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RESEARCH ARTICLE

Effect of UVC radiation on mouse fibroblasts deficient for FAS-associated protein with death domain

Lidija Begović^a, Maja Antunović^b, Igor Matic^b, Ivana Furčić^c, Ana Baricević^d, Valerija Vojvoda Parcina^e, Petra Peharec Štefanić^b, Biserka Nagy^b and Inga Marijanović^b

^aDepartment of Biology, University of J. J. Strossmayer in Osijek, Osijek, Croatia; ^bDivision of Molecular Biology, Faculty of Science, University of Zagreb, Zagreb, Croatia; ^cInstitute for Anthropological Research, Zagreb, Croatia; ^dRudjer Boskovic Institute, Center for Marine Research, Rovinj, Croatia; ^eCenter for Research and Knowledge Transfer in Biotechnology, University of Zagreb, Zagreb, Croatia

ABSTRACT

Purpose: Ultraviolet (UV) radiation-induced apoptosis enabled us to study the mechanism of DNA damage and to investigate how cells avoid consequences of damaged DNA. Cells with extensive DNA damage activate extrinsic and intrinsic pathways of apoptosis. The extrinsic pathway is coupled to a FAS-associated protein with death domain (FADD), an adaptor protein molecule necessary for mediating apoptotic signals through the cell.

Materials and methods: Viability and apoptosis of wild-type and FADD-deficient mouse embryonic fibroblasts were investigated 1, 3, 24 and 48 h after exposure to three doses (50, 75 and 300 J/m²) of UVC radiation. Morphological changes were observed using DNA binding dyes (Hoechst and propidium iodide) while biochemical changes were monitored using immunodetection of the poly (ADP-ribose) polymerase (PARP) protein cleavage and caspase-3 activity assay.

Results: Results showed that the difference in cell death response between wild-type and FADD-deficient cells depended on dose and incubation time after exposure to UVC radiation. FADD-deficient cells are more sensitive to UVC radiation. Even though FADD-deficient cells lack an adapter protein of apoptotic extrinsic pathway, higher doses of UVC triggered their apoptotic response, while wild-type cells die mainly due to necrosis. A different pattern of caspase 3 activity and PARP cleavage was observed 24 h after radiation between two cell lines confirming higher apoptotic response in FADD-deficient cells.

Conclusions: Wild-type cells can execute apoptosis via both, the mitochondrial and the receptor-mediated pathway whereas FADD-deficient cells can only activate the intrinsic pathway. There is a difference in UVC radiation response between two cell lines indicating the role of FADD in the selection of cell death modality.

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Apoptosis; FADD; mouse embryonic fibroblasts; necrosis; UVC radiation

Introduction

Ultraviolet (UV) radiation can induce apoptosis in different cell systems (Schwarz 1998). However, the molecular mechanism of apoptosis induced by UVC remains unclear. UVC light has the highest energy within UV spectrum with a strong carcinogenic and cytotoxic effect (Cadet et al. 2005) and for those reasons is the most damaging to biological tissues. It targets both membrane receptors and nuclear DNA, triggering apoptotic signals.

Cell death caused by UV is due to UV-induced DNA damage. Previous studies have shown that DNA repair-deficient cell types are more sensitive to UVC than wild-type cells (Dunkern et al. 2001). UVC radiation also activates CD95/Fas receptor without the binding of CD95/Fas ligand (Rehemtulla et al. 1997). Thus, it was suggested that UVC radiation directly triggers apoptosis by activation of the CD95/Fas receptor. This conclusion was based mainly on the finding that UVC

induces clustering of CD95R/FasR (Aragane et al. 1998). Two cell surface receptors of the TNFR family, TNFR-1 and CD95 (Fas/APO-1), act as death receptors and many proteins mediate apoptosis through these receptors (Weinlich et al. 2011). FADD (Fas-associated protein with death domain) or MORT1 (mediator of receptor-induced toxicity) is an adaptor protein bridging death receptors with initiator caspases (Tournier and Chiochia 2010) acting as a downstream effector of apoptosis (Chinnaiyan et al. 1995). To summarize the induction of extrinsic apoptotic pathway by binding of Fas or TNF (tumor necrosis factor) to their corresponding receptors leads to the formation of a death-inducing signaling complex (DISC) which is composed of the adaptor protein FADD and procaspase-8. This results in the release of active caspase-8 (von Haefen et al. 2011). Activated caspase-8 triggers a cascade of different downstream effector caspases resulting in apoptotic cell death. FADD is essential for TNF-induced apoptosis and has been shown to mediate TNF-dependent

activation of acid sphingomyelinase (A-SMase) (Schwandner et al. 1998), lipopolysaccharide-induced apoptosis (Choi et al. 1998), embryonic development (Yeh et al. 1998) and T-cell activation and development.

In the present study, we addressed the question of UVC-induced apoptosis through FADD signaling pathway in FADD-deficient versus wild-type mouse embryonic fibroblasts. FADD gene knockout (k/o) approach was employed to address the role of FADD in photodynamic therapy (PDT)-induced apoptosis (Nagy et al. 2001). Using the same genetic model we evaluated the role of FADD receptor pathway in apoptosis after UVC radiation.

