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Original Paper

Suppressive Subtractive Hybridisation Reveals Differential Expression of Serglycin, Sorcin, Bone Marrow Proteoglycan and Prostate-tumour-inducing Gene I (PTI-1) in Drug-resistant and Sensitive Tumour Cell Lines of Haematopoetic Origin

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The development of therapy-induced drug resistance is still one of the most important therapeutic limitations. Nevertheless, an integrating view of the molecular mechanisms underlying resistance development in general is missing. In order to shed some light on the network of this resistance development, we established drug-resistant (doxorubicin (DX), methotrexate (MTX), cisplatin (cisPt), vincristine (Vin)) derivatives of six tumour cell lines (Jurkat, U937, HL60, DoHH-2, K562 and ARH77) of haematopoetic origin. Differential gene expression of drug-sensitive parental cell lines and the drug-resistant derivatives thereof was analysed by suppressive subtractive hybridisation. After dot blot screening for differential expression and sequencing of the cloned PCR fragments, differential expression was confirmed by Northern blot analysis. In an attempt to discriminate for differentially expressed genes only related to one or the other of the investigated drugs, the cDNAs of various resistant sublines (doxorubicin-, methotrexate-, cisplatin-resistant Jurkat cells) were pooled and compared with the sensitive parental cell line. In addition, cDNAs of the resistant derivatives of the different haematopoetic tumour cell lines were pooled and compared with the pooled cDNAs of the corresponding sensitive haematopoetic cell lines to eliminate cell line to cell line variations that were not related to drug resistance. As a result of this screening, the following genes showed a higher (at least 2-fold) or exclusive expression in the drug-resistant variants: serglycin, sorcin, BMPG (bone marrow proteoglycan gene) and PTI-1 (prostate-tumour-inducing gene 1). In addition, elevated expression of hsp90, previously found by our group to be upregulated in the drug-resistant colon carcinoma cell line LoVo H67P was found to be overexpressed in drug-resistant HL60 cells. © **1999 Elsevier Science Ltd. All rights reserved.**

Key words: subtractive suppressive hybridisation, drug resistance, haematopoetic cell lines, sorcin, serglycin, bone marrow proteoglycan, proto-oncogene PTI-1
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INTRODUCTION

CANCERS THAT initially respond to standard chemotherapeutic agents often relapse, with the selective outgrowth of a subpopulation of tumour cells that are resistant to further treatment. This development of a therapy-induced drug resistance is a common problem for a successful treatment of cancer patients. Drugs used in the different therapy protocols for the treatment of various tumour entities but also restricted to the treatment of patients with haematological malignancies exhibit quite different modes of action [1, 2]. They either inhibit

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Table 1. *List of subtracted cDNA combinations of the haematopoetic cell lines investigated*

	'tester'-cDNA*	'driver'-cDNA†
Subtraction A	HL60 MTX^R	HL60
Subtraction B	Jurkat MTX^R, Jurkat DX^R, Jurkat $cisPt^R$ (pooled)	Jurkat
Subtraction B	Jurkat	Jurkat MTX^R, Jurkat DX^R, Jurkat $cisPt^R$ (pooled)
Subtraction C	ARH77 Vin^R, ARHD60 DX^R, U937 MTX^R, U937 Vin^R (pooled)	ARH77 and U937

*'tester' cDNA, cDNA containing specific overexpressed transript. †'driver' cDNA, reference cDNA.

protein synthesis by degrading the amino acid asparagine [3], interact with the cytoskeleton [4], intercalate into DNA or RNA or are incorporated into the nucleic acids thereby leading to strand breaks or transcriptional arrest [1]. Other drugs are targeting proteins involved in nucleic acid metabolism [5] including DNA replication [6] or proteins involved in signal transduction and gene expression [7]. Some very new drugs (e.g. flavopiridol) are designed to interfere with well-known components of the cells own suicide programme known as apoptosis.

Tumours frequently display resistance not only to the specific agent initially used in the treatment, but also to a variety of structurally and functionally unrelated agents. It is speculated that the very different initial modes of action of the above mentioned drugs may also lead to specific detoxification mechanisms such as target protein overexpression [8, 9], increased repair or increased drug export out of the cell [11, 12] which may also be sensed by a common cellular mechanism [13]. If this sensor signals that the damage caused by any single or combined drug regimen is moderate, a repair programme is initiated to allow the cell to eliminate dangerous modifications or mutations. Alternatively, cell differentiation or senescence may be initiated to avoid the proliferation of an aberrant cell. In response to a sensed damage which is too extensive to be repaired, and when differentiation or senescence are impaired, the cellular suicide programme is initiated and the damaged cells become apoptotic [13].

