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Research Article

Role of Topoisomerase IIβ in DNA Damage Response following IR and Etoposide

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The role of topoisomerase IIβ was investigated in cell lines exposed to two DNA damaging agents, ionising radiation (IR) or etoposide, a drug which acts on topoisomerase II. The appearance and resolution of γH2AX foci in murine embryonic fibroblast cell lines, wild type and null for DNA topoisomerase IIβ, was measured after exposure to ionising radiation (IR) or etoposide. Topoisomerase II-DNA adduct levels were also measured. IR rapidly triggered phosphorylation of histone H2AX, less phosphorylation was seen in TOP2$\beta^{-/-}$ cells, but the difference was not statistically significant. IR did not produce topoisomerase II-DNA adducts above control levels. Etoposide triggered the formation of topoisomerase II-DNA adducts and the phosphorylation of histone H2AX, the γH2AX foci appeared more slowly with etoposide than with IR. Topoisomerase II-DNA complexes in WT cells but not TOP2$\beta^{-/-}$ cells increased significantly at 24 hours with the proteasome inhibitor MG132, suggesting topoisomerase IIβ adducts are removed by the proteasome.

1. Introduction

Exogenous agents such as ionising radiation (IR) and ultraviolet light or endogenous agents such as free radicals produced within cells can damage the DNA of eukaryotic organisms. Diverse mechanisms have evolved to detect and repair DNA damage that threatens the integrity of the genome. Here, we study two DNA damaging agents used in the treatment of cancer, IR and the epipodophyllotoxin drug etoposide that acts on topoisomerase II.

Cellular DNA damage responses to IR exposure have been extensively investigated and several pathways exist within the cell to respond to double strand breaks (DSBs) induced by ionising radiation (IR). Histone H2AX is rapidly phosphorylated following IR, with foci observed within the first minute following exposure [1–3]. The phosphorylation of H2AX occurs over megabase regions of chromatin extending away from the site of DNA damage [1] and initiates assembly of several proteins involved in the DNA damage response [4]. H2AX phosphorylation has been shown to be essential for correct amplification of the DNA damage response [5–7]. At sublethal levels of DNA damage, phosphorylated H2AX (termed γH2AX) forms distinct foci within the cell nuclei. At less than 150 DSBs per nucleus, there exists a 1 : 1 relationship of γH2AX foci : DSBs [2]. At these levels of DNA damage, γH2AX can be used as an accurate and sensitive surrogate reporter of DNA DSB levels [8]. H2AX can also be phosphorylated in response to topoisomerase II-targeting agents [9–12].

Topoisomerase II is an enzyme that alters the topological state of DNA via a transient covalent enzyme-bridged double strand break in the DNA, through which a second DNA helix can pass. These protein associated breaks can be stabilised by drugs such as etoposide [13, 14]. Two isoforms of topoisomerase II exist, termed α and β [15], these are both targeted by etoposide [16–18]. The genotoxic effects of etoposide are generally considered to be mediated through conversion of stabilised protein-DNA complexes to protein free "frank" DSBs [19, 20], possibly via collisions between the drug-stabilised topoisomerase-DNA complex and RNA polymerase during transcription or with DNA replication forks, analogous to the situation seen with topoisomerase I [21, 22]. Frank DSBs may also be generated by proteolytic degradation of the topoisomerase II moiety

