

Effect of UVC irradiation on p53 in FADD^{+/+} and FADD^{-/-} cells

Igor Matić*, Maja Radnić, Inga Marijanović, Ivana Furčić and Biserka Nagy

University of Zagreb, Faculty of Science, Department of Biology,
Horvatovac 102a, HR-10 000 Zagreb, Croatia

Abstract. The dominant paradigm of tumor biology is that evasion from apoptosis is one of the crucial features of malignant diseases and that the efficiency of cancer therapy depends on P53-dependent apoptosis. Because of the importance of apoptotic pathways in protecting cells against malignant transformation, disruption of apoptosis is extremely common in cancer cells, and is frequently due to mutations in the P53 tumor suppressor gene.

Fas-associated death domain (FADD) is an adapter protein that is required for apoptosis induced by all known death receptors. FADD is implicated in death receptor-independent apoptotic response, such as DNA damage. We used embryonic fibroblasts derived from FADD knockout mice and their genetic counterparts.

We predicted that UV exposure induces a loss of FADD function and leads to mutations in P53. Loss of FADD expression causes deregulation of apoptosis and expansion of mutated cells and initiation of cancer. We predicted that FADD dysfunction may be potentially advantageous for tumor growth and that FADD can act as a tumor suppressor.

Cells were irradiated with UVC light (254 nm) using a germicidal lamp (Upland, CA). The culture media was drained before the irradiation and fresh media was added after. In the first experiment we irradiated cells with a dose of 25 J/m² and after 5 days we isolated genomic DNA but part of the cells were irradiated again with the same dose. After 5 days DNA were isolated so the cumulative irradiation dose was 50 J/m². In the second experiment cells were irradiated ones with the dose of 40 J/m² and DNA was isolated after 18 days. Lethal dosage for each cell line is 50 J/m². Genomic DNA was analyzed by allele-specific polymerase chain reaction (AS-PCR) for CC to TT mutation at codons 154-155 and 175-176 in exon 5 and for C to T mutations at codons 270 and 275 in exon 8 of the P53 gene. The mutant-specific forward primer was used for each mutation. The reverse primers for amplification of mutations were not mutant-specific. Allele-specific PCR detection of p53 in genomic DNA from UV-irradiated knockout and wild type cells were analyzed by gel electrophoresis.

The results indicated that the mutant-specific primers (154-155, 175-176 and 275) didn't amplified 119-, 55- and 134-base pair (bp) products, unexpectedly, from irradiated FADD^{+/+} and FADD^{-/-} cell DNAs as well as expected from non-irradiated FADD^{+/+} and FADD^{-/-} cell DNAs. In contrast, the mutant-specific primer for codon 270 amplified 134-base pair product from irradiated FADD^{+/+} and FADD^{-/-} cell DNAs. Because FADD is required for elimination of UV-irradiated cells, its loss could inhibit apoptosis and lead to expansion of apoptosis defective cells that contain p53 mutations. The data presented here suggest that loss of FADD expression and loss of wild type p53 function due to mutations can inhibit apoptosis and lead to the expansion of UV carcinogenesis.

KEYWORDS: UVC radiation, mutations in p53, Fas-associating protein with death domain (FADD), AS-PCR

1. Introduction

The p53 tumor suppressor gene is found to be mutated in a majority of human cancers. The p53 protein plays therefore an important role in several signalling pathways related to cellular responses to different mutagens and carcinogens. Nuclear p53 expression is elevated after UV irradiation, and following a genotoxic insult p53 is involved in cell cycle arrest and apoptosis [1]. P53 gene appears to bear point mutations with the exact features of UV-induced point mutations, i.e., associated with di-pyrimidinic sites, mostly C to T transition [2]. Fas-associated protein with death domain (FADD) is adapter molecule required for the induction of apoptosis and in a highly regulated fashion can transduce either a signal for survival or one that leads directly to apoptosis. The nuclear localization of FADD and its interaction with a DNA repair protein [3] suggest its implication in a number of growth inhibitory signalling pathways. We were therefore interested to determine whether FADD loss influence the UV-