However, the components and/or mechanism of such a common cellular damage sensor are still unknown, although inhibition of drug-induced apoptosis by different mechanisms might be itself a kind of superimposed drug resistance mechanism [14, 15]. In order to shed some light on this speculative superimposed cellular mechanism we established a variety of resistant sublines of six different haematopoetic tumour cell lines (Jurkat, HL60, U937, ARH77, DoHH-2 and K562) using selected drugs leading to more or less different resistance mechanisms: doxorubicin (DX), which is mainly related to P-glycoprotein overexpression [8], methotrexate (MTX), which is known to be related to an overexpression of the dehydrofolate reductase [9], cisplatin (cisPt), an alkylating agent resulting in DNA damage [10, 16] and vincristine (Vin), which besides increasing P-glycoprotein expression causes a disturbance of the cytoskeleton [4]. A variety of different combinations of drug-resistant cell lines established during this study and their sensitive counterparts (Table 1) were compared for genes which are exclusively or at least overexpressed in the drug-resistant cell lines.

MATERIALS AND METHODS

Cell culture conditions

The cell lines K562 (DSM ACC 10), HL60 (DSM ACC 3), U937 (DSM ACC 5) and DoHH-2 (DSM ACC 83) were obtained from the German collection of micro-organisms and cell cultures (DSM), Braunschweig, Germany. ARH77 and the doxorubicin-resistant variant ARHD60 Dx^R were a kind gift of Dr Bellamy, Institute of Pathology, Tucson, U.S.A. The Jurkat cell line was derived from the laboratory cell culture stock. All cells were grown in Click's RPMI-1640 medium supplemented with 10% FCS (fetal calf serum) (GibcoBRL, Eggenstein, Germany), 10 mM Hepes, 1% L-glutamine (Biowhitaker, Boehringer Ingelheim, Germany) at 37°C in an atmosphere of 5% CO_2 in air and 100% humidity. Cells were routinely checked for mycoplasma contamination, especially just prior to suppressive subtractive hybridisation (SSH) using DAPI staining (Roche Diagnostics, Mannheim, Germany).

Drugs and selection of drug resistant cells

MTX, Vin, DX and 5-fluorouracil (5-FU) were obtained from Lederle (Wolfratshausen, Germany) and cisPt from HexalPharm (Holzkirchen, Germany). Doxorubicin was diluted in 0.9% NaCl (w/v), for all other drugs phosphate buffered saline (PBS) was used. Cells were initially exposed to drug concentrations representing the respective IC_{80} value (MTT assay) previously determined. Fresh drugs were added to the medium whenever it was changed (approximately two to three times a week). Drug concentrations were gradually increased to the respective final concentrations given in Table 2. After selection of drug-resistant sublines, drugs were added only once a week to maintain the selective pressure. Cells were cultured in the presence of the respective drugs for a year before SSH was performed and could be regarded as stable resistant cell lines. Prior to each experiment, cells were maintained in a drug-free medium for 1 week.

Drug sensitivity assay

For the drug sensitivity assay, cells were harvested and cultured for 24 h in fresh medium, and were subsequently pipetted into 96-well microtitre plates (Nunc, Wiesbaden, Germany) at a density of 5×10^4 cells/ml. Sensitivity of the cell lines to

Table 2. *Drug concentrations used to maintain the resistant cell lines*

Cell line	Drug concentration (μg/ml)
ARH77 Vin^R	0.3
ARHD60 DX^R	1.0
DoHH-2 $cisPt^R$	3.0
HL60 MTX^R	6.0
HL60 $cisPt^R$	3.0
Jurkat MTX^R	0.75
Jurkat DX^R	0.3
Jurkat $cisPt^R$	2.0
K562 Vin^R	0.2
U973 MTX^R	6.0
U937 Vin^R	4.0

Table 3. Sensitivity of resistant cell lines to selected cytostatic drugs

	Resistance factors ('rf's)				
Cell line	Vincristine	Doxorubicin	Cisplatin	Methotrexate	5-fluorouracil
HL60 MTX^R	0.8	0.8	1.0	1214*	0.4
HL60 $cisPt^R$	0.8	1.1	3.1*	2.5	0.7
ARHD60 DX^R	400	43*	1.5	1.3	2.3
ARH77 Vin^R	1250*	22	1.9	1.3	3.2
Jurkat MTX^R	0.8	1.7	0.8	14*	2
Jurkat DX^R	1.3	13*	0.7	20	3
Jurkat $cisPt^R$	0.7	0.8	4*	0.5	2
U937 MTX^R	1.3	0.8	0.6	150*	1.0
U937 Vin^R	960*	12	0.3	0.7	0.1
DoHH-2 $cisPt^R$	1.5	9.3	16*	2.7	1.9
K562 Vin^R	83*	18	1.8	1.7	1.6

'rf's, IC_{50} value of drug-resistant cells/IC_{50} of the respective parental cell line. IC_{50} values were determined by MTT assay. *Cytostatic drug used for selection of the corresponding cell line.

