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Importance of Bak for celecoxib-induced apoptosis

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ABSTRACT

The selective cyclooxygenase-2 (COX-2) inhibitor celecoxib constitutes the prototype of pro-apoptotic agents acting through the intrinsic death pathway in a Bcl-2 independent manner. To gain further insight into celecoxib-mediated apoptosis regulation at the level of the mitochondria we tested in how far the crucial pro-apoptotic Bcl-2 proteins Bak and Bax were involved using clones of the Bax-deficient Jurkat T-lymphoma cell model either expressing Bak (Jurkat Bak positive) or being negative for Bak (Jurkat Bak negative), or overexpressing Bcl-2 (Jurkat Bcl-2). Celecoxib induced substantial apoptosis in Jurkat Bak-positive cells. Overexpression of Bcl-2 had only limited protective effects. However, loss of Bak-expression conferred almost complete resistance of Jurkat cells to celecoxib-induced apoptosis. Neither enhanced celecoxib-concentrations nor prolonged incubation times were sufficient to normalize apoptotic rates upon celecoxib-treatment in these Bak/Bax-negative cells. In line with that observation, siRNA-mediated silencing of Bak in the Bak-positive Jurkat cells largely reduced the extent of celecoxib-induced cell death. Interestingly, in celecoxib-sensitive Bak-positive cells, celecoxib-treatment resulted in down-regulation of the anti-apoptotic Bcl-2 protein Mcl-1 which may contribute to celecoxib-mediated activation of Bak-dependent apoptosis. Taken together our data clearly show for the first time the functional relevance of Bak for celecoxib-induced apoptosis in Bax-deficient Jurkat T-lymphoma cells.

1. Introduction

The selective cyclooxygenase-2 (COX-2) inhibitor celecoxib is a structural derivative of the nonsteroidal anti-inflammatory coxibes. Apart from their potent anti-inflammatory action coxibes interfere with tumor initiation and tumor cell growth. Epidemiological studies suggest a lower incidence of colonic polyps in patients with the hereditary familial adenomatous polyposis syndrome [1] and a decreased risk for cancers of the colon, breast, esophagus, and stomach upon continuous use of these NSAIDs [2]. Moreover, preclinical investigations demonstrate promising anti-tumor activity of COX-2 inhibitors in a variety of human cancers when given as single drug [3,4] or in combination with chemotherapy [5,6], radiation treatment [7,8] or chemoirradiation [6,9]. Thus, antineoplastic coxibs may represent attractive compounds for clinical use in cancer treatment [10]. However, the molecular mechanisms of their antineoplastic effects are not yet completely understood. There is increasing evidence that inhibition of cell growth and angiogenesis as well as induction of cell cycle arrest and apoptosis contribute to the anti-tumor activity of these drugs [4,11,12]. Since malignant cells are often characterized by aberrant apoptosis regulation that can contribute to resistance to chemotherapy and ionizing radiation, agents that induce cell death in those apoptosis-resistant tumor cells constitute promising drugs for the modulation of therapy response.

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However, for a rational use of those drugs the molecular mechanisms of drug-induced apoptosis and related resistance mechanisms have to be defined.

Several investigations implicate that with respect to its antineoplastic action, celecoxib constitutes at least a bifunctional drug: although in some cell types, inhibition of its target molecule COX-2 may contribute to its cytotoxic effects, celecoxib was recently recognized as a prototype of drugs that induce cell death independently from COX-2 involving a novel Bcl-2-independent mitochondrial death pathway. Based on these unique features of celecoxib-action, several attempts have been made to dissect structural requirements for COX-2-inhibitory and apoptosis-inducing activities of celecoxib [11,13] and to develop from the parent structure of celecoxib a new class of antineoplastic drugs without COX-2-inhibitory action. Currently, synthetic derivatives of celecoxib without COX-2-inhibitory action such as OSU-03012 and dimethyl-celecoxib (DMC) are under current investigation [13,14].

On the cellular level, apoptosis is characterized by the activation of specialized intracellular proteases, the caspases. These central executioners of apoptosis can be activated via two distinct but interconnected pathways, the extrinsic, death receptor-dependent, and the intrinsic, death receptor-independent death pathway. The extrinsic pathway is initiated at the cellular surface by ligation of death receptor ligands (e.g. CD95-ligand, tumor necrosis factor (TNF) related apoptosis-inducing ligand, TRAIL) to their respective receptors (CD95-receptor, TRAIL-receptor) [15]. Ligand binding triggers rapid multimerization of the receptor, recruitment of the adapter protein Fas-associated protein with death domain (FADD) and an initiator caspase (mostly pro-caspase-8) to form the so-called death-inducing signaling complex (DISC) at the cellular membrane. Proximity of multiple pro-caspase-8 molecules in the DISC facilitates their autoproteolytic cleavage leading to their activation. Active caspase-8 subsequently activates downstream effector caspases-3, -6 and/or -7 that cleave a multitude of intracellular substrates thereby provoking the apoptotic phenotype [16].

In contrast, treatment with chemotherapeutic drugs, ionizing radiation or hypoxia triggers activation of the intrinsic pathway that critically involves mitochondrial damage including breakdown of the mitochondrial membrane potential and release of cytochrome *c*. Cytoplasmic cytochrome *c* triggers the formation of a high molecular cytoplasmic death-inducing complex, the apoptosome, which is composed of cytochrome *c*, the adapter protein Apaf-1, dATP and pro-caspase-9. The apoptosome in turn enables the proteolytic activation of the initiator caspase-9 that subsequently triggers activation of the effector caspase cascade and finally apoptosis [17].

Members of the Bcl-2 protein family function as critical regulators of the intrinsic apoptosis pathway and either promote or prevent cell death. The Bcl-2 proteins are subdivided into three distinct subclasses based on the number of conserved Bcl-2 homology (BH) domains. The anti-apoptotic multidomain Bcl-2-like proteins Bcl-2, Bcl-x_L, Mcl-1 and Bcl-w share up to four BH-domains (BH1-4) and are critical for the maintenance of cell survival [18]. The pro-apoptotic Bax-like multidomain Bcl-2 proteins such as Bax, Bak and Bok share the BH-domains 1–3 and constitute the central effectors of mitochondrial permeability transition. In contrast, the pro-apoptotic so-called BH3-only proteins, e.g. Bid, Bad, Noxa and Puma, that share only the BH3-domain, function as sensors of cell stress and trigger apoptosis initiation upstream of Bax, Bak and Bok [19]. In this scenario, commitment of cells to apoptosis is mainly regulated by protein-protein interactions between these pro- and anti-apoptotic Bcl-2 family members. In the absence of death signals, Bax-like proteins are kept in check by their Bcl-2-like anti-apoptotic counterparts. However, cellular stress triggers transcriptional up-regulation and/or activation of BH3-only proteins by posttranslational modifications. Subsequent binding of BH3-only proteins to their multidomain counterparts leads to the activation and multimerization of Bax-like proteins in subcellular membranes such as the mitochondria thereby facilitating mitochondrial permeability transition, release of cytochrome *c*, caspase-activation and finally apoptosis. However, it is still controversial whether Bax-like proteins become activated by direct interaction with a subset of BH3-only proteins, the so-called ''activator BH3-only proteins'' [20,21] or indirectly as a consequence of the release of Bax-like proteins from their anti-apoptotic relatives upon competitive displacement by BH3-only proteins with higher binding affinity [22].

Up to now, the cellular pathways and molecular determinants of cell death induction by coxibs are still a matter of debate. As mentioned above, at least partially the effects are not related to the COX-2 inhibition [11,12,23–25]. Moreover, although some authors observed the activation of death receptor-dependent events upon celecoxib-treatment [26,27] most reports argue for the importance of the intrinsic death pathway [13,26,28,29]. Others and we could demonstrate that celecoxib induces apoptosis in lymphoma cells via an intrinsic pathway which relies on Apaf-1, caspase-9 activation and cytochrome *c* release, but is not abrogated by overexpression of Bcl-2 [28,29]. Similarly, Bcl-2 overexpression did not protect against celecoxib-induced apoptosis in oral cancer as well as in LNCaP and PC-3 prostate cancer cell lines although involving inhibition of the PDK-1/Akt signaling pathway upstream of the mitochondria [13,25,30]. Since cells from solid human tumors are often characterized by defective apoptosis pathways including up-regulated expression of anti-apoptotic Bcl-2-proteins, these observations highlight celecoxib and derivatives as an attractive approach for clinical use in the treatment of human malignancies, in particular in human tumors resistant against chemotherapeutic drugs and ionizing radiation. However, a rational use of celecoxib and related compounds without COX-2-inhibitory action in cancer therapy requires a better understanding of the molecular mechanisms of apoptosis initiation.

