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Increase of P-glycoprotein-mediated drug resistance by hsp 90β

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The expression of heat shock proteins hsp27, hsp60, hsp70, hsp90α and hsp90β in extracts of three cell lines (LoVo Dx^R, $KBCh^R8$-5 and S180 Dx^R) expressing the MDR (multidrug resistance) positive phenotype as well as in the sensitive parental lines has been investigated. We present evidence that heat shock protein hsp90β is associated with the P-glycoprotein (Pgp or P170) one of the most prominent components of the drug resistance machinery. In the doxorubicin-resistant cell line LoVo Dx^R, but not in the sensitive parental line, hsp90β is expressed constitutively as shown by Northern blotting. The expression of hsp90β in the sensitive LoVo cell line, however, can be induced by exposure of the doxorubicin-sensitive parental cell line to different stress factors (dexamethasone, doxorubicin, heat treatment or cadmium chloride). We were able to demonstrate that hsp90β can be co-precipitated along with Pgp and vice versa. In native agarose gels both proteins migrated together as one single band as shown by Western blot analysis. This intracellular protein protein interaction may present a mechanism for the modulation of Pgp function possibly by a stabilization of the protein which seems to be attributed to hsp90β (in the human colon carcinoma cell line and in the murine cell line S180). Antisense experiments with oligonucleotides directed against hsp90β and Pgp, respectively, showed a synergistic effect of the selected hsp90β antisense oligonucleotide in combination with the previously described Pgp antisense oligonucleotide in reducing the doxorubicin resistance. The hsp90β antisense oligonucleotide when applied in addition to the Pgp antisense oligonucleotide increased the doxorubicin sensitivity of the resistant human colon carcinoma cell line 2-fold. On the contrary, the hsp90β antisense oligonucleotide alone in contrast to the Pgp antisense oligonucleotide alone did not cause a reduction of the chemoresistance. Moreover, Pgp half-life was reduced in the presence of both antisense oligonucleotides as compared with an incubation with an anti-Pgp antisense oligonucleotide alone.

Key words: Antisense, drug resistance, heat shock, stress response.

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Introduction

The folding and assembly of nascent polypeptides are mediated *in vivo* by molecular chaperones, many of which are members of the heat shock protein family. This protein family is highly conserved throughout evolution. Members of the heat shock protein (hsp) 90 family have been implicated in the stabilization and folding of several cytosolic proteins like actin, tubulin, the aryl hydrocarbon receptor, the inactive cytoplasmic forms of the glucocorticoid and progesterone receptors, several protein kinases, and viral oncogene products.[1–6]

The role of heat shock or stress response proteins in relation to drug resistance is the subject of some controversy. Ciocca *et al.* deduced from their experiments with human breast cancer cell lines that heat-induced elevated expression of hsp27 and hsp70 is associated with doxorubicin resistance but not with P170 glycoprotein mRNA overexpression or resistance to other drugs of the multidrug resistance (MDR) group of drugs.[7] Moreover, drug-induced resistance was higher than heat-induced cross-resistance. In transfection experiments using an hsp27 expression vector introduced in Chinese hamster lung fibroblasts, Huot *et al.* were able to demonstrate a cross-resistance against the MDR-related drugs vincristine, adriamycin, daunorubicin, colchicine and actinomycin D.[8] No cross-resistance was observed against non-MDR drugs such as 5-fluorouracil.[9]

High-level expression of hsp60 predicts poor survival in patients treated with cisplatin-containing chemotherapy.[10] hsp60 expression has also been found to be correlated to cisplatin resistance in head and neck cancer cells.[11] However, in transfection experiments with hsp60 no increase in the cisplatin resistance could be demonstrated.

The expression of hsp27 and hsp60 is a relevant prognostic marker in mammary carcinoma, and ovarian and bladder carcinoma.[12] Glucocorticoid resistance seems to be related to abberant hsp90/hsp70 expression in leukemic cell lines.[13]

In the present paper we address the possibility

that hsps may play a role in p170-mediated drug resistance. Therefore, we investigated doxorubicin-resistant and -sensitive lines for differences in their expression pattern for the following mRNAs: c-*fos*, h-*ras*, c-*myc*, hsp27, hsp60, hsp70, hsp90α and hsp90β. In immuno-co-precipitation experiments hsp90β and P-glycoprotein (Pgp) could be co-precipitated and vice versa. Antisense experiments have been done to investigate the impact of a specific reduction of the expression of one or the other of these two genes on doxorubicin resistance in the resistant LoVo Dx^R cell line and on the half-life of the Pgp.

