Actinomycin D upregulates proapoptotic protein Puma and downregulates Bcl-2 mRNA in normal peripheral blood lymphocytes

Ivan Kalousek, Barbora Brodska, Petra Otevrelova and Pavla Röselova

We have examined the ability of actinomycin D to induce apoptosis in human peripheral blood lymphocytes. Run-On assays were performed to specify the primary molecular damage, reverse transcription-PCR, Western blots and flow cytometry studies were performed to ascertain which proteins of the apoptosis machinery were affected to cause actinomycin D-induced cell death. Expression of 23 apoptosis-related genes was investigated. The down-regulation of ribosomal RNA synthesis caused by actinomycin D induced a mitochondria-dependent apoptosis. Although the expression of the majority of examined genes remained indifferent against actinomycin D activity, the cellular level of p53 protein increased, subsequently upregulating both Puma mRNA and protein. Puma-mediated mitochondrial apoptosis was accompanied by nucleolin cleavage and Bcl-2 mRNA destabilization. The stability of the cellular level of Bcl-2 protein independent of a mRNA decrease suggests that protection of Bcl-2 protein against proteasomal degradation can moderate the apoptotic process. In peripheral blood lymphocytes cultured *in vitro*, the apoptosis induced by a low concentration of actinomycin D (10 nmol/l) is dependent on p53 and Puma activation. This apoptotic pathway is demonstrated in peripheral blood lymphocytes for the first time. A different apoptotic pathway induced in peripheral blood lymphocytes using this drug has, however, been previously revealed by other authors. The combination of cell specificity and dose-dependent effects can likely play a decisive role in apoptosis observed in peripheral blood lymphocytes after genotoxic drug application. *Anti-Cancer Drugs* 18:763–772

Anti-Cancer Drugs 2007, **18**:763–772

Keywords: actinomycin D, apoptosis, Bcl-2, peripheral blood lymphocyte, p53, Puma, ribosomal synthesis

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Received 1 December 2006 **Accepted** 17 January 2007

Introduction

Actinomycin D (ActD) is a cytotoxic agent which was successfully used against neoplastic disorders, i.e. malignant melanoma [1], nephroblastoma [2] and Ewing tumor [3]. The drug intercalates into DNA, and interferes with RNA polymerases and DNA topoisomerases. At high concentrations, ActD causes damage to the DNA and inhibits transcription from all three classes of RNA polymerase promoters, whereas at low concentrations, at the level up to 10 nmol/l, it is a strong and specific inhibitor of ribosomal gene transcription [4–7]. The suppression of rRNA synthesis causes a stress of ribosomal biogenesis and promotes the generation of complexes of some ribosomal proteins, i.e. L5 [8], L11 [9] and L23 [10,11], with oncoprotein HDM2. Inhibiting the HDM2-mediated p53 ubiquitination, the formation of such complexes stabilizes the tumor suppressor protein p53 against proteasomal degradation and increases its cellular level [8]. In addition, destabilizing the HDM2 binding to the transcription activation domain of p53, ribosomal proteins promote the ability of p53 to enhance the transcription of target genes. Growing evidence has shown that p53 is regulated primarily by its protein stability. The mechanisms of p53 stabilization and activation in response to DNA damage are contained in the p53 protein phosphorylation by DNA-PK or other protein kinases with similar specificity [12–14]. The method of p53 stabilization, however, triggered by rDNA specific damage likely incorporates the formation of complexes of HDM2 with ribosomal proteins because the induction of p53 under growth inhibitory conditions, such as low-dose ActD can be significantly attenuated by knocking down L11 [9]. In response to DNA damage p53 is capable of mediating growth arrest and genomic repair [15,16]. Depending on the nature and extent of injury, however, p53 can elicit the elimination of damaged cells by initiating the process of apoptosis [17].

The classical intrinsic or mitochondrial pathway of apoptosis proceeds through the suppression of activity of antiapoptotic members of the Bcl-2 protein family such as Bcl-2, Bcl-x_L, Bcl-w, Bfl-1, Boo and Mcl-1, and promotion of activity of proapoptotic members Bak and Bax [18]. These proteins permeabilize the mitochondrial outer membrane to allow for the release of caspase activator cytochrome *c* that through interactions with

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Apaf-1 induces caspase activity [19]. Bcl-2 proteins have been shown to interact and to be regulated by another group of proteins, the BH3-only subfamily [20], including Bad, Bid, Bim, Bik, Bnip3, Bok, Hrk, Moap-1, Noxa and Puma [21,22]. In response to stress signals, these proteins largely initiate a death cascade by antagonizing prosurvival Bcl-2-like molecules [23,24]. It appears they have evolved to recognize different stress stimuli. Whereas Bax and Bak play a central role in most forms of apoptosis, BH3-only proteins appear to modulate apoptosis through cell type-specific and signal-specific pathways [25,26]. It appears, however, that proapoptotic protein–protein interactions are not strictly dependent on Bcl-2 homology. Protein Siva was revealed [27] and, although it lacks any of the known BH domains, the protein specifically interacts with Bcl-x_L [28].

Another regulatory mechanism controls the apoptotic process at the level of caspase activity downstream of cytochrome *c* release or death receptor signalling [29]. Inhibitors of apoptosis (IAPs), proteins XIAP, c-IAP1, c-IAP2 and survivin, suppress the apoptosis by preventing procaspase activation and inhibiting mature caspase activity. The second mitochondria-derived activator of caspases Smac is a negative regulator of IAPs which is released from mitochondria with cytochrome *c* eliminating an inhibitory effect of IAPs [30,31].

It appears likely that different proteins are required to execute particular death responses in individual cell types. Some of them, e.g. Bak, Bax, Bad, Bid, Bim, Bnip3, Puma, Noxa and Siva, are transcriptionally regulated by p53 [32–36]. The extent of the p53-dependent induction of these genes varies significantly in different cell types and a role for individual proapoptotic proteins in DNA damage-induced p53-dependent apoptosis has yet to be clarified [19].