The present study was designed to define molecular determinants of the celecoxib-induced intrinsic death pathway in more detail with a focus on Bax-like proteins as central effectors of apoptosis at the level of the mitochondria.

2. Materials and methods

2.1. Reagents

Celecoxib was provided by Pharmacia-Pfizer (Erlangen, Germany) and dissolved in ethanol at a concentration of 100 mM

(stock solution). Hoechst 33342 (Calbiochem, Bad Soden, Germany) and propidium iodide (PI, Sigma–Aldrich, Deisenhofen, Germany) were dissolved in distilled water to 1.5 mM and 5 mg/ml stock solutions, respectively. Tetramethylrhodamine-ethylester-perchlorate (TMRE) was from Molecular Probes (Mobitec, Göttingen, Germany). The proton shuttle carbonylcyanide-m-chloro-phenylhydrazone (CCCP) was from Sigma–Aldrich (Deisenhofen, Germany).

Primary rabbit antibodies against Bax, caspase-3, cleaved caspase-3 (Asp175), Mcl-1, PARP, cleaved PARP (Asp214) and primary mouse antibody against p53 were purchased from Cell Signaling (New England Biolabs, Schwalbach/T., Germany). caspase-8 was detected using a mouse monoclonal antibody (BioCheck, Münster, Germany). The primary rabbit antibody against human Bak and the monoclonal mouse antibody recognizing the pro- and cleaved form of caspase-9 were from Upstate (Charlottesville, United States), Bid-specific rabbit IgG was from R&D-Systems (Minneapolis, United States) and primary mouse antibodies against Bcl-2 from Santa Cruz Biotechnology (Heidelberg, Germany). The monoclonal mouse antibody for COX-2 was supplied by Pharmingen (Becton-Dickinson, Heidelberg, Germany). Secondary antibodies: Horseradish peroxidase (HRP)-conjugated polyclonal-anti-rabbit and monoclonal-anti-mouse secondary antibodies were obtained from GE Healthcare/Amersham Biosciences (Freiburg, Germany). All other chemicals were purchased from Sigma–Aldrich (Deisenhofen, Germany) if not otherwise specified.

Bak siRNA (ON-TARGETplus SMARTpool) and non-specific siRNA (siCONTROL) were supplied by Dharmacon, Lafayette, USA and the 4-mm cuvettes were from Bio-Rad, München, Germany. Electroporation was performed with a rectangular pulse of 400 V × 10 ms (EPI2500, Fischer, Heidelberg, Germany).

2.2. Cell lines, cell culture and cellular treatment

Bak-negative Jurkat T-lymphoma cells (=Bak-negative JCAM 1.6 subclone, subsequently named Jurkat Bak negative) were obtained from A. Weiss (University of California, San Francisco, USA). Jurkat E6.1 and Bak-positive Jurkat T-lymphoma cells (=Bak-positive JCAM 1.6 subclone, subsequently named Jurkat Bak positive) were purchased from ATCC (Bethesda, MD, USA). Jurkat vector cells (Jurkat vector) as well as Jurkat Bcl-2 clone 3 with overexpression of wild type Bcl-2 (Jurkat Bcl-2 clone 3) were used as otherwise specified [31]. Jurkat vector was kindly provided by B. Leber (Ontario, Canada).

For all experiments, cells were grown in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum and maintained in a humidified incubator at 37 °C and 5% CO_2. Overall cell number was quantified by counting cells at 20× magnification with an inverse transmission light microscope (Hund; Wetzlar, Germany) employing a Neubauer counting chamber. Cells were treated for up to 24 h with 0–100 μM celecoxib. All experiments were performed with a solvent control (medium with 0.1% ethanol). The final ethanol concentration in the tissue culture experiments was always at or below 0.1% (v/v). Cells were irradiated with 6 MV photons from a linear accelerator (Precise Treatment System, Elekta, Stockholm, Sweden) with a dose rate of 5.8 Gy/min.

2.3. Determination of apoptotic nuclear morphology

Apoptotic cellular and nuclear morphologies were analyzed by fluorescence microscopy upon combined staining of the cells with Hoechst 33342 and PI to discriminate between apoptotic (chromatin condensation and/or fragmentation) and necrotic cells (rose stained cells without chromatin condensation or fragmentation). To this end, cells were incubated for 10 min with Hoechst 33342 and PI at final concentrations of 1.5 μM and 2.5 μg/ml, respectively. Morphology of stained cells was determined by fluorescence microscopy (Zeiss Axiovert 200, Carl Zeiss, Jena, Germany) using a G365/FT395/LP420 filter set. Cells were analyzed at 40× magnification and documented using a CCD camera device (Zeiss Axiocam MR, Jena, Germany).

2.4. Determination of nuclear fragmentation

For quantification of nuclear fragmentation, cells were submitted to FACS analysis upon staining with PI in a hypotonic buffer [32] using a FACS Calibur flow cytometer (Becton-Dickinson, Heidelberg, Germany). In brief, cells were washed, incubated for 60 min at room temperature in the dark in 0.1% (w/v) sodium citrate plus 50 μg/ml PI and 0.1% (v/v) Triton X-100 and subsequently subjected to FACS analysis.

2.5. Determination of mitochondrial alterations

The mitochondrial transmembrane potential ($\Delta\Psi m$) was analyzed by flow cytometry using the $\Delta\Psi m$-specific stain TMRE. Cells were loaded for 30 min at 37 °C with 25 nM TMRE and subsequently analyzed by flow cytometry. Preincubation with 1 μM of the proton ionophore CCCP was used as a positive control for complete depolarization.

2.6. Determination of caspase-activation

Caspase-activation was determined by Western blot analysis (see below) using antibodies against the active cleavage products of caspases-8, -9 and -3 as well as against the full length and cleaved caspase-3 substrate PARP.

2.7. Western blot analysis

For analysis of protein expression, cells (1×10^7/ml) were lysed for 10 min at 99 °C in CST lysis buffer (62.5 mM Tris–HCl (pH 6,8), 2% (w/v) SDS, 10% (v/v) glycerol, 50 mM DTT, 0.01% (w/v) bromphenolblue). Twenty micrograms lysates were separated by SDS-PAGE and blotted onto PVDF-membranes (Roth, Karlsruhe, Germany). Blots were blocked for 1 h in PBS buffer containing 0.05% (v/v) Tween 20 and 5% (w/v) non-fat dried milk. The membrane was incubated over night at 4 °C with the respective primary antibodies. After repeated washings with TBS/Tween 20 (0.05%, v/v) the membrane was incubated for 1 h at room temperature with the secondary antibody and again washed several times with TBS/Tween 20. Detection of antibody binding was performed by enhanced chemoluminescence staining (ECL Western blotting analysis system, GE Healthcare/Amersham-Biosciences, Freiburg, Germany). Equal protein loading was confirmed by Coomassie Brilliant

Blue stain (Fermentas GmbH, St. Leon-Rot, Germany) and β-Actin detection. Prestained protein markers and protein markers were from Fermentas GmbH, St. Leon-Rot, Germany. Quantification of Western blot analysis was performed with densitometric software ImageJ (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, USA, http://www.rsb.info.nih.gov/ij/, 1997–2007).

2.8. siRNA transfection

Cells were cultured in complete RPMI-1640 medium at a low density to ensure log phase growth at the time of transfection. Cells were collected and resuspended in RPMI-1640 w/o phenol red. For each transfection, 200 μl cell suspension were mixed with Bak siRNA (ON-TARGETplus SMARTpool, Dharmacon, Lafayette, USA) or non-specific siRNA (siCONTROL, Dharmacon, Lafayette, USA) and transferred to a 4 mm cuvette (Bio-Rad, München, Germany). The cells were electroporated with an EPI2500-electroporator (Fischer, Heidelberg, Germany) and immediately transferred to six-well plates with 6 ml pre-warmed RPMI. Seventy five micromolar Celecoxib was added 48 h after transfection. Transfection efficacy was measured by the uptake of a fluorescent siRNA (siGLO, Dharmacon, Lafayette, USA) in living cells.

3. Results

3.1. Celecoxib-induced apoptosis in Bax-deficient Jurkat cells depends on the expression of Bak

We have shown in an earlier investigation that celecoxib induces apoptosis in Jurkat T-lymphoma cells via an intrinsic, mitochondrial death pathway. It is now widely accepted that activation of the intrinsic apoptosis pathway is dependent on the presence of pro-apoptotic members of the multidomain Bax-family of Bcl-2 proteins, mainly Bax or Bak. However, Jurkat T-lymphoma cells have been shown to differ in the expression of Bax, Bak and the tumor suppressor and apoptosis regulator p53 depending on the respective cell line [33–35]. Therefore, in a first set of experiments, the expression-status of Bax, Bak and p53 was verified by Western blot analysis of lysates from the cell lines used in the present study (Fig. 1). In addition, expression of the anti-apoptotic protein Bcl-2 and COX-2, an assumed target protein of COX-2 inhibitors was also evaluated. As shown in Fig. 1A, all Jurkat cell lines used in the present study failed to express Bax while this pro-apoptotic protein was readily expressed in control cells from the human colon carcinoma line HCT-116. However, the second pro-apoptotic effector protein Bak, was only expressed in Jurkat E6.1 and Jurkat Bak-positive cell line whereas Bak was completely absent from lysates of the related Jurkat Bak-negative cell line (Fig. 1B). All Jurkat cell lines displayed endogenous Bcl-2 and were negative for p53 and COX-2 (Fig. 1A–C). As shown in Fig. 1C, treatment with celecoxib did not increase COX-2-protein levels in Jurkat cells (Fig. 1C).