Materials and methods

Cell lines and culture conditions

Cell lines H67P, LoVo doxorubicin resistant (Dx^R), KB 3-1, $KBCh^R$ 8-5, S180 and S180 Dx^R were used throughout this study. LoVo H67P and LoVo Dx^R were kindly provided by Dr Rivoltini (Milan),[14] cell lines S180 and S180 Dx^R by Dr Volm (DKFZ, Germany), and cell lines KB 3-1 and $KBCh^R$8-5 were obtained from the ATTC (Rockville, MD). Cells were grown in Clicks/RPMI medium (Gibco, Eggenstein, Germany) containing 100 U/ml penicillin, 100 mg/ml streptomycin, supplemented with 10% fetal calf serum (FCS). The MDR-resistant cell lines were transfered alternatingly in medium with and without doxorubicin at a concentration of 5 μg/ml (S180 Dx^R) or 1 μg/ml (LoVo Dx^R and $KBCh^R$8-5).

Stress treatment of the doxorubicin-sensitive LoVo cell line

For the various stress treatments 5×10^6 LoVo cells were seeded in a medium-sized culture flask (80 cm^2) and incubated overnight in the above-mentioned medium at 37°C in an atmosphere containing 5% CO_2. Then cells were either incubated at 43°C for 1 h, in the presence of 1 mM of dexamethasone for 3 h, 0.5 μg of doxorubicin for 1 h or in the presence of 200 μM $CdCl_2$. For the isolation of RNA cells were pelleted and subjected to the protocol of Chirgwin *et al.*[15]

Northern blotting

Total RNA from the respective cell lines was isolated according to Chirgwin *et al.*, run on denaturating agarose gels (40 μg/lane) and transfered to nylon membranes (Hybond N) as described by Chomczynski.[15–17]

Hybridization was performed in the presence of $5 \times$ SSC, $5 \times$ Denhardts solution, 25 mM $NaPO_4$ buffer, pH 6.5, 1% (w/w) SDS at 65°C for 12 h. After three washing steps [$0.1 \times$ SSC, 0.1% (w/w) SDS, 65°C], detection was performed with the DIG detection kit from Boehringer Mannheim (Mannheim, Germany) and AMPPD from Tropix (Serva, Heidelberg, Germany).

Immunoprecipitation

Cell lysates for the immunoprecipitation were prepared as follows: $2-5 \times 10^7$ cells were resuspended in 6 ml of a buffer (PBSTDS) containing 10 mM Na-phosphate (pH 7.2), 0.9% NaCl, 1% Triton X-100, 0.5% Na-deoxycholate, 0.1% SDS, 0.2% Na-azide, 0.004% Na-fluoride. After an incubation at 4°C for 10 min cells were passed through a needle to disrupt the cells. The suspension was centrifuged at 1500 r.p.m. for 15 min at 4°C. The supernatant was recovered and subjected to a second centrifugation step at 25 000 r.p.m. for 1 h at 4°C. After this centrifugation step the supernatant was incubated with the respective antibody [hsp90 (SPA8-45) or Pgp (C 219)] and Protein A agarose in the following amounts: 10 μl supernatant, 10 μl antibody [100 μg/ml] and 15 μl Protein A agarose. The reaction mixture was incubated for 24 h at 4°C on a rolling incubator. Subsequently, a centrifugation step was performed at 2500 r.p.m. for 15 min at 4°C. The pellet was washed four times with the above mentioned PBSTDS buffer and centrifugation was at 2500 r.p.m. for 15 min at 4°C. After the last washing step the pellet was resuspended in 40 μl buffer and boiled for 5 min. Protein concentration was determined according to the Lowry method (Sigma, Deisenhofen, Germany) following the manufacturers instructions. Discontinuous SDS gels were run at 100 V for 2–3 h, loaded with 20 μg/lane of total protein.[18] Protein transfer to nitrocellulose membranes was carried out for 2 h at 70 mA by use of a semidry blotting equipment (Phase, Lübeck, Germany). After protein transfer, additional binding sites on the nitrocellulose membrane were blocked either for 12 h at 4°C or for 1 h at room temperature under agitation in TBS-T buffer (20 mM Tris, pH 7.6, 137 mM NaCl, 0.1% Tween 20) containing 5% dry milk powder. After washing, the membranes were incubated for 12 h at 4°C with monoclonal antibody 830 (hsp90) from Biomol (Hamburg, Germany) or

