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Jasmonates induce nonapoptotic death in high-resistance mutant p53-expressing B-lymphoma cells

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1 Mutations in p53, a tumor suppressor gene, occur in more than half of human cancers. Therefore, we tested the hypothesis that jasmonates (novel anticancer agents) can induce death in mutated p53-expressing cells.

2 Two clones of B-lymphoma cells were studied, one expressing wild-type (wt) p53 and the other expressing mutated p53.

3 Jasmonic acid and methyl jasmonate (0.25–3 mM) were each equally cytotoxic to both clones, whereas mutant p53-expressing cells were resistant to treatment with the radiomimetic agent neocarzinostatin and the chemotherapeutic agent bleomycin.

4 Neocarzinostatin and bleomycin induced an elevation in the p53 levels in wt p53-expressing cells, whereas methyl jasmonate did not.

5 Methyl jasmonate induced mostly apoptotic death in the wt p53-expressing cells, while no signs of early apoptosis were detected in mutant p53-expressing cells. In contrast, neocarzinostatin and bleomycin induced death only in wt p53-expressing cells, in an apoptotic mode.

6 Methyl jasmonate induced a rapid depletion of ATP in both clones.

7 In both clones, oligomycin (a mitochondrial ATP synthase inhibitor) did not increase ATP depletion induced by methyl jasmonate, whereas inhibition of glycolysis with 2-deoxyglucose did.

8 High glucose levels protected both clones from methyl jasmonate-induced ATP depletion (and reduced methyl jasmonate-induced cytotoxicity), whereas high levels of pyruvate did not.

9 These results suggest that methyl jasmonate induces ATP depletion mostly by compromising oxidative phosphorylation in the mitochondria.

10 In conclusion, jasmonates can circumvent the resistance of mutant p53-expressing cells towards chemotherapy by inducing a nonapoptotic cell death.

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Abbreviations: 2ME, 2-mercaptoethanol; PS, phosphatidyl serine

Introduction

p53 is a tumor suppressor gene encoding a transcription factor. Its tumor-suppressive activity involves inhibition of cell proliferation through cell cycle arrest and/or apoptosis. Mutation in p53 occurs in more than half of human cancers (Willis & Chen, 2002). Cells harboring mutated p53 lose the ability to elicit the enzymatic DNA repair cascade, to inhibit cell proliferation and to induce programmed cell death, resulting in induction of uncontrolled proliferation and malignancy (Lowe & Ruley, 1993; Nelson & Kastan, 1994; Hansen & Oren, 1997; Levine, 1997). Various tumors consisting of mutant p53-expressing cells exhibit high resistance to radiation and chemotherapeutic drugs. Circumventing this abnormal resistance is a major challenge in cancer therapy (Wallace-Brodeur & Lowe, 1999; Bykov *et al*., 2002; Seeman *et al*., 2004).

In a nonstressed wild-type (wt) cell, p53 activity is very low. However, its activity is significantly increased upon a challenge to the cell, for example, UV and γ irradiation, starvation and introduction of cytotoxic drugs. On the other hand, mutant p53 is usually expressed at high levels that do not change in response to cytotoxic drugs (Cadwell & Zambetti, 2001).

We discovered that a group of plant stress hormones named jasmonates possess anticancer activities *in vitro* and *in vivo* (Fingrut & Flescher, 2002). Jasmonates increased significantly the survival of lymphoma-bearing mice and induced death in human leukemia, prostate, breast and melanoma cell lines (Fingrut & Flescher, 2002), as well as in leukemic cells from chronic lymphocytic leukemia patients (Rotem *et al*., 2005). The mechanism of action of jasmonates involves direct action on mitochondria, resulting in cell death (Rotem *et al*., 2005). Previously, we analyzed the jasmonate-induced cellular death process in Molt-4 human lymphoblastic leukemia cells and found that these plant compounds are capable of inducing both necrotic and apoptotic death (Fingrut & Flescher, 2002).

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Molt-4 cells express proteins encoded by both a wt and a mutant allele of the p53 gene (Rodrigues *et al*., 1990; Fu *et al*., 2001; Phoon *et al*., 2001). p53 mediates ionizing radiation-induced death in Molt-4 cells (Gong *et al*., 1999), suggesting that this cell line behaves as wt p53-expressing cells. Our aim was to analyze whether p53 plays a role in jasmonate-mediated cell death. To that end, we employed a system consisting of clones of the murine B-lymphoma 29M4.1 cell line, expressing either wt or mutant p53 protein (Aloni-Grinshtein *et al*., 1993). We found that jasmonates are capable of inducing cell death in the wt p53-expressing cells, as well as in the mutant p53-expressing cells. The latter were shown to be resistant to the radiomimetic neocarzinostatin, as well as to bleomycin. Thus, jasmonates can circumvent drug resistance induced by p53 mutations.

Methods

Cells

The 29M6.2 wt p53-expressing clone and 29M6.10 mutant p53-expressing clone were obtained *via* cloning and recloning of the 29M4.1 cell line (Aloni-Grinshtein *et al*., 1993). These murine B-lymphoma IgM-expressing cells are capable of differentiating to IgG-, IgE- and IgA-secreting cells upon B-cell stimulation (Aloni-Grinshtein *et al*., 1993). The 29M4.1 cells were derived from the I.29 murine B-cell lymphoma cell line (Sato *et al*., 1973). The mutant p53 protein is expressed in 29M6.10 cells constantly at high levels, is phosphorylated and acetylated, and confers high resistance to irradiation (Zan-Bar, personal communication). The 29M6.10 cells, expressing only mutant p53, are characteristic of most mutant p53-expressing human tumors that tend to lose the wt allele by deletion (Cadwell & Zambetti, 2001).

