as apoptosis inducing factor (AIF) could be involved. It was reported that release of AIF is a caspase-independent event (11). Some other compounds such as Staurosporine, have been shown to induce both caspase-dependent and – independent apoptotic pathways in melanoma and MCF-7 cell lines (5, 12).

ROS were reported to induce different kinds of cell death including necrosis, caspase-dependent and -independent apoptosis (13-15) and in view of this, generation of ROS as a cause of cell death was investigated in this study. The results showed that although ROS was produced in melanoma cells by treatment with RB, they did not appear to be involved in the induction of either non-apoptotic or apoptotic cell death as the antioxidant GSH inhibitted generation of ROS but was unable to prevent melanoma from toxicity induced by RB (Figure 2).

RB-induced cell death mechanisms in melanoma cells, are still unclear. Lysosome could be considered as a one of the proposed targets for RB-induced toxicity in melanoma cells. Rupture of lysosomes, leading to the release of their cathepsin content, has long been recognized as potentially harmful to the cell (16). Tumor cells may be preferentially sensitive to agents that trigger the lysosomal apoptosis pathway (17). The degree of lysosomal permeabilization may determine the amounts of cathepsins released into the cytosol. A complete breakdown of all lysosomes will result in necrosis, whereas partial breakdown may trigger apoptosis (18).

To our knowledge, the present study is the first to show toxicity of RB in melanoma cells in which, toxicity was partially dependent on caspase activation and independent of ROS production. Actually for understanding RB-induced cell death mechanisms in melanoma cells, more studies are needed to carry out.

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