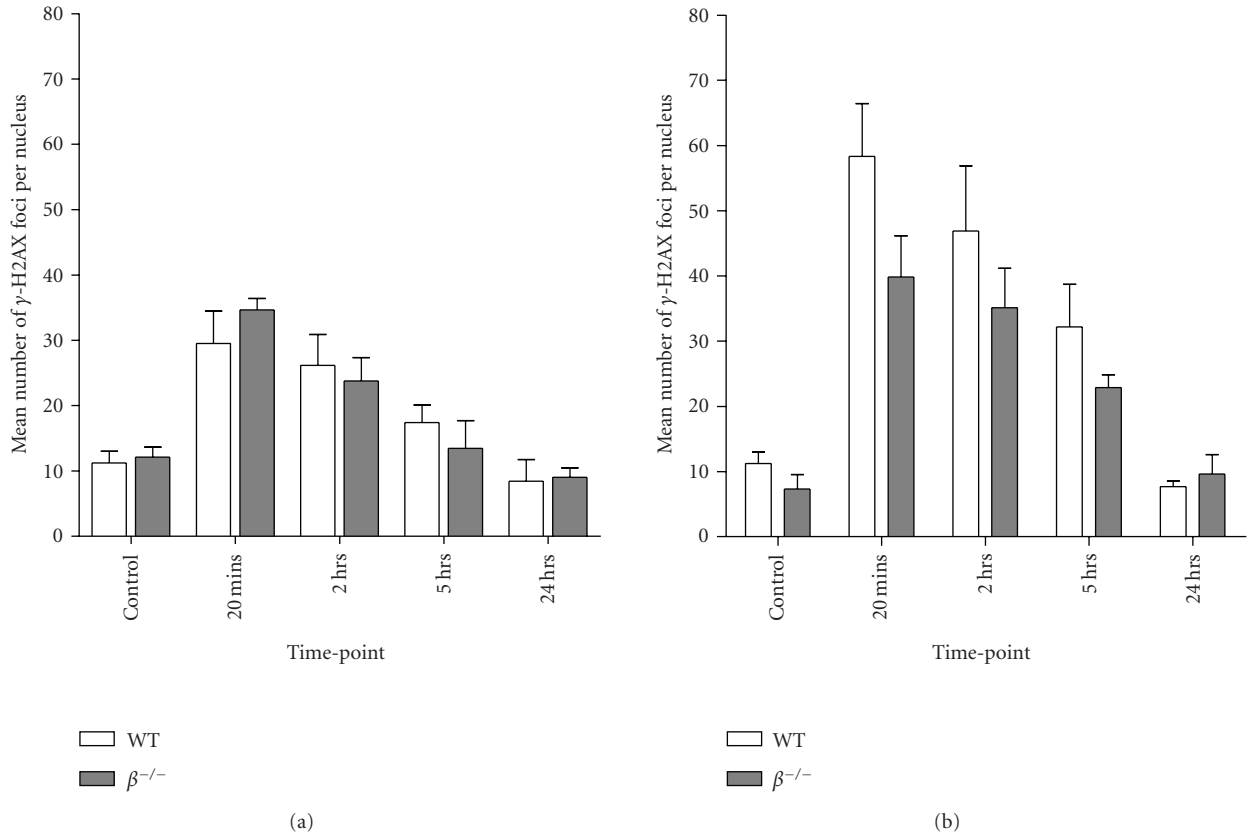

Figure 1: Effect of topoisomerase IIβ status on γH2AX focus formation in response to IR. WT and TOP2$\beta^{-/-}$ MEFs were exposed to 1 Gy IR (a) or 2 Gy IR (b) and γH2AX foci were counted at 20 minutes, 2 hours, 5 hours, and 24 hours after exposure. Data are derived from at least $n = 3$ independent experiments.

[23–26] and topoisomerase IIβ is thought to be preferentially degraded over the α isoform [26]. A reduction in etoposide induced DSB levels was reported in cells cotreated with the proteasome inhibitor MG-132 [26], suggesting that the proteasome has a role in converting etoposide-stabilised protein-DNA complexes into frank DSBs [26]. Additionally a 5′ tyrosyl DNA phosphodiesterase (TTRAP) has recently been identified that may play a role in generating frank DSBs ready for repair [27].

Topoisomerase II has been implicated in the cellular response to DNA DSBs. Down regulating topoisomerase IIα by siRNA altered the response to radiation [28] whilst topoisomerase IIβ has been reported to play a role in promoting DSB repair following peroxide damage [29]. The damage sensor TopBP1 was first identified as a topoisomerase IIβ interacting protein [30], and WSTF (Williams syndrome transcription factor) which regulates the H2AX DNA damage response [7] interacts with WINAC, a topoisomerase IIβ containing multi protein complex [31]. Thus topoisomerase IIβ may be directly involved in damage detection and signalling following IR via protein-protein interactions. Alternatively, topoisomerase IIβ may be required for proper regulation of genes involved in the damage responses. For example, cells downregulated for topoisomerase IIβ have been reported to express reduced peroxiredoxin 2 [32]. To investigate whether topoisomerase IIβ affects the cellular response to IR or etoposide induced DNA damage, we used WT and TOP2$\beta^{-/-}$ MEFs, we used H2AX assays and in parallel the trapped in agarose DNA immunostaining (TARDIS) assay to examine the kinetics of formation and removal of topoisomerase II-DNA complexes in response to IR or etoposide treatment.