The cells were maintained in a humidified atmosphere, at 37°C with 5% CO_2 in RPMI-1640 medium (Biological Industries, Beit Haemek, Israel) supplemented with 10% FCS, 10 mM Hepes, 50 μM 2-mercaptoethanol (2ME), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 1 mM NEAA (Aloni-Grinshtein *et al*., 1993). For the relevant experiments, we used RPMI-1640 without glucose. Glucose and pyruvate were supplemented at the appropriate concentrations.

Cell growth and viability assays

Cytotoxicity assay (Ordan et al.*, 2003)* The CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI, U.S.A.) was employed. Upon completion of a given experiment, MTS (a tetrazolium compound) + phenazine methosulfate were added for 1 h at 37°C. This allowed for the reduction of MTS in metabolically active cells. The soluble MTS formazan product was measured at 490 nm by the CERES 900 HDi ELISA reader (Bio-Tek Instruments, Inc., Highland Park, VT, U.S.A.). Optical density is directly proportional to the number of living cells in culture. Cytotoxicity (%) was calculated in the following way: [(OD of control cells − OD of drug-treated cells)/OD of control cells] × 100.

Flow-cytometric analysis of apoptotic death Upon completion of treatment with cytotoxic agents, we performed a flow-cytometric analysis of the mode of death. Rates of early apoptosis and late apoptosis/death were detected using an Annexin-V fluorescein apoptosis Kit (R&D Systems, Minneapolis, MN, U.S.A.) according to the manufacturer's instructions. Briefly, cells were stained with fluoresceinated Annexin-V and PI, and percentages of Annexin-V-FITC/propidium iodide positive cells were determined using a flow cytometer apparatus (FACScan, Beckton-Dickinson, San Jose, CA, U.S.A.), and ModFIT-LT2.0 software.

Externalization of phosphatidyl serine (PS) to the outer leaflet of the plasma membrane is a hallmark of early apoptosis. In the presence of calcium ions, FITC-labeled Annexin-V adheres to PS, resulting in green fluorescence of apoptotic cells (Overbeeke *et al*., 1998). Propidium iodide enters the cells only when the plasma-membrane integrity is lost, as occurs during cell necrosis. In cell culture (where apoptotic cells are not consumed by phagocytes), these apoptotic cells will eventually also lose membrane integrity and be stained with propidium iodide (Lockshin & Zakeri, 2004). After entry into the cells, propidium iodide binds to cellular DNA, resulting in red fluorescence of the nucleus (Overbeeke *et al*., 1998). Thus, early-apoptotic cells are stained with Annexin-V-FITC alone, whereas cells stained with both Annexin-V-FITC and propidium iodide are referred to as late-apoptotic or dead (Donner *et al*., 1999; Gogal *et al*., 2000; Suska *et al*., 2005).

Determination of total p53 levels

For analysis of total p53 levels, whole-cell lysates were extracted with a lysis buffer consisting of 20 mM Tris HCl, pH 8, 137 mM NaCl, 2 mM EDTA, pH 8, 1% Triton-X-100, 1 mM sodium orthovanadate, 0.02 mM leupeptin, 0.13 TIU ml^{-1} aprotinin and 1 mM phenylmethylsulfonyl fluoride, for 40 min on ice. The protein concentration of the samples was determined by Bio-Rad DC Protein Assay (Bio-Rad Laboratories Inc., Hercules, CA, U.S.A.). Protein extracts where mixed with sample buffer (2 : 1) consisting of 60 mM Tris, pH 6.8, 3% SDS, 10% glycerol, 5% 2ME and 0.05% bromophenol blue and boiled for 5 min. Protein samples (170 μg of whole-cell lysate) were separated by sodium dodecylsufate–polyacrylamide gel (12%) electrophoresis (SDS–PAGE), followed by immunoblotting at 4°C overnight, using a mixture of two specific antibodies against total p53: PAb 240 (1 : 50, prepared from hybridoma supernatant (Gannon *et al*., 1990) and PAb 421 (1 : 2000, prepared from ascitic fluid; Harlow *et al*., 1981). The antibodies bind both wt and mutant p53. p53–antibody complexes were stained with HRP-conjugated antibody (1 : 500,000, Jackson ImmunoResearch, PA, U.S.A.). Actin levels were used as a loading control. Goat polyclonal antiactin antibody was used as a primary antibody (1 : 500, Santa Cruz Biotechnology, CA, U.S.A.). Actin–antibody complexes were stained with HRP-conjugated antibody (1 : 2000, Santa Cruz Biotechnology, CA U.S.A.). Enhanced chemiluminescence (ECL) reagent was added, and the blots were exposed to ECL film (Eastman Kodak, Rochester, NY, U.S.A.).

Determination of ATP levels

The CellTiter-Glo™ Luminescent Cell Viability Assay (Promega, Madison, WI, U.S.A.) was employed. Upon completion of a given experiment, plates were equilibrated to room

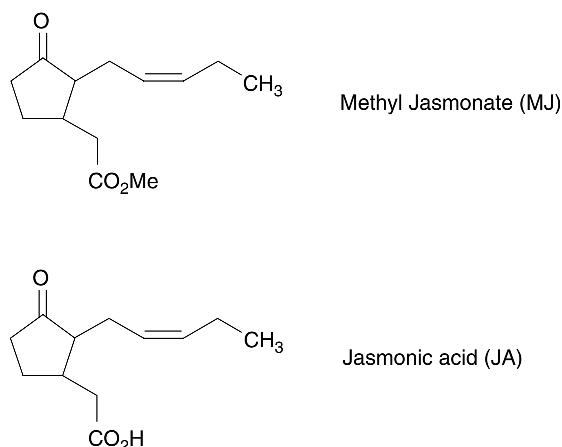

Figure 1 Structures of jasmonates.

temperature. CellTiter-Glo™ reagent containing luciferin and luciferase was added to each well and the plates were mixed on an orbital shaker for 2 min to induce cell lysis. In the presence of ATP, Mg^{2+} and molecular oxygen, mono-oxidation of luciferin is catalyzed by luciferase, generating a luminescence signal. Cells were then incubated for 10 min at room temperature to stabilize the luminescence signal. Luminescence was recorded using Kodak digital science – Image station 440 CD. Luminescence is directly proportional to ATP concentration. ATP depletion (% of control) was calculated in the following way: ((luminescence of control cells − luminescence of drug-treated cells)/luminescence of control cells) × 100.

