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Involvement of tyrosine kinase p56/Lck in apoptosis induction by anticancer drugs

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Abstract

Induction of apoptosis is a hallmark of the cellular response of human lymphocytes and lymphoma cells to treatment with anticancer drugs and irradiation. Both treatment modalities trigger apoptosis through intrinsic, mitochondrial apoptosis pathways resulting in the activation of caspases. We and others have shown that the tyrosine kinase p56/Lck is involved in the regulation of apoptosis induced by irradiation or treatment with ceramide but dispensable for death receptor triggered cell death. However, the role of p56/Lck for apoptosis induction in response to anticancer drugs is unclear. To elucidate the putative requirement of p56/Lck for apoptosis signaling of cytotoxic drugs, activation of caspases and alteration of mitochondrial functions were determined in Jurkat T cells, the p56/Lck deficient JCaM1.6 cells and the p56/Lck retransfected JCaM1.6/Lck cells in response to chemotherapeutic drugs with different targets of their primary action. Treatment with Doxorubicin, Paclitaxel or 5-Fluorouracil induced a breakdown of the mitochondrial membrane potential and apoptotic cell death in p56/Lck expressing Jurkat and the retransfected JCaM1.6/Lck cells within 48 h of treatment. However, almost no mitochondrial alterations and no induction of apoptosis could be detected in the p56/Lck deficient JCaM1.6 cells. Correspondingly, activation of caspases-9, -8, and -3 and cleavage of the caspase-3 substrate PARP (poly-(ADP-ribose)-polymerase) were almost completely absent in JCaM1.6 cells while present in p56/Lck positive Jurkat and JCaM1.6/Lck cells. In contrast, retransfection of the cells with the p56/Lck-related tyrosine kinase Src could not restore sensitivity to the treatment with cytotoxic drugs indicating a specific role of the tyrosine kinase p56/Lck in apoptosis signaling. Importantly, kinase-activity of p56/Lck may be dispensable for its pro-apoptoptic action since preincubation with the Src-kinase inhibitor PP2 did not reduce apoptosis induced by cytotoxic drugs. In conclusion, the tyrosine kinase p56/Lck is essential for apoptosis induction by Doxorubicin, Paclitaxel and 5-Fluorouracil regulating early steps of the mitochondrial apoptosis signaling cascade, including alteration of mitochondrial functions and caspase-activation.

Keywords: Apoptosis; Caspases; p56/Lck; Mitochondria; Src-like tyrosine kinase; Cytotoxic drugs

1. Introduction

Induction of apoptosis, a highly regulated form of cell death, is a common mechanism of the cytotoxic action of chemotherapeutic drugs. Apoptosis is characterized by the activation of caspases, a family of cystein proteases [1,2]. In general, caspases can be activated by two distinct but interconnected signaling cascades, the death receptor pathway which is triggered by death receptor ligands e.g. CD95-ligand, TRAIL and the death receptor independent mitochondrial pathway which is initiated upon application of cellular stresses (e.g. irradiation and treatment with chemotherapeutic drugs) [3–5].

Stimulation of death receptors (e.g. CD95, TNF-receptor, TRAIL-receptor) by their specific ligands causes autoproteolytic activation of caspase-8 at the receptor level within the so-called death inducing signaling complex (DISC). In this pathway, caspase-8 represents the key initiator caspase and directly triggers activation of the

Abbreviations: Apaf-1, apoptotic protease activating factor 1; Bax, Bcl-2-associated X-protein; Bcl-2, B-cell lymphoma gene 2; Caspase, cysteine-aspartate-directed protease; dATP, deoxy adenosine triphosphate; Erk, extracellular signal-regulated kinase; FACS, fluorescence-activated cell sorter; 5-FU, 5-Fluorouracil; PARP, poly-(ADP-ribose)-polymerase; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)-pyrazolo-[3,4-*d*] pyrimidine; TCR, T-cell receptor; TMRE, tetramethylrodaminethylester; TRAIL, TNFα-related apoptosis inducing ligand

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effector caspase cascade [6,7]. In this context it was suggested that—at least in some cell types—induction of apoptosis by chemotherapeutic agents may be dependent on drug-induced up-regulation of the CD95 death receptor ligand (CD95-L) and death receptor mediated activation of caspases [8,9]. However, further reports revealed that drug-induced cell death follows death receptor-independent, mitochondrial signaling pathways [5,10–14].

It is now widely accepted that cellular stresses including chemotherapeutic treatment mostly provoke alterations of mitochondrial functions with breakdown of the mitochondrial membrane potential and release of pro-apoptotic cytochrome *c* from the mitochondrial intermembrane space into the cytosol [11,12]. Subsequently, pro-caspase-9 is activated within a cytosolic complex composed of cytochrome *c*, dATP, and the adaptor molecule Apaf-1 [15,16]. Caspase-9 constitutes the initiator caspase of those intrinsic, death receptor independent mitochondrial death pathways and activates downstream effector caspases such as caspases-3, -6 and -7 [4,14,17–19]. In mitochondrial death pathways, caspase-8 is only activated downstream of the mitochondria and acts as an effector caspase [4,10].

Mitochondrial death pathways are regulated by pro- and anti-apoptotic Bcl-2 like proteins [20–22]. The complex interaction of pro-apoptotic Bcl-2 family members (Bax, Bak (Bcl-2 antagonist/killer), Puma, Noxa) leads to cytochrome *c*-release from mitochondria. In contrast, Bcl-2 and Bcl-x_L, two anti-apoptotic proteins of the Bcl-2-family inhibit mitochondrial pathways for the induction of apoptosis [20,22,23]. However, the up-stream signalling molecules of the mitochondrial death pathways are less defined.

In addition to the essential role of caspases, the mitochondria and proteins of the Bcl-2 family, the Src-like tyrosine kinase p56/Lck has been implicated in the regulation of apoptosis induced by the human immunodeficiency virus transactivator protein (HIV-TAT protein) [24], by ionising radiation [25] as well as by ceramide [26]. Furthermore, activation of p56/Lck by CD19 cross-linking has been shown to facilitate radiation-induced apoptosis in radioresistant B-lymphoma cells [27]. Similar to the role of p56/Lck for the radiation response in T cells, the B-cell receptor-associated Src-like kinase Btk and the Src-like kinase Lyn have been implicated in regulation of apoptosis in B cells in response to radiation or UV light, respectively [28,29].

In general, p56/Lck functions in the transduction of TCR signals [30]. Activation of p56/Lck in response to TCR stimulation provokes phosphorylation of critical residues of the TCR complex with subsequent initiation of downstream signaling events including calcium influx, activation of the transcription factor NFAT (nuclear factor of activated T cells) and activation of the mitogenic kinases Erk1 and Erk2 [30–32].

In a recent report we could demonstrate that p56/Lck controls early mitochondrial steps of radiation induced apoptosis [33]. Using p56/Lck-deficient JCaM1.6 and p56/Lck-transfected JCaM1.6/Lck cells, we could show that the radiation-induced breakdown of the mitochondrial transmembrane potential, the release of cytochrome *c* and the activation of caspases were severely impaired in the JCaM1.6 cells, while being present in p56/Lck-transfected Jcam1.6/Lck cells [33].

Since many chemotherapeutic drugs induce apoptosis via mitochondrial signaling pathways and p56/Lck was shown to be required for several stimuli acting via mitochondrial pathways, we aimed to specify the role of p56/Lck for drug induced apoptosis. To this end, apoptosis signaling was analysed in p56/Lck deficient JCaM1.6 and p56/Lck-transfected JCaM1.6/Lck cells upon treatment with Doxorubicin, Paclitaxel and 5-FU. Our data reveal that p56/Lck-deficiency strongly reduces sensitivity of JCaM1.6 cells to apoptosis-induction in response to treatment with those chemotherapeutic drugs acting on different primary intracellular targets.