2. Materials and Methods

2.1. Cell Culture. Wild-type mTOP2β-4 (containing both topoisomerase IIα and topoisomerase IIβ) [WT] and mtop2β-5 [TOP2$\beta^{-/-}$] immortalized mouse embryonic fibroblasts (MEFs) have been described previously [17] and were maintained as monolayers in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and penicillin (50 μg/mL)/streptomycin (50 μg/mL). Cells were grown at 37°C in a humidified atmosphere containing 5% CO_2. Cell culture reagents were obtained from Gibco BRL (Paisley, UK).

2.2. Chemicals and DNA Damaging Agents. All chemicals were obtained from Sigma-Aldrich (Poole, UK) and VWR International (Lutterworth, UK). Etoposide was obtained

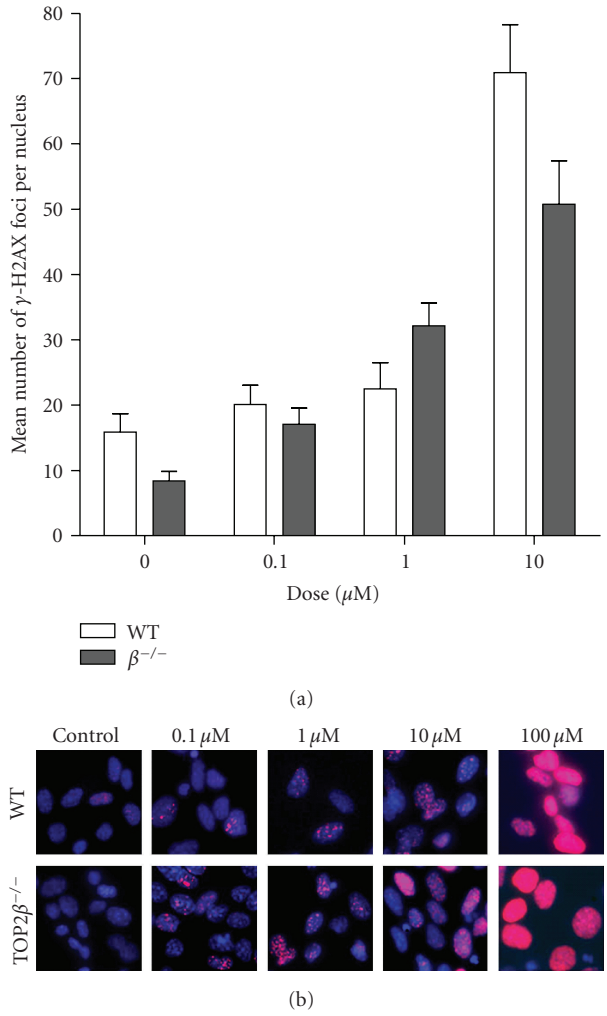

Figure 2: γH2AX foci formed in response to increasing doses of etoposide. WT and TOP2$\beta^{-/-}$ MEFs were exposed to 0.1, 1, 10, and 100 μM etoposide for 2 hours and then assayed for γH2AX foci. (a) Histograms of foci number ± SEM at each dose counted. 100 μM was not counted as foci were not distinct at this dose. Data derived from one experiment. (b) Representative images of γH2AX foci (red) in nuclei stained with DAPI (blue) in WT and TOP2$\beta^{-/-}$ MEFs.

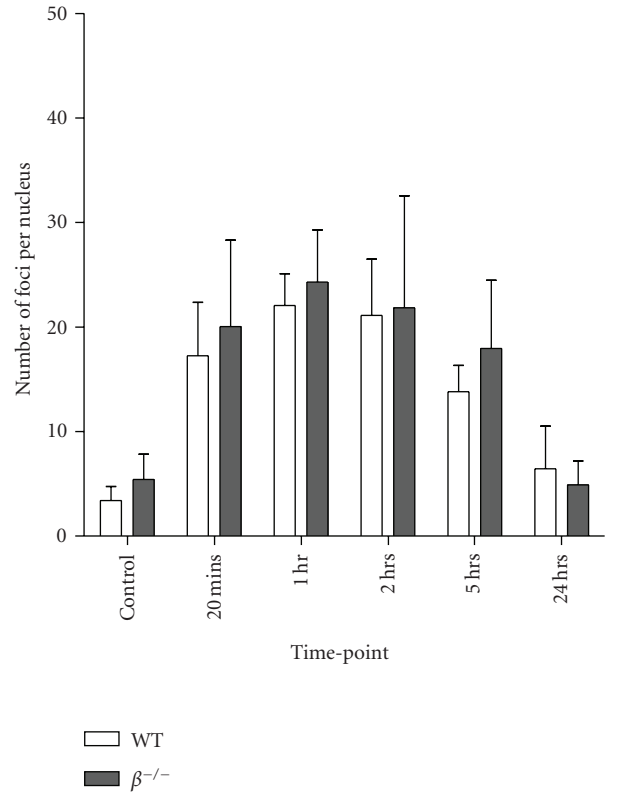

Figure 3: γH2AX foci following a 2 hour short-term exposure to etoposide. WT and TOP2$\beta^{-/-}$ MEFs were exposed to 1 μM etoposide and γH2AX foci were counted at the indicated time points after drug removal. The data are derived from at least $n = 3$ independent experiments, each data point shows the mean ± SEM.

