

Study of rose bengal-induced cell death in melanoma cells in the absence of light

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Abstract

Objective

Melanoma is among the top six cancers as a cause of death and morbidity. Unfortunately there has been little progress in the medical treatment of metastatic melanoma, because of its resistance to current chemotherapeutic agents. In view of this, there is much interest in the identification of new agents for the treatment of melanoma. Rose Bengal (RB) has been used as a systemic diagnostic of hepatic function, ophthalmic diagnostic and photosensitizer in photodynamic treatment. In the present study, effects of RB, not as a photosensitizer, was tested in melanoma cells in the absence of light.

Materials and Methods

Human melanoma cell lines, Me4405, Me1007, IgR3, Mel-FH, Mel-RM, Mel-CV, MM200, Sk-Mel-28 and fibroblast cells were cultured in DMEM medium. Cell death was quantitated by MTT assay. Apoptotic cells were determined using PI staining of DNA fragmentation by flow cytometry (sub-G1 peak).

Results

The result showed RB could induce pronounced cell death in different melanoma cell lines but not in fibroblast cells. This toxicity was predominantly induced by non-apoptotic cell death but in some cell lines, RB could also induce apoptotic cell death.

Conclusion

RB may be considered as a promising chemotherapeutic agent for the treatment of melanoma in the future.

Keywords: Melanoma, Rose Bengal, Cell Death, Apoptosis.

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Introduction

Melanoma continues to increase in incidence in many parts of the world and remains among the top six cancers as a cause of death and morbidity. 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 (1, 2).

Over the past decade, it has become known that many therapeutic agents kill cancer cells by inducing apoptosis (3, 4). Three major apoptotic pathways originating from separate subcellular compartments have been identified; the death receptor, mitochondrial and endoplasmic reticulum pathways (5, 6). 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 (7, 8). Apoptosis signaling mechanisms induced by many agents are however impaired in tumor cells, leading to resistance against therapy (9). In view of this, there is much interest in identification of agents for treatment of melanoma.

Rose bengal (4,5,6,7-tetrachloro-2',4',5',7'-tetraiodofluoresceindisodium) or RB is an anionic water soluble Xanthene dye capable of photo catalytic conversion of oxygen molecule to singlet oxygen upon irradiation with green light (10, 11). Its long history of safe use as a systemic diagnostic of hepatic function (12, 13), as well as a topical ophthalmic diagnostic (14, 15), suggests RB should have minimal side effects (16).

In an attempt it is sought to study the direct effect of different concentrations of RB, not as a photosensitiser, in melanoma cell lines in the absence of visible light. We report RB could induce pronounced cell death in melanoma cell lines. This toxicity was predominantly due to non-apoptotic cell death. In some melanoma cell lines, RB could also induce apoptosis.

These results suggest that RB could be considered as a promising chemotherapeutic agent for the treatment of melanoma in the future.

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. 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, Me1007, IgR3, Mel-FH, Mel-RM, Mel-CV, MM200, and Sk-Mel-28 (Dr. Hersey' laboratory, University of Newcastle, Australia) have been described previously (17). The cell lines were cultured in DMEM containing 5% FCS. Fibroblast cells were cultured in DMEM containing 10% FCS. Cells were seeded overnight, and then incubated with RB in the dark.

Cell Viability: Cell viability was measured by MTT cell proliferation assay (18) 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 24 h followed by treatment with RB (10, 50, 100 and 200 μ M) 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 (17). In brief, melanoma cells were cultured overnight in a 24-well plate and treated with RB or TRAIL for 24h 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).

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

Results

Rose Bengal Induces Cell Death in Melanoma Cells

Morphological changes

Different melanoma cell lines were cultured with increasing concentration of RB in the dark. As early as 30 min after the addition of RB at 200 µM, morphologic changes were observed in the Me 4405 cell line consisting of

reduction in volume and rounding over 6 hours until the nucleus constituted the majority of the cellular volume. After 6h nearly all of the Me 4405 were floating (Fig. 1A). Morphologic changes in SK-Mel-28 cells were the same but at slower rates starting 1h after addition of RB. After 24h of incubation with RB at 200 µM the majority of SK-Mel-28 cells were round. In contrast, there were no morphological changes in fibroblasts (Fig. 1A) at the same concentration and over the same period.

Cell viability

The impact of RB on cell viability was quantitated in MTT assays. As shown in Fig. 1B, RB decreased cell viability in melanoma cells but not in Fibroblast cells. Toxicity was evident at 100 µM and pronounced at 200 µM of RB. Time dependent of reduced viability is shown in Fig. 1C.