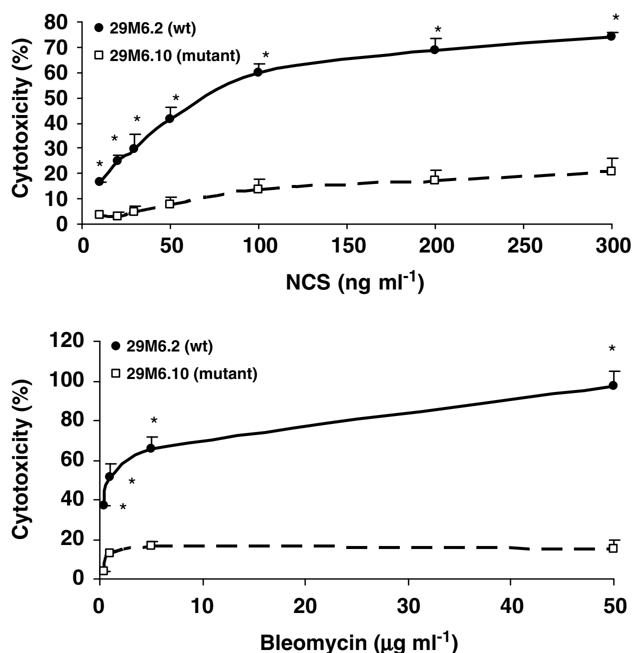

Figure 2 29M4.1 cells expressing mutant p53 are resistant to neocarzinostatin (NCS) and bleomycin treatment. 29M4.1 cells (at 2.5×10^4 per well) were seeded in 96-well plates, and the cytotoxic drug bleomycin was added for 24 h, at the indicated concentrations. NCS was added for 6 h at the indicated concentrations. Optical density representing viable cells was determined by the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay. Cytotoxicity is calculated as % of control untreated cultures, mean ± s.d., $n = 3$, $*P < 0.05$.

Materials

Methyl jasmonate and jasmonic acid (Figure 1) were purchased from Sigma-Aldrich Chemie GmbH, Steinheim, Germany. Bleomycin was purchased from Baxter oncology GmbH, Frankfurt, Germany. Neocarzinostatin (Goldberg & Kappen, 1995) was purchased from Kayaku Co. (Tokyo, Japan). The rest of the reagents used were purchased from Sigma (St Louis, MO, U.S.A.) unless otherwise stated. Jasmonic acid and methyl jasmonate were dissolved in ethanol to give a stock solution of 500 mM. Further dilutions were performed in culture medium. The final concentration of ethanol in cultures did not exceed 0.6%. Neocarzinostatin and bleomycin were dissolved in culture medium.

Statistical analysis

Results are presented as mean ± s.d. of n independent experiments. Statistical significance was assessed using the two-tailed Student's t-test. $P < 0.05$ was considered statistically significant.

Results

To demonstrate the different response of wt and mutant p53-expressing cells functionally, we exposed them to a standard DNA-damaging radiomimetic agent, neocarzinostatin, and to bleomycin. It should be noted that neocarzinostatin was administered at a concentration of 300 ng ml^{-1}, equivalent to 5 Gy, and was active throughout the incubation period. Thus, it delivered a much greater damage in comparison to a one-time γ irradiation hit of the same energy. Neocarzinostatin and bleomycin were practically devoid of any significant cytotoxic effect towards cells expressing the mutant p53 (Figure 2). Furthermore, we studied the difference in basal and stress-induced levels of p53, between wt and mutant p53-expressing cells. As expected (Wallace-Brodeur & Lowe, 1999), the basal levels of p53 were elevated in mutant p53-expressing cells. Neocarzinostatin and bleomycin induced an increase in the p53 levels of wt, but not of mutant, p53-expressing cells (Figure 3).

In order to analyze the role of p53 in the cytotoxic action of jasmonates, we exposed B-lymphoma cells expressing either wt or mutant p53 to two jasmonate derivatives: jasmonic acid and methyl jasmonate (Figure 1). These plant stress hormones were shown by us to induce death in various human and murine cancer cells (Fingrut & Flescher, 2002). Our original finding was that plant stress hormones in general exhibit anticancer effects (Fingrut & Flescher, 2002). One such plant stress hormone is salicylic acid, which is also a nonsteroidal anti-inflammatory drug. Therefore, we used in our former study (Fingrut & Flescher, 2002), as well as in the current one, a range of concentrations based on the highest nontoxic pharmacological blood concentration of salicylic acid in humans (approximately 3 mM; Katzung, 1998). Indeed, these concentrations can be achieved in the blood of mice administered methyl jasmonate i.v., without causing any overt toxicity (Flescher, personal communication).