from Sigma and was dissolved in methanol. IR exposure was carried out using a Gammacell 1000 irradiator with a [^{137}Cs] source (Nordion International, Inc.) and was delivered at a rate of approximately 3.08 Gy/min.

2.3. H2AX Focus Assay. The γH2AX focus assay was performed as described previously in detail by Watters and colleagues [12].

2.4. TARDIS (Trapped in Agarose DNA Immunostaining). WT and TOP2$\beta^{-/-}$ MEFs were seeded at 3×10^4 cells/well into 6-well tissue culture plates and grown to approximately 80% confluency. Cells were exposed to the appropriate DNA damaging agent (IR or etoposide) and harvested by trypsinisation at specific time-points as detailed in the text. Trypsinised cells were resuspended in 1 mL of ice-cold PBS, centrifuged at 1000 rpm for 3 minutes and then resuspended in 50 μL of ice-cold PBS. The slide preparation has been described in detail previously [17]. Slides were probed with an antibody that detects topoisomerase IIα and β.

Quantification of complex levels has been described previously [16]. Statistical analyses were carried out using Graphpad Prism 4 software (Cherwell Scientific, Oxford, UK.). Two-tailed, paired, and unpaired Students t-tests were generally used to compare data sets and data sets between cell lines; analysis of variance (ANOVA) was used for multiple comparisons where appropriate, as detailed in the text. All statistical analyses were calculated using a 95% confidence interval ($P < .05$).

3. Results and Discussion

The present study examined two DNA damaging agents, IR and etoposide. γH2AX formation was used as a surrogate marker for DSBs in parallel with topoisomerase II-DNA complex measurement in both wild-type and topoisomerase IIβ null murine embryo fibroblast cell lines (MEFs).

The response to three doses of IR (0.5, 1 and 2 Gy) was determined in MEFs wild type (WT) or null for topoisomerase IIβ (TOP2$\beta^{-/-}$). In response to 1 Gy, γH2AX

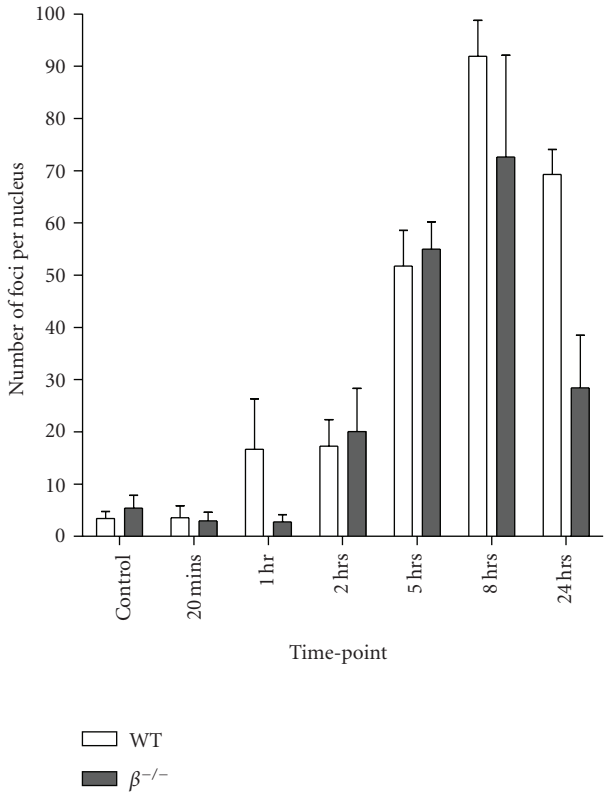

Figure 4: γH2AX foci during continuous exposure to etoposide up to 24 hours. WT and TOP2$\beta^{-/-}$ MEFs were exposed to 1 μM etoposide and γH2AX foci were counted at the indicated time points up to 24 hours. The data are derived from at least $n = 3$ independent experiments, each data point shows the mean ± SEM.