In a next step we tested in how far lack of Bak-expression would interfere with celecoxib-induced apoptosis in Bax-negative Jurkat cells. To this end we analyzed the extent of

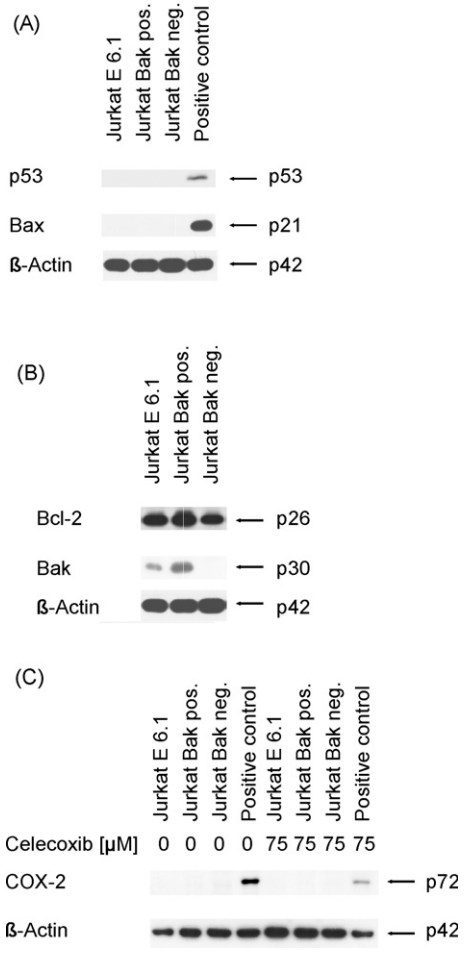

Fig. 1 – Classification of Jurkat-T-lymphoma cell clones by Western blot analyses of cell death relevant proteins. (A) Expression-levels of p53 and Bax were verified for the Jurkat T-lymphoma cell lines used in the present study (Jurkat E6.1, Jurkat Bak positive and Jurkat Bak negative). HCT-116 wildtype (wt) cells were used as positive controls for Bax- and p53-expression. (B) Verification of the expression levels of Bak and Bcl-2 in Jurkat E6.1, Jurkat Bak-positive and Jurkat Bak-negative cells. (C) Determination of COX-2 expression levels with and without treatment for 12 h with 75 μM celecoxib in Jurkat cell lines. Fadu cells were employed as positive control. Treatment with 75 μM celecoxib did not induce COX-2-expression. β-Actin was used as a loading control. Data show one representative experiment.

morphological alterations typical for apoptosis induction by fluorescence microscopy upon staining with Hoechst 33342 and PI. As shown in Fig. 2 high levels of cells with condensed chromatin and nuclear fragmentation were observed in the Bak-expressing cell line (Jurkat Bak positive). In contrast, in the Bak-deficient Jurkat Bak-negative cells almost no cells with apoptotic morphology could be detected even upon treatment with 100 μM celecoxib (Fig. 2).

To further corroborate these findings on the importance of Bak for celecoxib-induced cell death induction we next verified extent and kinetics of celecoxib-induced apoptosis in

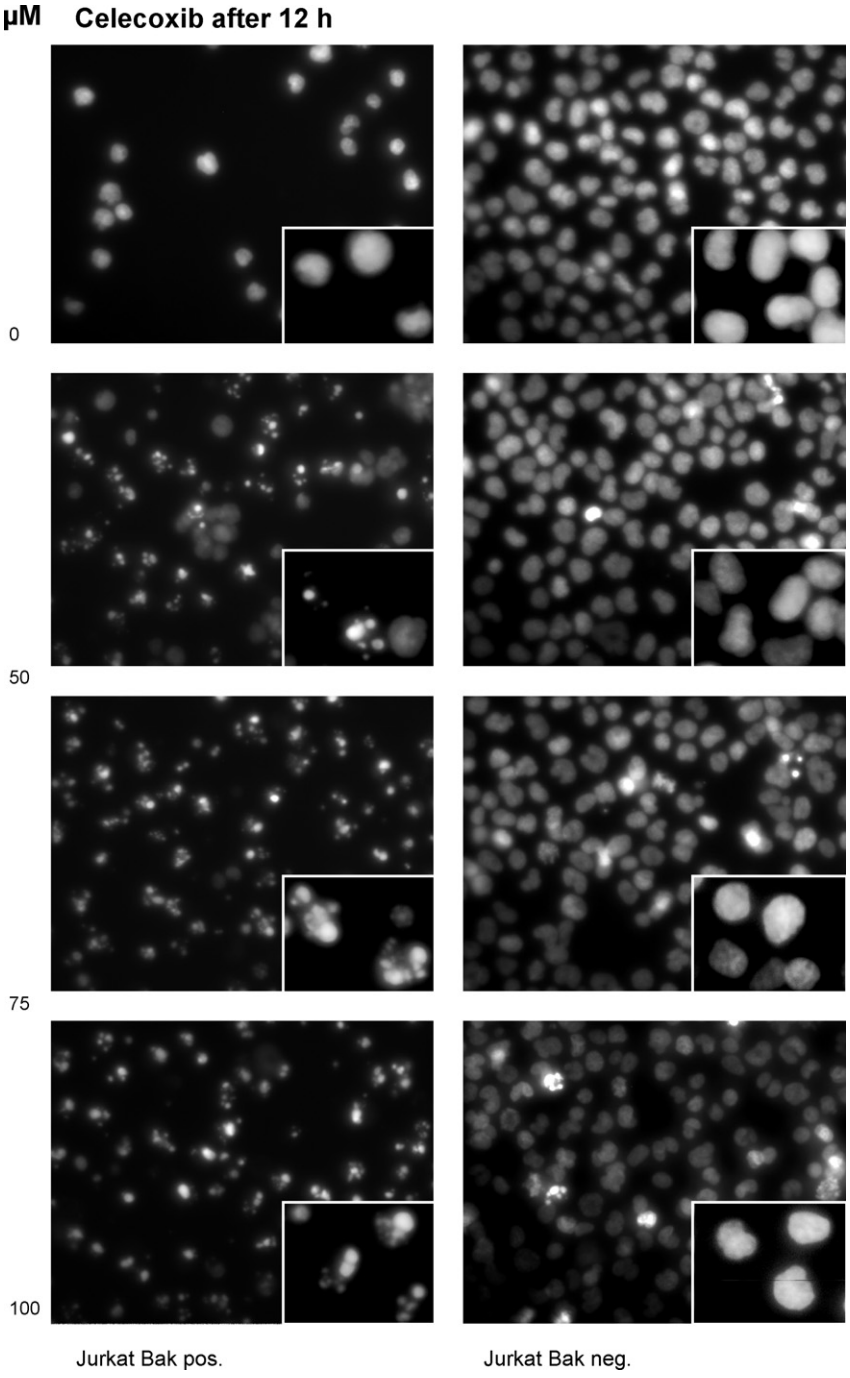

Fig. 2 – Celecoxib induces apoptosis-related nuclear changes only in Jurkat-T-lymphoma cells with Bak-expression. Jurkat Bak-positive and Jurkat Bak-negative cells were treated for 12 h with 0, 50, 75 or 100 μM celecoxib as indicated. Treatment-induced changes in nuclear morphology were evaluated by fluorescence microscopy upon staining of the cells with Hoechst 33342. Substantial chromatin condensation and fragmentation indicative for the induction of apoptosis were only found in Bak-expressing cells. The control cells in all experiments contained the solvent ethanol (0.1%). Data show representative figures.

Bak-positive and Bak-negative Jurkat cell lines by flow cytometry. As represented in Fig. 3, treatment with celecoxib-induced substantial time- and concentration-dependent apoptosis-related morphological changes (Fig. 3A and B) as well as nuclear fragmentation (Fig. 3C and D) in the two Bak-expressing cell lines Jurkat E6.1 and Jurkat Bak positive. In contrast, lack of Bak-expression in Jurkat Bak-negative cells led to almost complete resistance to celecoxib-induced morphological alterations and nuclear fragmentation (Fig. 3A–D). Even increased concentrations (100 μM) and long incubation times (12 and 24 h) failed to induce significant apoptosis in the Bak-negative Jurkat cells.