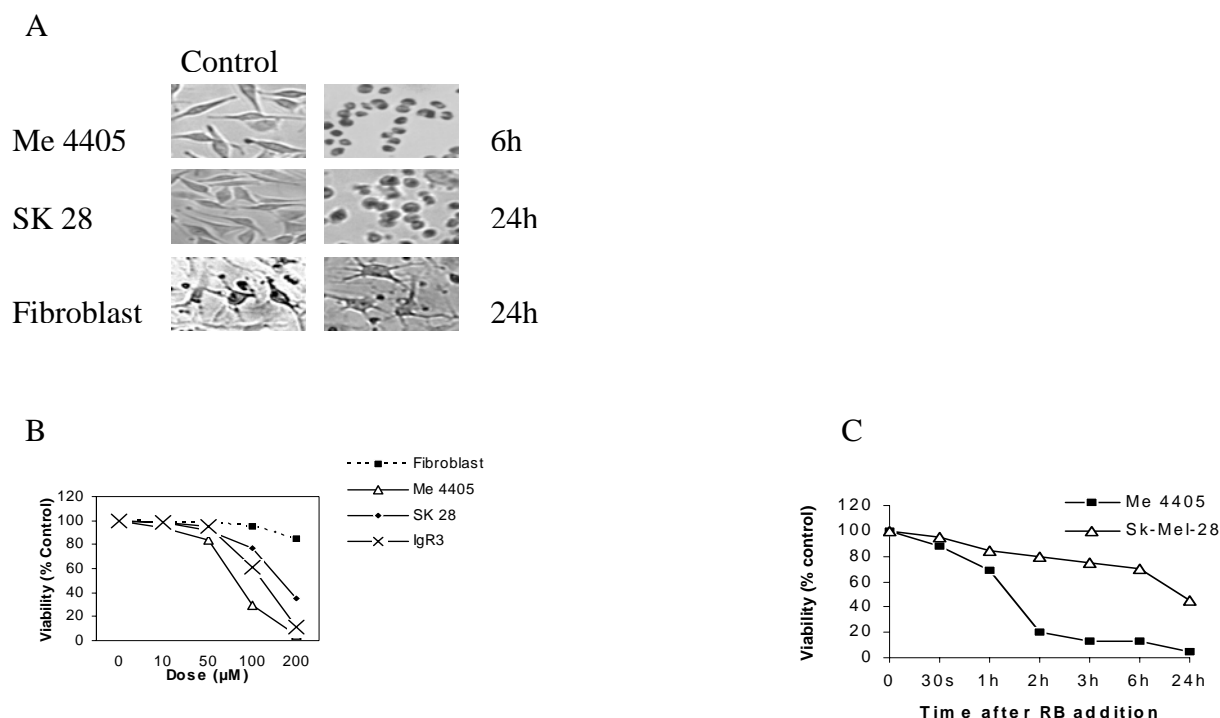


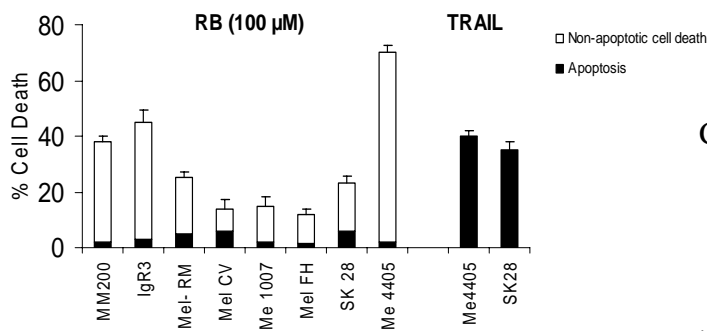
Fig. 1. RB induces morphologic changes and affects the viability of cultured melanoma cells. A., Cell lines Me 4405 and SK 28 and normal fibroblast treated with 200 µM of RB. While Me 4405 (6h incubation) and SK 28 (24h incubation) were completely round, fibroblast were unchanged even after 24h. B., normal fibroblast, and cell lines Me 4405, SK 28 and IgR3 were incubated with different concentration of RB for 24 h. While RB could not induce significant reduced viability in normal fibroblast, the maximum amount of cell death was induced at 200 µM of RB. Viability was quantitated by MTT assay. C, Effect of RB (200 µM) on Me 4405 and SK 28 was studied at different incubation time. Kinetics of toxicity in Me 4405 was so fast and after 6 h nearly all cells were dead.

RB Induces Both Non-apoptotic and Apoptotic Cell Death

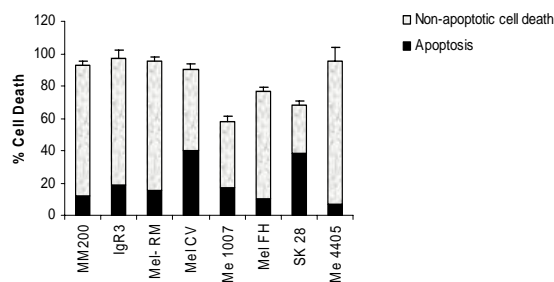
Different melanoma cell lines were treated with RB at 100 and 200 μ M for 24 h. Me 4405 and SK 28 cells were also treated with TRAIL (200 ng/ml for 24 h) a member of the TNF family that is known to induce apoptosis in the melanoma cell lines (17), as a positive control. As shown in Fig 2A although RB at 100 μ M affected the viability of melanoma cell lines, but apoptosis did not markedly contributed in the cell lines tested and non-apoptotic cell death was the main form of cell death. Fig 2B shows, treatment with RB (200 μ M) induced