focus numbers were similar between the two cell lines, with ~35 γH2AX foci per nucleus at 20 minutes (Figure 1(a)). γH2AX foci were most abundant at the 20 minute time-point after irradiation and decreased at each subsequent time-point investigated and returned to background levels at 24 hours (Figure 1(a)). Following 2 Gy (Figure 1(b)), focus numbers appeared higher in the WT cell line than the TOP2$\beta^{-/-}$ at 20 minutes, 2 hours, and 5 hours following exposure. The lower foci number in the TOP2$\beta^{-/-}$ cells suggests the initial DNA damage responses following IR are altered in these cells, however, the differences were not statistically significant by two way ANOVA or Students t-test. Nor was there any significant difference between the levels of apoptosis following IR between the two cell lines. The trapped in agarose DNA immunostaining (TARDIS) assay was used to detect topoisomerase II protein-DNA complex levels in these cell lines following IR. Topoisomerase II complex formation did not increase in response to IR in WT or TOP2$\beta^{-/-}$ cells when compared to the untreated cells, as previously reported for CEM cells [18] (data not shown), thus IR had no effect upon topoisomerase II-DNA complex levels in the 24 hours following exposure to 2 Gy.

In an etoposide "dose finding" experiment topoisomerase IIβ wild-type and null MEF cell lines were exposed to 0, 0.1, 1, 10, or 100 μM etoposide for 2 hours, after which time cells were placed in drug-free media for 20 minutes before being assayed for γH2AX foci. Foci numbers per nucleus increased in both cell lines with increasing dose of drug. At 100 μM, foci were no longer distinct and therefore uncountable (Figure 2). To ensure foci were within a countable range, subsequent experiments were performed using doses of 1 μM and 10 μM only.

WT and TOP2$\beta^{-/-}$ cells were exposed to 1.0 μM etoposide for 2 hours, cells were then resuspended in drug-free media and assayed for γH2AX at different time points after drug removal, 20 minutes, 2 hours, 5 hours, and 24 hours following removal of drug (Figure 3). The kinetics of focus formation and removal were comparable between the wild-type and TOP2$\beta^{-/-}$ cell lines, and by 24 hours postdrug removal foci numbers were similar to untreated controls. No statistically significant difference was found in focus numbers between the two cell lines, indicating that topoisomerase IIβ status did not affect the kinetics of disappearance of γH2AX phosphorylation following a 2 hour exposure to 1 μM etoposide, this is consistent with the evidence that the cytotoxic effect of etoposide is mainly mediated via topoisomerase IIα in these cells, since the IC50 for etoposide did not differ significantly between the two cell lines [17]. The topoisomerase II-DNA adducts levels measured by TARDIS after a 2 hour exposure to etoposide has previously been reported [17].

Wild-type and TOP2$\beta^{-/-}$ cells were also exposed continuously to 1.0 μM etoposide over the course of 24 hours, and samples removed to quantify γH2AX foci formation at various time points (Figure 4). After two hours of etoposide exposure the foci numbers were comparable to that seen after the two-hour short term exposure, numbers then increased further with continued exposure to etoposide. The maximal foci numbers were seen after 8 hours of etoposide, and they then decreased by 24 hours even though etoposide was still present. The focus numbers were statistically different between the two cell lines only at the 24 hours point ($P < .05$), when the WT cell line had approximately double the number of foci compared to the TOP2$\beta^{-/-}$ cell line.

Topoisomerase II-DNA complex levels in WT and TOP2$\beta^{-/-}$ cells were determined using the TARDIS assay at time-points over a 24 hour continuous exposure to etoposide at 1 μM (Figure 5(a)) or 10 μM (Figure 5(b)). At both drug doses and in both cell lines, treatment induced a time-dependent increase in topoisomerase II DNA adduct levels (FITC immunofluorescence) up to the 8 hour time-point followed by a decrease at the 24 hour time-point (Figures 5(a) and 5(b)). With 1 μM etoposide, the increase was significant at 8 hours in WT cells and at both 5 hours ($P < .05$) and 8 hours ($P < .01$) in TOP2$\beta^{-/-}$ cells. Although levels decreased at 24 hours, immunofluorescence was still significantly greater than background levels in both wild-type and TOP2$\beta^{-/-}$ cells ($P < .05$). When cells were exposed to 10 μM etoposide, immunofluorescence levels in WT cells became statistically significant at 2 hours after drug addition ($P < .05$) and remained elevated at all other time-points ($P < .01$). In TOP2$\beta^{-/-}$ cells, levels were significant at 1 hour ($P < .01$), 5 hours ($P < .05$), and 24 hours ($P < .001$). Notably immunofluorescence levels were greater in the TOP2$\beta^{-/-}$