2. Material and methods

Hoechst 33342 was purchased from Calbiochem. The proton shuttle carbonylcyanide-*m*-chloro-phenylhydrazone (CCCP) was from Sigma. Phytohemagglutinin-L was purchased from Sigma. PD98059 was from Cell Signaling and dissolved in DMSO as 10 mM Stock. The inhibitor of the Src family of protein tyrosine kinases, PP2, was received from Calbiochem and dissolved in DMSO as 5 mM Stock. All other biochemicals were from Sigma chemicals unless otherwise specified.

2.1. Cell culture and transfections

Jurkat E6 T-lymphoma cells were purchased from ATCC (Bethesda, Maryland, USA) and used upon vector transfection with pRC/cmv as described elsewhere [12,34]. The p56/Lck deficient Jurkat clone (JCaM1.6) and the p56/Lck cDNA expressing JCaM1.6/Lck clone were a kind gift from A. Weiss (University of California, San Francisco, USA). For all experiments, cells were grown in RPMI 1640 medium (Life Technologies) and maintained in a humidified incubator at 37 °C and 5% CO_2.

Expression vectors encoding wild-type and activated Src as well as the empty vector pUSEamp were obtained from Upstate. Wild-type Bcl-2 was kindly provided by B. Leber (McMaster University, Hamilton, Ont., USA) and cloned into the expression vector pRC/cmv (Invitrogen). JCaM1.6 cells stably expressing wild-type Src (JCaM1.6/Src.wt), activated Src (JCaM1.6/Src.act), the respective vector control cells (JCaM1.6/Vector) or wild type Bcl-2 (JCaM1.6/Bcl-2) were prepared by electroporation using a Gene pulser II (BioRad) and used as pool transfectants selected by supplementation of the medium with 800 μg/ml G418 (Life Technologies).

2.2. Treatment with anticancer drugs

Doxorubicin and 5-FU were purchased from Pharmacia-Pfizer and used in concentrations of 0.5 μg/ml and 10 μM, respectively. Paclitaxel was from Alexis and was used in a final concentration of 100 nM. For MEK-1 inhibition PD98059 was added at a concentration of 25 μM, 1 h prior to treatment with anticancer drugs. For p56/Lck inhibition, cells were preincubated for 1 h with 5 μM PP2 before stimulation with anticancer drugs.

2.3. Determination of cell proliferation

Assessment of cellular proliferation was performed using the Wst-1 cell proliferation reagent (Roche Molecular Biochemicals) as described elsewhere [35]. In brief, cells were seeded in 96-well plates in a final volume of 100 μl/well. The number of living cells was determined after 24, 48 and 72 h by addition of Wst-1 solution (10 μl/well). Absorbance at 450 nm (λ reference: 620 nm) was determined upon incubation of the plates for 120 min at 37 °C using an Anthos 2010 plate reader (Anthos).

2.4. Determination of apoptosis

Cell death was determined by propidium iodide-exclusion (staining with 0.1 μg/ml propidium iodide). Apoptosis was quantified with a FACS Calibur flow cytometer (Becton Dickinson) analysing light scatter characteristics. In addition, apoptosis was quantified upon staining of the cells with Hoechst 33342 (1.5 μM for 15 min) and subsequent fluorescence microscopy (Zeiss Axiovert 135, Carl Zeiss) using an excitation wavelength filter of 380 nm. Analysis was documented using an CCD camera device (Zeiss Axiocam MRm). Cells with intense chromatin condensation and nuclear fragmentation were considered as apoptotic.

2.5. Immunoblotting

Cells were lysed for 30 min at 4 °C in a lysis buffer containing 25 mM HEPES, 0.1% SDS, 0.5% deoxycholate, 1% Triton X-100, 10 mM EDTA, 10 mM NaF and 125 mM NaCl. Insoluble material was removed by centrifugation for 10 min at 13,000 rpm. After denaturation for 5 min at 100 °C in SDS-loading buffer, 20 μg of the lysate was separated by SDS–PAGE. Blotting was accomplished employing a tank blotting apparatus (Bio-Rad) onto Hybond C membranes (Amersham). Ponceau S staining (Sigma) confirmed equal protein loading. Blots were blocked for 15 min at RT in TBS buffer containing 0.05% Tween 20 and 5% fetal calf serum. Caspase-8 was detected using a mouse monoclonal antibody (BioCheck) directed against the p18 as described previously [4]. Caspase-9, -3 and PARP were detected using cleavage specific antibodies from Cell Signaling Technology (New England Biolabs). Bcl-2 expression was determined using an Bcl-2 antibody (Santa-Cruz) and p56/Lck expression was tested using an antibody against the C-terminus of p56/Lck (Becton Dickinson Transduction-Labs). The monoclonal mouse anti-Src antibody was from Upstate (Biomol). Procaspase-3 and Bcl-x_L expression were analysed using Caspase-3 and Bcl-x_L antibodies (Becton Dickinson Transduction-Labs). Activation of Erk1/2 was determined using an activation specific antibody as described previously [36].

After repeated washings with TBS/Tween 20 (0.05%) (TBST) the membrane was incubated with an alkaline phosphatase coupled secondary antibody (Santa-Cruz-Biotech) in TBST for 1 h at room temperature and washed three times with TBST. The detection of antibody binding was performed by enhanced chemoluminescence (CSPD, Tropix).

2.6. Determination of the mitochondrial membrane potential

Mitochondrial transmembrane potential ($\Delta\psi_m$) was analysed using the potential sensitive dye TMRE (Molecular probes, Mobitech). At the indicated time points cells were stained for 30 min at 37 °C in PBS supplemented with 25 nM TMRE. Staining was quantified by FL2 employing a FACS Calibur flow cytometer (Becton Dickinson).

3. Results

3.1. JCaM1.6 cells lack expression of functional p56/Lck

To verify p56/Lck expression in JCaM1.6 cells, JCaM1.6/Lck and Jurkat T cells, protein levels of p56/Lck were analysed by Western blotting with an antibody against the C-terminus of p56/Lck [37,38]. While JCaM1.6/Lck and Jurkat T cells showed approximately equal expression of p56/Lck, no intact p56/Lck was detectable in JCaM1.6 cells (data not shown).

Earlier investigations had shown that the normal response to TCR stimulation required p56/Lck function provoking phosphorylation and thus activation of Erk1/2 kinases [12]. Therefore, to show functional activity of p56/Lck in the cell lines used in the present study, Erk1/2 activation upon stimulation of the CD3/TCR with phytohemagglutinin-L (PHA-L, 50 μg/ml) was examined using an antibody specific for the phosphorylated Erk1/2 kinases. As expected, phosphorylation of Erk1/2 was significantly reduced in JCaM1.6 while present in p56/Lck-transfected JCaM1.6/Lck and Jurkat cells (Fig. 1). These data demonstrate expression and functional activity of p56/Lck in JCaM1.6/Lck and Jurkat cells as well as deficient expression and activity of p56/Lck in JCaM1.6 cells.

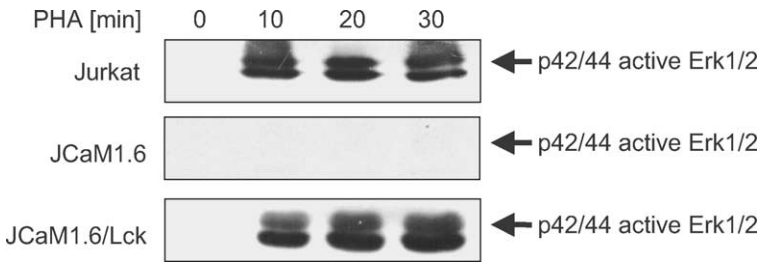

Fig. 1. Functional activity of p56/Lck in T-lymphoma cells. Functional activity of p56/Lck was analysed by determination of Erk1/2 activation in response to T-cell receptor stimulation with 50 μg/ml phytohaemagglutinin (PHA). Data show one representative of three independent experiments.