* Presenting author, E-mail: imatic@zg.biol.pmf.hr

induced mutations in p53 gene. Several studies have shown that the p53 tumor suppressor gene is a target for UV-induced mutations and plays a critical role in induction of skin tumors. In addition, defects in receptor signalling death pathway of apoptosis may play an important role in the development of UV-induced skin tumors. In addition, loss of receptor pathways of apoptosis correlate with progression in colon carcinoma and melanoma and the wild-type of p53 protein can up-regulate Fas/FasL interaction [4]. We recently found that FADD was not essential for the induction of apoptosis in UV-irradiated cells. In this study we investigated the appearance of p53 mutations in UV-irradiated cells without and with FADD expression. The aim of this study was to find out whether FADD could reduce or prevent the induction of p53 mutations in UV-irradiated cells.

2. Materials and Methods

2.1 Cell culture

Cells were cultured at 37°C in a 5% CO₂ atmosphere with DMEM containing 10% fetal calf serum, 2mM glutamine, 4.5g/l glucose, 100units/ml penicillin, and 100µg/ml streptomycin. Cells were sub-cultured in every 3-4 days using Trypsin-EDTA.

2.2 UV irradiation of cells

Cells were irradiated with UVC light (254 nm) using a germicidal lamp (Upland, CA). The culture media was drained before the irradiation and fresh media was added after. In the first experiment, we irradiated cells with a dose of 25 J/m² and isolated genomic DNA after 5 days; part of the cells were irradiated again with the same dose (the cumulative irradiation dose was 50 J/m²) and DNA was isolated after 5 days. In the second experiment, cells were irradiated once with the dose of 40 J/m² and DNA was isolated after 18 days. Lethal dosage for each cell line was 50 J/m².

2.3 Allele-specific polymerase chain reaction, AS-PCR

Genomic DNA was analyzed by AS-PCR for CC to TT mutation at codons 154-155 and 175-176 in exon 5, and for C to T mutations at codon 270 and 275 in exon 8 of the p53 gene. The universal reverse primers used for amplification of mutations hybridize to both wild type and mutant 53 alleles: 5'GAGGGCTTACCATCACCATC3' (20-mer) for codons 154-155 and 175-176 and 5'GCCTGCGTACCTCTCTTTGC3' (20-mer) for codons 270 and 275. The upstream mutant-specific primer 5'CCTCCAGCTGGGAGCCGTGCTT'3 hybridize to mutant codon 154-155 but not wild type sequences because the last three nucleotides (CTT) correspond to mutant bases present in UV-induced tumors. Similar mutant-specific primers for detecting tandem TT mutations at codon 175-176 and C to T mutation at the codon 270 and codon 275 were used (5'TCGTGAGACGCTGCCCCCATT3', 5'GGACGGGACAGCTTTGAGGTTT3' and 5'GTGTTTGTGCCTGCCT3', respectively).

Genomic DNA (360ng) from UV-irradiated knockout and wild type cells were amplified by AS-PCR in a 50µL reaction mixture containing 0,55µM of each primer, 2,8mM MgCl₂, 0,12mM dNTPs and 1,85U of AmpliTaq Gold® DNA Polymerase (Applied Biosystems). Cycling conditions were: 6 minutes (min) denaturation at 95°C; followed by 35 cycles of 1min denaturation at 94°C, 1min annealing at 63°C (for codon 175-176), 65°C (for codon 275) or 69°C (for codons 154-155 and 270), and 1min extension at 72°C; with final extension 7min at 72°C. PCR products were analyzed on 2.0% agarose gels.

Mutant-specific primers amplified 119 bp product from cells having mutations at codon 154-155, 55 bp product detected mutations at codon 175-176, 134 bp product detected mutations at codon 270 and 112-bp product detected mutations at the codon 275.