In this report we have examined the apoptotic effect induced by specific rDNA damage causing the attenuation of ribosomal RNA synthesis in normal peripheral blood lymphocytes (PBL). On the normal population of PBL cultured in the presence of 10 nmol/l ActD, we have investigated the way in which DNA damage accompanied by ribosomal stress may trigger mitochondrial dysfunction and caspase activation. We would like to contribute to understanding the mechanism by which this drug, in addition to the beneficial therapeutic effect, causes noxious toxicity in normal tissue leading to lymphopenia [37,38].

Methods

Cell cultures and viability

Human mononuclear cells were isolated from buffy coats of healthy volunteers using density gradient centrifugation on Histopaque 1077 (Sigma-Aldrich, St Louis, Missouri, USA) at 300 *g* for 25 min at 20°C. Monocytes were depleted by culture in plastic flasks for 45 min in RPMI 1640 (Sigma) and by harvesting nonadherent cells [38]. Lymphocytes were resuspended at 1×10^6 cells/ml in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum. Then, 10 nmol/l ActD (Sigma) was added and cells were cultured at 37°C in a 5% CO_2 humidified atmosphere for time frames up to 72 h. The viability (mitochondrial activity) of cells was monitored using the MTT Kit I (Roche Diagnostics, Indianapolis, Indiana, USA). Cells were grown for time frames up to 72 h in the absence or presence of 10 nmol/l ActD in microplates under the conditions mentioned above. Once the incubation period was over, the MTT was added to each well for 6 h. A solubilization solution was then applied overnight. Absorbance at 580 nm was measured using a microplate MRX ELISA reader (MTX Lab Systems, Vienna, Virginia, USA).

Nuclear run-on transcription of primary rRNA transcript

Nuclei from 1×10^7 PBL treated with 10 nmol/l ActD for up to 72 h were isolated as described previously [39,40] and incubated with 20 μCi of [α-^{32}P-] GTP (MP Biochemicals, Irvine, California, USA) in a buffer containing 25 mmol/l Tris–HCl (pH 8.0), 12.5% glycerol, 2.5 mmol/l Mg-acetate, 4 mmol/l $MgCl_2$, 60 mmol/l KCl, 1 mmol/l of ATP, cytidine 5-triphosphate (CTP), uridine 5-triphosphate and 0.1 mmol/l guanosine triphosphate, 0.05 mmol/l ethylenediaminetetraacetic acid, 2.5 mmol/l dithiothreitol (DTT), 10 μl/ml of protease inhibitor cocktail (Sigma) and 1 unit/μl of human placental RNase inhibitor (GE Healthcare, Buckinghamshire, UK) in a total volume of 100 μl for 40 min at 25°C. Radiolabelled RNA was isolated using an RNeasy Mini Kit (Qiagen, Valencia, California, USA) and used in hybridizations. Two micrograms of both plasmids pB containing a 5.8-kbp *Eco*RI fragment B of human ribosomal gene cloned into pBR322 and pBR322 alone as negative control were denaturated and immobilized on the Hybond-N membrane (GE Healthcare). Specific ribosomal RNA transcripts were detected on nylon strips subjected to hybridization in accordance with the manufacturer's protocol for RNA probes. Filmless autoradiography was performed on Fujifilm imaging plates. After 4 h of strip exposure, transcription signals were visualized and quantified by scanning autoradiograms (PhosphorImager FLA 2000; Fuji Medical Systems, Stamford, Connecticut, USA).

Isolation of mitochondria

Cytosolic and mitochondrial fractions were prepared from PBL cultured for up to 72 h in the absence or presence of 10 nmol/l ActD using the Cell Mitochondria Isolation Kit (Sigma) according to the instructions of the manufacturer. In brief, after 10 min incubation on ice in cytosol extraction buffer containing protease inhibitor, the cells were subjected to homogenization with Dounce homo-

genizer. Homogenates were transferred to a microcentrifuge tube and centrifuged at 600*g* for 10 min at 4°C. The supernatants were then transferred to a fresh microcentrifuge tube and centrifuged at 11 000*g* for 10 min at 4°C. In this step, supernatants were collected as cytosolic fractions and used for cytochrome *c* determination by immunoblots. Pellets were boiled for 10 min in 100 µl Laemelli buffer and were then saved as mitochondrial fractions.

Active caspases detection

The activation of executive caspase was investigated using the Vybrant FAM Caspase-3 and 7 Kit (Invitrogen, Carlsbad, California, USA) on the basis of fluorescent inhibitor caspases (FLICA) methodology. PBL at 1×10^6 cells/ml were cultured for up to 72 h in pure RPMI 1640 or in medium containing 10 nmol/l ActD. Harvested cells, resuspended in 300 µl of pure media, were added with 10 µl of 30 × FLICA (FAM-DEVD-FMK) reagent and incubated for 60 min at 37°C in 5% CO_2 protected from light and mixed twice during incubation. Cells were washed twice in the wash buffer provided with the kit and analyzed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, California, USA). The activation of caspase-3 and caspase-9 was also investigated in the immunoblots described below.

Extraction of RNA and reverse transcription-PCR

The extraction of RNA from PBL cultured for up to 72 h in the absence or presence of 10 nmol/l ActD was performed using the RNeasy Mini Kit according to the manufacturer's instructions. Briefly, 10^7 frozen cells were dissolved in 0.6 ml of Qiagen RNeasy lysis buffer. The cell-lysis buffer mixtures were further dissociated by passage through a Qiashredder column (Qiagen). The total RNA was precipitated with ethanol, washed three times with Qiagen washing buffers on an RNeasy spin column and eluted with 0.1 ml of RNase-free water. The purified RNA was stored at −75°C until it was needed.