3.2. p56/Lck is required for the induction of apoptosis by Doxorubicin, Paclitaxel and 5-FU

To specify the requirement of p56/Lck for the cellular response to treatment with chemotherapeutic drugs, induction of apoptosis was analysed in JCaM1.6 cells, JCaM1.6/Lck and Jurkat cells using Doxorubicin, Paclitaxel or 5-FU, three anticancer drugs with different primary intracellular targets. Apoptotic phenotypes were visualized by fluorescence microscopy upon staining of the nuclei with Hoechst 33342. Our data reveal that all drugs readily induce chromatin condensation and DNA fragmentation in Jurkat and JCaM1.6/Lck cells. However, almost no signs of nuclear apoptosis could be detected in the p56/Lck-deficient JCaM1.6 cells (Fig. 2).

Lck constitutes the most common Src-like tyrosine kinase in T-lymphocytes while other tyrosine kinases are only expressed at low levels. To test, whether over-expression of the p56/Lck-related tyrosine kinase Src would restore sensitivity of the Lck-deficient JCaM1.6 cells to cytotoxic drugs, cells were transfected with wild-type as well as activated Src (Fig. 3D) and tested to their sensitivity to drug-induced apoptosis. However, in contrast to retransfection with p56/Lck, Src-transfection did not increase sensitivity of JCaM1.6 cells to the induction of apoptosis by cytotoxic drugs (Fig. 3A–C). Thus, in JCaM1.6 cells Src cannot substitute for p56/Lck in regard to apoptosis induction.

To further elucidate apoptosis signaling of JCaM1.6 cells upon drug exposure, induction of apoptosis was

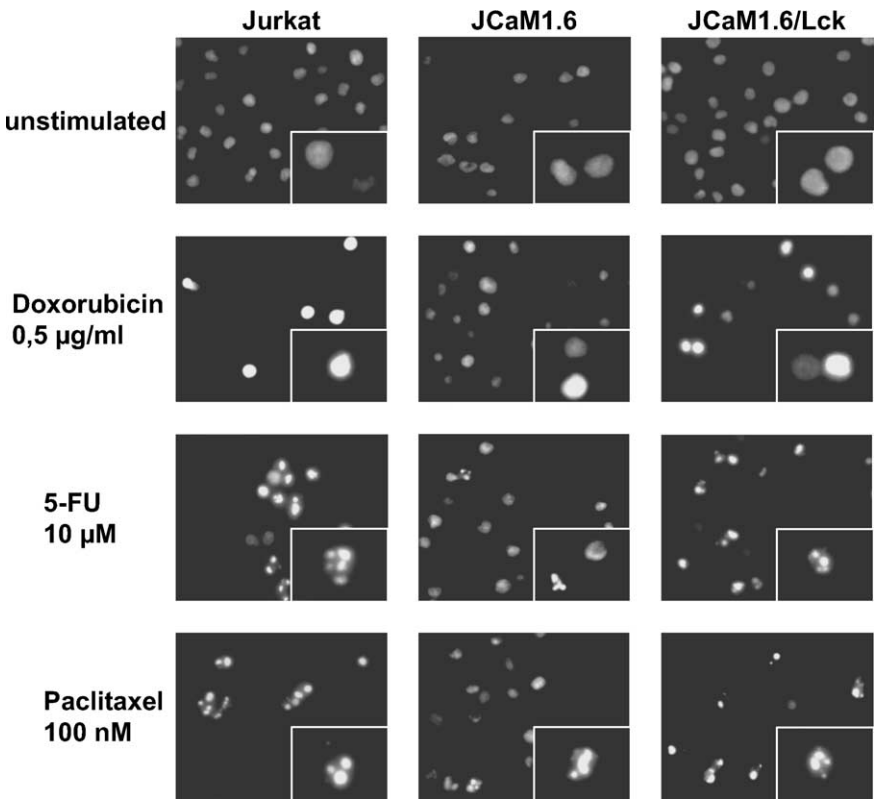

Fig. 2. Induction of apoptotic nuclear morphology in Jurkat T-lymphoma cells is dependent on the expression of p56/Lck. The induction of an apoptotic nuclear morphology is almost completely abrogated in p56/Lck deficient JCaM1.6 cells. Jurkat, JCaM1.6 and JCaM1.6/Lck cells were treated for 36 h with the indicated concentrations of Doxorubicin, 5-FU or Paclitaxel. Nuclear morphology was analysed by fluorescence microscopy upon staining with Hoechst 33342. Cells with the typical apoptotic morphology showing chromatin condensation and nuclear fragmentation were considered apoptotic.

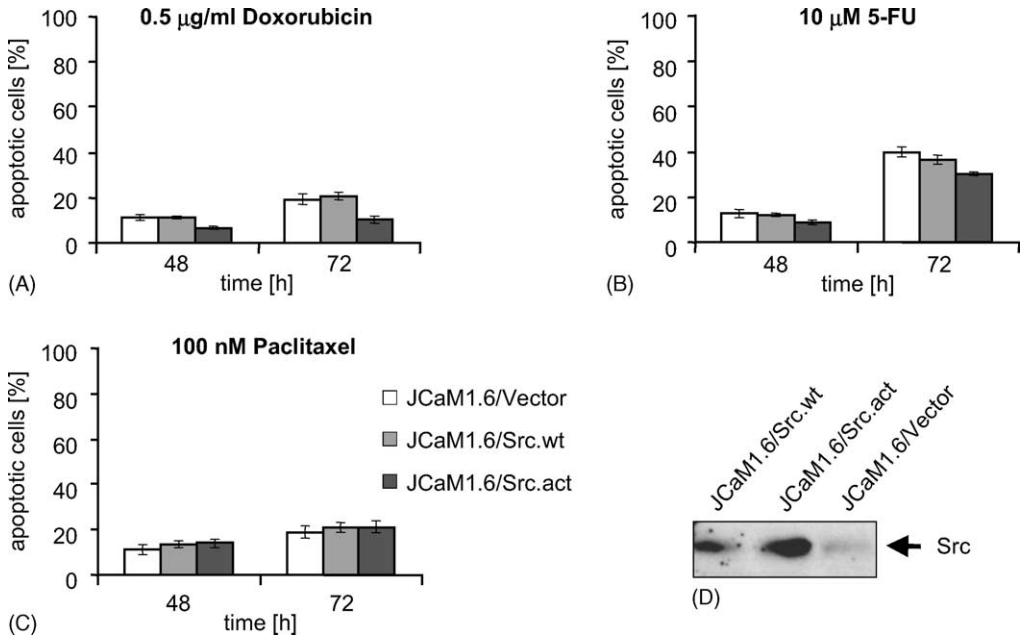

Fig. 3. The function of p56/Lck during apoptosis induction cannot be replaced by the tyrosine kinase Src. Lck-deficient JCaM1.6 cells were transfected with wild type Src (JCaM1.6/Src.wt), active Src (JCaM1.6/Src.act) or the respective empty vector (JCaM1.6/Vector). Stably transfected cells were treated for 48–72 h with (A) 0.5 µg/ml Doxorubicin, (B) 10 µM 5-FU or (C) 100 nM Paclitaxel. Apoptosis induction was quantified by FACS analysis using light scatter characteristics. Data show means ± S.D. ($n = 3$). (D) Expression of Src was tested by Western blotting cytosolic extracts of JCaM1.6/Vector, JCaM1.6/Src.wt and JCaM1.6/Src.act cells with a Src-specific antibody.

quantified by FACS analysis using light scatter characteristics 24–72 h after treatment with 0.5 µg/ml Doxorubicin, 100 nM Paclitaxel or 10 µM 5-FU (Fig. 4A–C). Treatment with 0.5 µg/ml Doxorubicin induced significant apoptosis in JCaM1.6/Lck and Jurkat cells within 24 h of treatment yielding maximal apoptosis rates already after 48 h. In contrast, JCaM1.6 cells did only undergo delayed cell death with almost no signs of apoptosis after 48 h and only 47.5% apoptotic cells after 72 h of treatment (Fig. 4A).