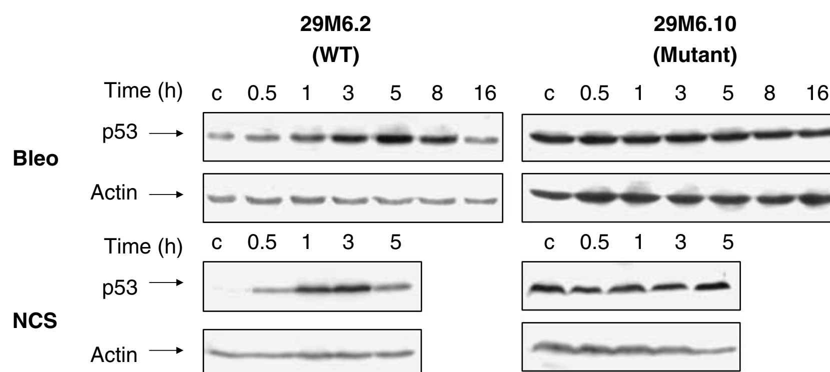

Figure 3 NCS and bleomycin induce a time-dependent elevation of p53 levels, only in wt p53-expressing cells. Wt (the 29M6.2 clone) or mutant (the 29M6.10 clone) 29M4.1 cells (at 6×10^6 per flask) were seeded in 25 cm^2 flasks and treated with or without (control = C) NCS (300 ng ml^{-1}) or bleomycin (Bleo, 50 μg ml^{-1}) for the indicated periods of time. For analysis of p53 levels, whole-cell lysates were prepared and proteins were separated by SDS–PAGE, followed by immunoblotting using specific antibodies against p53. In addition, we performed immunoblotting using specific antibodies against actin as a control for protein loading. Antigen–antibody complexes were stained with HRP-conjugated antibodies and enhanced chemiluminescence reagent, and exposed to ECL film. A typical experiment is shown, representing three experiments with similar results.

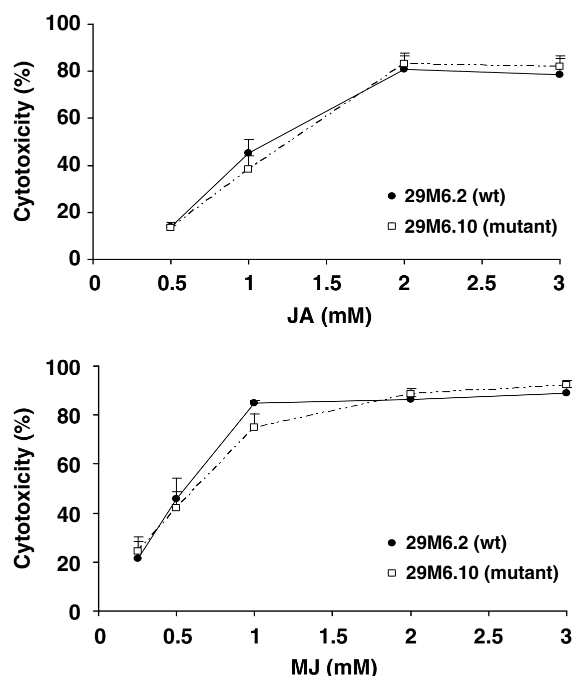

Figure 4 Jasmonates are cytotoxic towards 29M4.1 cells regardless of their p53 status. Wt p53-expressing (the 29M6.2 clone) and mutant p53-expressing (the 29M6.10 clone) cells (at 2.5×10^4 per well) were seeded in 96-well plates, and jasmonic acid (JA) or methyl jasmonate (MJ) at the indicated concentrations was added for 24 h. Optical density representing viable cells was determined as described in the legend for Figure 2. Cytotoxicity is calculated as % of control untreated cultures, mean ± s.d., $n = 3$.

As can be seen in Figure 4, both jasmonates were cytotoxic to the lymphoma cells. Most importantly, jasmonates were cytotoxic towards the B-lymphoma cells to the same extent, regardless of whether they expressed wt or mutant p53. Note that methyl jasmonate did not induce a rise of p53 levels in wt cells (Figure 5).

Next, we assessed potential differences between the jasmonate-induced cell death in wt and mutant p53-expressing cells. Since mutations in p53 often compromise the ability of cells to undergo apoptosis, we analyzed the mode of cell death involved. As can be seen in Figure 6, early apoptosis (Annexin-V + /propidium iodide−) could be detected in the wt p53-expressing cells 2 h after treatment with neocarzinostatin and bleomycin. The percentage of early-apoptotic cells continued to rise, reaching a peak at 6 h. In bleomycin-treated cells, late-apoptotic/dead cells (Annexin-V + /propidium iodide +) were detectable after 8–16 h. Again, treatment with neocarzinostatin or bleomycin did not induce death in mutant p53-expressing cells (Figure 6), demonstrating the high resistance induced in the cells by the p53 mutation. Methyl jasmonate induced apoptotic death in the wt cells. Early-apoptotic wt p53-expressing cells were detected at 2–8 h of methyl jasmonate treatment. Late-apoptotic/dead cells began to appear at 6 h of treatment, reaching a peak at 16 h (Figure 6). On the other hand, early apoptosis was not observed in methyl jasmonate-treated mutant p53-expressing cells (Figure 6). Dead cells that have lost membrane integrity began to appear at 2 h of methyl jasmonate treatment, reaching a peak at 16 h. Since no signs of early apoptosis were detected in mutant cells treated with methyl jasmonate, it is probable that the plasma-membrane permeabilization observed starting at 2 h of methyl jasmonate treatment is the result of a nonapoptotic mode of death.

Mitochondrial oxidative phosphorylation is a major source of cellular ATP. Given the known direct effects of jasmonates on mitochondria isolated from transformed cells (Rotem *et al*., 2005), we evaluated the effect of methyl jasmonate on the levels of ATP in wt *versus* mutant p53-expressing cells. We found that the basal ATP levels of the two clones were similar (5.01 ± 0.68 nmol ATP per 10^6 cells in 29M6.2 cells *versus* 5.47 ± 1.44 nmol ATP per 10^6 cells in 29M6.10 cells). Treatment with methyl jasmonate induced a rapid time- and dose-dependent decrease in cellular ATP levels in both p53 wt and mutant cells (Figure 7). There was a correlation between the inability of neocarzinostatin and bleomycin to induce death in the mutant p53-expressing clone (Figure 2), and the limited degree of cellular ATP depletion induced by the agents in these cells (Figure 7).