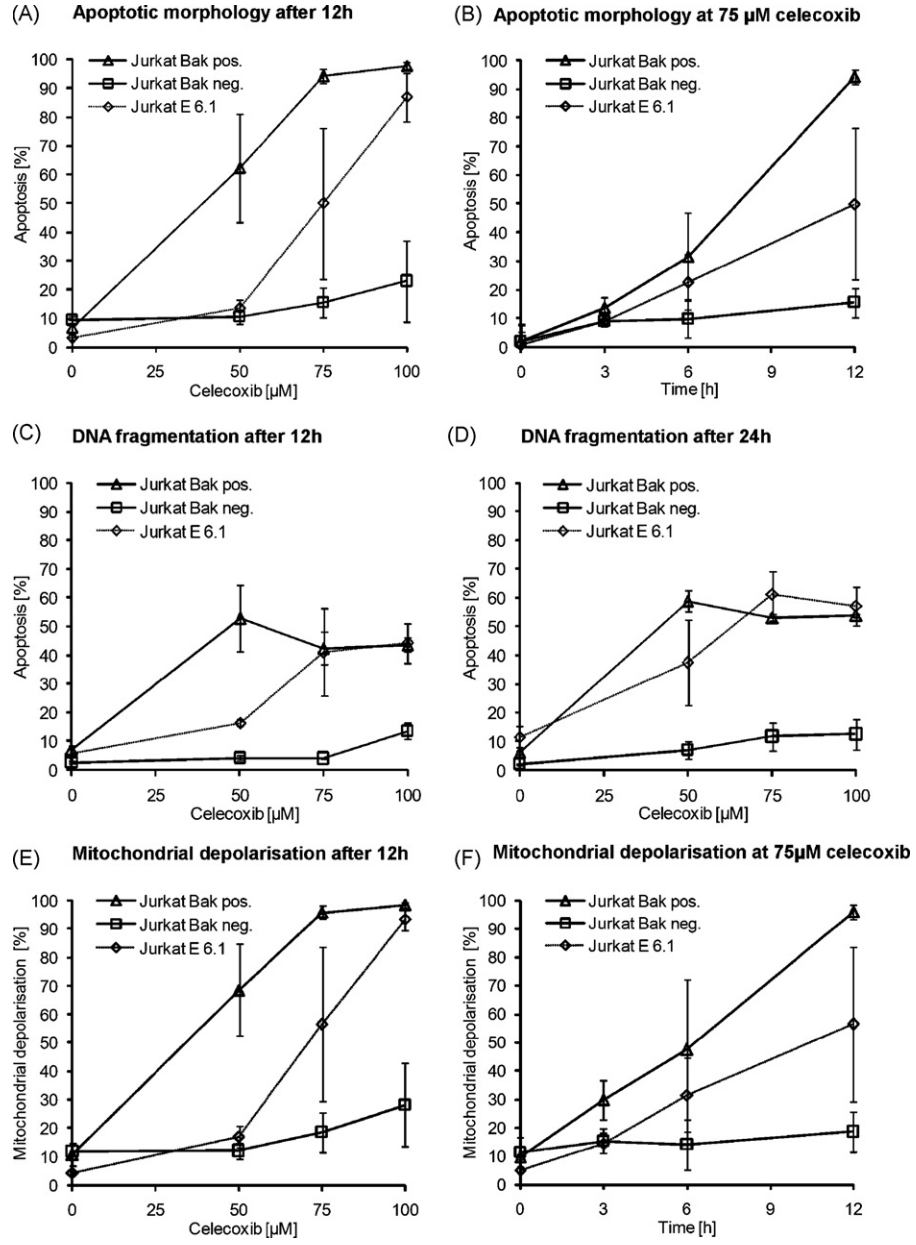

Fig. 3 – Celecoxib-induced apoptosis in lymphoma cells depends on Bak. Jurkat E6.1, Jurkat Bak-positive and Jurkat Bak-negative cells were treated for 0, 3, 6 or 12 h with 0, 50, 75 or 100 μM celecoxib as indicated. (A) Concentration- and (B) time-dependent changes in cell morphology were examined by flow cytometry using FSC/SSC. Time-dependent apoptosis-related loss in DNA (Sub-G1 peak) was determined (C) 12 h and (D) 24 h after celecoxib-treatment by flow cytometry upon staining of the cells with propidium iodide (PI) in a hypotonic buffer. Finally, (E) concentration- and (F) time-dependent depolarization of the mitochondrial membrane potential was analyzed by flow cytometry upon staining of the cells with the potential sensitive dye TMRE. Significant dose- and time-dependent apoptosis induction could only be measured in Bak-positive Jurkat-cells whereas Bak-deficient Jurkat cells showed almost no apoptosis induction. Control cells in all experiments were incubated in medium supplemented with the solvent ethanol (0.1%). Data show means ± S.D. of three independent experiments.

Up to now, these data indicated that celecoxib induces cellular shrinkage, chromatin condensation and DNA fragmentation only in Bak-positive cell clones, while these changes could not be observed in Bak-deficient Jurkat cells. As shown earlier, celecoxib-induced apoptosis signaling involves depolarization of the mitochondrial membrane potential and caspase-activation. In this regard, in the paradigmatic intrinsic mitochondrial death pathway breakdown of mitochondrial transmembrane potential ($\Delta\Psi m$) and release of cytochrome *c* constitute prerequisites for activation of caspase-9 and the effector caspase cascade downstream of the mitochondria. Therefore, in a next set of