Similar to the observations with Doxorubicin, treatment with 100 nM Paclitaxel clearly induced rapid apoptosis at high rates in JCaM1.6/Lck and Jurkat cells reaching maximal levels within 48 h. Again, Paclitaxel almost completely failed to induce apoptosis in the p56/Lck deficient JCaM1.6 cells within 48 h and only yielded 33.8% apoptotic cells after 72 h of treatment (Fig. 4C).

Furthermore, incubation with 10 µM 5-FU readily triggered apoptosis in Jurkat and JCaM1.6/Lck cells coming to a maximum after 72 h. Similar to the observations with Doxorubicin and Paclitaxel, apoptosis rates in JCaM1.6 cells were reduced when compared to the p56/Lck expressing Jurkat and JCaM1.6/Lck cells (Fig. 4B). However, despite reduced apoptosis rates, p56/Lck deficient cells were not fully resistant to drug-induced apoptosis but underwent delayed cell death after prolonged exposure (72 h) to cytotoxic drugs (Fig. 4A–C). To test, whether the phenomenon of delayed apoptosis in the p56/Lck-deficient cells may be blocked by over-expression of Bcl-2, JCaM1.6 cells were transfected with Bcl-2 (Fig. 4D, right panel) and tested for their sensitivity to apoptosis induced by 5-FU, Doxorubicin and Paclitaxel. As shown in Fig. 4D (left panel), over-expression of Bcl-2 leads to a small decrease of delayed apoptosis upon treatment with 100 nM Paclitaxel and 10 µM 5-FU but did not cause complete abrogation of delayed apoptosis of JCaM1.6 cells.

To test whether kinase activity of p56/Lck was required for apoptosis induction, we preincubated JCaM1.6/Lck cells with 5 µM of the Src-like kinase inhibitor PP2 for 60 min prior to the treatment with the cytotoxic drugs. While pretreatment with PP2 inhibited subsequent PHA-stimulated Erk-activation (Fig. 4E, right panel), PP2 did not reduce apoptosis rates induced by Doxorubicin, Paclitaxel or 5-FU (Fig. 4E, left panel).

Since Erk kinases have been implicated in apoptosis regulation, we wondered whether inhibition of the downstream kinase Erk may influence apoptosis rates in JCaM1.6/Lck cells. To this end, JCaM1.6/Lck cells were pretreated for 60 min with the MEK-1 inhibitor PD98059 that prevents Erk-activation by inhibition of its upstream activating kinase MEK-1. After the preincubation period cells were treated with 0.5 µg/ml Doxorubicin, 10 µM 5-FU or 100 nM Paclitaxel and apoptosis induction was quantified upon 48 and 72 h of drug exposure. However, pretreatment with PD98059 did not alter the rates of apoptotic cell death induced by cyotoxic drugs (Fig. 4F).

Furthermore, to rule out the possibility that an altered proliferation rate may be causative for the apparently reduced sensitivity of p56/Lck-deficient JCaM1.6 cells