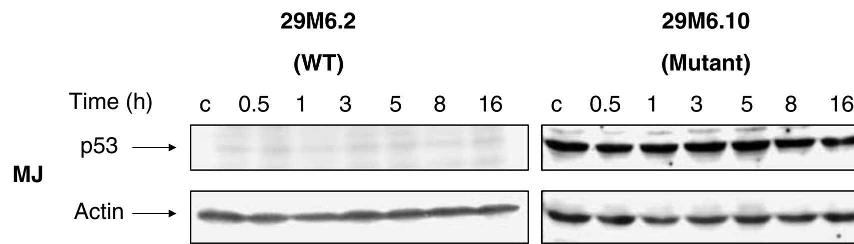

Figure 5 Methyl jasmonate does not induce a rise in the p53 levels of either wt or mutant p53-expressing cells. Mutant (the 29M6.10 clone) or wt (the 29M6.2 clone) 29M4.1 cells (at 6×10^6 per flask) were seeded in 25 cm^2 flasks and treated with or without (control = C) methyl jasmonate (MJ; 1 mM) for the indicated periods of time. p53 and actin levels were analyzed, see the legend for Figure 3. Actin levels were used as a loading control. A typical experiment is shown, representing three experiments with similar results.

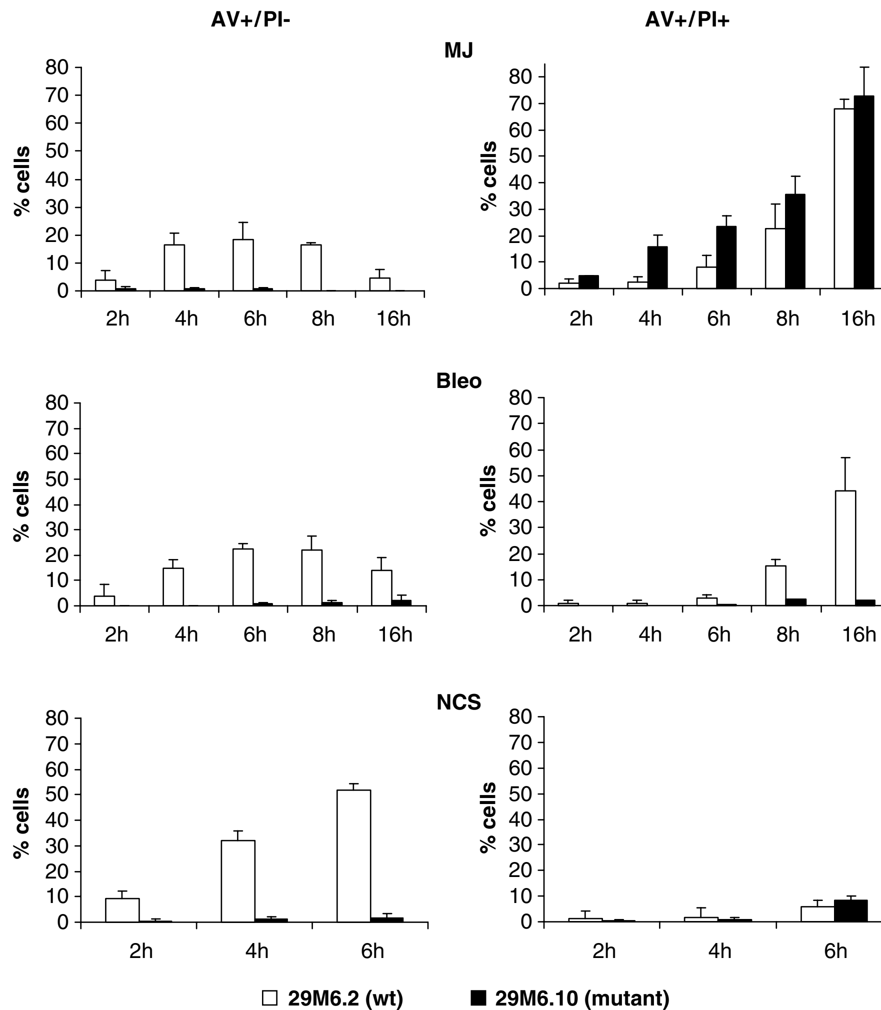

Figure 6 Differential mode of death induced by methyl jasmonate in cells expressing wt *versus* mutant p53. Wt (the 29M6.2 clone) and mutant (the 29M6.10 clone) p53-expressing 29M4.1 cells (at 3×10^5 per well) were seeded in six-well plates for the indicated periods of time, with or without methyl jasmonate (MJ; 1 mM), NCS (300 ng ml^{-1}) or bleomycin (Bleo, 50 μg ml^{-1}). Annexin-V-FITC (AV) and PI-positive cells were detected using the Annexin-V fluorescein apoptosis kit. Cells were analyzed in a flow cytometer apparatus and the percentages of AV- and/or PI-positive cells were calculated using ModFIT-LT2.0 software. Results are presented as mean ± s.d., $n = 3$.

We further investigated the mechanism by which methyl jasmonate-induced ATP depletion varies in the two clones. Cells have two major ATP-generating mechanisms, one *via* oxidative phosphorylation in the mitochondria, using a variety of substrates including pyruvate, and the other by glycolysis occurring in the cytosol, using glucose as a substrate. To assess the importance of glycolysis *versus* oxidative phosphorylation in generating ATP in the two clones (under normal conditions and upon treatment with methyl jasmonate), cells were preincubated for 3 h with 5 μg ml^{-1} oligomycin (Lyamzaev

