All-trans retinoic acid (atRA) differentially induces apoptosis in matched primary and metastatic melanoma cells – a speculation on damage effect of atRA via mitochondrial dysfunction and cell cycle redistribution

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All-trans retinoic acid (atRA) has been suggested to exert its cytotoxicity via apoptosis but the mechanisms behind the damage effects have not been fully understood. In this study, we investigated the cytotoxic effects of atRA in eleven primary and matched metastatic cutaneous melanoma cell lines. All the primary and metastatic melanoma cell lines examined expressed the retinoic acid receptors. The cultured melanoma cells treated with atRA showed dysfunction of mitochondria and altered cell cycle distribution, inhibited cell proliferation and apoptosis. The cytotoxic effects of atRA were dose- and time-dependent. The dysfunction of mitochondria and induction of apoptosis were more pronounced in the primary tumor cells than in the metastatic cell lines from the same patients. The data indicate that the cytotoxic effect of atRA was mediated through dysfunction of mitochondria, alterations in cell cycle and induction of apoptosis. Melanoma in early stage may have better response to atRA adjuvant therapy than the melanoma in late stage, suggesting the early utility of atRA in melanoma chemotherapy.

Introduction
Retinoic acids have effects on cellular proliferation and differentiation in various cancers including melanoma (1–3). It has a capability to inhibit cell growth in both normal and malignant cells by induction of growth arrest in the G0/G1 phase of the cell cycle (4–6) and by inducing apoptosis (6–8).

The cytotoxic effect of atRA on cell growth and induction of apoptosis is mediated via specifically binding and activating retinoic acid receptors, such as RARα, RARβ, RARγ and the 9-cis-RA receptor, RXRα (9,10). In breast cancer, when the human RARβ gene is introduced into RARβ negative cell lines the transduced cells undergo G1 arrest and apoptosis after exposure to atRA (9).

There is a great variation in the sensitivity to atRA. For example, in most human breast cancer cell lines, a lack of the expression of RARβ, makes these cells insensitive to atRA-induced cell cycle arrest and apoptosis. By introducing the human RARβ gene into RARβ-negative breast cancer cell lines, Seewaldt and colleagues showed that these RARβ-transduced clones undergo cell growth inhibition with G1 arrest and apoptosis after 4–6 days of treatment with 1 µM atRA. They concluded that the cell growth arrest and apoptosis are mediated by RARβ in the breast cancer cells (9). Apoptotic cells are also observed in embryonal carcinoma cells after exposure of atRA at physiological concentrations (6) but high concentration (10 µM) of atRA and relatively long exposure time is required to induce apoptosis in human hepatoma Hep3B cells (7). No apoptotic cells are observed in human leukemia cells even after 8 days of exposure to retinoids (8), and normal human mammary epithelial cells do not show apoptosis after exposure to atRA although the cells have normal function of retinoic acid receptors (5). In melanoma, the synthetic retinoid CD437 has been demonstrated to induce DNA damage, cell growth arrest and retinoic acid receptor-independent apoptosis (11).

Mitochondria is considered as a central target during a drug-induced apoptosis through changing the balance of pro- and anti-apoptotic proteins in bcl-2 family, releasing cytochrome C, and activating caspases. Several genes have been described to be involved in the process of retinoic acid-induced apoptosis. Bax protein (12) and the nuclear transcription factor, TR3 (13) have been shown to be translocated from cytosol to mitochondria to initiate apoptosis, and bcl-2 is down-regulated during the retinoic acid-induced apoptosis (14).

The detailed mechanism for drug-induced cell damage remains unclear although the cytotoxic effect has been suggested through the apoptosis. Whether atRA induces apoptosis on melanoma cells, whether mitochondria serves as a key target in the cytotoxic effect of atRA, and whether retinoic acid receptors play an essential role in the apoptosis are still not fully understood. In this study, we have investigated the cytotoxic effect of atRA on matched primary and metastatic melanoma cell lines and shown that atRA may be cytotoxic to primary and metastatic melanoma cells mediated by the dysfunction of mitochondria, and cell cycle re-distribution has been also involved in the effect of atRA. This may subsequently lead to cell cycle arrest and apoptosis.