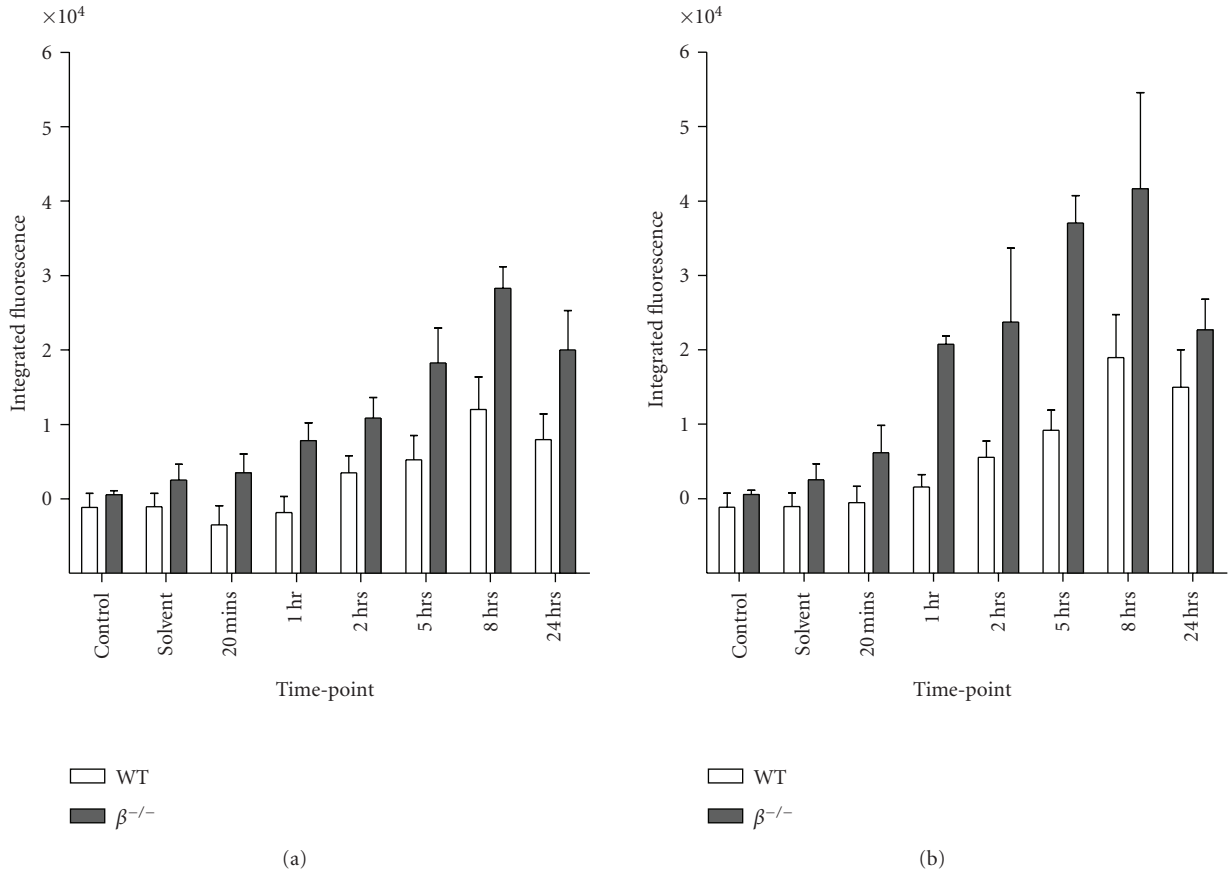

Figure 5: TARDIS analysis of cells treated with etoposide continuously for 24 hours. Topoisomerase II-DNA adducts were detected using an antibody that detects both isoforms in WT and TOP2$\beta^{-/-}$ MEFs following exposure to 1 μM (a) or 10 μM (b). Plots show the mean of the median FITC fluorescence from at least three independent experiments.

cells than the wild-type cells (Figure 5) at both drug doses and all time-points (all P-values <.05). The higher complex levels seen in cells lacking topoisomerase IIβ may result from the longer half life of topoisomerase IIα complexes [16] or be due to a role of topoisomerase IIβ in sensing and/or promoting repair [29] or alternatively to downregulation of peroxiredoxins in TOP2$\beta^{-/-}$ cells [32, 33].