Materials and methods

Cell lines

Wild-type mouse embryonic fibroblasts (MEF) were derived from wild-type embryos while FADD-deficient were derived from FADD-deficient embryos at 9.5 days of gestation. FADD-deficient animals have been previously described (Yeh et al. 1998) and generated by Dr W. C. Yeh (Toronto, Canada). Wild-type and FADD-deficient MEF were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco Laboratories, Gaithersburg, MD, USA) with 4.5 g/l glucose and 2 mM L-glutamine (Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum (FBS, Gibco Laboratories, Gaithersburg, MD, USA), 100 U/ml of penicillin and 100 µl/ml of streptomycin (Sigma-Aldrich, Taufkirchen, Germany) at 37 °C in a humidified atmosphere containing 5% CO₂. The cells were subcultured every 3–4 days using trypsin-ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich, Taufkirchen, Germany).

Induction of cell death by UVC radiation

To establish confluent cell culture, 1×10^6 cells were seeded in 10 cm Petri dishes, 24 h before treatment. Cells (80% confluent) were exposed to UVC radiation by using a germicidal lamp (model HNS 15W OFR G13, Osram, Zagreb, Croatia) to induce cell death. Radiation dose was measured using UVC dosimeter (UVItec Ltd, Cambridge, U.K.). The culture medium was aspirated, and the cells were exposed to 50, 75 and 300 J/m² of UVC radiation. Afterwards, fresh medium was added, and the cells were incubated at 37 °C with 5% CO₂ for 1, 3, 24 and 48 h. Control cells were handled in an identical manner but without the exposure to UVC radiation.

MTT assay

The viability of FADD-deficient and wild-type cells after exposure to UVC radiation was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, Taufkirchen, Germany) assay. Briefly, in a 96-well microplate containing 5000 cells in each well, 40 µl of 0.5 mg/ml MTT solution in DMEM containing 10% FBS was added to each well and incubated at 37 °C under 5% CO₂. After 4 h, cells were lysed and blue formazan was dissolved by adding 170 µl of dimethyl sulfoxide (DMSO, Sigma-Aldrich,

Taufkirchen, Germany) for 24 h at room temperature. The absorbance was measured at 570 nm using microplate reader (GloMax[®]-Multi Detection System, Promega, Madison, WI, U.S.A.). Non-irradiated cells were used as a positive control. All analyzes were performed in quadruplicates and results are presented as % of control.

Treatment with TNF-alpha

To induce CD95/Fas-receptor mediated apoptosis both cell lines were incubated for 24 h with tumor necrosis factor alpha (TNFα, Sigma-Aldrich, Taufkirchen, Germany) in the presence of the protein synthesis inhibitor cycloheximide (CHX, Sigma-Aldrich, Taufkirchen, Germany). Final concentrations of TNF-alpha were 25 and 50 ng/ml while final concentration of CHX was 10 µg/ml. Control cells were treated only with 10 µg/ml of CHX.

Determination and quantification of apoptosis and necrosis

For semi-quantitative assessment of apoptosis, nuclear apoptotic changes were visualized using DNA-binding fluorochrome-bis-benzimide trihydrochloride (Hoechst 33258, Sigma-Aldrich, Taufkirchen, Germany) and propidium iodide (Sigma-Aldrich, Taufkirchen, Germany). All cells were harvested (floating and adhered), washed twice in phosphate buffered saline (PBS, Gibco Laboratories, Gaithersburg, MD, U.S.A.), centrifuged at 1500 rpm for 5 min and fixed for 30 min in 3.7% formaldehyde/PBS at room temperature. After centrifugation, cells were resuspended in 10 µl of Hoechst 33258 (100 µg/ml) and 10 µl of propidium iodide (2 µg/ml). Cells displaying typical apoptotic nuclear morphology: crescent shaped, nuclear periphery, condensed chromatin, apoptotic bodies were used for calculation of apoptotic index (number of apoptotic cells over the number of total cells counted expressed as a percentage). The necrotic index (number of necrotic cells over the number of total cells counted expressed as a percentage) was calculated by counting cells displaying typical necrotic morphology. Apoptotic and necrotic features of the cells were assessed using fluorescence microscope (Olympus BX51) and each time triple determinations were performed counting at least 500 cells.

Total protein extraction and quantification

Following the UVC radiation, cells were collected, washed twice with ice-cold PBS (Gibco Laboratories, Gaithersburg, MD, U.S.A.) and centrifuged for 5 min at 400 g and 4 °C. The pellet was resuspended in 50 µl of chilled Cell Lysis Buffer (Invitrogen, Carlsbad, CA, U.S.A.) without a protease inhibitor. Samples were incubated on ice for 10 min and then centrifuged for 1 min at 10,000 g and 4 °C. The supernatant was collected and stored at –80 °C for further analysis. Protein concentrations were measured by using bicinchoninic acid (BCA) protein assay (Pierce, Thermo Fisher Scientific, Waltham, MA, U.S.A.) following the manufacturer's protocol. Absorbance

was measured using microplate reader (Labsystems Diagnostics, Vantaa, Finland) at 570 nm.