Material and methods
Cell culture medium, chemicals and antibodies
RPMI 1640 cell culture medium, fetal calf serum (FCS), l-glutamine (200 mM), penicillin (5000 IU/ml), streptomycin (5000 mg/ml) and trypsin-EDTA were purchased from Life Technologies ( Paisley, UK). atRA, propidium iodide (PI) and 3,3-diaminobenzidine tetrahydrochloride (DAB) were from Sigma Chemical Co (MO, USA). WST-1 and Annexin V were from Roche Diagnostics GmbH (Mannheim, Germany). Phosphate-buffered saline (PBS) was from EC Diagnostics AB (Uppsala, Sweden) and 4% buffered formaldehyde from Produktion and Laboratorium (Gothenburg, Sweden). Hydrogen peroxide (H2O2) and methanol were from Merck (Darmstadt, Germany). Apop-Tag in situ apoptosis detection kit was from Oncor, Gaithersburg (MD, USA). Rabbit polyclonal primary antibodies against RARα, RARβ, RARγ and RXRα receptors were from Santa Cruz (CA, USA). Rabbit immunoglobulin and peroxidase-antiperoxidase (PAP) were from DAKO (Glostrup, Denmark).

Melanoma cell lines and culture conditions
Eleven human cutaneous melanoma cell lines were used in this study. Matched primary and metastatic melanoma cell lines (WM164-451Lu; WM793-1205Lu; WM278-WM1617; WM115-WM239A; and FM55P-FM55M1-FM55M2) were derived from the same patients. The metastatic melanoma cell lines (451Lu and 1205Lu) were experimentally induced in mice from...
The matched primary (WM164, WM793, WM278 and WM115) and metastatic (451Lu, 1205Lu, WM1617 and WM239A) melanoma cells were exposed to atRA at concentrations ranging from 0.0001 to 10 µM at 37°C for 3 days. Mitochondria function was estimated with WST-1-ELISA technique. The absorbance of WST-1 from each well was measured by an ELISA reader at 450 nm with a reference wavelength of 750 nm. The values were expressed as percentage of controls and presented as mean ± SEM from three separate experiments. Statistical significance between the primary and metastatic tumor cells is marked (*P < 0.01). The mitochondria function was reduced by increasing the amount of atRA in all the cell lines. The primary tumor cells were more vulnerable to exposure of the atRA than the matched metastatic cells.

Human primary melanomas (WM164 and WM793, ref. 15). The cells were harvested from the original cell cultures by trypsinization and seeded in 96-well cell culture plates, 35 mm dishes or on microscopy slides in dishes with RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (complete RPMI 1640) for 24 h. The medium was replaced by 200 µl serum-free RPMI 1640 medium in the incubator at 37°C for different periods of time (1–6 days). The medium with atRA was replaced every other day. Parallel controls were incubated in serum-free medium without atRA. The cells were collected by trypsinization, washed in PBS and stained with PI. Ten thousand events were collected from each sample and the data were analyzed by a cell cycle analysis software (ModFit LT V3.0).

Detection of apoptosis
After 24 h incubation in complete RPMI 1640 medium, the cells (5 × 10^5 cells/dish) were treated with atRA (0.0001–10 µM) at 37°C for different periods of time (1–6 days). The medium with atRA was replaced every other day. The controls were incubated in serum-free medium. The cells were collected by trypsinization, washed in PBS and centrifugation at 1000 c.p.m. The pellet was re-suspended in 250 µl PBS and 100 µl Annexin V solution (20 µl Annexin V in 1000 µl of 10 mM HEPES buffer, 140 mM NaCl, 5 mM CaCl_2 at pH 7.4 with 20 µl of 50 µg/ml PI) and incubated at room temperature in the dark for 15 min. Additional HEPES buffer (200 µl) was added to the labeled cell suspension. Subsequently, both signals from Annexin V and PI were simultaneously recorded from 10^5 cells in each sample. The data were analyzed by using Becton Dickinson CELLQuest software.

Apoptosis was confirmed by in situ TUNEL assay
Melanoma cells in culture were treated with 10 µM atRA for up to 6 days. The cell suspensions were harvested on microscopy slides and air-dried. Apoptag in situ apoptosis detection kit was used for detecting apoptosis. Briefly, the air-dried slides were pre-incubated in 0.5% H₂O₂ in methanol for 3 min in order to inactivate endogenous peroxidase. After washing in PBS (3 × 5 min), the slides were incubated with equilibration buffer for 10 s and then with pre-warmed TdT enzyme in a humidified chamber at 37°C for 60 min. The reaction was stopped by incubation of the slides in pre-warmed (37°C) strength stop wash buffer at room temperature for 10 min and washed in PBS (3 × 5 min). Subsequently, the slides were incubated with anti-digoxigenin-peroxidase for 30 min, washed in PBS (3 × 5 min) and developed in DAB with H₂O₂ in PBS. After careful washing in distilled water the slides were counterstained in methyl green for 10 min, washed in distilled water, dehydrated using increasing concentrations of ethanol, cleared and mounted with coverslips.