Semiquantitative reverse transcription (RT)-PCR was performed using 2 µg of total RNA and One-Step RT-PCR kit (Qiagen). Sequences of specific primers (Invitrogen) collected in Table 1 were designed utilizing the website program http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi. The RT-PCR program applied was: 30 min at 50°C for reverse transcription, 15 min at 95°C for HotStarTaq DNA polymerase activation, Omniscript and Sensiscript reverse transcriptases inactivation and the cDNA template denaturation followed by 30 cycles consisting of 1 min denaturation at 94°C, 1 min of annealing at 60°C and 2 min of extension at 72°C. The program was terminated by a final extension of 10 min at 72°C. Amplified products were detected by electrophoretic analysis on 2% agarose gels stained with ethidium bromide (0.5 µg/ml) followed by examination under UV light. The integral intensities of fluorescence bands were obtained using the Molecular Imager Gel Doc XR System (Bio-Rad, Hercules, California, USA) and image analyzer Software AIDA 1D v. 4.08 (Raytest, Straubenhardt, Germany).

Immunoblots

PBL cultured for up to 72 h in the absence or presence of 10 nmol/l ActD harvested from cultures and washed in PBS were boiled for 5 min in 200 µl of Laemelli buffer. After centrifugation at 40 000*g* in RPMI for 3 h and 4°C supernatants were subjected to 12% sodium dodecyl

Table 1 Reverse transcription-PCR primer sequences

Gene	Accession	Forward primer	Reverse primer
β-Actin	BC016045	GGCATCCTCACCCTGAAGTA	AGGGCATACCCCTCGTAGAT
Bad	NM_004322	CGAGTGAGCAGGAAGACTCC	CCACCAGGACTGGAAGACTC
Bak	NM_001188	TTTTCCGCAGCTACGTTTTT	ACAAACTGGCCCAACAGAAC
Bax	NM_004324	TTTGCTTCAGGGTTTCATCC	GGAGGAAGTCCAATGTCCAG
Bcl-2	NM_000633	GGGTACGATAACCGGGAGAT	ACAGTTCCACAAAGGCATCC
Bcl-w	NM_004050	GCTGAGGCAGAAGGGTTATG	AAAAGGCCCCTACAGTTACC
Bcl-x_L	NM_138578	GGAGCTGGTGGTTGACTTTC	GGATCCAAGGCTCTAGGTG
Bfl-1	U27467	TTACAGGCTGGCTCAGGACT	CCCAGTTAATGATGCCGTCT
Bid	AF087891	CAGAGAGCTGGACGCACTG	GAGGGATGCTACGGTCCAT
Bik	NM_001197	AAGACCCCTCTCCAGAGACA	GGGGATCTCCAGAACCTCAT
Bim	BC033694	TGGCAAAGCAACCTTCTGAT	TCTTGGGCGATCCATATCTC
Bnip-3	NM_004052	CTGGACGGAGTAGCTCCAAG	AGCAGCAGAGATGGAAGGAA
Bok	NM_032515	AGATCATGGACGCCTTTGAC	TGTGCTGACCACACACTTGA
Boo	NM_020396	GAAGAAGTGGGGCTTCCAG	GAAAGGGGGTCCTGAAGAAG
Hrk	NM_003806	CTAGGCGACGAGCTGCAC	CACAGCCAAGGCCAGTAGGT
c-IAP1	NM_001165	CCTGTGATTAATGCTGCCGT	TCTCTTGCTTGTAAAGACG
Mcl-1	NM_021960	TAAGGACAAAACGGGACTGG	ACCAGCTCCTACTCCAGCAA
Moap-1	NM_022151	CAGAAATCGAGGAGGCTCTG	CCTCAGCTGCCTCATAATCC
Noxa	NM_021127	AAGAAGGCGCGCAAGAAC	CGTGCACCTCCTGAGAAAAC
p53	NM_000546	CCCTTCCCAGAAAACCTACC	CCTCATTCAGCTCTCGGAAC
Puma	NM_014417	GTCCTCAGCCCTCGCTCT	CTAATTGGGCTCCATCTCG
Siva	NM_021709	GTGAGCCAGAGGGAGTTGA	ATGTCACTGCAGTCCACGAG
Smac I	NM_019887	GGAGCCAGAGCTGAGATGAC	CCAGCTTGGTTTCTGCTTTC
Survivin	AY795969	GGACCACCGCATCTCTACAT	GGAAAGTCCAGAGCCTTCCT
XIAP	NM_001167	GTGCGGTGCTTTAGTTGTCA	AGGGTTCCTCGGGTATATGG

sulfate–polyacrylamide gel electrophoresis and the separated proteins were semidry blotted onto Hybond P, Amersham's PVDF transfer membranes (GE Healthcare). Blots were blocked for 2 h with 5% nonfat dried milk in PBS–0.05% Tween 20, 0.01% NaN_3, then primary antibodies were added to the final dilution ranging from 1:500 to 1:2000 (for anti-β-actin antibody 1:20 000) and blots were incubated overnight. After washing three times for 5 min with PBS–0.05% Tween, the horseradish peroxidase-conjugated secondary antibodies (GE Healthcare) diluted 1:20 000 in PBS–0.05% Tween were added for 2 h. After another three washes, the blots were developed and visualized using the ECL plus Western blotting detection system and Hyperfilm ECL (GE Healthcare). Signals were quantified using the Power Look III scanner (UMAX Technologies, Dallas, Texas, USA) and image analyzer Software AIDA 1D. Primary antibodies against Bak (R&D Systems, Minneapolis, Minnesota, USA), Bax, Bcl-2, Bcl-x_L, Bfl-1, Bid, Bim, (Santa Cruz Biotechnology, Santa Cruz, California, USA), Moap-1 (Axxora, San Diego, California, USA), Mcl-1 (Sigma), nucleolin, p53 and poly(ADP ribose) polymerase (PARP) (Santa Cruz), and Puma (Axxora) were rabbit polyclonal sera. Antibodies against β-actin (Sigma), Apaf-1 (R&D), BNIP3 (Sigma), caspase-3 (R&D), caspase-9 and IAP-1 (BD), cytochrome c (BD), Noxa (Axxora), and Smac and XIAP (BD) were mouse monoclonal antibodies. Antibody against Siva (Abnova, Heidelberg, Germany) was mouse polyclonal serum.