To investigate whether the decrease in topoisomerase II-DNA complexes between the 8 hour and 24 hour time-points was due to proteasomal degradation of topoisomerase II, WT and TOP2$\beta^{-/-}$ cells were incubated in the presence of the proteasome inhibitor, MG-132, for 30 minutes prior to and during 24 hour exposures to 1 μM and 10 μM etoposide. As shown in Figure 6(e), when treated with MG-132 alone, topoisomerase II-DNA complex levels were only significantly elevated above background levels at the 24 hour time-point. Immunofluorescence levels were comparable in the WT and TOP2$\beta^{-/-}$ cells at all time-points considered. In cells cotreated with etoposide and MG-132, topoisomerase II-DNA complex levels did not decrease between the 8 hour and 24 hour time-points, as seen in cells treated with etoposide alone. Figure 6(a)–6(d), demonstrate that cotreatment led to increased immunofluorescence at the 24 hour time-point, in both cell lines and at both drug doses. This increase was most dramatic in WT cells, where cotreatment led to a 6-fold increase in immunofluorescence in cells treated with 1 μM etoposide and a 15-fold increase in cells treated with 10 μM etoposide. In the TOP2$\beta^{-/-}$ cells, the increase was roughly 2-fold at both doses of drug. This indicates that topoisomerase II removal at 24 hours is mediated via the proteasome, and that the effect is greatest on topoisomerase IIβ, in agreement with previous studies [23, 25, 26].

4. Conclusions

In response to IR phosphorylation of histone H2AX was triggered immediately, and slightly less phosphorylation was seen in TOP2$\beta^{-/-}$ cells, which may indicate altered DNA damage sensing. As previously reported, IR did not produce topoisomerase II-DNA adducts. In response to etoposide both γH2AX foci and topoisomerase II-DNA adducts were formed, with foci appearing 1 hour after treatment in WT cells and after 2 hours in TOP2$\beta^{-/-}$ cells. This was slower than following treatment with IR, and presumably reflects the need to remove the topoisomerase II adduct to produce a frank DSB to trigger phosphorylation of histone H2AX. In both instances, levels become maximal at the 8 hour time-point and subsequently decrease at 24 hours.