Detection of PARP cleavage by Western blotting

Five micrograms of proteins were separated on 4–20% polyacrylamide gels (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, U.K.) as described in Antunovic et al. (2015). Briefly, after transfer to the nitrocellulose membrane (GE Healthcare Life Sciences, Little Chalfont, U.K.) membrane was blocked in Tris-buffered saline (20 mM Tris-HCl, 150 mM NaCl) containing 0.1% Tween 20 and 5% non-fat milk. Anti-PARP-1 (Santa Cruz Biotechnology, Dallas, TX, U.S.A.) or anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH) (Sigma-Aldrich, Taufkirchen, Germany) antibodies were diluted 1:1000 and 1:5000, respectively. After overnight incubation at 4 °C, the membrane was washed in TBST and incubated for 1 h at room temperature with the anti-rabbit HRP-linked secondary antibody (Cell Signaling Technology, Danvers, MA, U.S.A.) diluted 1:2000. Blots were developed by Clarity Western ECL Substrate (Bio-Rad, Hercules, CA, U.S.A.) and Hyperfilm ECL (GE Healthcare Life Sciences, Little Chalfont, U.K.). The film was developed using Kodak processing chemicals for autoradiography films (Sigma-Aldrich, Taufkirchen, Germany).

Caspase -3 activity assay

Caspase-3 Colorimetric Protease Assay (Invitrogen, Carlsbad, CA, U.S.A.) was used to detect caspase-3 activity in wild-type and FADD-deficient cells after UVC radiation (doses 0, 50, 75, 300 J/m²) according to the manufacturer's instructions. Fold-increase in caspase activity was determined by direct comparison to the level of the wild-type control. All analyzes were performed in duplicates.

Statistical analyses

Changes in caspase-3 activity following UVC radiation of wild-type and FADD-deficient cell lines were evaluated by two-way ANOVA. Changes were compared to control (non-irradiated cells) for each cell line separately as well as between these two cell lines, using Tukey's post test, with $p < 0.05$ considered statistically significant. Results are presented as the mean value of two to three experiments \pm standard deviation (SD). Different letters represent statistical difference at $p \leq 0.05$. All statistical analyzes were done with Statistica 12 Software (StatSoft Inc., U.S.A.).

Results

Cell viability is decreased in dose- and time-dependent manner after UVC exposure

Cell viability was determined by the MTT assay. The viability of all cells was decreasing with the dose and post-exposure time (Figure 1(A)–(D)). However, the FADD-deficient cells were more sensitive to UVC radiation. The viability of both cell

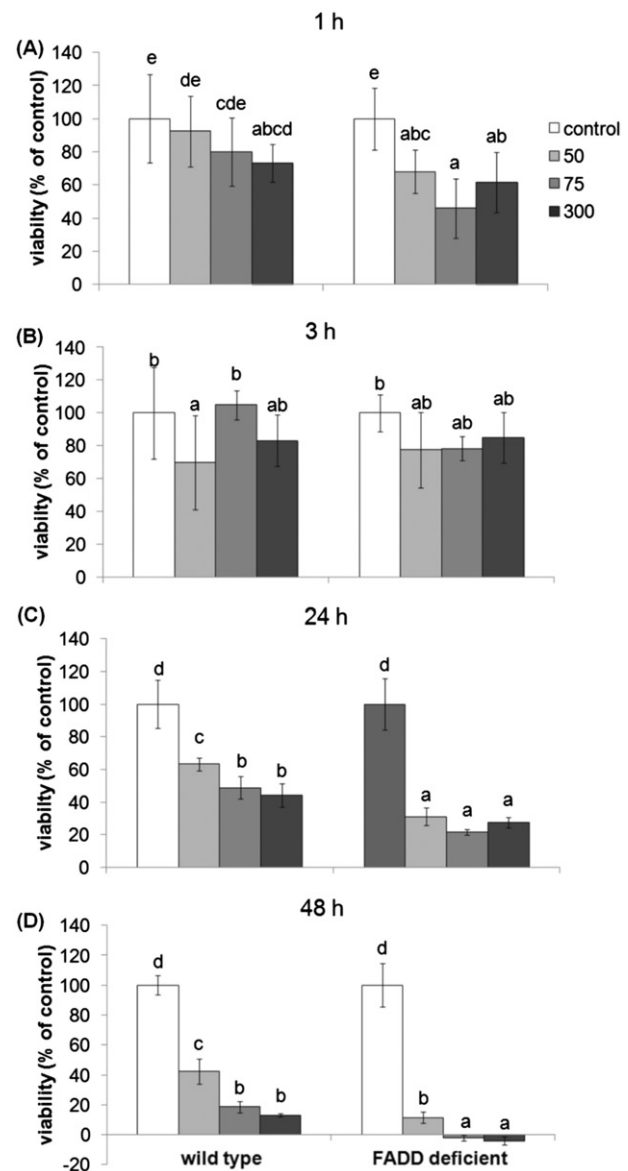


Figure 1. The effect of UVC radiation on the viability of wild-type and FADD-deficient cells after (A) 1 h, (B) 3 h, (C) 24 h and (D) 48 h of exposure to UVC radiation (50, 75 and 300 J/m²) measured using MTT assay. Cells exposed to increasing doses of irradiation showed a dose-dependent decrease in viability. Results are represented as percentage of viable cells compared to untreated cells (control). Different letters represent statistical difference at $p \leq 0.05$ between control and treated cells and between two cell lines for each exposure time separately. Error bars represent \pm SD of mean values of three experiments ($n = 3$).

lines, 24 h after exposure, decreased significantly, but to a higher extent in the FADD-deficient cell line (Figure 1(C)). The viability of FADD-deficient cells, 48 h after exposure to all three doses, significantly decreased by 80% at 50 J/m². Higher doses induced severe decrease, even up to 100%, when compared to control (Figure 1(D)). The viability of wild-type cells was also significantly decreased compared to control, but to a lesser extent than it was observed in the FADD-deficient cell line.