Statistical analysis

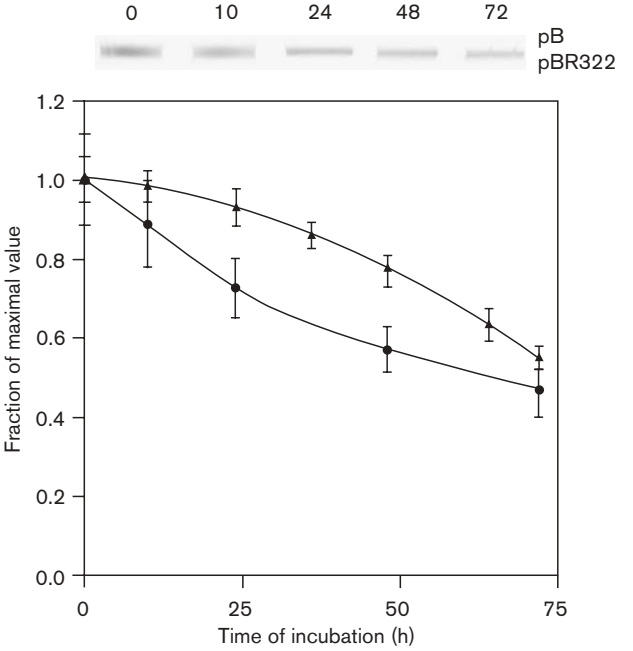
Signals from the run-on assay, nucleic acid electrophoresis and protein immunoblotting were evaluated as integrated peak intensities using AIDA software. All experiments, including MTT test of viability, were repeated four times and representative data are shown. In diagrams arithmetic means with SEM error bars were plotted. Significance levels (P values from Student's paired t-test) were determined using InStat Software (GraphPad Software, San Diego, California, USA). A value of 0.05 or lower was considered a statistically significant difference between the groups compared.

Results

Treatment of lymphocytes with 10 nmol/l actinomycin D downregulates ribosomal genes transcription and induces cell death

Ribosomal RNA synthesis in nuclei isolated from PBL cultured in normal RPMI 1640 and in medium added with 10 nmol/l ActD was examined using Run-On Assay. The total transcribed and ^{32}P labelled RNA was hybridized to slot-blotted rDNA and filmless autoradiograms were scanned and quantified. The data collected in Fig. 1 suggests that incubation of PBL with 10 nmol/l ActD attenuates the ribosomal transcription. Within 24 h of incubation the radiolabelling signal fell to 70% and within 72 h to 50% of its original value. The viability of cells treated with 10 nmol/l ActD was monitored through the activity of mitochondrial enzymes of viable cells. The amount of PBL containing functional mitochondria despite drug application decreased with the time of incubation, and the rate of transformation of monitoring substrate dropped to 90% within 24 h and to 60% within 72 h. Analysis of our data suggested that the ribosomal synthesis attenuation preceded the mitochondrial dysfunction ($P = 0.006$).

Fig. 1

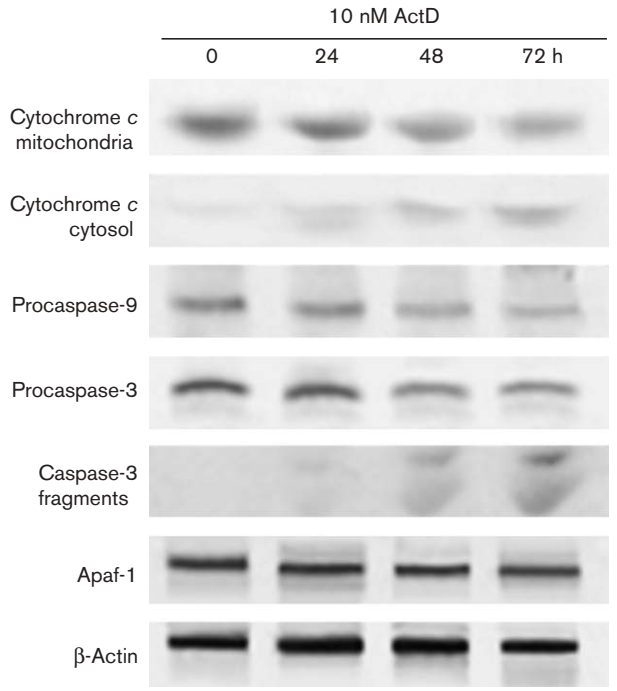


The attenuation of ribosomal RNA synthesis precedes the mitochondrial dysfunction in peripheral blood lymphocytes treated with 10 nmol/l actinomycin D. –●– Run-on assay data demonstrating the rate of nucleolar synthesis of ribosomal RNA. –▲– MTT test data demonstrating the rate of mitochondrial transformation of monitoring substrate. The upper part of the graph shows the representative experimental data. Radiolabelled RNA from run-on transcription assays was hybridized to a slot-blotted fragment of rDNA gene in pBR322.