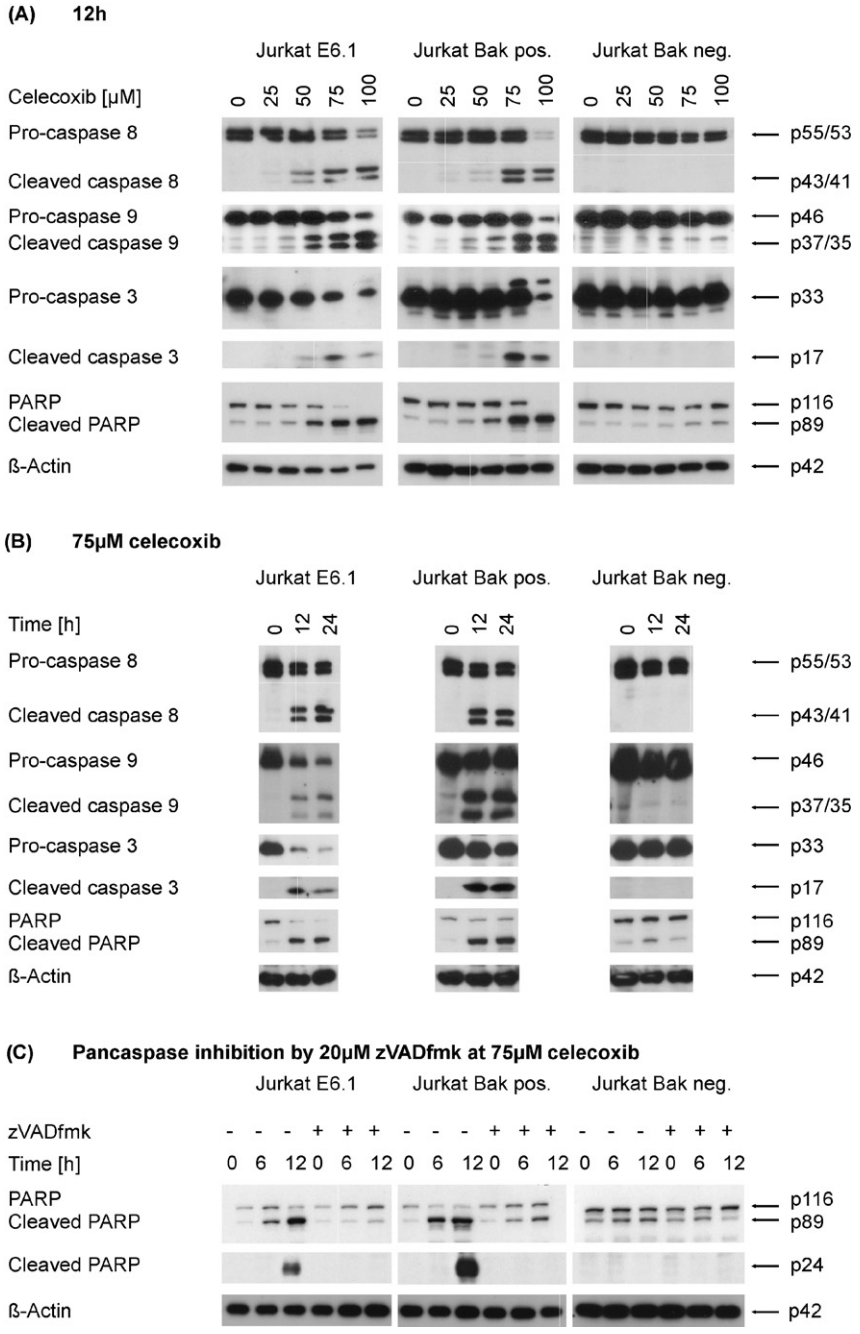

Fig. 4 – Celecoxib-induced apoptosis involves Bak-dependent caspase-activation. Jurkat E6.1, Jurkat Bak-positive and Jurkat Bak-negative cells were treated (A) for 12 h with 0, 25, 50, 75 and 100 μM celecoxib or (B) for 0, 12 or 24 h with 75 μM celecoxib. Activation of caspases was determined by Western Blot analysis of cytosolic extracts using antibodies specific for the inactive pro-caspases and the active cleavage products of caspase-8, -3 and -9, respectively. In addition processing of the caspase-3 substrate PARP was also evaluated. Control cells (0 μM) were incubated in the presence of medium supplemented with the solvent ethanol. While Bak-proficient cells show strong activation of caspases in response to celecoxib, Bak-deficient cells fail to show significant caspase-activation upon celecoxib-treatment and represented only a slight pro-caspase 9 cleavage and PARP processing in contrast to Bak-positive cells. (C) Jurkat E6.1, Jurkat Bak positive and Jurkat Bak-negative cells were pretreated for 10 min with 20 μM of the pan-caspase-inhibitor zVAD-fmk and subsequently treated for 0, 6 and 12 h with 75 μM celecoxib. PARP-cleavage in the presence or absence of pan-caspase-inhibition by zVAD-fmk was quantified by Western Blot analysis. β-Actin was used as a loading control (A–C). (D and E) Jurkat E6.1, Jurkat Bak-positive cells were pretreated for 10 min with 20 μM of the pan-caspase-inhibitor zVAD-fmk and subsequently treated for 0, 6 and 12 h with 75 μM celecoxib. Levels of celecoxib-induced apoptosis in the presence or absence of pan-caspase-inhibition by zVAD-fmk were measured by flow cytometry using (D) determination of mitochondrial membrane depolarization (TMRE) and (E) DNA fragmentation (Sub-G1 peak). Inhibition of caspase-activation does not inhibit celecoxib-induced depolarization of the mitochondrial membrane potential but abrogates apoptosis execution.

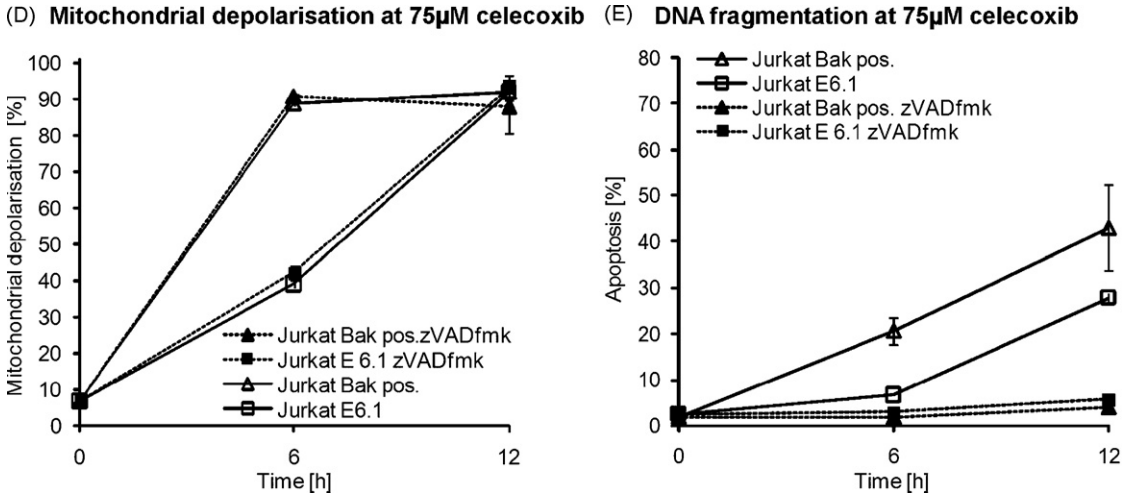

Fig. 4. (Continued).

experiment we analyzed in how far Bak-deficiency would interfere with celecoxib-induced mitochondrial damage as determined by FACS-analysis upon loading of the cells with the fluorescent potential sensitive dye TMRE. As shown in Fig. 3E and F celecoxib-concentrations of 50 and 75 μM were sufficient to induce rapid depolarization of the mitochondrial membrane potential in Bak-expressing cell lines yielding 30–50% of cells with low $\Delta\Psi m$ within 6 h. In contrast, in Bak-deficient cells celecoxib almost completely failed to trigger breakdown of the mitochondrial membrane potential even upon incubation with high concentrations (100 μM) and after extended incubation times (12 h) supporting the above-mentioned results on morphological alterations and nuclear fragmentation.

To further substantiate our findings on the importance of Bak for celecoxib-induced apoptosis we investigated activation of caspases-9, -3 and -8 by Western blot analysis (Fig. 4). As expected, celecoxib induced a concentration- and time-dependent processing of pro-caspase-9 and pro-caspase-3 in the two Bak-positive Jurkat cell lines. This is indicated by decreased levels of pro-caspases-9 and -3 as well as by increased levels of the respective cleavage products upon celecoxib-treatment (Fig. 4A and B). In line with this observation celecoxib induced cleavage of the caspase-3 substrate PARP into 89 kDa cleaved PARP. Processing of the 55/53 kDa pro-caspase-8 isoforms into the corresponding 41/43 kDa cleavage fragments occurred with similar kinetics. In contrast, no substantial cleavage of pro-caspases -9, -3 and -8 or of the caspase-3 substrate PARP could be detected in the Bak-negative Jurkat cell line (Fig. 4A and B).

To demonstrate the importance of caspase-activation for celecoxib-induced cytotoxicity apoptosis rates were quantified in cells pretreated with the pan-caspase-inhibitor zVAD-fmk. Our data reveal that pre-treatment with zVAD-fmk potently reduces celecoxib-induced caspase-activation (Fig. 4C). Moreover, although inhibition of caspase-activation did not inhibit celecoxib-induced depolarization of the mitochondrial membrane potential (Fig. 4D) pre-incubation with the pan-caspase-inhibitor zVAD-fmk completely abrogated apoptosis-related nuclear fragmentation in the Bak-positive Jurkat E6.1 and Jurkat Bak-positive cells (Fig. 4E). Similar to our earlier observations these results place celecoxib-induced caspase-activation downstream of the mitochondria.

3.2. *Molecular mechanisms of celecoxib-induced apoptosis at the level of the mitochondria*

Up to now we could demonstrate that celecoxib fails to induce mitochondrial damage, caspase-activation and apoptosis in Jurkat-cells without Bak-expression. In general apoptosis induction by Bax and Bak is counteracted by anti-apoptotic Bcl-2 and related proteins. Since in earlier investigations overexpression of Bcl-2 was almost without effect on celecoxib-induced apoptosis we wondered about the mechanisms of celecoxib-induced effects at the mitochondria.

In a first step we verified the lack of inhibitory effects of increased expression of Bcl-2 on celecoxib-induced apoptosis by using a Bcl-2 overexpressing Jurkat cell clone (Fig. 5) [36]. Similar to the other cell lines used in the present study, Jurkat vector and Bcl-2 overexpressing Jurkat Bcl-2 cells were characterized by the presence of pro-apoptotic Bak, but lack of Bax-, p53- and COX-2-expression (Fig. 5A). As expected, levels of Bcl-2 were found to be increased in Jurkat Bcl-2 compared to Jurkat vector cells. Pro-apoptotic Bax remained undetectable during celecoxib-treatment (data not shown). Similar to our earlier observations, celecoxib induced almost similar rates of caspase-activation (Fig. 5B), mitochondrial damage (Fig. 5C) and DNA fragmentation (Fig. 5E) in the Bcl-2 overexpressing Jurkat cells compared to Jurkat vector cells. In contrast, increased levels of Bcl-2 readily protected Jurkat cells against pro-apoptotic effects of ionizing radiation proving functional relevance of the inhibitory action of Bcl-2 against a paradigmatic stimulus of the mitochondrial death pathway (Fig. 5D).