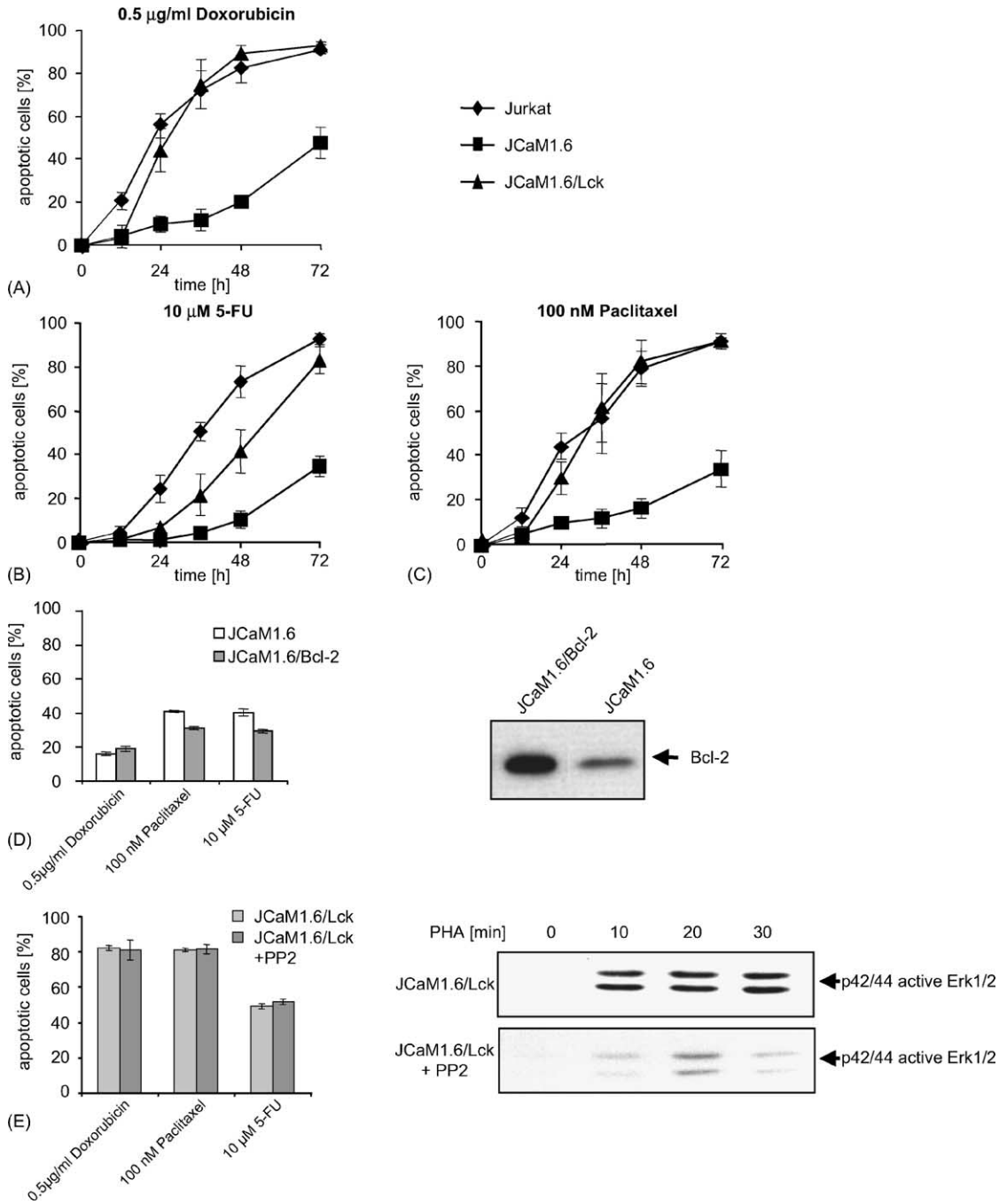

Fig. 4. Kinetics of apoptosis induction upon treatment with Doxorubicin, Paclitaxel or 5-FU depend on the expression of p56/Lck. Jurkat, JCaM1.6 and JCaM1.6/Lck cells were treated for 24–72 h with the indicated concentrations of Doxorubicin, Paclitaxel or 5-FU. Apoptosis induction in response to (A) 0.5 μg/ml Doxorubicin, (B) 10 μM 5-FU or (C) 100 nM Paclitaxel was quantified by FACS analysis using light scatter characteristics. Data show means ± S.D. ($n = 3$). (D) Bcl-2 does not abrogate delayed apoptosis in JCaM1.6 cells. Left panel: JCaM1.6 cells were transfected with Bcl-2 (JCaM1.6/Bcl-2). Cells were treated for 72 h with 0.5 μg/ml Doxorubicin, 100 nM Paclitaxel or 10 μM 5-FU and apoptosis induction was determined by FACS analysis using light scatter characteristics. Data show means ± S.D. ($n = 3$). Right panel: Expression of Bcl-2 was verified by Western blotting cytosolic extracts of JCaM1.6 and JCaM1.6/Bcl-2 cells using a Bcl-2-specific antibody. (E) Inhibition of p56/Lck kinase activity does not interfere with drug-induced apoptosis in JCaM1.6/Lck cells. Left panel: Jurkat, JCaM1.6 and JCaM1.6/Lck cells were pretreated with 5 μM PP2 or medium for 60 min and subsequently incubated for 48 h with 0.5 μg/ml Doxorubicin, 100 nM Paclitaxel or 10 μM 5-FU. Apoptosis induction was quantified by FACS analysis using light scatter characteristics. Data show means ± S.D. ($n = 3$). Right panel: JCaM1.6/Lck cells were pretreated with 5 μM PP2 or medium and subsequently incubated for the indicated times with 50 μg/ml PHA. PHA-stimulated activation of Erk1/2 was determined by Western blotting using antibodies against active Erk. (F) Inhibition of Erk does not interfere with drug-induced apoptosis in JCaM1.6/Lck cells. JCaM1.6/Lck cells were pretreated with 25 μM PD98059 for 60 min before the addition of 0.5 μg/ml Doxorubicin, 100 nM Paclitaxel or 10 μM 5-FU. Apoptosis induction was quantified by FACS analysis using light scatter characteristics after 48 and 72 h of continuous exposure to the drugs. Data show means ± S.D. ($n = 3$). (G) Deficiency of p56/Lck does not alter cell proliferation. Cells were seeded at initial concentrations of 2500, 5000, 7500 or 10,000 cells/well in 96 well plates. The number of viable cells was analysed 24, 48 and 72 h after seeding using the Wst-1 cell proliferation reagent. Data are means ± S.D. ($n = 3$).

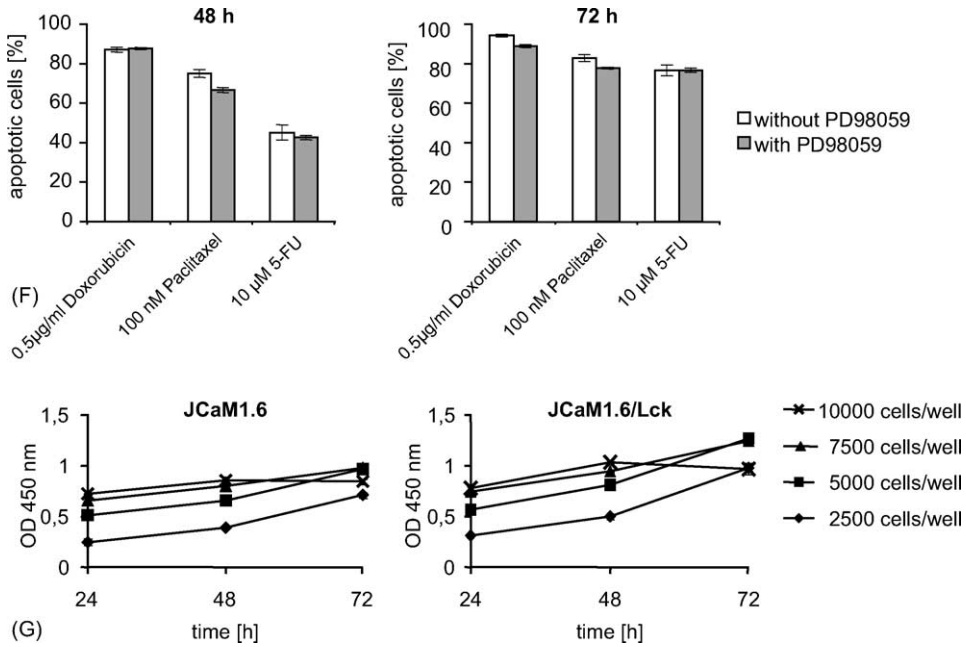

Fig. 4. (*Continued*).

to drug-induced apoptosis, we compared the basal proliferation rates of untreated JCaM1.6 cells with those of JCaM1.6/Lck cells (Fig. 4G). The data show that p56/Lck-deficiency did not result in reduced cellular proliferation. Thus, apoptosis resistance was not due to inappropriate T-cell receptor mediated proliferation of JCaM1.6 cells.

3.3. Deficiency of p56/Lck interferes with drug induced mitochondrial apoptosis signaling pathways

Up to this point our data revealed that p56/Lck deficiency strongly interferes with apoptosis induction upon treatment with Doxorubicin, Paclitaxel and 5-FU. Since p56/Lck has been shown to control early steps of radiation induced apoptosis such as mitochondrial alterations and caspase-activation [33], we wondered at which point of the apoptotic signaling cascade p56/Lck would interfere with drug induced apoptosis. To specify the localization of p56/Lck within drug-induced apoptosis signaling cascades, we analysed the influence of p56/Lck deficiency on drug induced caspase cleavage and mitochondrial alterations.

To determine in how far the absence of p56/Lck prevents the activation of caspases upon drug treatment, the time course of caspase processing was analysed by Western blotting cytosolic extracts of the Doxorubicin-, Paclitaxel- and 5-FU treated JCaM1.6- and JCaM1.6/Lck cells using specific antibodies against the typical cleavage fragments.

Treatment of p56/Lck expressing JCaM1.6/Lck cells with Doxorubicin clearly induced activation of caspases-9, -3 and -8 within 24 h, as indicated by the appearance of the active subunits as well as the cleavage of the caspase-3 substrate PARP. In contrast, no activation of caspases could be detected in the p56/Lck-deficient JCaM1.6 cells even after 72 h of treatment with Doxorubicin (Fig. 5A).

Similarly, caspase-activation and cleavage of PARP were completely absent in p56/Lck deficient JCaM1.6 cells up to 72 h after treatment with 100 nM Paclitaxel. In contrast, in p56/Lck expressing JCaM1.6/Lck cells activation of caspases-9, -3 and -8 as well as cleavage of PARP could already be detected within 24 h of treatment (Fig. 5C).

As shown in Fig. 5B, 5-FU readily induced caspase-activation and PARP cleavage in the p56/Lck-transfected JCaM1.6/Lck cells with similar kinetics as observed for apoptosis induction. In contrast, treatment of the p56/Lck-deficient JCaM1.6 cells with 10 µM 5-FU for up to 72 h failed to induce significant activation of caspases. However, cleavage of the caspase-3 substrate PARP was observed after 48 and 72 h of treatment, pointing to a delayed induction of a p56/Lck-independent apoptosis in 5-FU-treated cells.

Taken together, these data reveal that p56/Lck deficiency strongly interferes with drug induced caspase-activation (Fig. 5A–C).