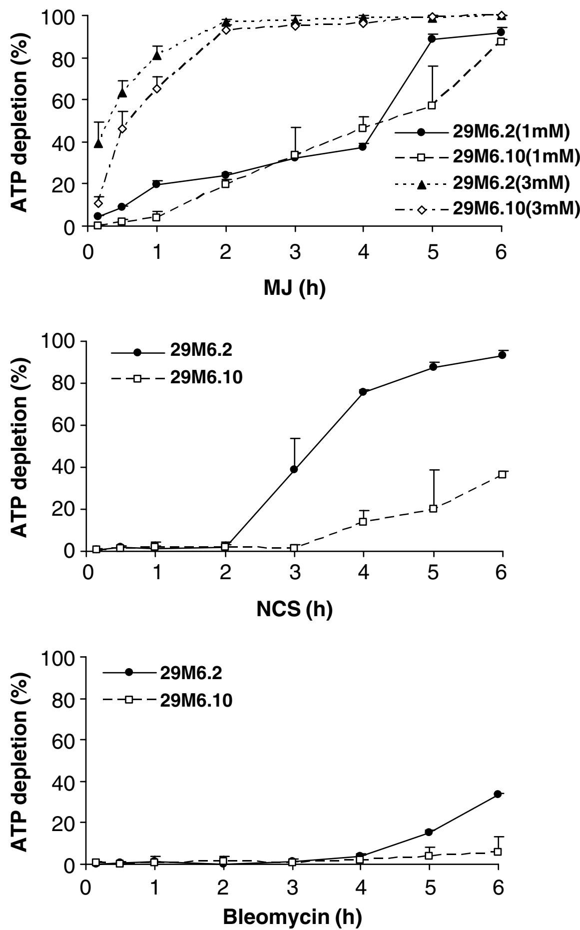

Figure 7 Methyl jasmonate induces rapid ATP depletion in 29M4.1 cells. Wt (29M6.2) or mutant (29M6.10) 29M4.1 cells (at 1×10^4 per well) were seeded in 96-well opaque-walled plates and methyl jasmonate (MJ; at 1 or 3 mM), NCS (at 300 ng ml^{-1}) or bleomycin (at 50 μg ml^{-1}) was added for the indicated periods of time at 37°C. Untreated cells incubated in culture medium for 6 h were used as control. ATP levels were determined by a luciferase-based assay. ATP depletion is calculated as % of control untreated cultures, mean $\pm$ s.d., $n = 3$.

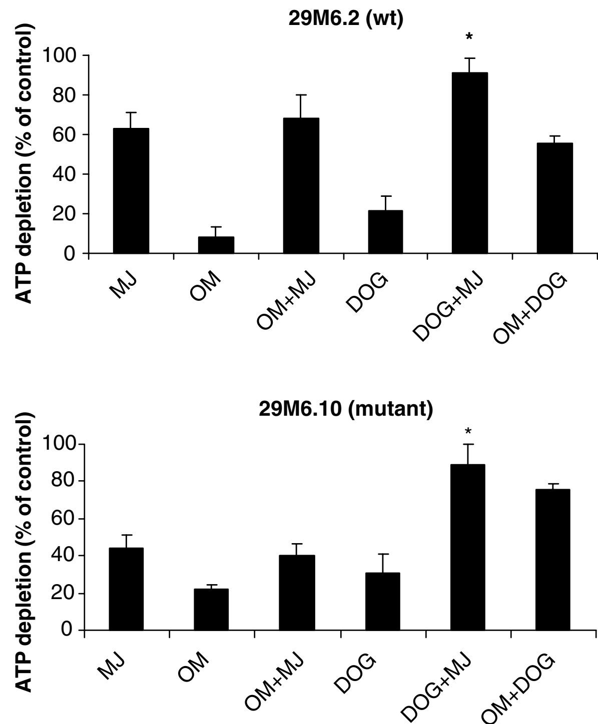

Figure 8 Methyl jasmonate reduces ATP levels by compromising oxidative phosphorylation in the mitochondria. Wt or mutant 29M4.1 cells were seeded in 96-well opaque-walled plates (at 1×10^4 per well) and incubated in culture medium supplemented with the following: none, 5 μg ml^{-1} oligomycin (OM) or 5 mM 2-deoxyglucose (DOG) for 3 h at 37°C. Cells were then either untreated or treated with methyl jasmonate (MJ) at 3 mM for 10 min at 37°C. Cells incubated in culture medium with no supplements for 10 min at 37°C were used as control. ATP levels were determined by a luciferase-based assay. ATP depletion is calculated as % of control untreated cultures, mean $\pm$ s.d., $n = 3$. *$P < 0.05$ comparing ATP depletion following treatment with methyl jasmonate and (methyl jasmonate $\pm$ 2-deoxyglucose).

et al., 2004; Zong *et al.*, 2004) or with 5 mM 2-deoxyglucose (Lyamzaev *et al.*, 2004), before treatment with 3 mM methyl jasmonate for 10 min. Oligomycin inhibits oxidative phosphorylation by inhibiting mitochondrial ATP synthase (Salomon *et al.*, 2000). 2-Deoxyglucose is an inhibitor of glycolysis (Maschek *et al.*, 2004). Incubation of the cells with oligomycin or 2-deoxyglucose induced only a moderate decrease in ATP levels, suggesting that when either glycolysis or oxidative phosphorylation is inhibited there can be partial compensation by the other metabolic pathway (Figure 8). In both p53 wt and mutant clones, preincubation with oligomycin did not increase ATP depletion induced by methyl jasmonate (Figure 8). These results suggest that methyl jasmonate and oligomycin act on the same ATP-generating pathway, that is, the mitochondria, and that methyl jasmonate perturbs these organelles to an extent that renders the oligomycin effect irrelevant. On the other hand, inhibition of glycolysis by 2-deoxyglucose enhanced the effect of methyl jasmonate on ATP levels, yielding a drastic depletion of 90–91% in cellular ATP levels in both clones. This decrease in ATP levels was significantly greater than the ATP depletion caused by methyl jasmonate alone (Figure 8).