C219 (Pgp) from CIS Isotopen Diagnostic (Dreieich, Germany) diluted in TBS. Subsequent detection of the murine primary antibody was performed using a mouse antibody detection kit (Amersham) according to the manufacture's instructions. For non-denaturating electrophresis the boiling step of the resuspended immunoprecipitate was omitted and electrophoresis was run in 3% (w/v) agarose gels in a Tris bicine buffer system (100 mM, pH 8.0). Blotting was performed as described above. The membrane was divided in two pieces and stained as above using the hsp90 antibody for one part of the membrane and the Pgp antibody for the other.

Antisense experiments

For the antisense experiments cells were harvested and washed three times with the above-mentioned medium without FCS. Cells were adjusted to 5×10^4 ml and seaded in a final volume of 100 μl/well in microtiter plates. S-ODNs were added to the desired final concentration (2 μM) in a total volume of 10 μl medium without FCS. Only a single dose of phosphorothioate antisense oligonucleotide (S-ODN) or the respective controls was applied. After an additional incubation for 12 h, FCS was added to a final concentration of 10%. After a total incubation time of ~72 h cells were subjected to the chemosensitivity assays.

In vitro drug assays

Modulation of doxorubicin cytotoxicity by S-ODNs was monitored by a colorimetric assay utilizing the tetrazolium salt MTT as described.[19] Briefly, cells were incubated in the presence of the respective S-ODN and doxorubicin at different concentrations (0–100 μg/ml) in microtiter plates. After an incubation period of ~72 h the tetrazolium salt (5 mg/ml stock solution) was added at 25 μl/well; after 2 h of incubation at 37°C, 100 μl of the extraction buffer [20% w/v SDS; 50% DMF; 50% H_2O, pH 4.7 (2.5% of 80% acetic acid; 2.5% 1 N HCl)] was added. Absorption was measured after 12 h of incubation at 37°C using a Dynatech 96-well multiscanner at 570 nm. Medium without cells subjected to the same treatment was used as a blank. Data are expressed as the percentage of surviving of cells without doxorubicin calculated from the absorbance corrected for background absorbance.

Drugs and oligonucleotides

Doxorubicin and MTT were obtained from Sigma. Oligonucleotides were synthesized by NAPS (Göttingen, Germany).

Results

Two human and one murine doxorubicin-resistant and -sensitive cell lines [resistance factors (rf) for the human cell lines were 8-fold ($KBCh^R8$-5) and 50-fold (LoVo Dx^R), for the murine 150-fold (S180 Dx^R)] have been investigated for differences in the expression of c-*fos*, h-*ras*, c-*myc*, hsp27, hsp60, hsp70, hsp90α and hsp90β. In Northern blot experiments no differences in the expression of c-*fos*, h-*ras*, c-*myc*, hsp27, hsp60, hsp70 and hsp90α were found between the resistant cell lines and the sensitive parental lines (data not shown). However, of the cell lines tested, only the resistant cell line LoVo Dx^R, but not the sensitive one, expresses hsp90β constitutively (Figure 1). In S180/S180 Dx^R also the sensitive couple expresses hsp90β but to a smaller extent than the resistant one; however, no difference could be found for the cell line KB 3-1/$KBCh^R8$-5 (Figure 2). The hsp27 expression is more pronounced in the doxorubicin-resistant $KBCh^R8$-5 line than in the sensitive KB 3-1 line; hsp27 is present in the resistant cell line LoVo Dx^R but not in the sensitive parental line (data not shown). Interestingly, hsp27 is not detectable in the murine sarcoma cell line S180/S180 Dx^R. Exposure of the doxorubicin-sensitive human colon carcinoma cell line LoVo to various stress stimuli such as dexamethasone, doxorubicin, cadmium chloride or heat treatment induced the expression of hsp90β (Figure 3), which under standard cell culture conditions is not expressed in the sensitive LoVo line.