3. Results

Effect of UV irradiation on p53 mutations

In addition to loss of activation of receptor pathway, loss of p53 function has been shown to inhibit apoptotic cell formation following UV irradiation. We therefore analyzed genomic DNA isolated from UV-irradiated FADD⁺ and FADD^{-/-} cells. Using AS-PCR assay we were looking for CC to TT hot spot mutations at p53 codons 154-155 and 175-176 and for C to T mutations at codons 270 and 275. The cells were irradiated with three UV doses, 25 J/m², 40 J/m² and 50 J/m². DNA was extracted from un-irradiated control cells and from UV-irradiated cells. Representative gel electrophoresis data are shown in Figures 1-4. The results indicate that the primer specific for p53 mutant sequences at codon 270 amplified genomic DNA from the irradiated cells of both cell lines while the primers specific for p53 mutants at codons 154-155, 175-176 and 275 did not amplified DNA from any of tested cell lines. Dose dependent mutation event was not observed within the used dose range.

Figure 1: Detection of p53 mutations at codons 154-155 and 270 in FADD^{+/+} and FADD^{-/-} cell lines irradiated with UVC (254 nm) with the dose of 40 J/m². DNA was extracted from normal and UV-irradiated cells and analysed by allele-specific polymerase chain reaction (AS-PCR). The mutant-specific primer for codon 154-155 did not amplify 119-base pair product from normal and UV-irradiated FADD^{+/+} and FADD^{-/-} cells DNAs (lanes 1-8). In contrast, C→T base changes at codon 270 were detected in all DNAs. The primers designed to detect mutation at codon 270 amplified 134-base pair product (lanes 10-17).

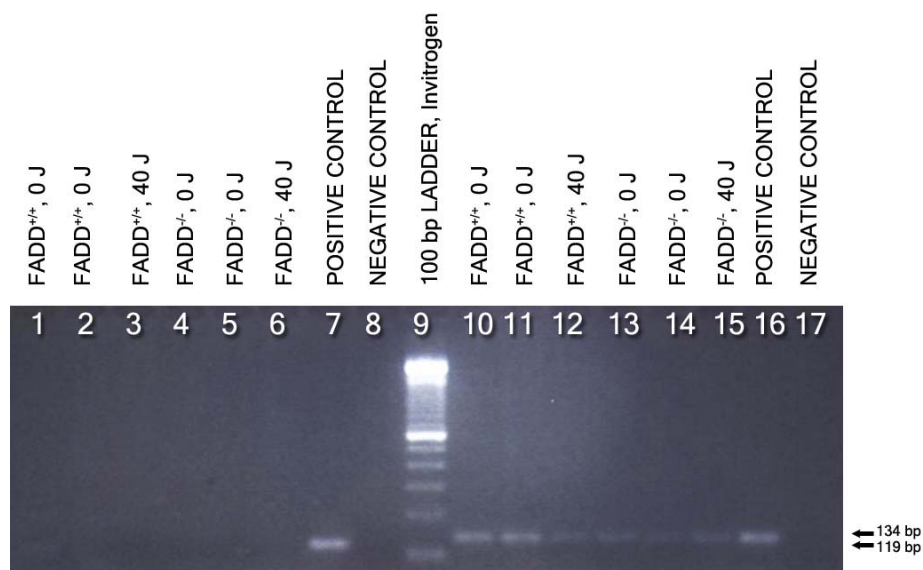


Figure 2: Detection of p53 mutations at codons 275 and 175-176 in FADD^{+/+} and FADD^{-/-} cell lines irradiated with UVC (254 nm) with the dose of 40 J/m². C→T base changes at codon 275 were not detected using mutant-specific primer which amplify 112-base pair product (lanes 1-8). The mutant-specific primer for CC→TT mutations at codon 175-176 did not amplify 55-base pair products (lanes 10-17).