Induction of apoptosis with TNF-alpha

To verify that increased number of apoptotic cells in the FADD-deficient cell line was a consequence of UVC radiation

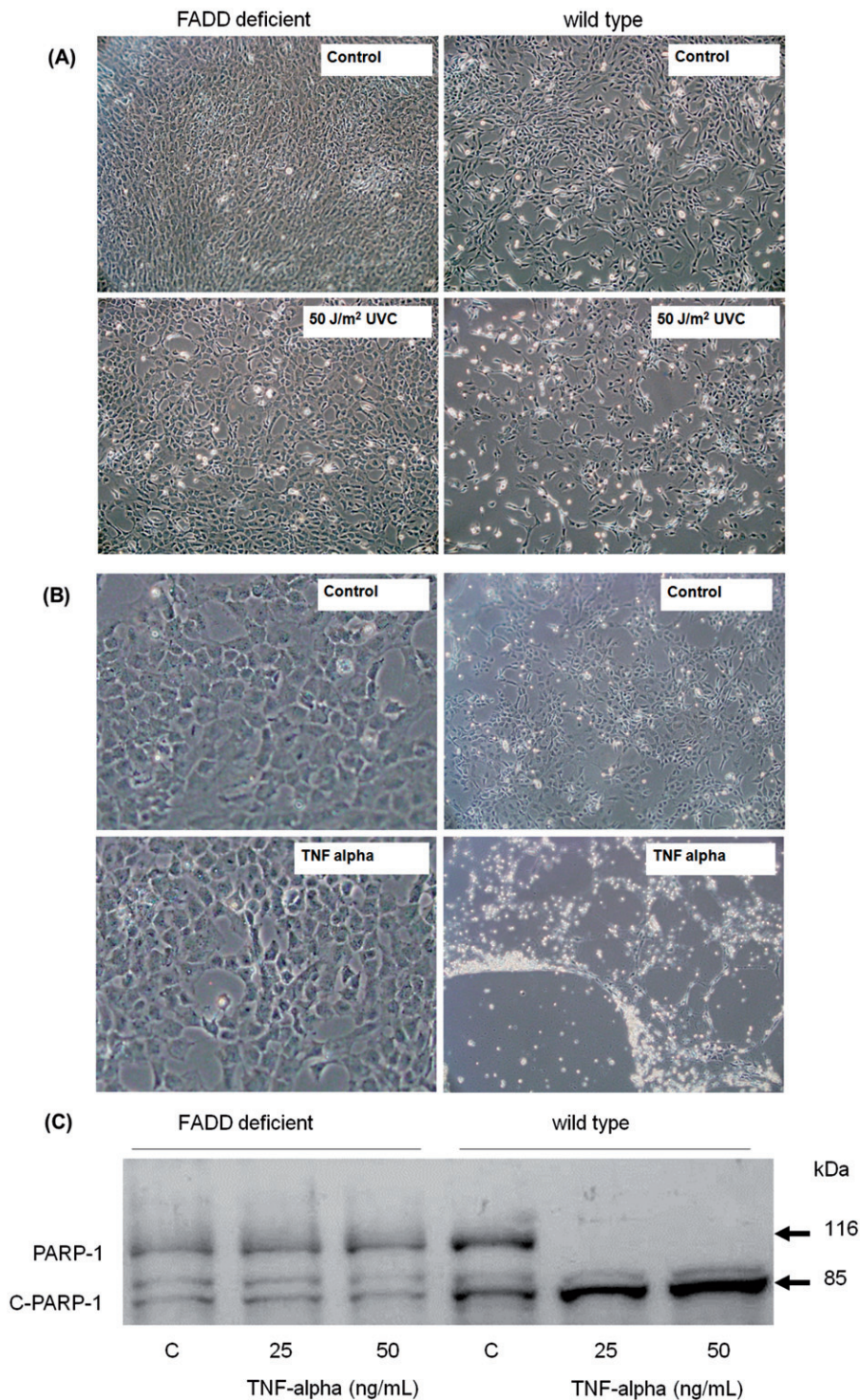


Figure 2. (A) UVC-induced apoptotic morphology 24 h after UVC radiation in wild-type and FADD-deficient cell lines. (B) TNF-alpha-induced apoptosis only in wild-type cells. Cells were collected 24 h after treatment with TNF-alpha (25 ng/ml) and cycloheximide (10 µg/ml) and examined under a light microscope (original magnification 100×). Control cells were treated with cycloheximide only. Apoptotic cells detached from the plate. (C) Cleavage of poly (ADP-ribose) polymerase (PARP) protein in wild-type and FADD-deficient cells 24 h after treatment with TNF-alpha (25 and 50 ng/ml) with the addition of cycloheximide (10 µg/ml). Control cells (C) are treated with 10 µg/ml cycloheximide. Arrows indicate 116 kDa (whole protein) and 85 kDa (cleaved fragment).