Since mitochondrial alterations, such as breakdown of the mitochondrial transmembrane potential ($\Delta\psi_m$) and release of cytochrome *c* constitute key events of the mitochondrial apoptosis signaling cascade [15,19], we analysed the influence of p56/Lck deficiency on $\Delta\psi_m$ breakdown. The FACS data reveal that treatment with 0.5 µg/ml Doxorubicin induced rapid breakdown of $\Delta\psi_m$ within 24 h in the p56/Lck expressing cells yielding 64.7 and 60.2% of JCaM1.6/Lck and Jurkat cells with depolarized $\Delta\psi_m$, respectively (Fig. 6A). In contrast, no significant mitochondrial changes could be observed in the p56/Lck-deficient JCaM1.6 cells up to 48 h of treatment. However, prolonged treatment (72 and 96 h) led to the appearance of

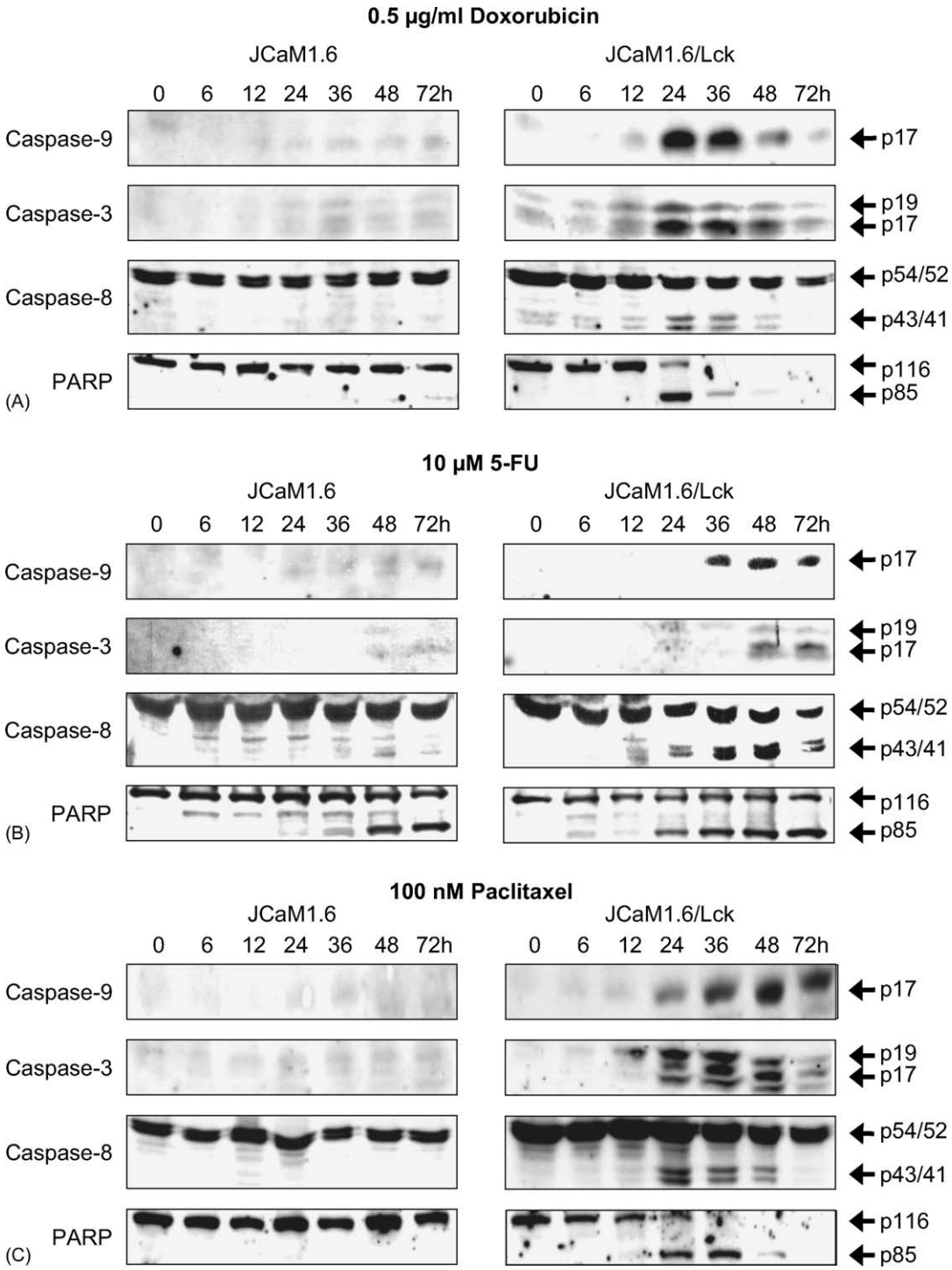

Fig. 5. Caspase-activation upon drug treatment requires the expression of p56/Lck. In contrast to rapid caspase-activation in p56/Lck expressing JCaM1.6 cells, almost no cleavage of caspases-9, -3 and -8 or the caspase-3 substrate PARP was detectable in p56/Lck deficient JCaM1.6 cells. Caspase-activation was determined by Western blot analysis of cytosolic extracts from JCaM1.6 cells (left panel) and JCaM1.6/Lck cells (right panel) 6–72 h after treatment with (A) 0.5 µg/ml Doxorubicin, (B) 10 µM 5-FU or (C) 100 nM Paclitaxel. Data show one representative of three independent experiments.

cells with depolarized mitochondrial membrane potential in the p56/Lck deficient JCaM1.6 cells pointing to some p56/Lck independent drug effects on mitochondria.

Similar to Doxorubicin, treatment with 10 µM 5-FU and 100 nM Paclitaxel failed to induce significant breakdown of the mitochondrial transmembrane potential within 48 h of treatment in the p56/Lck-deficient JCaM1.6 cells while inducing rapid and almost complete depolarization within 24–48 h in the p56/Lck expressing Jurkat and JCaM1.6/Lck cells (Fig. 6B and C). Again, a small amount of mitochondrial depolarisation could be observed in the JCaM1.6 cells upon prolonged treatment (72 h).

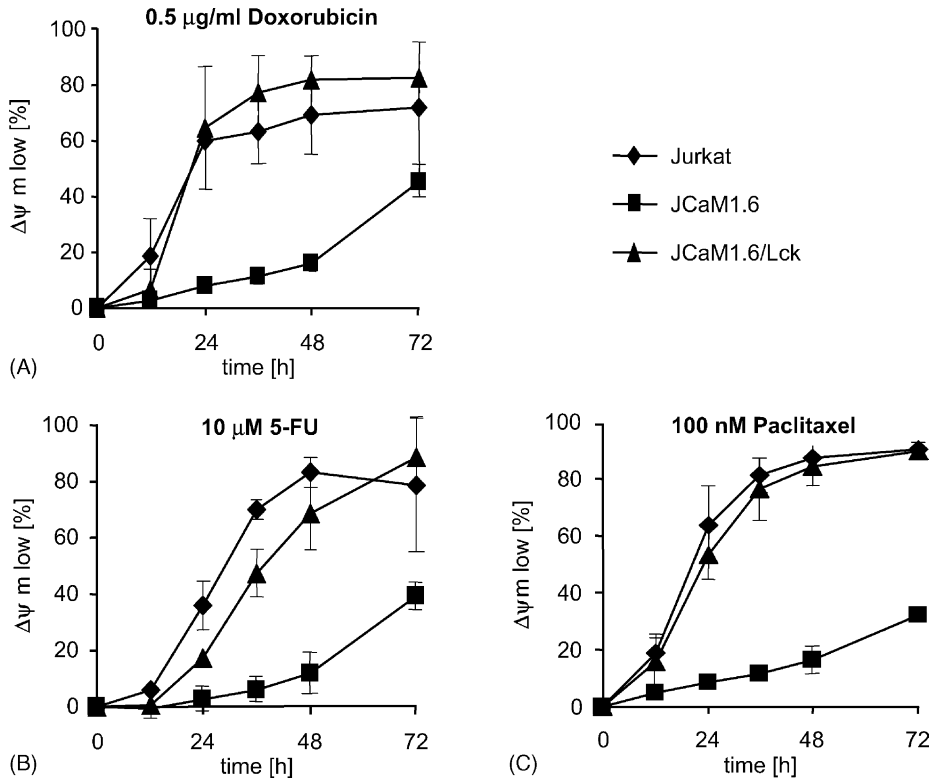

Fig. 6. Time kinetics of drug induced mitochondrial alterations during apoptosis depend on the expression of p56/Lck. Drug-induced breakdown of the mitochondrial membrane potential is almost completely abrogated in p56/Lck deficient cells. Jurkat, JCaM1.6 and JCaM1.6/Lck cells were treated for 24–72 h with (A) 0.5 µg/ml Doxorubicin, (B) 10 µM 5-FU or (C) 100 nM Paclitaxel. Integrity of the mitochondrial membrane potential was then determined by FACS analysis of TMRE stained cells. Data show means ± S.D. ($n = 3$).