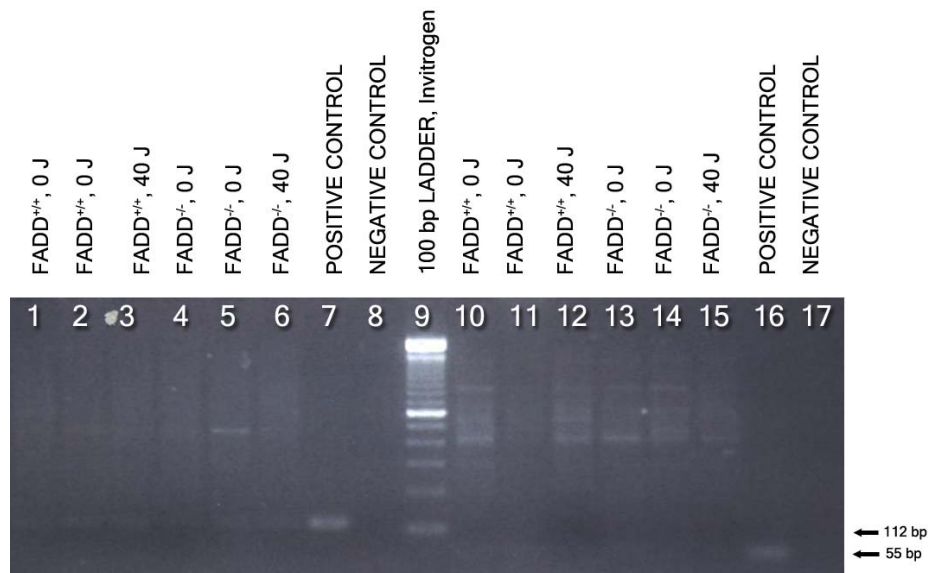


Figure 3: Detection of p53 mutations at codons 175-176 and 154-155 in FADD^{+/+} and FADD^{-/-} cell lines irradiated with UVC (254 nm) with a dose of 25 J/m² and with the cumulative irradiation dose of 50 J/m². DNA was extracted from normal and UV-irradiated cells and analysed by allele-specific polymerase chain reaction (AS-PCR). To detect p53 mutations we used mutant-specific primers for CC→TT mutations at codons 175-176 (lanes 1-8) and 154-155 (lanes 10-17). The result indicated that mutant-specific primers did not amplify 55- and 119-base pair products.

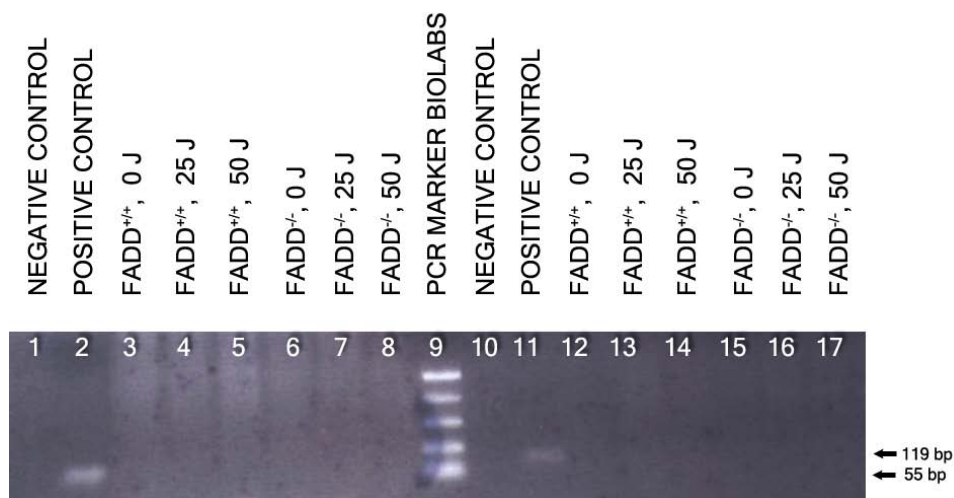


Figure 4: Detection of p53 mutations at codons 270 and 275 in FADD^{+/+} and FADD^{-/-} cell lines irradiated with UVC (254 nm) with a dose of 25 J/m² and with the cumulative irradiation dose of 50 J/m². The mutant-specific primer 270 amplified 134-base pair product from normal and UV-irradiated FADD^{+/+} and FADD^{-/-} cells DNAs (lanes 1-8). The primers designed to detect mutation at codon 275 did not amplify 112-base pair product (lanes 10-17).