both non-apoptotic and apoptotic cell death in melanoma cell lines. The main form of cell death was non-apoptotic death, however, in some cell lines such as Sk-Mel-28 and Mel-CV apoptosis was also markedly involved. Flow cytometry detection of DNA fragmentation (17) induced by RB in the cell line SK 28 but not in Me 4405 is shown in Fig 2C. While nearly all of 4405 cells were dead, there was no marked sub-G1 peak in flow cytometry histogram of these cells indicating non-apoptotic cell death has the main role in RB-induced toxicity in Me 4405 cells.

A



B



C

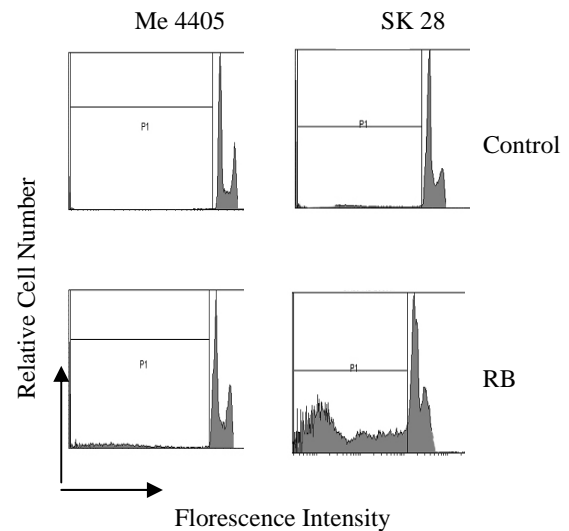


Fig. 2. RB induces both non-apoptotic and apoptotic cell death in melanoma cells. A, Different melanoma cell lines were treated with RB at 100 μ M for 24h. Me 4405 and SK 28 cells were also treated with TRAIL (200 ng/ml) for 24 h as an inducer of apoptosis and positive control. The proportion of apoptotic cells was measured with PI staining of DNA fragmentation by flow cytometry. B, RB-induced apoptotic and non-apoptotic cell death at 200 μ M for 24h in the same melanoma cell lines. C, Flow cytometry histograms of apoptosis assays by the PI method. SK 28 and Me 4405 were treated with 200 μ M of RB for 24 h. Columns, mean of three individual experiments; bars, S.E.M.

Discussion

RB has been explored as a photodynamic sensitiser for cancer chemotherapy (19) and for inactivation of either viruses (20) gram-positive bacteria, or protozoa (21) and can induce photohemolysis and photothrombosis (22). It was reported that low concentrations of RB after exposure to visible light could induce apoptosis of human epidermal carcinoma A431 cells. At these low concentrations (5-10 μM), RB did not induce any cell death itself (23), but in this study, for the first time the direct effect of higher concentrations of RB (10-200 μM) was explored in melanoma cell lines and normal fibroblast in the absence of visible light. The above results indicate that RB induced cell death in all of the cultured melanoma cell lines tested but not in fibroblast at concentrations of 100 and 200 μM . Toxicity was evident at 100 μM and pronounced at 200 μM of RB. The main form of cell death was non- apoptotic cell death, most probably necrosis. In some cell lines (Sk-Mel-28 and Mel-CV) at 200 μM of RB apoptosis was also markedly involved in induction of cell death.

RB-induced cell death mechanisms in melanoma cells are not clear. Lysosome could be considered as one of the proposed targets for the selective RB-induced toxicity in melanoma cells. Rupture of lysosomes, leading to the release of their cathepsin content, has long been recognized to be potentially harmful to the cell (24). Tumor cells may be preferentially sensitive to agents that trigger the lysosomal apoptosis pathway.

Different possible mechanisms underlies this preferential sensitivity including increased levels of several cysteine cathepsins (25) as well as of cathepsin D (26), in human tumors leading to release of larger amounts of cathepsins in tumor cells upon lysosomal rupture (27). 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 (28). The relative uptake of RB into fibroblasts and melanoma cells could be also considered for the selective RB-induced toxicity in melanoma cells.

The significance of non-apoptotic forms of cell death in chemotherapy and the mechanism(s) by which they are induced by chemotherapeutic drugs remain, largely, unclear. It is however noteworthy that, the non-apoptotic cell death is often observed under conditions in which apoptosis is inhibited. In addition to inducing apoptosis, a number of chemotherapeutic agents have been reported to induce non-apoptotic forms of cell death (29).

To our knowledge, the present study is the first to show toxicity of RB for melanoma cells. Actually for understanding RB-induced cell death mechanisms in melanoma cells, more studies are needed to be carried out.

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Rose Bengal induces cell death in melanoma

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