MTX, DX, Vin, 5-FU and cisPt was determined after 96 h of drug exposure using a MTT assay [17]. The IC_{50} represents the drug concentration resulting in 50% growth inhibition and was calculated from the linear transformation of the dose–response curves. The resistance factors (rfs) were calculated from the ratio of the IC_{50} of the drug-resistant cell line and the IC_{50} of the sensitive parental cell line (Table 3).

RNA extraction and Northern blot analysis

Total cellular RNA of drug-resistant, -sensitive or -stressed sensitive cells was prepared by cell lysis using guanidine isothiocyanate and subsequent centrifugation through a caesium chloride cushion [18]. Stress treatment was performed as described elsewhere [19]. Briefly, cells were either exposed at 43°C, to 1 μg/ml cisPt, to 200 μM cadmium sulphate for 1 h each or to 1 μM dexamethasone for 3 h. Absorbance at 260 nm was used for RNA quantitation. Total cellular RNA (10 μg/lane), was denatured at 70°C for 20 min in 50% formamide; 6.5% formaldehyde; 1×MOPS buffer (20 mM morpholino-propansulphonic acid MOPS, 5 mM sodium acetate, 1 mM EDTA); 0.7% sodium-dodecylsulphate (SDS); 3.3% glycerine; 0.007% bromophenol blue, and 2 μg ethidium bromide and subjected to electrophoresis in MOPS buffer on a 1.5% (w/v) denaturating agarose gel in the presence of 5% (v/v) formaldehyde. RNA was transferred to a nylon membrane (Roche Molecular Biochemicals GmbH, Mannheim, Germany) in 20× SSC (3 M NaCl, 0.3 M sodium citrate) for approximately 8 h according to ref. [20] and immobilised by baking the nylon membrane at 120°C for 30 min. Filters were prehybridised in high-SDS buffer (7% SDS; 50% formamide; 5× SSC; 0.05 M phosphate buffer; 2% blocking reagent (Roche Molecular Biochemicals); 0.01% laurylsarcosine; 50 μg/ml salmon sperm DNA) for 20 min at 68°C. Hybridisation was carried out at 68°C for 16 h with digoxigenin-labelled RNA probes complementary to the respective mRNA target (see Table 4). RNA probes were generated by *in vitro* transcription (MEGAshortscript Kit, ITC Heidelberg, Germany) of PCR products containing a T7-promoter at the 3′-primer. Membranes were washed twice in 2× SSC, 0.1% SDS for 15 min at room temperature, and twice in 0.3× SSC, 0.1% SDS for 15 min at 68°C. After an incubation in blocking solution (1% blocking reagent (Roche Molecular

Table 4. Primers used for PCR analysis and generation of RNA probes

Name	Sequence	Blast accession number
rsp	5′-CAG GAA ACA GCT ATG ACC-3′	M13 reverse primer*
usp	5′-GTA AAA CGA CGG CCA GTG-3′	M13 forward (-20) primer*
Serglycin 5′	5′-CCT GTT CCA TTT CCG TTA G-3′	J03223
Serglycin 3′-T7	5′-GGC TAA TAC GAC TCA CTA TAG GGA GAC CTG TTC CAT TTC CGT TAG-3′	J03223
Sorcin 5′	5′-CAG GAT GGG CAG ATA GAT G-3′	L12387
Sorcin 3′-T7	5′-GGC TAA TAC GAC TCA CTA TAG GGA GAG GTG ATC TTT CCA TTG GTG C-3′	L12387
Bone marrow proteoglycan (BMPG)-5′	5′-GGA TGA GGA GAC ACC AGA G-3′	X65787
Bone marrow proteoglycan (BMPG)-3′-T7	5′-GGC TAA TAC GAC TCA CTA TAG GGA GAG AGC AGC CCA GTA CGC AAA G-3′	X65787

*rsp and usp primers correspond to the M13 reverse and the M13 forward (-20) primer of the TA-cloning kit (Invitrogen, Heidelberg, Germany).