Taken together, deficiency of p56/Lck strongly impaired the breakdown of $\Delta\psi_m$ triggered in JCaM1.6 cells by the treatment with Doxorubicin, Paclitaxel or 5-FU compared to p56/Lck expressing Jurkat and JCaM1.6/Lck cells. Again, these data nicely corroborate the data obtained on quantification of apoptosis (Fig. 4A–C) and on caspase-activation (Fig. 5).

To exclude any role of indirect effects caused by an up-regulation of anti-apoptotic proteins, such as Bcl-2 and related proteins in JCaM1.6 cells, we tested the expression of Bcl-2, Bcl-x_L and MCL-1 (myeloid cell leukemia gene 1) by Western blotting. However, no relevant increase in the basal expression levels of either molecule was observed in the p56/Lck deficient JCaM1.6 cells compared to the p56/Lck expressing Jurkat and JCaM1.6/Lck cells (data not shown).

4. Discussion

Our data show for the first time that the Src-like tyrosine kinase p56/Lck is crucial for apoptosis induced by chemotherapeutic drugs with different primary targets of their cytotoxic action. Furthermore, the results indicate that p56/Lck controls key steps of the drug induced apoptosis signaling cascade involving alteration of mitochondrial functions and caspase-activation. These conclusions are based on the findings that apoptosis induced by Doxorubicin, 5-FU and Paclitaxel was significantly reduced in p56/Lck deficient JCaM1.6 cells compared to p56/Lck expressing JCaM1.6/Lck cells. In addition, neither a significant breakdown of $\Delta\psi_m$ nor a significant activation of caspases could be detected in the p56/Lck-deficient cells upon 24–48 h of drug treatment.

This is reminiscent of our own observations on the necessity of p56/Lck expression for radiation induced apoptosis [25,33] as well as of reports on the importance of p56/Lck for apoptosis induction by ceramide and the HIV-TAT protein [24,26]. Since apoptosis induction by chemotherapeutic drugs, ceramide or radiation has been shown to follow mitochondrial apoptosis signaling pathways and may even converge at the mitochondrial level [4,11,14,39], these findings make a more general role of the tyrosine kinase p56/Lck for the control of early steps of mitochondrial apoptosis pathways very likely.

In contrast, death receptor mediated apoptosis signaling pathways had been shown to be executed almost independently of p56/Lck since deficiency of p56/Lck only partially attenuated apoptosis induction by death receptor ligands such as TRAIL or CD95-ligand [33,40]. In this context, the altered time course of death receptor induced apoptosis observed in p56/Lck-deficient cells was attributed to an inadequate activation of the mitochondrial amplification loop in the p56/Lck-deficient cells that is

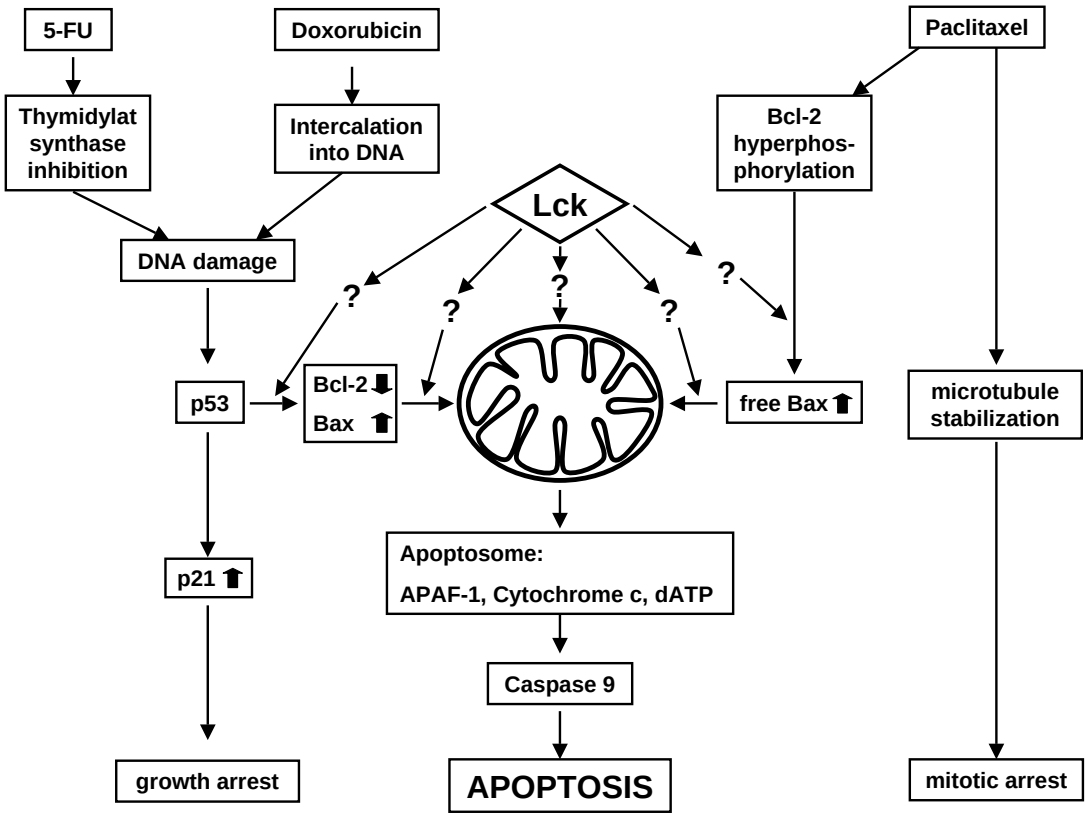

Fig. 7. Signaling pathways. Apoptosis pathways induced by cytotoxic drugs converge at the level of the mitochondria. The DNA intercalating drug Doxorubicin as well as the antimetabolite 5-FU induce DNA damage leading to activation and stabilization of the tumor suppressor p53. Dependent on the cellular context, p53 induces growth arrest via upregulation of the cyclin dependent kinase inhibitor p21 or apoptosis via increased expression of the pro-apoptotic Bcl-2 protein family member Bax. In contrast, the microtubule damaging agent Paclitaxel that causes microtubule stabilization, induces mitotic arrest in the G2/M phase of the cell cycle and apoptosis. Putatively, Bcl-2 hyperphosphorylation leading to augmented levels of free Bax may be required for the subsequent activation of mitochondrial apoptosis steps, e.g. membrane permeabilization with breakdown of the mitochondrial transmembrane potential and release of pro-apoptotic mitochondrial proteins like cytochrome *c*. Cytochrome *c* together with Apaf-1 and dATP is required for the activation of pro-caspase 9 in the cytosolic death inducing complex, called apoptosome. Caspase-9 in turn triggers the effector caspase cascade that is required for the execution of apoptosis. In this scenario, p56/Lck may directly or indirectly influence the Bax/Bcl-2 ratio, thereby facilitating the induction of mitochondrial alterations.

required for the amplification of apoptotic signals in cells with low initial death receptor mediated caspase-8 activation [41].

Importantly, our results indicate that not only drugs that directly or indirectly induce DNA-damage, but also agents with a distinct primary target of their cytotoxic action are all dependent on expression of functional p56/Lck to trigger apoptotic cell death.

It is well established that DNA-damaging drugs, e.g. the thymidylate synthase inhibitor 5-FU, induce apoptosis via p53-dependent up-regulation of pro-apoptotic Bax (Fig. 7) [42]. However, 5-FU has also been shown to induce p53-independent cell death [43,44]. In this context, a role of up-regulated CD95-ligand expression has been suggested [45]. Further reports indicate that the relative levels of Bcl-2, Bcl-x_L and Bax may determine sensitivity of tumor cells to 5-FU-induced apoptosis [44,46].