but not cell hypersensitivity for apoptotic pathway activation, we induced the cell death via extrinsic pathway. Both cell lines, wild-type and FADD-deficient, were treated with TNF-alpha (Figure 2). In contrast to UVC-radiated cells (Figure 2(A)), TNF-alpha-treated FADD-deficient cells did not undergo apoptosis (Figure 2(B)) as wild-type cells indicating that the FADD-deficient cells are not sensitized to undergo apoptosis

upon death receptor apoptotic pathway, but only upon the induction of UV-induced DNA damage. Also, treatment with TNF-alpha-induced cleavage of poly(ADP) ribose polymerase (PARP) in wild-type cells which resulted in the appearance of 85 kDa protein fragment, which was not observed under identical treatment conditions by TNF-alpha (25 and 50 ng/ml) in FADD-deficient cells (Figure 2(C)).

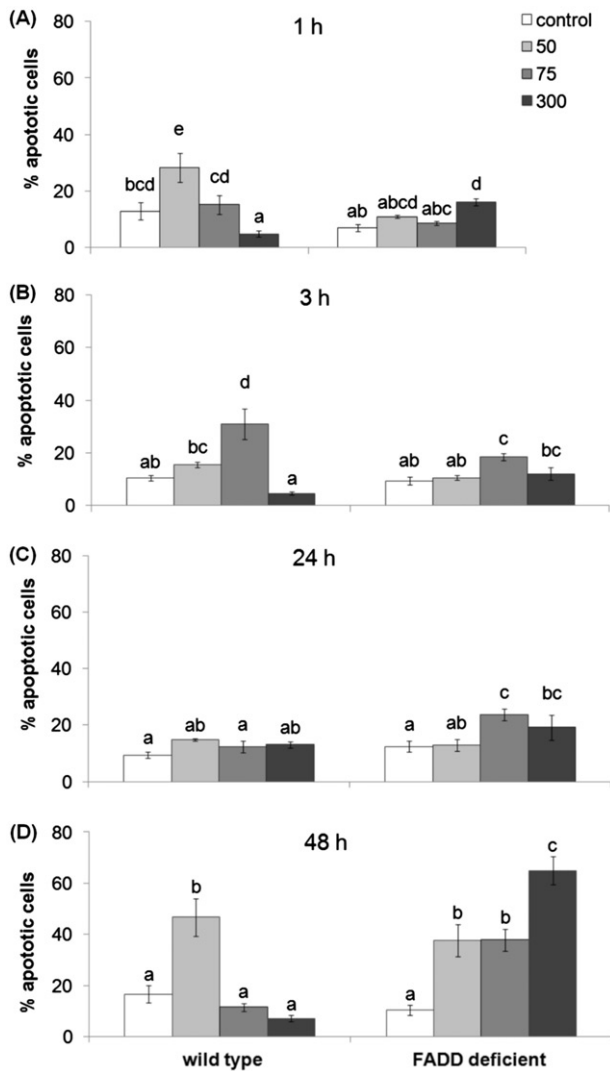


Figure 3. Percentage of cells displaying apoptotic morphology in wild-type and FADD-deficient cell lines after 1 h (A), 3 h (B), 24 h (C) and 48 h (D) of UVC radiation at 50, 75 and 300 J/m². Different letters represent statistical difference at $p \leq 0.05$ between control and treated cells and between two cell lines for each exposure time separately. Error bars represent \pm SD of mean values of three experiments ($n = 3$).

Apoptosis is the main cell death mode in the FADD-deficient cell line

To elucidate whether decreased cell viability after exposure to UVC radiation was due to the induction of apoptosis, we have quantified apoptotic and necrotic cells in both cell types. Cell death due to apoptosis was first observed 1 h after UVC radiation in both cell types (Figure 3(A)), but the percentage of apoptotic cells over time differed due to different cell death modalities in FADD-deficient and wild-type cells (Figures 3 and 4). A number of FADD-deficient apoptotic cells reached 65%, 48 h after exposure to 300 J/m² of UVC (Figure 3(D)). However, less than 10% of wild-type cells died due to apoptosis under the same conditions. Altogether, 48 h after exposure, FADD-deficient cells were more sensitive to apoptotic cell death induced by UVC radiation at higher doses (75 and 300 J/m²) compared to wild-type cells. Furthermore, the frequency of necrotic death was higher in wild-type cells 48 h after exposure to 300 J/m² (Figure 4(D)). The percentage of