Biochemicals) in maleinic acid buffer: 0.1 M maleinic acid, 0.15 M NaCl, pH 7.5) for 30 min at room temperature, the nylon membranes were incubated with an alkaline phosphatase (AP) conjugated anti-digoxigenin antibody (1:10 000) in 1% blocking solution for 30 min. Two additional washings for 15 min in 0.3% Tween-20 in maleinic acid buffer of the membranes were followed by a 5 min incubation with the CDP-Star substrate (Roche Molecular Biochemicals) diluted 1:1000 in detection buffer (100 mM Tris, 100 mM NaCl, pH 9.5). Subsequently, the nylon membranes were exposed to X-ray films (Amersham Life Science, Braunschweig, Germany) for 2–30 min. Densitometric analysis was carried out using WinCam 2.2 software of ImagePro V (Cybertech, Berlin, Germany). To normalise this analysis for RNA-loading differences, membranes were also hybridised with a human β-actin probe (Roche Molecular Biochemicals).

SSH

mRNA was synthesised from 100 μg of total RNA of the corresponding cells using the Dynal kit for mRNA synthesis (Deutsche Dynal, Hamburg, Germany). SSH was carried out using the Clontech PCR-Select cDNA Subtraction Kit (Clontech, Heidelberg, Germany). For analysis of differentially expressed mRNAs 'driver' cDNAs were subtracted from 'tester' cDNAs as indicated in Table 1. The subtracted cDNA was amplified in a Thermal Cycler (MJ-Research, Biozym, Hameln, Germany) for 30 cycles: 94°C, 60 sec; 60°C, 45 sec; and 72°C, 90 sec using KlenTaq polymerase mix and buffer (Clontech). The nested PCR was performed using 12 and 16 cycles, respectively (94°C, 60 sec; 68°C, 30 sec; and 72°C, 90 sec). After 12 cycles of nested PCR no products were detectable in an agarose gel. Therefore, an aliquot of the PCR reaction was removed after 12 cycles and an additional four cycles were run with the remaining reaction mixture. An aliquot of both nested PCR products was ligated into the pCRII-vector of the TA-Cloning Kit (Clontech) and transformed into competent *Escherichia coli* DH5α cells.

For a rapid screening of the obtained clones containing PCR fragments derived from putative differentially expressed mRNAs before sequencing, 96 clones were cultured in 100 μl Luria Bertani (LB)-medium supplemented with ampicillin (100 μg/ml) in a 96-well microtitre plate. One microlitre of bacterial suspension was denatured in PCR buffer (AmpliTaq buffer with 3 mM $MgCl_2$, and 1 U AmpliTaq polymerase/25 μl buffer) for 10 min at 98°C. Amplification of cDNA inserts was done using a Thermal-Cycler (MJ Research, Biozym, Hameln, Germany) and the reverse (rsp) and universal sequencing primer (usp) indicated in Table 4 for 35 cycles: 95°C, 30 sec; 68°C, 3 min, an additional extension period at 72°C for 5 min was added. Positive PCR products were ethanol precipitated, dotted onto three nylon membranes (Roche Molecular Biochemicals, Germany) and cross-linked to the membrane by baking at 120°C for 30 min. Membranes were hybridised with three probes synthesised from digoxigenin-labelled cDNAs of the sensitive and the resistant cell line, respectively, and digoxigenin-labelled subtracted cDNAs. Labelling of cDNAs of resistant and sensitive cell lines was performed with 15 μg of cDNA and 2 μg of subtracted cDNA, respectively. cDNA was prepared using the Gibco/BRL Superscript™ Pre-amplification System with 200 μM dATP, 200 μM dCTP, 200 μM dGTP, 100 μM dTTP, 100 μM DIG-11-dUTP, 3 mM $MgCl_2$, Tfl-buffer, 1 μl random hexamer (50 ng), and 2 U Klenow-fragment at 37°C for 16 h. PCR products were ethanol precipitated and the adaptor sequence was removed by restriction digestion to prevent probe hybridisation related to the adaptor sequences. To minimise false-positive clones, positive PCR products were hybridised with labelled cDNA probes of resistant and sensitive cell lines, respectively. Only PCR products showing different signals with regard to the 'tester' or 'driver' probe indicating overexpression of a given cDNA-fragment in the drug-resistant variant were sequenced on an ABI 373 A sequencer (Applied Biosystems, Weiterstadt, Germany) using the ABI-DNA-sequencing kit. The sequences obtained were compared with reported sequences (GenBank) using the BlastN network service (ncbi-blast-search).

RESULTS

Drug resistance profile of resistant cells

In order to study potentially new molecular mechanisms of drug resistance development in haematopoetic cell lines a panel of drug-resistant variants of the cell lines HL60, ARH77, U937, Jurkat, DoHH-2 and K562 was established and to determine their relative resistance (rf) to the respective drugs the *in vitro* resistance factors were calculated (Table 3).