If the doxorubicin resistance in the resistant LoVo Dx^R cell line is due in part to cooperation of Pgp and the hsp90β proteins it should be possible to co-precipitate both proteins. Using the immunoprecipitation technique, we therefore looked for protein complexes of hsp90β and Pgp in the three different sensitive and doxorubicin-resistant cell lines. After immunoprecipitation with an anti-hsp90 antibody and gel electrophoresis of the precipitate in an SDS gel under denaturating conditions a protein with the appropriate molecular weight of 170 kDa could be detected with an anti-Pgp antibody. On the other hand, using the anti-Pgp antibody for the precipitation step resulted in the detection of a protein with the anti-hsp90 antibody after SDS–gel electrophor-

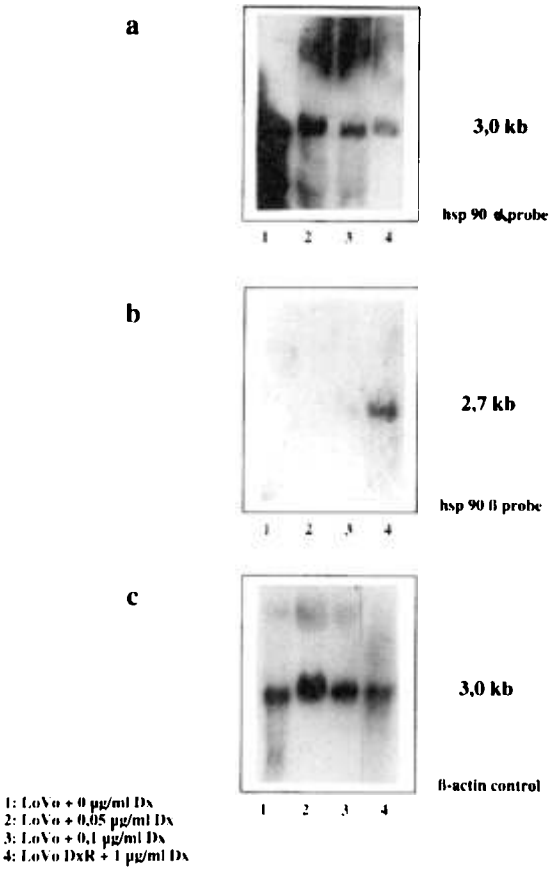

Figure 1. Northern blot analysis of hsp90 mRNA expression in the doxorubicin-sensitive and -resistant colon carcinoma cell line LoVo and LoVo Dx^R, respectively. (a) With a hsp90α probe. (b) With a hsp90β probe. (c) With a β-actin probe as a control for RNA load. The doxorubicin-resistant cell line LoVo Dx^R was usually grown in the presence of 1 μg/ml doxorubicin (according to Rivoltini *et al.*[16]). LoVo Dx^R grown in the absence of doxorubicin also showed the expression of hsp90β (data not shown). Doxorubicin-sensitive LoVo cells were either grown in the absence of doxorubicin (lane 1) or exposed to 0.05 or 0.1 μg/ml doxorubicin for 1 h before they were harvested (lane 2 and 3, respectively). No expression of hsp90β could be found under these conditions.