To further illuminate the role of glycolysis *versus* oxidative phosphorylation in the response of the two clones to methyl jasmonate, we selectively supplemented the culture medium with substrates of the two major ATP-generating pathways. The concentrations of glucose and pyruvate in the cells' normal culture medium were 11 and 1 mM, respectively. We preincubated the cells for 3 h in: culture media containing glucose at low (2 mM) or high (28 mM) concentration, in the absence of pyruvate; or in culture media containing pyruvate at a low (1 mM) or high (10 mM) concentration, in the absence of glucose. Cells were treated with 3 mM methyl jasmonate for 30 min and ATP levels were determined. High glucose levels protected both wt and mutant p53-expressing cells from methyl jasmonate-induced ATP depletion (Figure 9a). Most importantly, high levels of pyruvate did not protect the cells against methyl jasmonate-induced ATP depletion (Figure 9a). These results suggest again that, in the presence of methyl jasmonate, mitochondria are unable to utilize pyruvate in order to generate ATP *via* oxidative phosphorylation. As a result, ATP levels in both clones drop by 94–95%. To rule out the possibility that the cells cannot inherently utilize pyruvate as a substrate for ATP synthesis, we measured the basal ATP

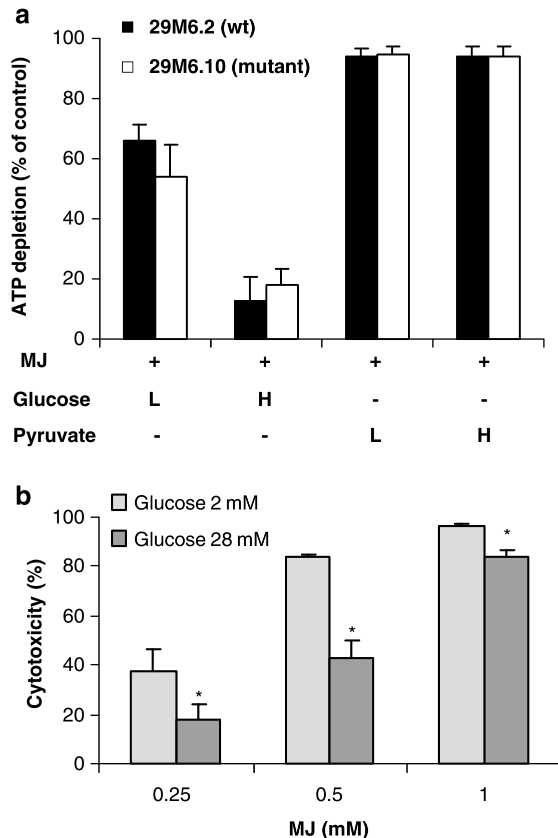

Figure 9 High glucose levels inhibit methyl jasmonate-induced ATP depletion and cytotoxicity. (a) 29M4.1 cells were seeded in 96-well opaque-walled plates (at 1×10^4 per well) and incubated in culture medium containing glucose at 2 mM (Glucose L) or 28 mM (Glucose H) in the absence of pyruvate, or in culture medium containing sodium pyruvate at 1 mM (Pyruvate L) or 10 mM (Pyruvate H) in the absence of glucose, for 3 h at 37°C. Cells were then treated with methyl jasmonate at 3 mM for 30 min at 37°C. Untreated cells incubated in the appropriate culture medium were used as control. ATP levels were determined by a luciferase-based assay. ATP depletion is calculated as % of control untreated cultures, mean ± s.d., $n = 3$. (b) 29M6.2 wild-type cells (at 2.5×10^4 per well) were seeded in 96-well plates, and incubated in culture medium containing glucose at 2 mM (Glucose L) or 28 mM (Glucose H) in the absence of sodium pyruvate for 3 h at 37°C. Cells were then treated with methyl jasmonate at the indicated concentrations for 24 h at 37°C. Untreated cells incubated in the appropriate culture medium were used as control. Optical density representing viable cells was determined as described in the legend for Figure 2. Cytotoxicity is calculated as % of control untreated cultures, mean ± s.d., $n = 3$, $*P < 0.05$.

levels of cells incubated for 3 h in culture media containing glucose or pyruvate as described above. We found that cells incubated in pyruvate (at low or high concentrations) did not exhibit lower ATP levels than cells incubated with glucose. On the contrary, in both clones, average basal ATP levels in cells incubated with pyruvate were slightly higher than in those incubated with glucose (e.g., average basal ATP levels in the 29M6.10 clone were: 5.45 nmol ATP per 10^6 cells in cells cultivated in 28 mM glucose *versus* 6.1 nmol ATP per 10^6 cells in cells cultivated in 10 mM sodium pyruvate). Decreased ATP depletion, in culture medium containing high levels of glucose, correlated with decreased cytotoxicity of methyl jasmonate (Figure 9b). In conclusion, our results suggest that ATP synthesis and its depletion by methyl jasmonate are similar in the wt and mutant p53-expressing B-lymphoma cells under study.

Discussion

We report here on the ability of plant stress hormones belonging to the jasmonate group to kill B-lymphoma cells expressing mutant p53, in contrast to the practical inability of bleomycin and the radiomimetic agent neocarzinostatin to kill these cells. While neocarzinostatin and bleomycin induced a time-dependent increase in the p53 levels of B-lymphoma cells expressing wt p53, methyl jasmonate failed to do so. Furthermore, whereas the cell death induced by methyl jasmonate in wt p53-expressing cells was mostly apoptotic, no signs of early apoptosis were detected in the mutant cells. Methyl jasmonate induced a rapid ATP depletion in both wt and mutant p53-expressing cells. In both clones, inhibition of oxidative phosphorylation by preincubation with oligomycin did not increase methyl jasmonate-induced ATP depletion, whereas inhibition of glycolysis by preincubation with 2-deoxyglucose did. Supplementing the culture medium with high glucose levels had a protective effect against methyl jasmonate-induced ATP depletion and cytotoxicity, whereas high levels of pyruvate did not prevent ATP depletion in either of the clones.