Actinomycin D-induced death of lymphocytes is mediated by cytochrome c release followed by caspase activation

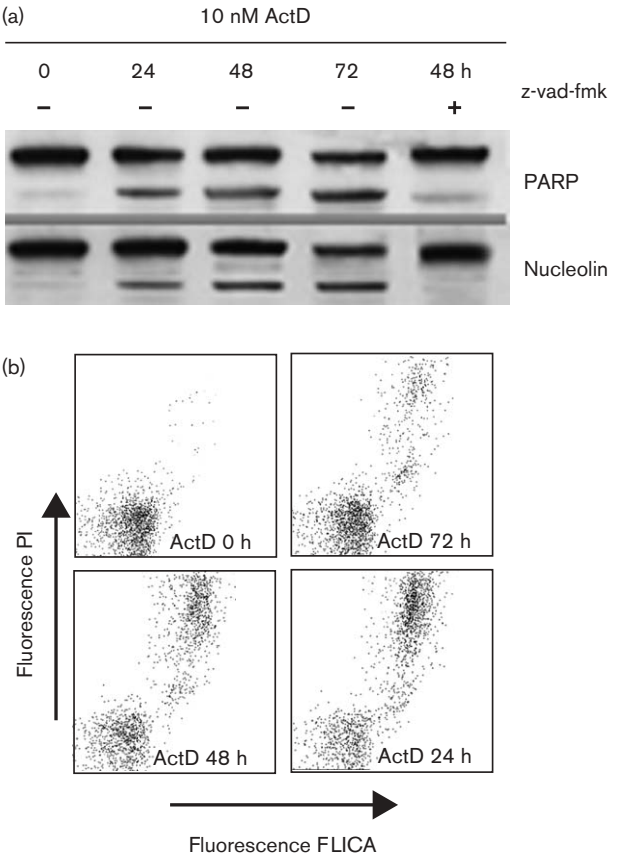
Cytochrome c release from mitochondria, Apaf-1 expression and caspase-9 and caspase-3 activation in PBL incubated with 10 nmol/l ActD was examined in immunoblots (Fig. 2). An accumulation of cytochrome c was detected in the cytosol with a concomitant decrease of cytochrome c in the mitochondrial fraction ($P = 0.02$) The expression of Apaf-1 appeared to be independent of the presence of 10 nmol/l ActD. Activation of caspase-9 was detected in immunoblots, indicating a decrease of cellular level of inactive procaspase ($P = 0.01$). Activation of caspase-3 was accompanied by decrease of

Fig. 2



Actinomycin D (ActD; 10 nmol/l) initiates intrinsic mitochondrial way of apoptosis in peripheral blood lymphocytes. Immunoblot data demonstrating the outflow of mitochondrial cytochrome *c* to cytoplasm, the activation of apical caspase-9 accompanied by the diminution of inactive procaspase, the activation of executive caspase-3 accompanied by the diminution of inactive procaspase-3, and appearance of active fragments and the expression of proapoptotic protein Apaf-1.

Fig. 3



Increase in activity of executive caspase-3 induced in peripheral blood lymphocytes by 10 nmol/l actinomycin D (ActD) is accompanied by proteolytic cleavage of specific substrates and increasing amount of dying cells. (a) Immunoblot data demonstrating that the proteolysis of poly(ADP ribose) polymerase and nucleolin is inhibited with pancaspase inhibitor z-vad-fmk. (b) Flow cytometry two-parameter dot-plots demonstrating increasing amount of cells accumulating the fluorescent inhibitor of activated caspase-3 and propidium iodide (PI).

procaspase-3 ($P = 0.01$) signal, and formation of 18- and 12-kDa subunits of the mature caspase-3. Increasing activity of executive caspase induced by 10 nmol/l ActD and leading to the proteolytic cleavage of the PARP and nucleolin is demonstrated in Fig. 3a as well as the fact that caspase-3- dependent generation of the 85-kDa proteolytic fragment of PARP and 80-kDa fragment of nucleolin was abrogated by pan-caspase inhibitor 50 µmol/l z-vad-fmk. Data from flow cytometry shown in Fig. 3b indicate an increasing amount of cells dying in response to the 10 nmol/l ActD treatment, and accumulating propidium iodide and the fluorescent inhibitor of activated caspase-3.

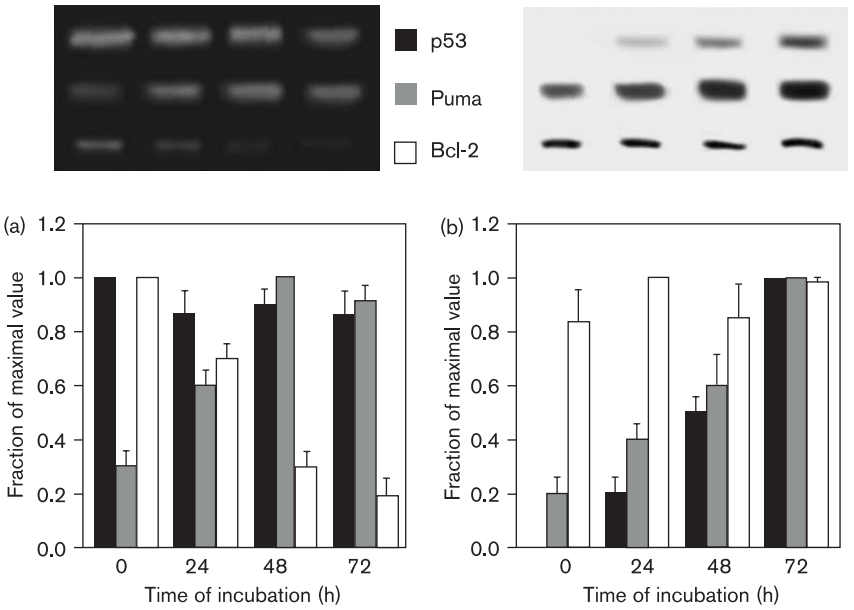
Treatment of lymphocytes with 10 nmol/l actinomycin D upregulates proapoptotic protein p53

The cellular content of p53 mRNA in PBL treated with 10 nmol/l ActD was examined in ethidium bromide-stained RT-PCR products separated on agarose gel. Data in Fig. 4a demonstrate that the cellular level of p53 mRNA was independent of the presence of 10 nmol/l ActD ($P = 0.85$). An increase in cellular content of protein p53 in PBL treated with 10 nmol/l ActD was detected in immunoblots (Fig. 4b). Data indicate the total absence of p53 in untreated PBL and time-dependent increase of the p53 level in cultures containing 10 nmol/l ActD ($P = 0.003$). A comparison of mRNA and protein expression data suggests that p53 protein upregulation did not originate in p53 mRNA elevated expression or stability.