Doxorubicin and vincristine have been associated with the overexpression of the P-glycoprotein and the development of a multidrug resistance (MDR) phenotype [8]. Therefore, a cross-resistance between these two drugs is often reported [21] and was also found for the DX-resistant ARH D60 Dx^R cell line investigated in this study (rf Vin: 400) as well as for the newly established Vin-resistant variant of ARH77 cell line (ARH Vin^R rf DX: 22) (Table 3). This cross-resistance to DX was also found for U937 Vin^R (rf DX: 12) and K562 Vin^R (rf DX: 18) cell lines. No significant cross-resistances to the investigated antimetabolites MTX and 5-FU or the alkylating agent cisPt were found in these three Vin/DX-resistant cell lines.

However, unexpected cross-resistances were observed for drug-resistant variants of Jurkat, HL60 and DoHH-2 cell lines. Beside a resistance against the drug used for selection of the respective resistant subline, a cross-resistance against drugs exhibiting a different mechanism of cell poisoning was found. The DX (rf: 13)-resistant Jurkat cells are also resistant to MTX (rf: 20) but not to Vin (rf: 1.3), the cisPt resistant DoHH-2 variant (rf: 16) is also resistant to DX (rf: 9.3) and the cisPt-resistant HL60 derivative (rf: 3.1) exhibits a moderate resistance towards MTX (rf: 2.5). None of the MTX-resistant sublines showed a significant cross-resistance to 5-FU, although cross-resistances of this type are often reported [22] and the resistance factor of the MTX-resistant variant is rather high (rf up to 1214 for HL60 MTX^R). MTX-resistant variants also showed no cross-resistance to any of the other drugs tested. Therefore, the established panel of drug-resistant haematopoetic cell lines offers the possibility to study candidate genes for common steps in drug resistance development.

Differential expression of heat shock genes in drug-resistant and -sensitive haematopoetic cell lines

There is increasing evidence for a correlation between stress-induced expression of heat shock genes and therapy-induced drug resistance [23]. In Northern blot experiments it was shown that the DX-resistant colon cancer cell line LoVo Dx^R expresses hsp 90β constitutively to a high amount,

whereas in the sensitive cell line no constitutive expression of hsp 90β was found [19]. However, expression of hsp 90β could be induced using different stress stimuli such as elevated temperature, heavy metal or drug exposure. In order to investigate if heat shock proteins are also involved in drug resistance of the newly-established drug-resistant haematopoetic cell lines, the expression of hsp 90α and hsp 90β was analysed by Northern blot. The level of the hsp 90β mRNA as well as the hsp 90α in RNA in HL60 cells resistant to MTX or cisPt is approximately 2–3-fold higher than the level in the drug-sensitive parental cell line (Figure 1). For the other drug-resistant cell lines established during this study no overexpression of hsp 90α or hsp 90β was detectable.

In addition to the identified heat shock proteins exhibiting a differential expression in drug-resistant and -sensitive haematopoetic cell lines, prostatic tumour inducing gene 1 (PTI-1) was identified as a candidate gene in the pooled fraction of Jurkat cell lines resistant to DX, MTX or cisPt (Table 5). Northern blot analysis confirmed PTI-1 overexpression in the MTX-resistant Jurkat cell line (data not shown). Interestingly, PTI-1 had been found previously to be differentially expressed in the DX-resistant and -sensitive colon carcinoma cell line LoVo and LoVo DX^R, respectively [19] as well as in prostatic carcinoma [24]. Beside PTI-1 also CAPL (S100A4), a calcium binding protein involved in tumour metastasis, was found to be overexpressed in DX-resistant LoVo DX^R cell lines ([19]; this paper, data not shown). Therefore, the established panel of drug-resistant haematopoetic cell lines was also analysed for the expression of CAPL (S100A4) by Northern blot. Most of the drug-resistant and -sensitive cell lines investigated showed no expression of CAPL (S100A4), the only exception being Vin-resistant U937 variant (data not shown). In contrast, elevated levels of CAPL (S100A4) were detected in several colon cancer cell lines (HT29, WiDr, SW480 and Colo205, data not shown) without a remarkable drug resistance.

Table 5. Candidate genes obtained after SSH from cDNAs of resistant and sensitive haematopoetic cell lines

	Size of cDNA (bp)	% Identity of the SSH-PCR product to the genebank sequence
Subtraction A*		
Serglycin†	200	94
Thymosin β4	69	84
MCP 1	80	93
TafII31	253	86
TCTP	310	97
TCP-1	44	97
U32	44	100
Subtraction B*		
PTI-1†	217	83
Glycyl-t-RNA-synth.	295	88
Hexokinase 1	383	89
Hsc70	286	95
E6-AP-ubiq.-protein-ligase	212	93
Subtraction C*		
Sorcin†	312	92
Bone marrow proteoglycan†	167	84
L5	19	100

*See Table 1. †Genes exhibiting a higher expression in the resistant variants by Northen blot analysis.