These data underline that celecoxib induces apoptosis in Jurkat cells via a mitochondrial death pathway which is not abrogated by overexpression of Bcl-2. As already mentioned

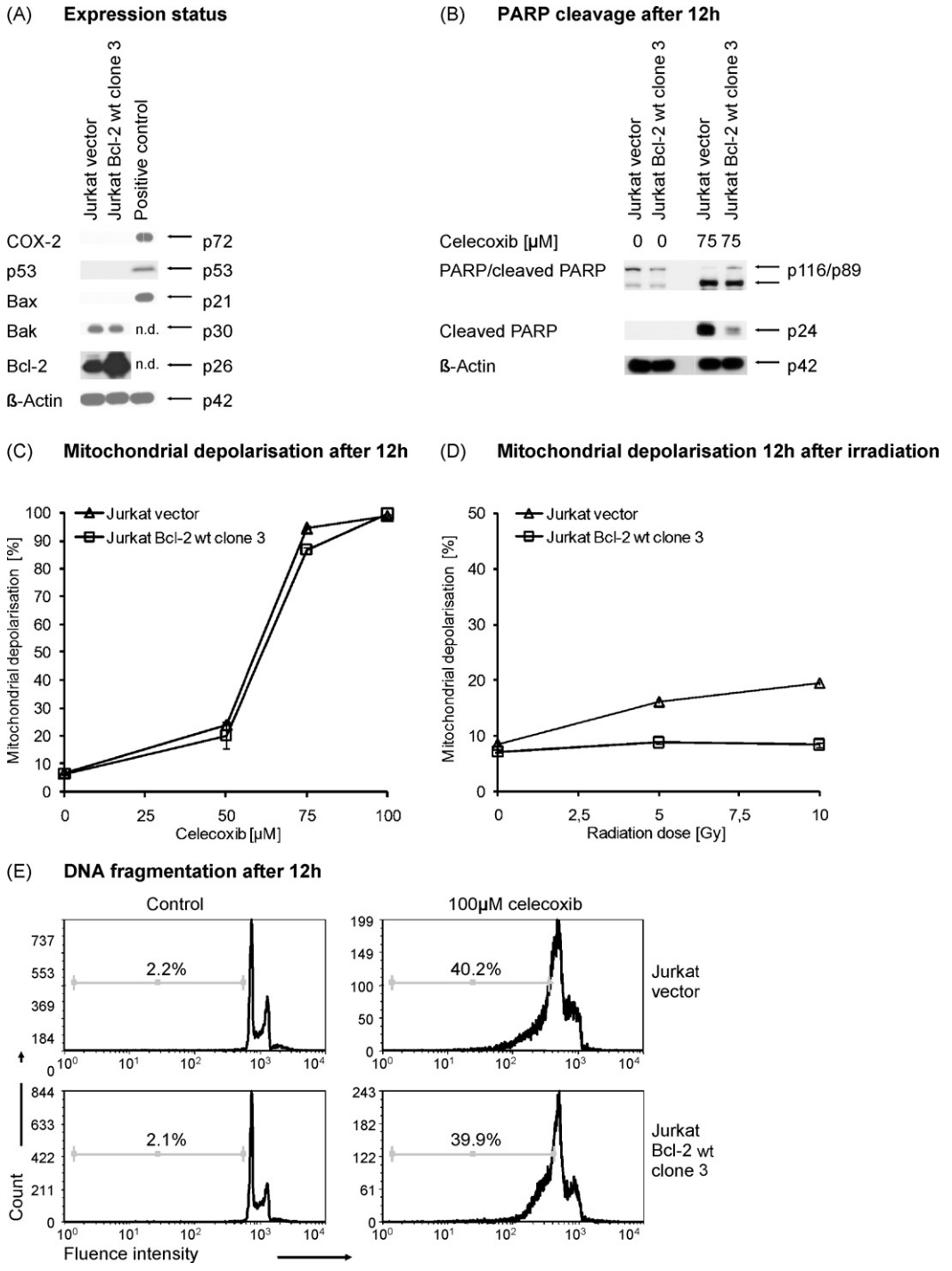

Fig. 5 – Overexpression of Bcl-2 does not abrogate celecoxib-induced apoptosis. (A) Expression of COX-2, p53, Bak, Bax, Bcl-2 and β-Actin as loading control as well as (B) cleavage of the caspase-3 substrate PARP upon treatment with 75 μM celecoxib were verified for Jurkat T-lymphoma cell lines with (Jurkat Bcl-2 wt clone 3) and without (Jurkat vector) Bcl-2 overexpression by Western blot analysis. Activation of caspases as indicated by decrease of full length PARP and increase of the cleaved large and small PARP fragments were observed in Jurkat vector as well as Bcl-2 overexpressing cells. Depolarization of the mitochondrial membrane potential indicative for apoptosis induction was determined in both cell types (C) 12 h after incubation with 0, 50, 75 and 100 μM celecoxib and (D) 12 h after irradiation with 0, 5 and 10 Gy by flow cytometry (TMRE). While both cell types revealed almost similar levels of celecoxib-induced apoptosis irrespective of the expression levels of Bcl-2, apoptosis levels upon irradiation were decreased in Bcl-2 overexpressing cells, proving Bcl-2-independent pro-apoptotic effects of celecoxib despite radioprotective effects of Bcl-2. Data show means ± S.D. of three independent experiments. (E) Similar characteristics were observed when analyzing DNA fragmentation (Sub-G1 peak) 12 h after celecoxib-treatment by flow cytometry. Representative histograms after treatment with 100 μM celecoxib and respective controls were demonstrated.