Treatment of lymphocytes with 10 nmol/l actinomycin D upregulates proapoptotic protein Puma

Cellular content of Puma mRNA in PBL treated with 10 nmol/l ActD was examined by RT-PCR (Fig. 4a). Data demonstrate that the level of Puma mRNA in PBL was dependent on the presence of 10 nmol/l ActD and increased significantly the longer it was incubated for ($P = 0.002$). An increase to the cellular content of protein Puma in PBL treated with 10 nmol/l ActD was detected in immunoblots as shown in Fig. 4b. A low level of Puma was revealed in untreated PBL and a time-dependent

Fig. 4



Variations in expression of p53, Puma and Bcl-2 genes induced by 10 nmol/l actinomycin D in peripheral blood lymphocytes. (a) mRNA levels from semiquantitative reverse transcription-PCR, (b) protein levels from immunoblots. In both graphs the black bars represent p53, the gray bars represent Puma and white bars represent Bcl-2.

increase to Puma was indicated in cultures containing 10 nmol/l ActD ($P = 0.003$).

Bcl-2 mRNA but not antiapoptotic Bcl-2 protein is reduced in lymphocytes treated with 10 nmol/l actinomycin D

A significant decrease ($P = 0.001$) in the cellular content of Bcl-2 mRNA in PBL treated with 10 nmol/l ActD was detected in the electrophoretograms of RT-PCR products (Fig. 4a). The examination of immunoblots developed with anti-Bcl-2 antibody intriguingly showed that the cellular content of Bcl-2 protein was independent ($P = 1$) of ActD presence (Fig. 4b).

Cellular levels of the majority of Bcl-2 family proteins are independent of 10 nmol/l actinomycin D treatment

Using semiquantitative RT-PCR, the expression of the Bcl-2 gene family, proapoptotic Bad, Bak, Bax, Bid, Bik, Bim, Bnip3, Bok, Hrk, Moap-1, Noxa and Siva (lacking Bcl-2 homology), and antiapoptotic Bcl-x_L, Bcl-w, Bfl-1, Boo and Mcl-1 was examined in PBL treated with 10 nmol/l ActD. The data collected in Fig. 5 suggest that the cellular levels of the Bax mRNA slightly increased ($P = 0.041$) the longer it was incubated for in 10 nmol/l ActD. mRNA levels of Bak, Bid, Bim, Bnip3, MOAP-1, Noxa, Siva, Bcl-x_L, Bfl and Mcl-1 were, however, in time frames up to 48 h, independent of ActD presence. RT-PCR signals of Bik and Bcl-w could hardly be detected, and signals of Bad, Bok, Hrk and Boo could not be detected in the background (data not shown). The expression of proteins Bak, Bax, Bid, Bim, Bnip3, Moap-1, Noxa and Siva and proteins Bcl-x_L, Bfl-1 and Mcl-1 in PBL treated with 10 nmol/l ActD was investigated in immunoblots. The data in Fig. 6 suggest that immunoblot signals of all the proteins examined were independent of ActD presence over a time frame of 48 h. Extremely low or no signals of expression of Bim, Bnip3, Moap-1, Noxa, Siva and Bfl-1 proteins were detected (data not shown).

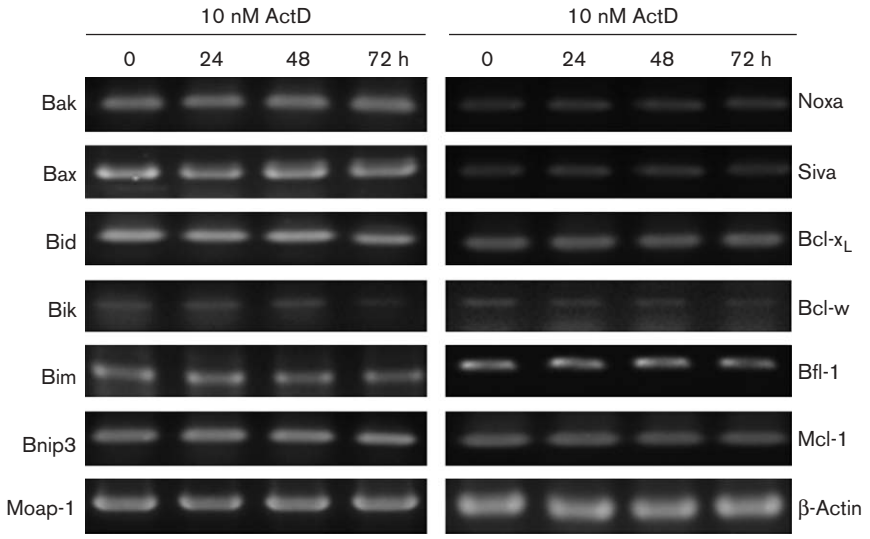
Cellular levels of intrinsic inhibitors of apoptosis and their antagonist are unaffected by 10 nmol/l actinomycin D treatment

The expression of the IAP gene family and the expression of the Smac gene were examined in PBL treated with 10 nmol/l ActD. Data collected in Fig. 7a suggest that cellular levels of mRNA of c-IAP1, XIAP and Smac were independent of ActD presence over a time frame of 48 h. The RT-PCR product of survivin could not be detected. Cellular levels of proteins c-IAP1, XIAP and Smac were independent of ActD presence in time frames up to 48 h (Fig. 7b).