Similarly, the anthracycline Doxorubicin induces DNA-damage and subsequent apoptosis via multiple mechanisms including intercalation into the DNA, inhibition of topoisomerase II and generation of reactive oxygen species [47]. Furthermore, Doxorubicin-induced apoptosis typically involved Bak and Bax-dependent cytochrome *c* release from mitochondria with subsequent caspase-activation and was blocked by over-expression of anti-apoptotic Bcl-2 proteins (Fig. 7) [48–51].

In contrast, Paclitaxel constitutes a microtubule-damaging agent that stabilizes the microtubules thereby abrogating the function of the mitotic spindle apparatus. Consequently, Paclitaxel provokes cell cycle arrest in the mitotic phase. However, Paclitaxel also promotes apoptotic cell death [52,53]. It has been shown, that similar to DNA-damaging drugs and radiation, Paclitaxel induces a mitochondrial signaling cascade involving loss of the mitochondrial membrane potential, release of cytochrome *c* from the mitochondrial intermembrane space and cytochrome *c*- and Apaf-1-dependent activation of caspases-9, -3 and -8 downstream of the mitochondria [54]. This is consistent with the observation that anti-apoptotic Bcl-2 family proteins are involved in the protection against

apoptosis induced by microtubule damaging drugs [55]. In this context, Paclitaxel-induced apoptosis appears to require inactivation of Bcl-2 by hyperphosphorylation that has been attributed either to the activation of kinase-pathways such as the c-*Jun* N-terminal kinase (JNK) pathway or the inactivation of protein phosphatases [52,56–58]. Most studies indicate that phosphorylation of Bcl-2 inactivates its anti-apoptotic function [59,60]. Phosphorylation may prevent its heterodimerization with Bax leading to increased levels of free Bax and subsequent activation of the mitochondrial pathway (Fig. 7) [61,62].

Our data clearly show that in addition to its role in cell death induced by irradiation and ceramide-treatment, p56/Lck also interferes with apoptosis induced by cytotoxic drugs that have all been shown to trigger mitochondrial death pathways. However, the exact mechanisms of the p56/Lck action are not completely understood. Since mitochondrial apoptosis pathways are mostly regulated by pro- and anti-apoptotic members of the Bcl-2 protein family, p56/Lck may either directly or indirectly be involved in the regulation of the activity of Bcl-2 proteins. However, it has been demonstrated that p56/Lck is not involved in the regulation of Bcl-2 function by hyperphosphorylation [63]. On the other hand, it has been suggested that p56/Lck may be critical to induce conformational changes of Bax that are required for its interaction with the mitochondrial membrane [33]. In our hands deficiency of p56/Lck did not alter basal expression levels of Bcl-2, Bcl-x_L or MCL-1.

Alternatively, p56/Lck may modulate the apoptotic threshold for anticancer drug induced apoptosis by interfering with up-stream signaling molecules involved in apoptosis regulation. Previous studies revealed that p56/Lck participates in phosphoregulation of phosphatidylinositol-3-kinase, mitogenic Erk1 and Erk2 [31,32] or GTPases of the Rho-family, all being involved in the control of proliferation and/or apoptotic cell death [64,65]. Furthermore, p56/Lck has been shown to function in the regulation of ion transport systems, including Cl^-, K^+, and Ca^{2+}, that are important regulators of cell proliferation and apoptosis [66–69].

However, in our hands inhibition p56/Lck kinase activity by the Src-like kinase inhibitor PP2 as well as inhibition of Erk activation by the MEK-1 inhibitor PD98059 failed to interfere with drug-induced apoptosis. These data suggest that the kinase activity of p56/Lck and downstream activation of Erk may be dispensable for the pro-apoptotic action of p56/Lck. Consequently, the defective apoptosis signaling in JCaM1.6 cells with deficiency of functional p56/Lck may not be caused by defective p56/Lck kinase activity/respective Erk activation but by the lack of some p56/Lck-mediated kinase-independent pro-apoptotic effects. Alternatively, decreased sensitivity of Lck-deficient JCaM1.6 cells to drug-induced apoptosis may not be due to the lack of pro-apoptotic activity of p56/Lck but to the existence of some kinase-independent anti-apoptotic effects of the truncated p56/Lck in the JCaM1.6 cells which could be overridden by expression of functional p56/Lck in the retransfected JCaM1.6/Lck cells. In a recent report, it was suggested that a cleaved form of the Src-like kinase Lyn that was generated by caspase-cleavage upon B-cell receptor engagement would behave as a negative regulator of mitochondrial apoptosis pathways. The inhibitory function of cleaved Lyn was attributed to a modulation of c-*myc* expression [70]. While high levels of Myc correlated with apoptosis induction, low levels of c-*myc* expression caused apoptosis resistance. Since JCaM1.6 cells are not completely deficient of p56/Lck but express a truncated protein devoid of the kinase domain, a similar inhibitory effect of truncated p56/Lck on c-*myc* expression may also be operative in these cells. In this context, peptides containing the soluble SH2 domain of Lck that is required for its interaction with phosphorylated protein structures have been shown to interfere with apoptosis induction [71,72]. Since truncated p56/Lck retains the SH2 domain [30] it may act as a dominant negative protein modulating c-*myc* expression and thus, sensitivity to apoptotic cell death.

Overall, our data reveal that lack of functional p56/Lck is sufficient to cause a significantly decreased sensitivity of JCaM1.6 cells to drug-induced apoptosis. Interestingly, the p56/Lck-related tyrosine kinase Src could not substitute for p56/Lck in regard to apoptosis signaling.

Importantly, Doxorubicin, 5-FU and Paclitaxel caused delayed cell death in JCaM1.6 cells after 72 h of drug exposure. Thus, p56/Lck deficient JCaM1.6 cells are not completely resistant to apoptosis induced by the treatment with cytotoxic drugs. Kinetics of delayed apoptosis were similar to the kinetics of the breakdown of the mitochondrial membrane potential pointing to a mitochondria-dependent late apoptosis pathway. However, over-expression of Bcl-2 did not abrogate delayed apoptosis in JCaM1.6 cells. Thus, undefined Bcl-2 independent late effects may be operative.

Furthermore, the mechanisms of stress-induced activation of p56/Lck is still unclear. It has been shown that membrane compartimentation of signaling molecules may function as key regulatory mechanism for cellular signaling pathways. In this context, application of cellular stress, e.g. treatment with cytotoxic drugs or irradiation induces the formation of the cellular second messenger ceramide. Subsequently, ceramide-induced alterations of membrane lipid composition [73] can facilitate clustering of signaling molecules such as cell surface receptors in specialized cholesterol- and sphingolipid-rich cell membrane domains, termed rafts, thereby triggering their activation [74,75]. In addition to specific surface receptors, e.g. CD95, membrane rafts were found to be enriched in signaling molecules, including Src-like phosphotyrosine kinases such as p56/Lck [76–78]. Thus, p56/Lck may become activated upon drug treatment or irradiation by ceramide mediated clustering of up-stream receptor molecules within lipid rafts.

Taken together, the data presented point to a key role of p56/Lck in the regulation of drug-induced apoptosis in T-lymphoma cells. Since the sensitivity to drug and/or radiation induced apoptosis has been correlated with the treatment response, functional inactivation of p56/Lck may directly contribute to decreased efficiency of anticancer treatment. Therefore, Src-like tyrosine kinases may constitute a novel target for anticancer drug development.

Note added in proof

Meanwhile, similar observations have been published by Jonghwa Won and co-workers [79] regarding Rosmarinic Acid induced cell death. The authors show that Rosmarinic Acid induces apoptosis in Jurkat T-lymphoma cells via a mitochondrial pathway involving p56/Lck. Consistent with our own data the proapoptotic function of p56/Lck was not dependent on p56/Lck kinase activity. In contrast, Rosmarinic Acid induced apoptosis relied on p56/Lck SH2 domain.

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