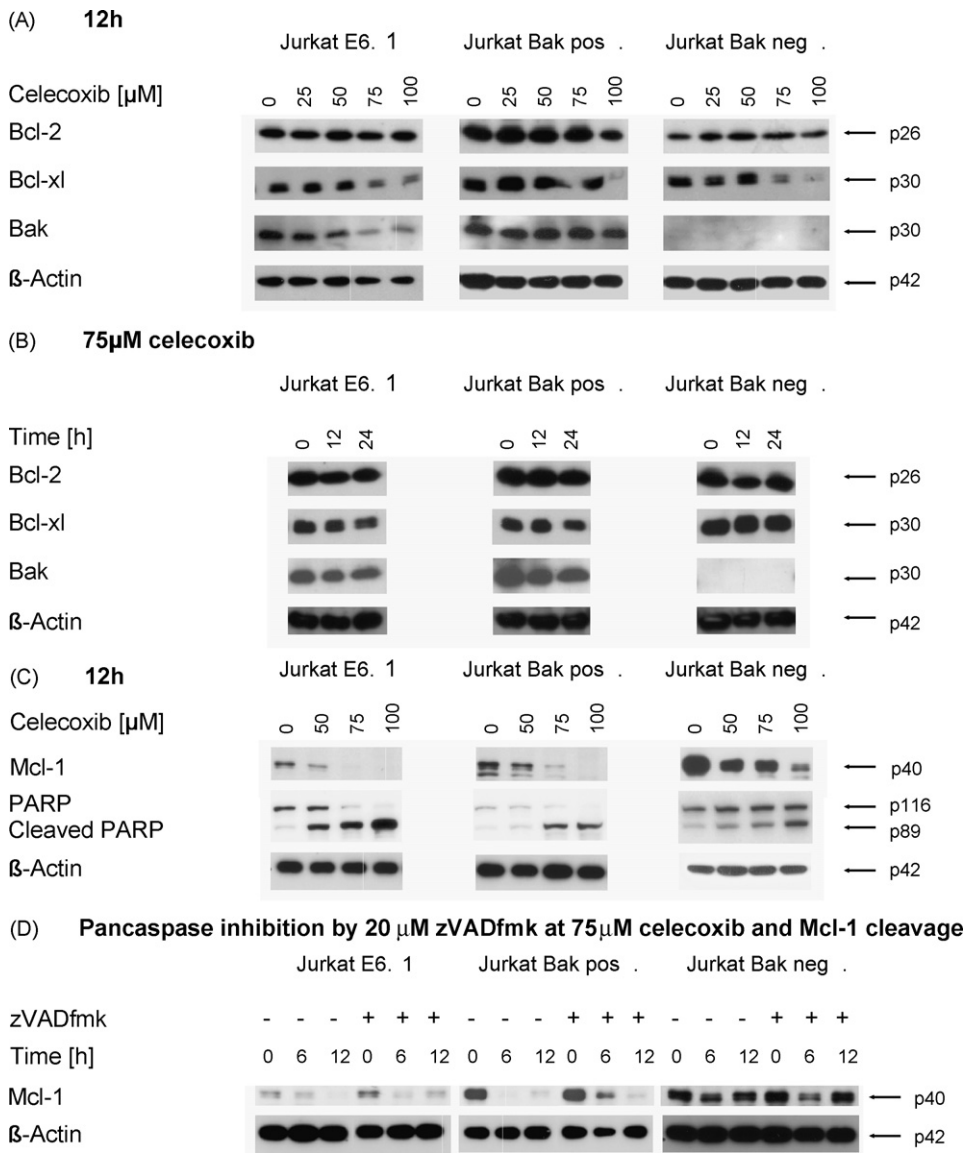

Fig. 6 – Interaction of celecoxib with pro- and anti-apoptotic Bcl-2 proteins. (A–C) Bak-expressing Jurkat Bak positive and Jurkat E6.1 cells as well as Bak-negative Jurkat cells were treated for 0, 12 or 24 h with 75 μM or for 12 h with 0, 50, 75 and 100 μM celecoxib as indicated. Protein levels of Bcl-2, Bcl-x_L, Bak, Mcl-1, uncleaved and cleaved PARP were subsequently evaluated by Western Blot analysis. Data show (A) concentration- and (B) time-dependent effects of celecoxib-treatment on expression of Bcl-2, Bcl-x_L and Bak, (C) concentration-dependent effects of celecoxib-treatment on Mcl-1 and PARP-levels. Even though Mcl-1 levels of untreated Bak-negative cells were increased compared to their Bak-positive counterparts a concentration-dependent constant reduction of Mcl-1 levels was in all Jurkat cell lines detectable (C). (D) Jurkat cells were pretreated for 10 min with 20 μM of the pan-caspase-inhibitor zVAD-fmk and subsequently treated for 0, 6 and 12 h with 75 μM celecoxib to study whether the observed decrease in Mcl-1 levels (Fig. 6C) was a caspase-mediated epiphenomenon or a functional event upstream of the mitochondria necessary for the onset of apoptosis. Western blots were performed demonstrating even in the presence of zVAD-fmk a massive celecoxib-mediated down-regulation of Mcl-1 in Bak-positive cells although the extent of Mcl-1-decrease was lower in the presence of zVAD-fmk. Moreover, celecoxib-mediated down-regulation of Mcl-1 was only transient in Bak-negative Jurkat cells pointing to a combination of caspase-dependent and -independent effects of celecoxib on Mcl-1 levels. β-Actin was used as a loading control (A–E). (E–G) Bak-positive Jurkat cells were transfected with Bak siRNA, non-targeting siRNA (nt siRNA) by electroporation and subsequently treated for 3, 6, and 12 h with 75 μM celecoxib 48h after transfection. (E) Effects of transfection on Bak protein expression were tested 48h after electroporation. Significant reduction of Bak-expression was only detectable with siRNA for Bak. Quantification of Western blot analysis was performed with densitometric software ImageJ (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, USA, http://rsb.info.nih.gov/ij/, 1997–2007). Effects of siRNA-mediated down-regulation of Bak on celecoxib-induced mitochondrial membrane depolarization ΔΨ (F) and nuclear fragmentation (Sub-G1 peak) (G) was determined 12 h after celecoxib-treatment with 75 μM by flow cytometry. Data (F and G) show means ± S.D. of three independent experiments.

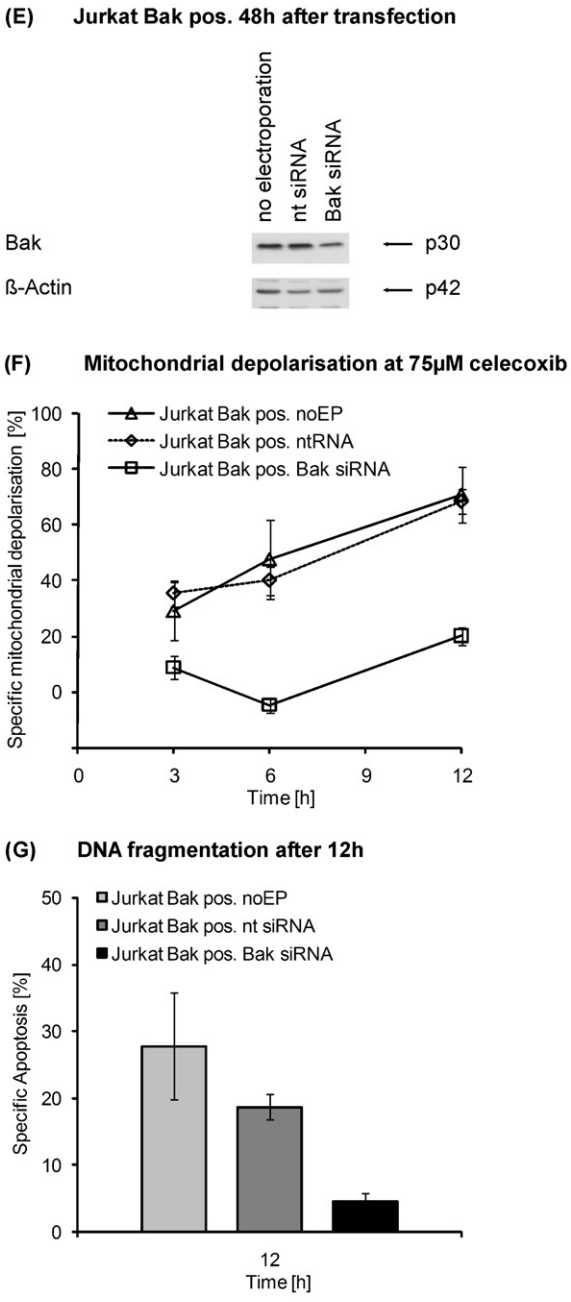

Fig. 6. (Continued).

above, the Jurkat cell lines used in the present study did not show expression of COX-2 in the absence or presence of celecoxib making inhibitory effects on COX-2-mediated expression of Bcl-2 highly unlikely (Fig. 1C). However, to exclude that the potent celecoxib-effects in Bcl-2 overexpressing cells may be executed via inhibitory effects on Bcl-2-expression, we subsequently evaluated to what extent celecoxib would interfere with the expression of anti-apoptotic Bcl-2 or related proteins (Fig. 6). As shown in Fig. 6A and B treatment with celecoxib did not alter the protein levels of Bcl-2 in all cell lines tested. In contrast, a tendency to decreased levels of Bcl-x_L expression was observed at least at increased concentrations (Fig. 6A). However, since apoptosis was already executed at earlier time points and lower drug concentrations, we hypothesize that the celecoxib-induced decrease in Bcl-x_L expression may not be the major trigger of the observed effects. In line with that interpretation, decreased protein levels of Bcl-x_L were also observed in Bak-negative celecoxib-resistant Jurkat cells. Moreover, decrease of Bcl-x_L paralleled the decrease of Bak levels in the apoptosis competent cells.

Interestingly, in the two Bak-positive cell lines, celecoxib-treatment resulted in prominent down-regulation of the anti-apoptotic Bcl-2 protein Mcl-1. Mcl-1 was almost undetectable 12 h after treatment with 50 and 75 μM celecoxib, respectively. Mcl-1 levels were also down-regulated in the Bak-negative Jurkat cells. However, since Mcl-1 levels were increased in untreated Bak-negative Jurkat cells compared

to their Bak-positive counterparts, increased concentrations of celecoxib were necessary to obtain a similar level of down-regulation (Fig. 6C). Since in addition to Bcl-x_L pro-apoptotic Bak is kept in check by Mcl-1, these data suggest that down-regulation of Mcl-1 may at least partially be responsible for celecoxib-mediated activation of Bak-dependent apoptosis [37].

Since in addition to Bcl-x_L pro-apoptotic Bak is kept in check by Mcl-1, these data suggest that down-regulation of Mcl-1 may at least partially be responsible for celecoxib-mediated activation of Bak-dependent apoptosis [37]. To study in how far the observed decrease in Mcl-1 levels would be an upstream event being necessary for the onset of apoptosis or rather a consequence of caspase-mediated Mcl-1 cleavage downstream of mitochondrial damage, the effect of celecoxib on Mcl-1 levels was determined in the presence or absence of pan-caspase inhibition by zVAD-fmk (Fig. 6D). Our data clearly show that even in the presence of zVAD-fmk celecoxib was able to induce massive down-regulation of Mcl-1 in Jurkat E6.1 and Jurkat Bak-positive cells (Fig. 6D left and middle panel) although the extent of the Mcl-1-decrease was somewhat reduced in Jurkat Bak-positive cells in the presence of zVAD-fmk. Moreover, celecoxib-mediated down-regulation of Mcl-1 was only transient in Bak-negative Jurkat cells (Fig. 6D right panel). Altogether these results point to a combination of caspase-dependent and caspase-independent effects of celecoxib on Mcl-1 levels.

In a last set of experiments we determined the influence of siRNA-mediated down-regulation of Mcl-1 on survival of Jurkat cells. As already observed earlier, siRNA-mediated down-regulation of Mcl-1 was sufficient to trigger massive apoptosis in our Jurkat cell model revealing functional relevance of celecoxib-mediated effects on Mcl-1 (data not shown) [38].