Discussion

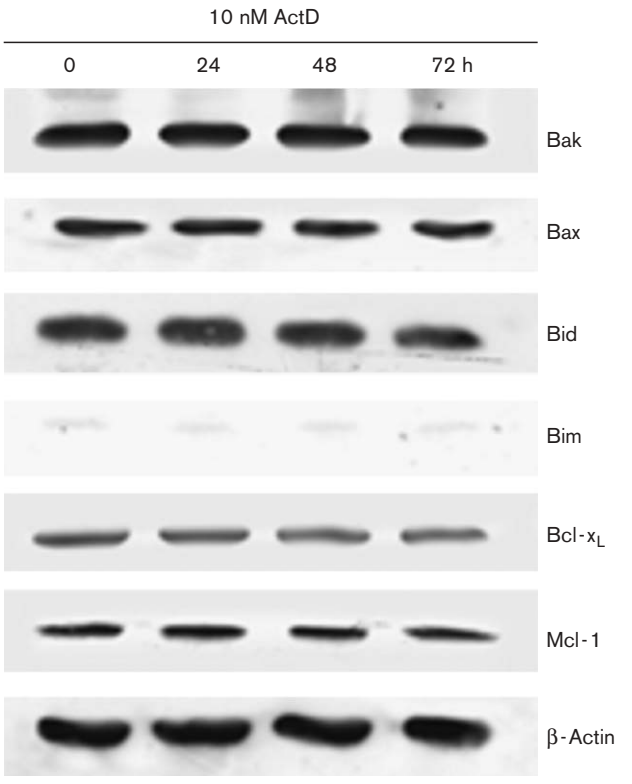
In this study we have examined the effect of 10 nmol/l ActD on ribosomal gene transcription and the transcription of 23 genes coding for apoptosis-related proteins in PBL. Our data suggest that whereas the rate of ribosomal gene transcription fell to 70% of its original value within

Fig. 5



Expression of proapoptotic and antiapoptotic genes of the Bcl-2 family in peripheral blood lymphocytes treated with 10 nmol/l actinomycin D (ActD). mRNA levels from semiquantitative reverse transcription-PCR.

Fig. 6



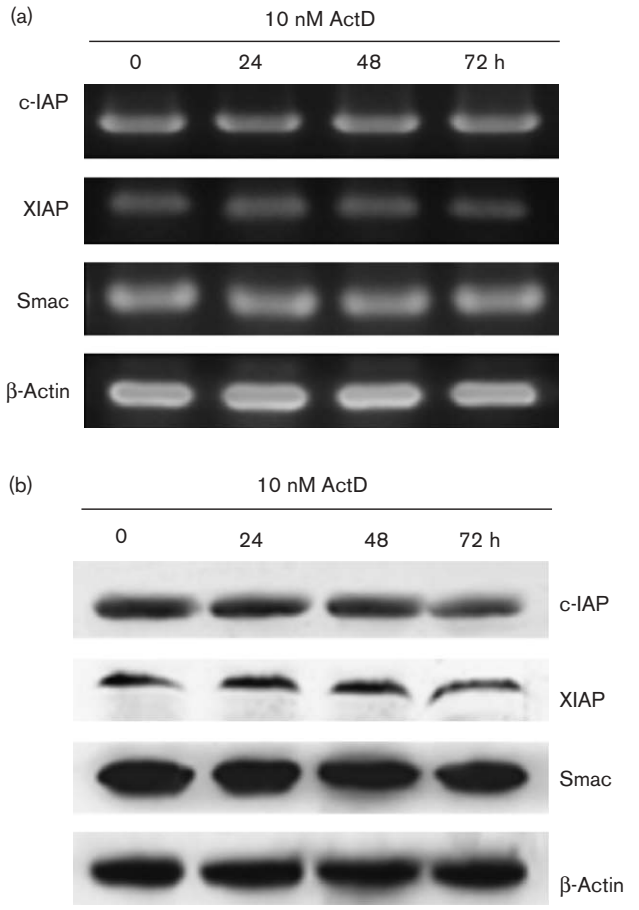
Expression of proapoptotic and antiapoptotic genes of the Bcl-2 family in peripheral blood lymphocytes treated with 10 nmol/l actinomycin D (ActD). Protein levels from immunoblots.

24 h of incubation with 10 nmol/l ActD (preceding significantly mitochondrial dysfunction) the cellular levels of mRNA coding for the majority of examined proteins were independent of the presence of low concentrations of ActD over a 48-h time frame. Our data agree with the findings of other authors [4,11] that a low concentration of ActD specifically inhibits the ribosomal transcription.

Decreasing cellular levels of procaspase-9 induced by incubation of PBL with 10 nmol/l ActD together with cytochrome *c* release and Apaf-1 protein expression suggest that the apical caspase-9 was activated through apoptosome formation [41–43]. Subsequent caspase-9-mediated activation of executioner caspase-3 is documented by decreasing cellular levels of procaspase-3 linked with increasing levels of caspase-3 fragments in anticaspase-3 immunoblots as well as by immunoblots of cleaved PARP and nucleolin [44]. Our flow cytometry two-parameter dot-plots demonstrate the relationship between cellular death and executive caspase activity.

The activation of tumor suppressor protein p53 as a consequence of ActD-induced ribosomal stress is a well-documented effect [45–47]. Our anti-p53 immunoblot data also show increasing levels of p53 protein depending on time of ActD treatment, despite a p53 mRNA indifference to ActD arising from RT-PCR data. These results match the mechanism by which the inhibition of HDM2-mediated ubiquitination results in an inhibition of proteasomal degradation of p53 [8–11,48].

Fig. 7



Expression of intrinsic inhibitors of apoptosis and their antagonist Smac in peripheral blood lymphocytes treated with 10 nmol/l actinomycin D (ActD). (a) mRNA levels from semiquantitative reverse transcription-PCR, (b) protein levels from immunoblots.