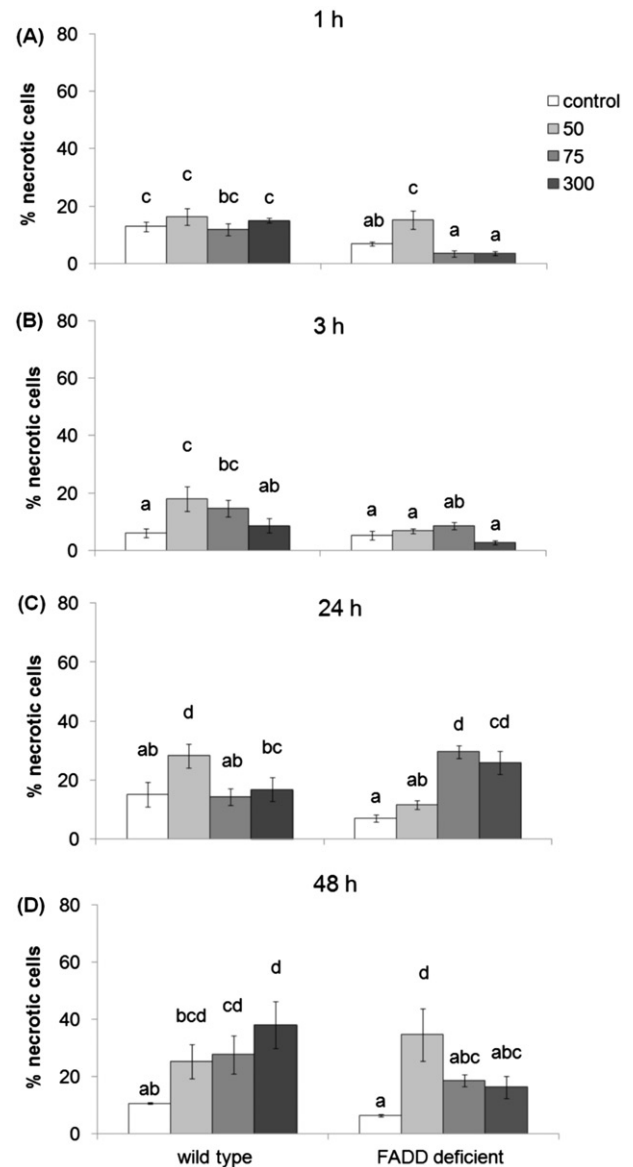


Figure 4. Percentage of cells displaying necrotic morphology in wild-type and FADD-deficient cell lines after 1 h (A), 3 h (B), 24 h (C) and 48 h (D) of UVC radiation at 50, 75 and 300 J/m². Different letters represent statistical difference at $p \leq 0.05$ between control and treated cells and between two cell lines for each exposure time separately. Error bars represent \pm SD of mean values of three experiments ($n = 3$).

wild-type necrotic cells was 40% and FADD-deficient 20% while at lower doses, cell lines did not differ significantly (Figure 4).

UVC radiation activates caspase-3 and consequently cleaves PARP in time- and dose-dependent manner

Induction of apoptosis in wild-type and FADD-deficient cells by UVC was further confirmed and quantified by caspase-3 activity assay and caspase-3 cleavage of poly (ADP) ribose polymerase (PARP). The activity of caspase-3 in FADD-deficient and wild-type cells increased in a time-dependent manner after exposure to 50, 75 and 300 J/m² (Figure 5). Activity was higher in FADD-deficient cells when compared to

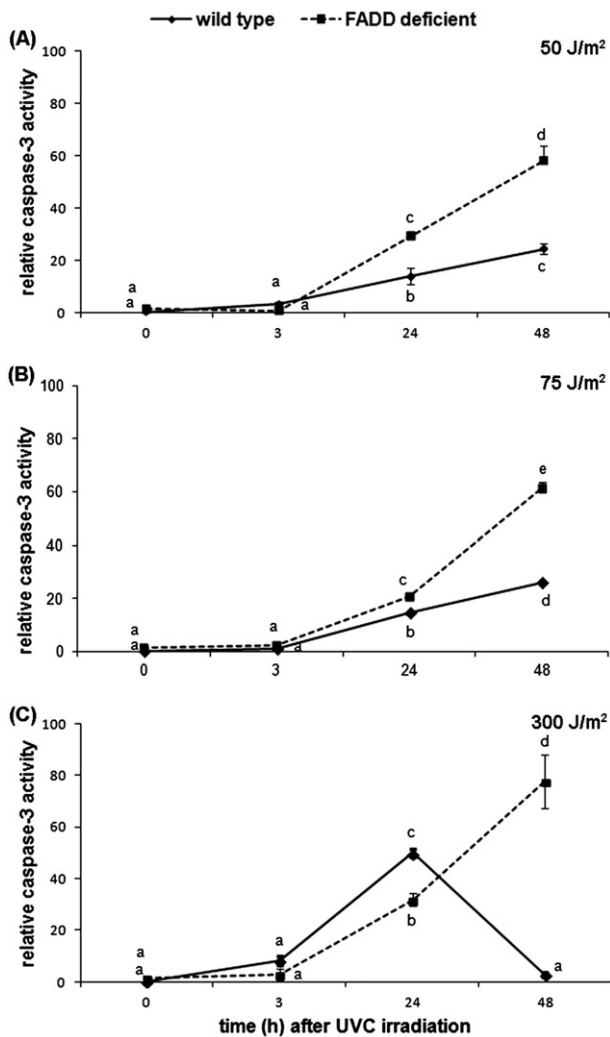


Figure 5. Measurement of caspase-3 activity in wild-type and FADD-deficient cells after 0, 3, 24 and 48 h of UVC radiation at (A) 50, (B) 75 and (C) 300 J/m². Different letters represent statistical difference at $p \leq 0.05$ between control and treated cells and between two cell lines for each UVC dose separately (50, 75 and 300 J/m²) 0, 3, 24 and 48 h after UVC exposure. Error bars represent \pm SD of mean values of three experiments ($n = 3$).