3.3. *Down-regulation of Bak by siRNA abrogates celecoxib-induced apoptosis*

Up to now our data suggested that expression of Bak is essential for celecoxib-induced apoptosis. To prove functional relevance of Bak-deficiency for celecoxib-resistance we tested in how far siRNA-mediated down-regulation of Bak in Bak-positive Jurkat cells would abrogate celecoxib-induced apoptosis and thus mimick the phenotype of the Bak-negative cells in respect to celecoxib-sensitivity. To this end, Bak-positive Jurkat cells were transiently transfected with siGlo-, non-target as well as Bak siRNA and subsequently treated with celecoxib 48 h after transfection. As shown in Fig. 6F transfection with Bak siRNA largely suppressed the time-dependent celecoxib-induced depolarization of the mitochondrial membrane potential compared to control cells without electroporation (no EP) and cells transfected by non-target siRNA (nt siRNA). Moreover, siRNA-mediated down-regulation of Bak resulted in an even more prominent reduction of DNA fragmentation in response to 75 μM celecoxib (Fig. 6G). Thus, siRNA-mediated down-regulation of Bak resulted in reduced sensitivity of the cells to celecoxib-induced apoptosis further corroborating our findings on the importance of Bak for celecoxib-induced apoptosis.

Successful uptake of siRNA (siGLO) in living (PI-negative) cells (90% after 24 h) was confirmed by flow cytometry (data not shown). As shown in Fig. 6E, transfection with Bak siRNA but not with non-target siRNA (nt siRNA) decreased Bak protein expression in Bak-positive Jurkat cells by 67.2% within 48 h (Jurkat Bak positive).

In our hands, partial down-regulation of Bak by was sufficient to almost completely block celecoxib-induced apoptosis (Fig. 6E). This observation underlines the importance of the balance of the complete set of pro- and anti-apoptotic proteins within a damaged cell and suggests that the low levels of Bak may be neutralized by concerted action of endogenous Bcl-2 and Bcl-x_L and some remaining Mcl-1.

4. Discussion

In this report we show for the first time that celecoxib-induced apoptosis in Bax-negative Jurkat cells depends on the expression of Bak. Functional relevance of Bak-deficiency for celecoxib-resistance was demonstrated by almost complete abrogation of celecoxib-induced apoptosis in Bak-deficient Jurkat cells as well as largely decreased apoptosis rates in Bak-positive Jurkat cells upon RNAi-mediated down-regulation of Bak-expression. Thus, while lack of Bax did not abrogate celecoxib-induced apoptosis, combined loss of Bak and Bax completely blocked the apoptotic cascade triggered by celecoxib-treatment. From these data we conclude that in Bax-deficient cells Bak is essential for the onset of apoptosis. In our hands celecoxib did not alter the expression levels of Bak but rather its activation state. This is in contrast to a recent report suggesting that celecoxib may affect apoptosis sensitivity by increasing Bak-expression in cervical cancer cells [39]. Celecoxib-mediated increase of Bak-expression and apoptosis rates were reduced by siGADD153 suggesting that in those cells GADD153 may play a key role in celecoxib-triggered Bak-expression and apoptosis. Unfortunately, the authors did not mention the Bax-expression status in their cells precluding final conclusions about the relative importance of Bak in the presence or absence of Bax. Nevertheless, both studies emphasize the emerging role of Bak to mediate celecoxib-induced apoptosis.

However, in Bax-expressing hepatocellular carcinoma cells treatment with celecoxib led to an increase in pro-apoptotic Bax and a subsequent shift in the Bax/Bcl-2 ratio resulting in growth inhibition and apoptosis induction [40]. This association of increased levels of Bax and celecoxib-action has also been observed by others [26,41]. In line with these observations, in human colorectal or esophageal cancer cells, apoptosis induction by other nonsteriodal anti-inflammatory drugs depended on the expression of Bax [42,43]. However, in neither study functional relevance of increased Bax levels or the relevance of its homologue Bak had been tested.

Together with the broad resistance of Bax/Bak-double knockout cells to many if not all death stimuli-inducing apoptosis via the intrinsic pathway these observations further confirm a functional redundancy of Bax and Bak. These two complementary pro-apoptotic Bcl-2 proteins of the Bax-family are essential for the activation of the intrinsic death pathway and can substitute each other in cell lines with defective

expression or function of their functional relative [44,45]. Since celecoxib and related compounds induce apoptosis via a mitochondrial death pathway it may be expected that dependent on the cell type cell death may be preferentially be mediated by either Bax or Bak [46,47], and that in Bax-deficient Jurkat cells Bak may be responsible for initiation of the death cascade [35].

Similar to our earlier observations, overexpression of Bcl-2 was not sufficient to abrogate celecoxib-induced apoptosis in the Jurkat T-lymphoma cells [28]. In line with this observation, Bcl-2 overexpression did not protect against apoptosis induced by celecoxib or derivatives in chronic lymphocytic leukemia [13], oral cancer [25], LNCaP and PC-3 prostate cancer cell lines [30]. Moreover, in our hands treatment with celecoxib did not alter the expression levels of Bcl-2 or Bcl-x_L. Therefore, in contrast to the results obtained by Subhashini and co-workers down-regulation of anti-apoptotic Bcl-2 and Bcl-x_L was not responsible for celecoxib-induced apoptosis [41,48,49].

In conjunction with the observation that Jurkat cells do not express the suggested target protein of coxibes, COX-2, in the absence of celecoxib and do not up-regulate that protein during treatment, our findings argue against an involvement of inhibitory effects of celecoxib on COX-2-mediated Bcl-2 expression in celecoxib-induced apoptosis, at least in Jurkat cells. Thus, our data corroborate earlier findings on COX-2-independent pro-apoptotic effects of celecoxib [11,12,23–25]. However, this does not exclude that in COX-2-expressing cells, inhibition of COX-2 may contribute to the antineoplastic effects of celecoxib e.g. by regulation of the levels of pro-and anti-apoptotic Bcl-2 family members [50,51]. In this regard, increased COX-2 expression in normal enterocytes from rat ileum transformed by anti-sense Bak rendered the cells sensitive to the pro-apoptotic effects of celecoxib [52,53]. Taking into consideration that COX-2-overexpression is associated with poorer prognosis [54–59] and that celecoxib can mediate both COX-2-dependent and COX-2-independent pro-apoptotic effects we speculate that despite the suggested increased risk of heart attack and stroke upon long term exposure, celecoxib may be of value for short treatment of COX-2-expressing tumors as single drug or in combination with classical chemotherapeutic drugs or ionizing radiation. In contrast, pro-apoptotic celecoxib-derivatives without COX-2-inhibitory action such as OSU-03012 or DMC may be safer for anticancer treatment of COX-2 negative tumors.

Interestingly, celecoxib-treatment was associated with a rapid loss of anti-apoptotic Mcl-1. Similarly, decreased levels of anti-apoptotic Mcl-1 in response to celecoxib-treatment have recently been observed by others in hepatocellular carcinoma as well as human T-cell leukemia virus-1-associated adult T-cell leukemia [26,29]. Since a selective interaction of Bak with Mcl-1 and Bcl-x_L in healthy cells has been proposed to prevent Bak-activation [60,61], celecoxib-induced down-regulation of Mcl-1 may contribute to celecoxib-induced Bak-activation and apoptosis. In our hands, celecoxib was still able to induce Mcl-1 decrease even in the presence of zVAD-fmk. Together with the finding that celecoxib-mediated down-regulation of Mcl-1 was largely reduced and only transient in Bak-negative Jurkat cells our results point to a combination of caspase-dependent and caspase-independent effects of celecoxib on Mcl-1 levels. Moreover, siRNA-mediated down-regulation of Mcl-1 was sufficient to trigger apoptosis in the absence of further stimuli. These observations confirm functional relevance of decreased Mcl-1-levels for the onset of apoptosis upon celecoxib-treatment, and confirm earlier findings on the role of Mcl-1 for Jurkat cell survival [38].

Based on these findings, we conclude that the pro-apoptotic Bcl-2 homologue Bak is essential for COX-2-independent celecoxib-mediated apoptosis in Bax-deficient Jurkat T-lymphoma cells at the level of the mitochondria. We hypothesize that celecoxib-mediated down-regulation of Mcl-1 is causative for the release of Bak from this essential pro-survival factor, Bak-mediated mitochondrial damage and subsequent Bak-dependent apoptosis.

Together with our earlier findings our novel observations underline that celecoxib and related compounds are potent antineoplastic agents with a unique mechanism of action. It is of special importance to notice that molecular requirements for celecoxib-induced apoptosis seem to differ from those of most DNA-damaging antineoplastic drugs and ionizing radiation. This may offer the opportunity for increased efficacy of combined treatment approaches using celecoxib in combination with chemotherapeutic drugs or ionizing radiation in cellular systems with loss of p53 and high expression levels of a special subset of anti-apoptotic Bcl-2-family members.

Conflicts of interest

None.

Acknowledgements

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