We have examined the expression of apoptosis-related proteins from the Bcl-2 family, and we revealed a huge increase of cellular levels of Puma mRNA and protein with time of incubation of PBL at 10 nmol/l ActD. We also noticed a slight increase in Bax mRNA; however, the differences in Bax protein amounts were insignificant. Data from different laboratories suggest that the involvement of individual Bcl-2 family proteins in p53-dependent cell death is cell-type and stimulus specific [33, 49–51], and significant differences in their proapoptotic properties were revealed. Kaeser and Iggo [52] found differences in the affinity of p53 to target promoters and suggested that the Puma promoter was relatively strong, whereas the Bax promoter was substantially weaker. Chen *et al.* [53] have shown that Puma and Bim inhibited all prosurvival proteins, whereas Bad and Noxa cooperated to induce killing. Villunger *et al.* [54] observed in mice with either Noxa or Puma disrupted a decreased number of dying cells in DNA damaged fibroblasts, although only loss of Puma protected lymphocytes. Erlacher *et al.* [49] demonstrated that the absence of Puma efficiently protected lymphoid cells from γ-radiation-induced apoptosis, whereas loss of Bim provided significantly lower protection and Noxa deficiency had no impact. These data imply prominence of Puma in an intrinsic pathway of apoptosis activation in lymphoid cells. On the other hand, findings indicating a key role of other BH3-only proteins in apoptotic control of homeostasis were collected and Bim has proven to be an important regulator of B-lymphocytes in the bone marrow [55,56]. Recently, Alves *et al.* [57] suggested that Noxa and Mcl-1 are key regulators of apoptosis that restrains lymphocyte expansion. Our data, however, suggest that the variations in expression of p53 and Puma form the main biochemical events implicated in the process of rDNA damage-initiated apoptosis in PBL. These speculations are supported by the findings indicating the absence or very low expression of other p53 inducible proteins Bad [58] and Bim [59] in PBL or Noxa [60] in healthy cells independently of ActD presence (our data).

Intriguing variations of Bcl-2 mRNA dramatically decreasing with time of incubation suggest that an effect upregulating or stabilizing Bcl-2 mRNA decreased with the progress of apoptosis. Donnini *et al.* [61] and Luzi *et al.* [62] have suggested that proteins promoting mRNA stability regulated the expression of the Bcl-2 gene. Sengupta *et al.* [63] suggested that nucleolin was involved in Bcl-2 mRNA stabilization and a cleavage of nucleolin in some apoptotic cells was indicated [64,65]. Our data which demonstrated cleavage of a 110-kDa band of nucleolin accompanying a significant decrease of Bcl-2 mRNA suggests that the proteolytic inactivation of extranucleolar nucleolin may be implicated in Bcl-2 mRNA downregulation. Our anti-Bcl-2 immunoblots, however, indicate that the cellular level of Bcl-2 protein is independent of ActD proapoptotic activity over a 48-h time frame. In PBL, induced to cell death with 10 nmol/l ActD, thus the diminution of Bcl-2 mRNA stability does not significantly intervene to promote apoptosis. Another effect stabilizing Bcl-2 protein likely moderates the progress of apoptosis. Bcl-2 degradation is mainly mediated via the ubiquitin–proteasomal pathway and accumulating evidence indicates that protein–protein interactions participate in the control of Bcl-2 stability. Mei *et al.* [66] revealed that Puma/Mcl-1 interaction moderates degradation of antiapoptotic Mcl-1, and Chen *et al.* [53] demonstrated Puma targets Bcl-2 and Mcl-1 proteins to have comparable affinity. Our data indicate suppressed expression of Bcl-2 targeting candidates Bad, Bik, Bim, Hrk and Noxa in PBL, and suggest the possibility for Puma/Bcl-2 interactions to play a role in Bcl-2 protein stability. We have also examined the possible drug-induced modification of the regulatory

system that controls caspase activity through inhibitory action of IAP family proteins c-IAP1, XIAP and survivin, and their mitochondrial antagonist Smac. We did not find any significant changes in total cellular expression of these proteins in PBL treated with 10 nmol/l ActD in time frames up to 48 h. Survivin remained unexpressed independently of ActD presence.

Understanding the mechanism by which anticancer drugs cause toxicity in normal tissue is of principal importance for optimizing effective strategies in chemotherapy. One of the noxious side effects contributing to the development of lymphopenia is drug-induced apoptosis of PBL. Stahnke *et al.* [38], studying apoptosis in mature peripheral mononuclear cells from patients undergoing chemotherapy including ActD administration, indicated Bax upregulation, mitochondrial alterations and caspase-8 activation independent of p53 and independent of activation of CD95. Our data intriguingly showed that the molecular interactions leading to p53 protein-mediated Puma upregulation and its proapoptotic activity were implicated in caspase activation in PBL. Significant differences of experimental conditions, however, are evident. In the experiment of the above authors, apoptosis was initiated *in vivo* using a clinically relevant dose of cytostatic drug. Our experiments were performed *in vitro* using a relatively low concentration of ActD. Despite the fact that no evidence of activation-induced CD95-mediated death was found by Stahnke *et al.* in the acute phase of chemotherapy-induced lymphocyte depletion and that the death receptor-independent cleavage of the proximal caspase-8 was also found by other authors [67], an influence of different environmental factors on the course of apoptosis during in-vivo and in-vitro experiments cannot be ruled out. Dose-dependent decrease in the selectivity of genotoxic action of ActD targeting in higher drug concentration, not only ribosomal genes but broad spectrum of transcribed genes, can also favorize another apoptotic way.

We conclude that in PBL cultured *in vitro* the apoptosis induced by a low concentration of ActD causing almost exclusively damage of rDNA and subsequent ribosomal stress is dependent of p53 and Puma activation. This apoptotic pathway, demonstrated in PBL for the first time, is not the only pathway inducible in PBL by this cytotoxic agent and by other genotoxic drugs. The combination of cell specificity and dose-dependent effects can likely play a decisive role in apoptosis observed in PBL after genotoxic drug application.

Acknowledgements

The authors thank J.E. Sylvester (Hahnemann University, Philadelphia) for the clone pB. This work was supported by Grant NR7998-3 from the Ministry of Health of the Czech Republic.

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