the wild-type cells except 48 h after exposure to 300 J/m² when activity dramatically decreased (Figure 5(C)). These data are in accordance with the necrotic phenotype of wild-type cells observed 48 h after exposure to 300 J/m² of UVC radiation (Figure 4(D)). Caspase-3 activity can be observed through PARP cleavage resulting in the appearance of 85 kDa protein fragment. Cleavage of PARP was detected 24 h after UVC radiation by Western blotting in the FADD-deficient and wild-type cells (Figure 6(C)) which is in accordance with the activity of caspase-3 (Figure 5(A)–(C)) at all three doses of UVC. Twenty-four hours after radiation two cell lines exhibited major differences in PARP protein expression. FADD-deficient cells had higher expression of PARP and cleaved PARP fragment when compared to the wild-type cells (Figure 6(C)). After 48 h of exposure to three different doses of UVC (50, 75 and 300 J/m²) cleavage of PARP protein was observed in both cell lines (Figure 6(D)). In accord with their necrotic status, wild-type cells irradiated with UVC dose of 300 J/m² did not exhibit clear bands (Figure 6(D)).

Discussion

Previous studies demonstrated that UVC efficiently induces apoptosis in many cell types (Sheikh et al. 1998, Dunkern and Kaina 2002, Feng et al. 2012, Radhakrishnan et al. 2012, Lee et al. 2014). It was shown that UVC can activate the CD95/Fas receptor independently without binding to CD95/Fas ligand (Rehemtulla et al. 1997, Aragane et al. 1998, Dunkern et al. 2001). Also, UV-induced apoptosis is possible in caspase-8-deficient Jurkat T-lymphocytes and mouse fibroblasts that are resistant to CD95/Fas-induced apoptosis (Juo et al. 1998, Varfolomeev et al. 1998). Therefore, the activation of caspase-8 may not play an important role in UVC-induced apoptosis.

Since FADD death signaling has not been required for apoptosis after UVC radiation in mouse embryonic fibroblasts, the question remains whether the lack of FADD will have an impact on cell death modality. It is reasonable to conclude that apoptosis is the main consequence of UVC-induced cytotoxicity, both in wild-type and FADD-deficient cells. UVC radiation activates the mitochondrial damage pathways in both cell lines. However, the cell death due to induction of apoptosis was higher in FADD-deficient cells, supporting the model of a direct activation of caspase cascade, independently of ligand binding. Possible ways how UVC radiation utilizes apoptosis are: (a) It does not require expression of FADD adaptor protein; (b) it causes PARP proteolysis and caspase activation; and (c) it causes different responses on cell proliferation in wild-type and FADD-deficient cells. We could assume that in FADD-deficient cells, the high level of non-repaired DNA damage forces signaling by upregulation of mitochondrial pathway, leading to enhanced UVC-induced apoptosis. Therefore, in FADD-deficient cells the mitochondrial pathway appeared to be dominant because the receptor/ligand triggered pathway is blocked. In wild-type cells, UVC-induced apoptosis can be executed via both, the mitochondrial and receptor-mediated pathways. In FADD-deficient cells, corresponding with the cellular viability determined by the MTT assay, a high level of UVC radiation led to enhanced apoptosis. We found that the response of two cell lines to UVC radiation was highly dependent on the dose. It was shown that low and high doses of UVC induce DNA damage and generate distinct sets of genetic responses (Gentile et al. 2003). For example, 48 h after the exposure of wild-type cells to the highest dose (300 J/m²) of UVC, viability decreased and necrosis was the dominant cell death mode. In contrast, the viability of FADD-deficient cells was significantly higher with apoptosis as a dominant cell death mode. This suggests that necrotic cell death needs the presence of the FADD adapter protein leading to the conclusion that necrosis in wild-type cells after higher doses of UVC is a regulated form of cell death, known as necroptosis. Such results indicated that FADD might have a role in the selection of cell death modality and confirms the possibility that necrosis can be a FADD-mediated and regulated process.

It was also documented that UVC directly activates death receptors in a ligand-independent way by inducing receptor clustering. Activated death receptors trimerize and transduce the apoptotic signal via their intra-cytoplasmic death domain (Schulze-Osthoff et al. 1998, Sartorius et al. 2001). UV induces