Expression of retinoic acid receptors
 Cultured cells grown on microscopy slides as above for 24 h were carefully washed in PBS ×3 and fixed in 4% buffered formaldehyde for 5 min. After rinsing in PBS, the cells were pre-incubated in methanol with 0.5% H₂O₂ for 5 min. Rabbit polyclonal primary antibodies against RARα, RARβ, RARγ and RXRα receptors in PBS (1:100) were applied and incubated at 4°C overnight. Further incubation was done with rabbit immunoglobulins (1:150) in PBS for 60 min, followed by rabbit PAP (1:150) in PBS for 60 min. The slides were rinsed in PBS, three times between each step. The slides were then incubated with 0.05% DAB plus 0.02% H₂O₂ in PBS for 8 min. The
Cytotoxic effect of retinoic acid on melanoma cells

Fig. 3. Cell cycle was measured by flow cytometry after exposed to 10 µM atRA for 3 days. The primary melanoma cells (WM115) showed a diploid DNA content histogram (a large G1 peak at channel 40 and a small G2 peak at channel 80) with a slightly-increased G2 peak and 55% apoptotic cells. The metastatic tumor (WM239A) from the same patient showed a diploid DNA histogram with only 18% apoptotic cells.

reaction was stopped by PBS × 3 and running distilled water for 10 min. To improve nuclear visualization the cells were counterstained in hematoxylin for 1 min. Following a rinse in running distilled water for 10 min, the cells were dehydrated through ethanol and xylene and mounted. All procedures were performed at room temperature unless otherwise indicated. All slides were taken through the procedure at the same time. Samples known to show positive immunostaining for each antibody were included in each assay as positive controls. As negative controls, primary antibodies were replaced by PBS on known positive samples, and the known negative samples were included. In all staining procedures, the positives showed clear staining and the negatives showed no staining. The experiments were performed in triplicate. The entire slides were scanned and then scored under a microscope.

Results

Effect of atRA on mitochondria

Following 24 h pre-incubation melanoma cells were exposed to atRA at various concentrations (0.0001–10 µM) at 37°C for 1–6 days. The experiments were performed in all these matched primary and metastatic melanoma cell lines, and the data were representative. Mitochondrial viability was estimated by the WST-1-ELISA technique. No effect on mitochondrial function was noticed during the first 2 days of atRA exposure. From day 3, the melanoma cells (WM164-451Lu, WM793-1205Lu, WM278-WM1617 and WM115-WM239A) showed decreased absorbance of WST-1 after exposure to atRA at increasing concentrations as a sign of reduced mitochondrial function (Figure 1). The mitochondria viability was decreased by increasing the atRA concentrations. Both primary (FM55P) and metastatic (FM55M1 and FM55M2) tumor cells revealed gradually decreased mitochondrial function over time after exposure to as low as 0.01 µM atRA for up to 6 days (Figure 2). When primary and matched metastatic melanoma cells were compared, the primary tumors showed higher susceptibility to atRA than the matched metastatic melanomas.
Effect of aTRA on cell cycle
The experiments were performed in all these matched primary and metastatic melanoma cell lines, and the data were representative. When melanoma cells were exposed to aTRA (0.0001–5 µM) no sign of alterations in cell cycle distribution was observed. After exposure to 10 µM aTRA, the melanoma cells were labeled with PI and the DNA content was measured by flow cytometry and the cell cycle was calculated. Primary tumor cells (WM115) showed a more pronounced decrease in the number of cells in G1/G0 and increase in apoptotic cells than did the metastatic cells (WM239A) from the same patients (Figure 3). An increase in cells in G2 phase was observed at the end of day 2, cells in subG1 phase appeared by day 3 and increased in number up to day 5. At day 6, a large number of cells (FM55P) in G2 phase were observed as well as an increase in the number of apoptotic cells (Figure 4). The controls without exposure to aTRA showed similar histograms from the first to the last day.

Effect of aTRA on apoptosis
The experiments were performed in all these matched primary and metastatic melanoma cell lines, and the data were representative. Melanoma cells were collected for flow cytometry analysis after exposure to 0.0001–10 µM aTRA for up to 6 days. No sign of apoptosis was observed when the melanoma cells were exposed to aTRA (0.0001–5 µM). After exposure to 10 µM aTRA both the apoptotic and necrotic cells were observed. Figure 5 showed that the fraction of melanoma cells in early apoptosis (labeled with Annexin V alone) as well as in late apoptosis/necrosis (labeled with Annexin V and PI) was much larger in the primary melanoma cells (WM164) than in the matched metastatic cells (451Lu). The early apoptotic cells were gradually increased from 1.8% to 51% during the first 5 days exposure of the FM55P cells to aTRA. On day 6, the number of early apoptotic cells decreased from 51% to 27% with a corresponding increase of late apoptotic/necrotic cells from 1.3% to 48% (Figure 6). The late apoptotic cells were confirmed by in situ TUNEL technique (data not shown).