4. Discussion

In this study, we used wild-type (wt), FADD deficient (Fadd^{-/-}) and p53 mutations in order to elucidate the role of non-repaired UVC lesions in apoptotic signalling. Although it is known that UVC light induces apoptosis in various cell types, the mechanism still needs to be elucidated. It is not known how important are the DNA damage and the receptor triggered pathway for a given cell type and how UVC-induced DNA damage triggers the response. UV-induced nuclear DNA damage leads to apoptosis via activation of p53 [5]. In addition, UV activates death receptors expression on the cell surface via induction or up-regulation of death ligands [6]. Several line of evidence indicates that p53 activates the CD95/Fas pathway in response to DNA damage by anticancer drugs [7] and by UV [8]. One might argue that the apoptotic pathway is evoked specifically in FADD proficient cells. This is not the case. In this study, we demonstrate the apoptotic response to UVC irradiation of FADD knock out cells, indicating that DNA damage and activation of Fas receptor contribute independently to apoptosis.

UV irradiation preferentially mutates p53 and in this way diminished p53-mediated apoptotic cell death. In this way UV exerts a selective pressure for mutated, damage resistant cells, allowing these cells to clonally expand and form cancer. We show that UV induces p53 mutations equally in FADD proficient and in FADD deficient cells. Activation of cell receptors on the cell surface is not required for UV induced apoptosis neither for UV-induced p53 mutations.

REFERENCES

- [1] de Gruijl FR, van Kranen HJ, Mullenders LH. UV-induced DNA damage, repair, mutations and oncogenic pathways in skin cancer. *J Photochem Photobiol B.* (2001) Oct;63(1-3):19-27.
- [2] Ziegler A, Leffell DJ, Kunala S, Sharma HW, Gailani M, Simon JA, Halperin AJ, Baden HP, Shapiro PE, Bale AE, et al. Mutation hotspots due to sunlight in the p53 gene of nonmelanoma skin cancers. *Proc Natl Acad Sci U S A.* (1993) May 1;90(9):4216-20.
- [3] Screaton RA, Kiessling S, Sansom OJ, Millar CB, Maddison K, Bird A, Clarke AR, Frisch SM. Fas-associated death domain protein interacts with methyl-CpG binding domain protein 4: a

- potential link between genome surveillance and apoptosis. *Proc Natl Acad Sci U S A.* (2003) Apr 29;100(9):5211-6. Epub 2003 Apr 17.
- [4] Ouhtit A, Gorny A, Muller HK, Hill LL, Owen-Schaub L, Ananthaswamy HN. Loss of Fas-ligand expression in mouse keratinocytes during UV carcinogenesis. *Am J Pathol.* (2000) Dec;157(6):1975-81.
- [5] Daniels F, Brophy D, Lobitz WC. Histochemical responses of human skin following ultraviolet irradiation. *J Invest Dermatol.* (1961) Nov;37:351-7.
- [6] Kumakiri M, Hashimoto K, Willis I. Biologic changes due to long-wave ultraviolet irradiation on human skin: ultrastructural study. *J Invest Dermatol.* (1977) Oct;69(4):392-400.
- [7] Müller M, Wilder S, Bannasch D, Israeli D, Lehlbach K, Li-Weber M, Friedman SL, Galle PR, Stremmel W, Oren M, Krammer PH. p53 activates the CD95 (APO-1/Fas) gene in response to DNA damage by anticancer drugs. *J Exp Med.* (1998) Dec 7;188(11):2033-45.
- [8] Kulms D, Schwarz T. Molecular mechanisms of UV-induced apoptosis. *Photodermatol Photoimmunol Photomed.* (2000) Oct;16(5):195-201.