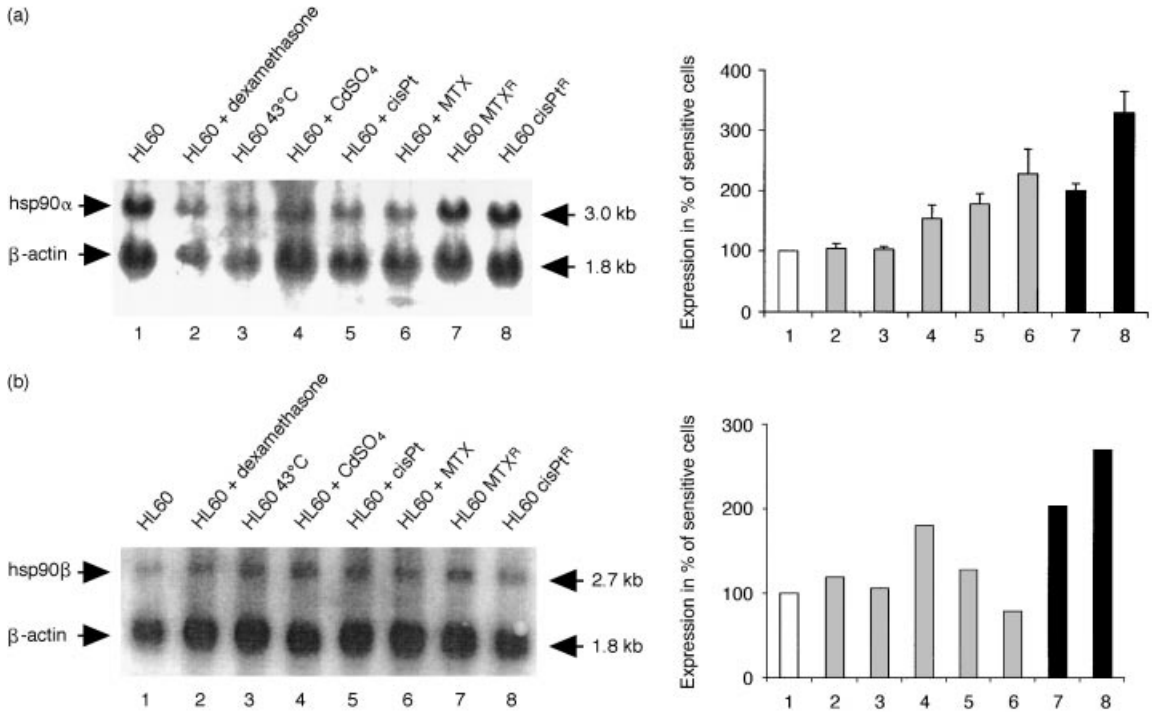

Figure 1. Northem blot analysis and densitometric quantification of heat shock protein mRNA expression in drug-resistant (methotrexate, cisplatin), sensitive and stressed (heat, heavy metal and cytotoxic drugs)-sensitive HL60 cells. Data are shown as mRNA expression in % of the sensitive parental HL60 cells: (a) hsp 90α; (b) hsp 90β. Data are expressed as mean values of different experiments ± standard deviation ($n = 6$ for hsp 90α); for hsp 90β two representative hybridisation experiments were analysed.

Screening for differentially expressed genes and Northern blot analysis

In an attempt to investigate new genes that might be involved in drug resistance development in haematopoetic cell lines, the cDNA of HL60 cells was subtracted from the cDNA of the methotrexate-resistant HL60 variant by SSH. In addition, to eliminate cell to cell variations in gene expression which might not be related to the development of drug resistance, several drug-resistant cell lines as well as their sensitive counterparts were pooled for SSH (Table 1). PCR products of the differentially expressed cDNA fragments were prescreened for false-positives, sequenced and candidate cDNA fragments identified using the ncbi advanced BLAST search are listed in Table 5. Genes exhibiting a higher expression in the resistant variants verified by Northern blot analysis are highlighted.

Further analysis of the candidate genes revealed a 3-fold increased expression of thymosin β4 in the Vin-resistant U937 cell line compared with the -sensitive line. All other haematopoetic cell lines showed a nearly uniform expression level of thymosin β4 (data not shown), only in the DX-resistant colon carcinoma cell line LoVo Dx^R a 4-fold increase of thymosin β4 was observed in the resistant cell line compared with the sensitive cell line.