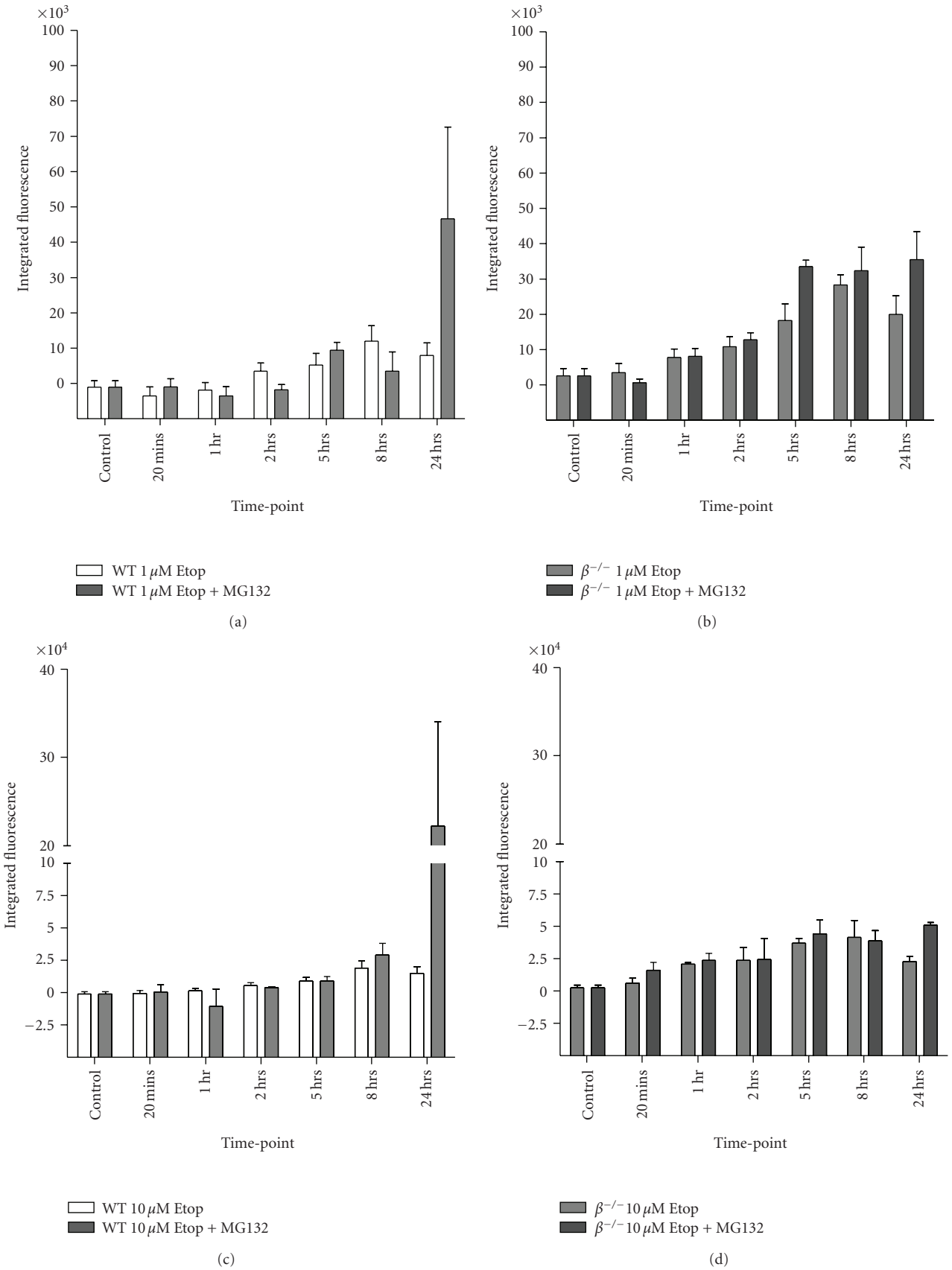

Figure 6: Continued.

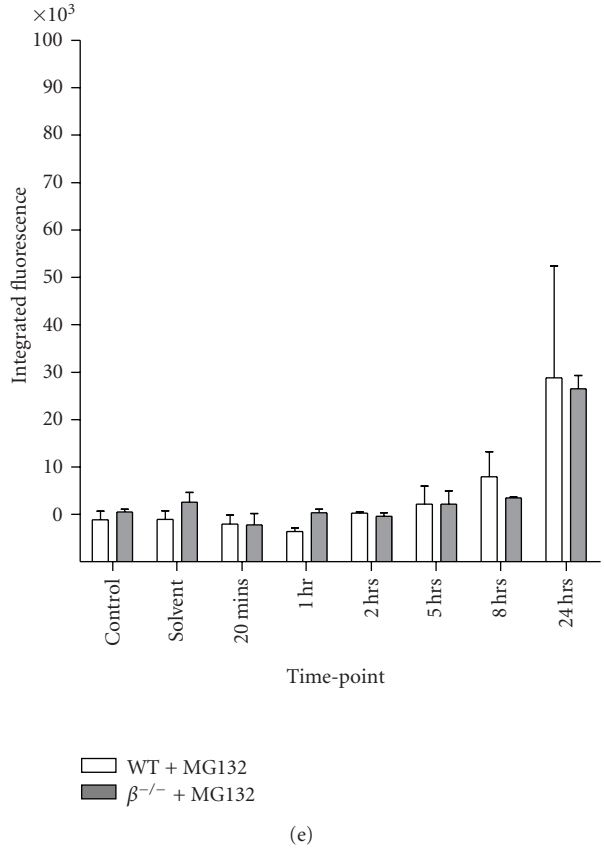

Figure 6: TARDIS analysis of cells treated with etoposide continuously for 24 hours plus and minus MG132 or with MG132 alone. Topoisomerase II-DNA adducts were quantified using an antibody that detects both isoforms. WT MEFs following exposure to 1 μM ± 2 μM MG132 (a), Top2$\beta^{-/-}$ MEFs following exposure to 1 μM ± 2 μM MG132 (b), WT MEFs following exposure to 10 μM ± 2 μM MG132 (c), Top2$\beta^{-/-}$ MEFs following exposure to 10 μM ± 2 μM MG132 (d), or WT and TOP2$\beta^{-/-}$ MEFs following exposure to 2 μM MG132 alone (e). Plots show the mean of the median FITC fluorescence.

Topoisomerase II complexes in WT cells but not TOP2$\beta^{-/-}$ cells increased significantly at 24 hours when the proteasome was inhibited, suggesting that topoisomerase IIβ adducts are removed by the proteasome.

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