Neocarzinostatin and bleomycin served here as positive controls for compounds that kill preferentially cells expressing wt p53. The most straightforward interpretation to the finding that jasmonates are equally cytotoxic towards cells expressing either wt or mutant p53 is that jasmonates can act upon the B-lymphoma cells in a p53-independent manner. The inability of methyl jasmonate to induce a rise in p53 levels of wt p53-expressing cells suggests further that p53 is probably not involved in the mechanism of action of jasmonates. On the other hand, the differences in the mode of death between the two clones suggest the contrary. We propose that the apoptotic death induced by methyl jasmonate in wt p53-expressing cells is p53-independent. Since p53 is a transcription factor, the p53-independent nature of the jasmonate cytotoxic effect is in line with our recent findings indicating that jasmonate cytotoxicity is independent of mRNA and protein *de novo* synthesis (Rotem *et al*., 2003).

The proposed p53-independent mechanism of action of methyl jasmonate raises another question: why is methyl jasmonate unable to induce early apoptosis in the mutant cells? Mutation of p53 generally results in the inactivation of its tumor suppressor function, conferring in many tumors the ‘gain-of-function’ phenotype, resulting in augmented cell growth and tumorigenic potential (Cadwell & Zambetti, 2001). In addition, the ‘gain-of-function’ causes high resistance to irradiation and to many chemotherapeutic reagents/drugs, and can impact chemotherapeutic outcomes (Li *et al*., 1998; Blandino *et al*., 1999; Wallace-Brodeur & Lowe, 1999; Murphy *et al*., 2000; Cadwell & Zambetti, 2001). We suggest that the mutant p53 may induce a gain-of-function which prohibits a complete apoptotic pathway. Consequently, methyl jasmonate overcomes this block by inducing a form of cell death that is nonapoptotic and involves a disruption of membrane integrity. Of note, it is commonly perceived that apoptosis and necrosis are not mutually exclusive processes, but rather can be a continuum of pathways leading to cell death. In fact, the same

stimulus at different concentrations can commit the same cells to either apoptotic or necrotic pathways. Moreover, cells beginning to die by apoptosis might switch to necrosis if unable to complete the apoptotic cascade (Fantin & Leder, 2004; Lockshin & Zakeri, 2004). Autophagy is another form of death that may be part of a cascade of interchanging death forms (Lockshin & Zakeri, 2004). Apoptotic death is a common outcome of radiation and many chemotherapeutic drugs. However, drug resistance occurs upon mutations which activate antiapoptotic genes and/or inactivate proapoptotic genes (Igney & Krammer, 2002). Therefore, the ability of methyl jasmonate to bypass apoptotic blocks by inducing a nonapoptotic cell death bears great clinical significance. Interestingly, a recent report suggests that carcinoma cells resistant to apoptotic stimuli due to overexpression of Bcl-2 die by necrosis when treated with the mitochondriotoxic compound F16 (Fantin & Leder, 2004). Thus, cells exhibiting abnormal expression of apoptosis-regulating genes (e.g., p53 mutation in our system) may bypass their inability to die by apoptosis, by dying through an alternative death mode, in response to cytotoxic agents.

It was recently found that jasmonates induce cell death by a direct effect on mitochondria, mediated by opening of the permeability transition pore complex (Rotem *et al*., 2005). This kind of mitochondrial damage can cause ATP depletion due to interference with oxidative phosphorylation. Consequently, we evaluated the effects of methyl jasmonate on ATP levels, aiming to identify possible differences between the wt and mutant cells. It was hypothesized that the mode of death of an individual cell could be decided by the availability of energy and the metabolic condition of the cell. Depletion of cellular ATP levels may shift the mechanism of cell death from apoptosis to necrosis (Leist *et al*., 1997; Gonzalez *et al*., 2001). Death induced by methyl jasmonate in the wt cells appeared to be mostly apoptotic, whereas death in the mutant p53-expressing cells lacked signs of early apoptosis and involved a rapid loss of membrane integrity. However, methyl jasmonate induced a similar decrease in both clones. Therefore, depletion of total ATP levels in the cells cannot explain the differences in the mode of death between the two clones. In both p53 wt and mutant clones, preincubation with oligomycin did not increase ATP depletion induced by methyl jasmonate. Therefore, we propose that, in both cell types, methyl jasmonate affects the same metabolic processes as oligomycin, namely oxidative phosphorylation in the mitochondria. In contrast, 2-deoxyglucose and methyl jasmonate had an additive effect on ATP depletion in both clones. The additive affect of methyl jasmonate and 2-deoxyglucose implies that the effects of the two compounds on ATP levels are mediated *via* inhibition of different mechanisms of ATP synthesis. In accordance with our hypothesis that methyl jasmonate causes rapid ATP depletion mainly by compromising ATP synthesis in the mitochondria, high glucose concentration protected the cells from methyl jasmonate-induced ATP depletion and cytotoxicity, whereas high levels of pyruvate did not. Taken together, these results suggest that the mechanism by which methyl jasmonate reduces ATP levels is probably identical in both cell types and results from interference with ATP synthesis in the mitochondria *via* oxidative phosphorylation. Recent evidence suggests that glycolysis has an unexpectedly important role in providing ATP required for sperm motility (Mukai & Okuno, 2004). Interestingly, methyl jasmonate was not toxic towards human sperm cells *in vitro* (Flescher, personal communication). The relative independence of sperm cells from oxidative phosphorylation as a supplier of ATP may account for the insensitivity of these cells to methyl jasmonate.

In conclusion, a novel group of anticancer agents, the jasmonates, are capable of killing B-lymphoma cells expressing wt as well as mutant p53, overcoming the phenotypic resistance to chemotherapy- and irradiation-induced cell death. Jasmonate-induced death of B-lymphoma cells involves severe ATP depletion. These findings support the importance of studying jasmonates as anticancer agents that can potentially eradicate tumors resistant to radiation and to currently available chemotherapy.

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