High expression levels of serglycin (CD44-ligand) in MTX- and Vin-resistant U937 cells were confirmed by Northern blot analysis, while in sensitive U937 cells serglycin mRNA was barely detectable (Figure 2). A similar low level of serglycin mRNA was found in Vin-resistant and -sensitive ARH77 cells. However, serglycin expression was not detected either in drug-resistant or -sensitive Jurkat cells.

The calcium binding protein sorcin is expressed in the resistant variants K562 Vin^R, ARH77 Vin^R and ARHD60 DX^R, while in the sensitive parental cell lines expression of sorcin was barely detectable (Figure 2). Furthermore, the Vin-resistant variant of U937, as well as the DX-resistant Jurkat clone do not express detectable amounts of sorcin.

The bone marrow proteoglycan (BMPG) was expressed in the resistant variants U937 MTX^R, HL60 MTX^R and HL60 $cisPt^R$ (Figure 2). In contrast, no BMPG expression was detected in either the other MTX- or cisPt-resistant sublines or in any of the six drug-sensitive haematopoetic cell lines investigated.

DISCUSSION

In this study, we established drug-resistant haematopoetic cell lines resistant to drugs used in anticancer chemotherapy which are known to be cytotoxic via different mechanisms [1, 2]. The aim of the work was to compare the expression profile of genes potentially related to the development of these drug-resistant phenotypes using SSH. We wanted to look for a potentially common cellular sensor for drug poisoning and to decipher parts of the cellular regulatory network sensoring drug-induced damage. Based on the read-out of this speculative cellular sensor the cell has to 'decide' between cell cycle arrest allowing for damage repair and differentiation, senescence or even active programmed cell death called apoptosis [13, 14].

Evidence was found for some unexpected cross-resistances (Table 3) indicating mechanisms of drug resistance development not only related to the well-known endpoints of resistance development such as the multiple drug resistance phenotype (MDR) or overexpression of the target gene of the selective drug used establishing the respective resistant cell lines [8, 9]. Using various combinations of 'tester' and 'driver' cDNAs (Table 1) during SSH we tried to eliminate genes which might be found due to cell-to-cell variations and genes which might be related to the resistance development for only a single drug by combining cDNAs of different resistant sublines (e.g. Jurkat MTX^R, $cisPt^R$ and Dx^R). A couple of genes which are upregulated in the drug-resistant variants or which are exclusively expressed in the resistant derivatives were identified and differential expression was confirmed by Northern blot analysis (Table 5, Figure 2).

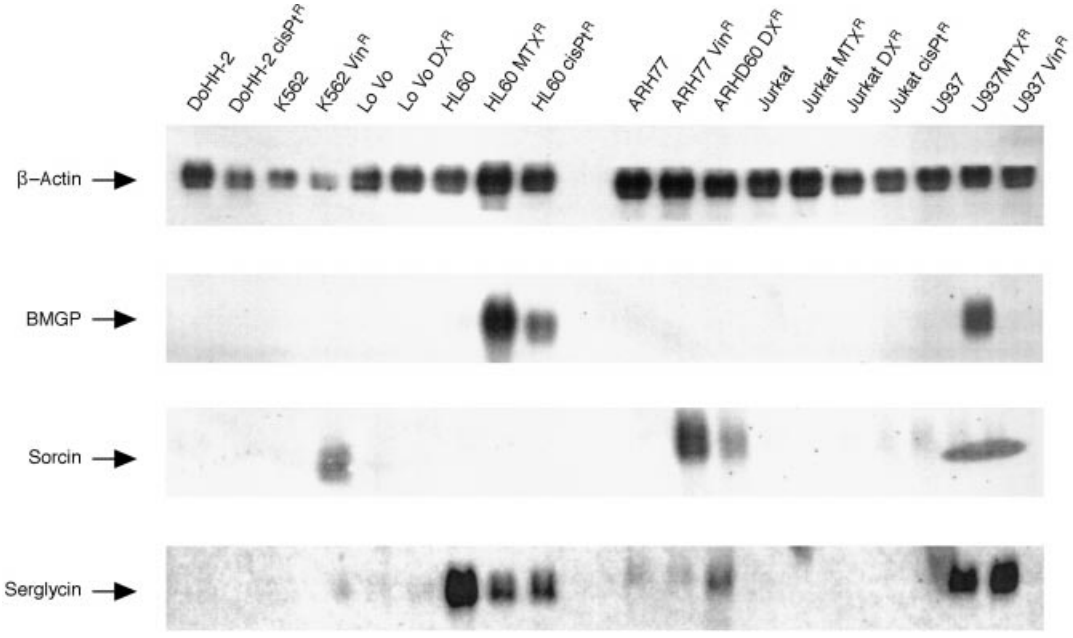

Figure 2. Northern blot analysis of the expression of sorcin, serglycin and bone marrow proteoglycan (BMPG) in different drug-resistant and -sensitive haematopoetic cell lines as well as in the doxorubicin-resistant and -sensitive colon cancer cell line LoVo H67P (LoVo and LoVo Dx^R, respectively).