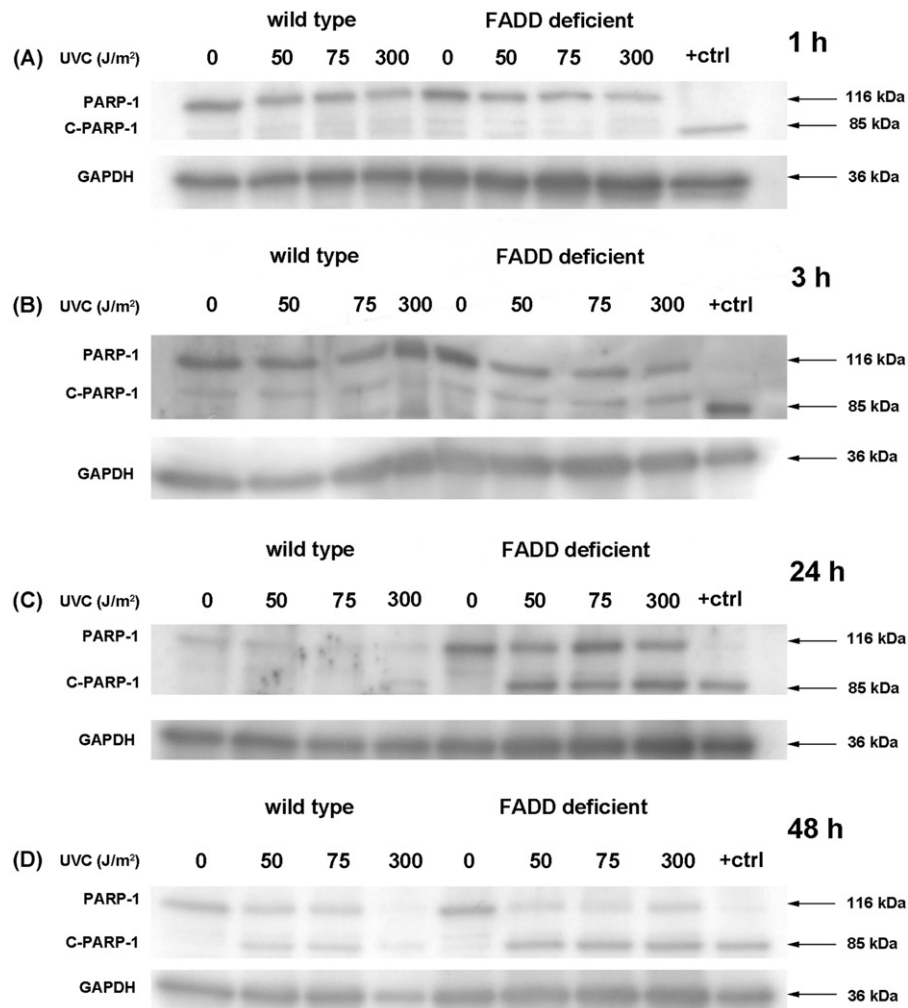


Figure 6. Poly (ADP-ribose) polymerase protein (PARP) cleavage in wild-type and FADD-deficient cells treated with UVC light after (A) 1 h, (B) 3 h, (C) 24 h and (D) 48 h. Arrows indicate 116 kDa (whole protein) and 85 kDa (cleaved fragment). 0, untreated control; +ctrl, positive control (wild-type cells treated with TNF-alpha). GAPDH is loading control.

the release of cytochrome c from mitochondria (Mootha et al. 2001). Whether UV affects the mitochondria directly or by receptor activation, or the induced apoptosis is a consequence of DNA damage, remains to be determined.

PARP is a nuclear protein involved in the repair of DNA damage caused by ionizing radiation, alkylating agents, and free radicals. PARP cleavage by caspases, generating 24 and 85 kDa-fragment, is one of the earliest signs of apoptosis (Boulares et al. 1999). In our study, PARP cleavage was detected upon exposure to UVC in both cell lines. Cleavage of PARP protein is a consequence of caspase-3 activation as well as DNA fragmentation (Enari et al. 1998). It was proposed that PARP cleavage might be indirectly involved in triggering apoptosis. This suggestion was based on inhibition of PARP homodimerization by the C-terminal cleavage product that reduced cellular PARP activity which is important for defense against UVC-mediated apoptosis (Kim et al. 2000).

It has been reported that UV-induced DNA damage leads to apoptosis via activation of p53 (Latonen et al. 2001). Also, UV activates death receptors expression on the cell surface via induction or up-regulation of death ligands (Lee et al. 2012). Several line of evidence indicates that p53 activates the CD95/Fas pathway in response to DNA damage by

anticancer drugs (Müller et al. 1998) and by UV (Kulms and Schwarz 2000). Lackinger and Kaina (2000) showed that p53-deficient mouse fibroblasts were more sensitive to UVC light and alkylating agents than wild-type cells.

One might argue that the apoptotic pathway is evoked specifically in wild-type cells. In this study, we demonstrated that FADD-deficient cells die by apoptosis triggered with UVC that indicated that DNA damage and activation of Fas receptor independently contribute to the programmed cell death. Although embryonic fibroblasts from FADD knockout mice were resistant to apoptosis mediated by TNF-alpha, they retained sensitivity to UVC radiation.

To summarize, wild-type cells can execute apoptosis via both, the mitochondrial and the receptor-mediated pathway whereas FADD-deficient cells can only activate the intrinsic pathway. There is a difference in UVC radiation response between two cell lines with the major difference presented 48 h after the exposure to 300 J/m² when FADD-deficient cells extensively die due to apoptosis while wild-type cells have better viability and die mainly due to necrosis. Thus, we conclude that FADD, although required for death receptor-mediated pathways, is dispensable in other modalities of programmed cell death as well as cell survival.

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Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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