Expression of retinoic acid receptors
The expression of RARα, RARβ, RARγ and RXRα receptors in the melanoma cells was examined by immunocytochemistry. All four receptors were positively expressed in the cytoplasm
Fig. 7. Expression of RARα, RARβ, RARγ, and RXRα receptors in melanoma cell lines were examined by immunohistochemistry. All the primary and metastatic melanoma cells tested expressed positively the four receptors at various levels. RARα was expressed in the primary (WM164, A) and the matched metastatic (451Lu, B) cells; RARβ in FM55P (C) and FM55M1 (D); RARγ in the primary (FM55P, E) and metastatic (FM55M1, F) melanoma cells; RXRα in WM164 (G) and 451Lu (H) cells. Original magnification, ×600.
and/or in the nuclei of all tested primary and metastatic melanoma cell lines. There was no systematic staining difference between the primary and metastatic tumors (Figure 7).

Discussion

In the present study, we used an experimental model system with matched primary and metastatic melanoma cells and experimentally derived metastatic cell lines from human melanoma to investigate the cytotoxic effect of atRA. This experimental system provides an opportunity to examine different cytotoxic drug responses of primary and metastatic melanomas.

Melanoma cells produce their own growth factors and grow well in serum-free medium for more than one week. We found that atRA at low concentration (0.0001 µM) caused a decline in mitochondria viability of the melanoma cells. Primary melanoma cells had lower capacity to cleave tetrazolium salts than the matched metastatic cells. The reduction of mitochondrial function expressed in percentage of control was more pronounced for the primary melanoma cells, suggesting that mitochondrial dehydrogenases in the primary melanoma cells were more susceptible to atRA resulting in a reduced enzyme activity. These results indicated that atRA, even at very low concentration, could initiate the cytotoxic effect on mitochondria in melanoma cells. The dysfunction of mitochondria was followed by alterations of the cell cycle distribution, and a decrease in cells in G1/G0 and increase in G2 phase were observed both in primary and metastatic melanoma cells. Mitochondria have been suggested to play an important role in apoptosis. Multiple stimuli may trigger mitochondria to release caspase-activating proteins, such as cytochrome c to the cytosol and initiate apoptosis (13,14,16–20). When cells were exposed to atRA at high concentration (10 µM), a clear apoptotic phase was observed in both primary and metastatic melanoma cells although the increase in the apoptotic phase was more pronounced in the primary cells than in the metastatic cells. Unlike many other cytotoxic agents that rapidly induce cell damage and cell death, we found that atRA-caused apoptosis in melanoma cells seemed to require a relatively long exposure time (3 days) and very high concentration (10 µM). This is comparable with a previous study of retinoic acid on leukemia HL-60 cells, which showed that >50% of the cells became apoptotic when exposed to 10 µM retinoic acid for 4 days (21).

There are two pathways described for drug-induced apoptosis, i.e. the death receptor pathway and the mitochondria pathway. Which of these mechanisms that is operating obviously depends on whether death receptors are present and function in the specific cells or on the tolerance of the mitochondria with respect to the drugs (20). In this study, we found that atRA first induced functional changes of mitochondria and cell cycle alterations, and finally leading to apoptosis. Therefore, we propose that the mechanism behind atRA-induced apoptosis may be due to a mitochondria-cell cycle-apoptosis pathway. We are working on the apoptotic, mitochondrial and cell cycle related proteins to further confirm this hypothesis.

In breast cancer RARβ has shown to play a critical role in mediating cell growth arrest and apoptosis (9). A previous study by RT-PCR examining the transcription levels of retinoic acid receptors (RARα, RARβ and RARγ) and 9-cis-RA receptor, RXRα in normal human melanocytes and several melanoma cell lines has shown that both melanocytes and melanoma cells expressed these receptors (22). In line with this, all melanoma cells examined in this study expressed RARα, RARβ, RARγ and RXRα receptors. Therefore, it is likely that presence of the receptors may be a common event in melanoma cells. These receptors might play a role in atRA-induced cytotoxic effects on melanoma cells, but not in mediating the difference in susceptibility of atRA between the primary and metastatic melanoma tumors.

We concluded that atRA induced cellular growth inhibition on both primary and metastatic melanoma cells. The cytotoxic effect on melanoma was mediated through the dysfunction of mitochondria and alterations in cell cycle followed by induction of apoptosis. Primary tumor cells were relatively more susceptible to this effect as compared to the matched metastatic melanoma cells, suggesting the possibility to utilize atRA in the treatment of early human melanoma.

Acknowledgements

This work was supported by the Swedish Cancer Foundation and the Welanders Finsen Foundation, Sweden.

References

different stages of progression and their biological and molecular analyses. Melanoma Res., 7 (Suppl. 2), S35–42.


Received July 24, 2002; revised and accepted September 24, 2002