Surprisingly, within the drug-resistant haematopoetic cell lines differentially expressed genes (PTI-1, hsp 90β and CAPL) were found which had been previously detected in the DX-resistant colon cancer cell line LoVo Dx^R [19]. PTI-1 was originally identified in a prostatic carcinoma by comparison with normal prostate tissue [24]. It has a 94% identity to the elongation factor EF-1α, only differing in its 5′-part. Expression of PTI-1 in contrast to EF-1α could not be detected in normal tissue using Northern blot analysis. However, a strong expression was observed especially in colon cancer cell lines with a further increase in drug-resistant variants [19]. Nevertheless, PTI-1 overexpression was only found in the MTX-resistant Jurkat variant but not in the other established drug-resistant haematopoetic cell lines compared with the sensitive parental lines. Another candidate gene found in the DX-resistant colon cancer cell line, the calcium binding protein CAPL (S100A4), which is known to be involved in cancer metastasis, was also detectable in only one of the drug-resistant haematopoetic cell lines (U937 Vin^R). Finally, hsp 90β which again was found to be highly overexpressed in the doxorubicin-resistant colon cancer cell line LoVo DX^R was also found to be overexpressed in MTX- and cisPt-resistant HL60 cells. However, overexpression was not as pronounced (2–3-fold) and was not detected in the other drug-resistant haematopoetic cell lines investigated in this study.

The screening performed during this study revealed some additional candidate genes (Table 5) which are thymosin β4 [25, 26], serglycin [27], sorcin [28] and bone marrow proteoglycan [29]. Sorcin was found to be overexpressed in the Vin-resistant cell line HOB1/VCR 1.0 [30] and members of the thymosin β family are involved in tumour metastasis [31]. Serglycin is one of the ETS transcription factor target genes [27], which is involved in haematopoeisis, angiogenesis and organogenesis and also in neoplasia. In contrast, bone marrow proteoglycan plays a role in intracellular calcium homeostasis in basophilic granulocytes [32].

Nevertheless, these candidate genes are not uniformly upregulated in all drug-resistant haematopoetic cell lines investigated. Although six different haematopoetic cell lines were selected for resistant subclones raised against four different drugs with rather different modes of action and known resistance mechanisms, and although a strategy of SSH with different combinations of drug-resistant and -sensitive cell lines (Table 1) was used, no common candidate gene was identified. Taken together, no hints were found in the direction of a common cellular sensor of drug-induced damage to trigger the subsequent 'decisions' either leading to repair or to cell elimination to protect the whole organism against a malfunctioning and possibly resistant hyperproliferative cell clone [13].

Although the identified genes upregulated in drug-resistant haematopoetic cell lines do not support a common cellular drug-induced response, some of the genes are involved in the interaction of the cell with its environment: serglycin (a CD44 ligand), bone marrow proteoglycan, CAPL (S100A4) and perhaps also thymosin β4 and sorcin [25–29]. Therefore, it is tempting to speculate that at least one aspect of cellular drug resistance development is a dysregulated communication of the resistant cell and its environment [33, 34]. This might be due to the upregulation of proteins interacting with the extracellular matrix, for example it was found that drug-resistant Jurkat cell lines exhibit an increased synthesis of metalloproteinases (Bertram and colleagues, data not shown). On the other hand, these drug-resistant Jurkat cell lines show a decreased expression of the FAS/APO-1 receptor (Bertram and colleagues, data not shown) which might be due to a metalloproteinase catalysed shedding of the external domain of the receptor resulting in an impaired response of the drug-resistant Jurkat cells to apoptotic stimuli via the FAS/APO-1 receptor. It is well known that shedding of the FAS-ligand is involved in an altered responsiveness of metalloproteinase overexpressing cell lines to apoptotic stimuli [35–37]. A recent paper reports on a co-selection of resistance to FAS-mediated apoptosis by selection for drug resistance [15]. Work is in progress to decipher the possible impact of some selected candidate genes overexpressed in the drug-resistant haematopoetic variants establishing stable transfectants of the sensitive parental cell lines overexpressing hsp 90β and serglycin.

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