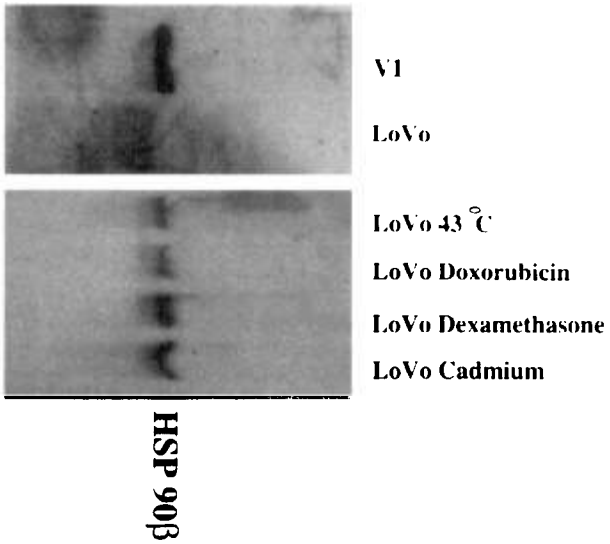

Figure 3. Induction of hsp90β after exposure of doxorubicin-sensitive LoVo cells to various stress factors shown by Northern blot analysis. RNA samples from the doxorubicin-resistant cell line (lane 1), the sensitive parental line (lane 2), as well as from the sensitive cell line after heat (lane 3), doxorubicin (lane 4), dexamethasone (lane 5) or cadmium chloride (lane 6) treatment were analyzed.

esis. Thus both proteins were co-precipitated in lysates of the resistant cells, suggesting an intracellular cooperation between hsp90β and Pgp (Figure 4). Electrophoretic separation under non-denaturating conditions in an agarose matrix revealed a single band which could be detected with the anti-hsp90 antibody as well as with the anti-Pgp antibody (Figure 5a and b). The hsp90β protein was absent in the doxorubicin-sensitive LoVo line (Figure 5a, lane 5).

In a previous study, we demonstrated that the doxorubicin resistance in LoVo Dx^R cells can be reduced by *mdr1* antisense phosphorothioate oligonucleotides or ribozymes.[20] Consequently, for a

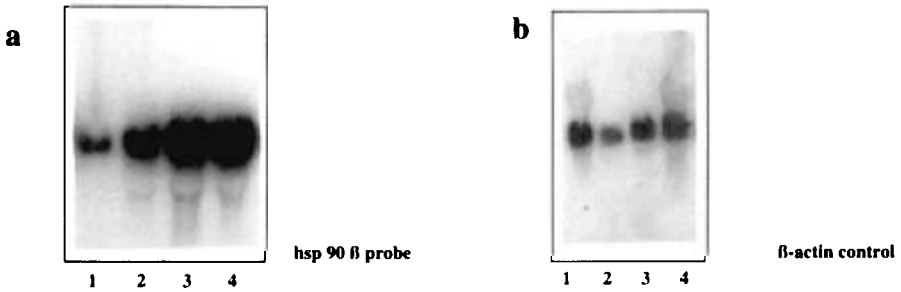

Figure 2. Detection of hsp90β mRNA expression in cell lines S180/S180 Dx^R and KB/$KBCh^R$8-5, respectively (a: lane 1, S180; lane 2, S180 Dx^R; lane 3, KB; lane 4, $KBCh^R$8-5); RNA load (40 μg/lane) was evaluated using a β-actin probe as a control (b, lanes as in a).

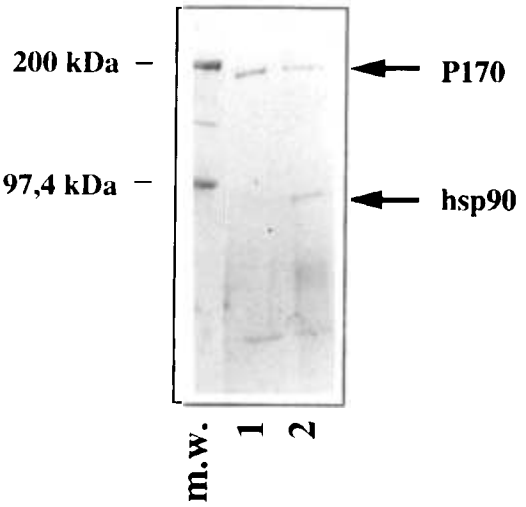

Figure 4. Detection of hsp90 and Pgp (P170) after immunoprecipitation of cell lysates with an anti-Pgp antibody (C219) and separation on SDS PAGE (7.5% v/v). Lane 1 (LoVo) and lane 2 (LoVo Dx^R). To evaluate the specificity of the immunoprecipitation, controls were run using either precipitations without an antibody or using control sera. Under the conditions used only very faint bands could be seen (data not shown).

further confirmation of an inter-relation between Pgp-related drug resistance and stress response (e.g. hsp90β expression), the effect of a specific reduction of the hsp90β expression on the doxorubicin resistance was evaluated in a drug-sensitivity assay using cell lines LoVo and LoVo Dx^R after an incubation in the presence of antisense oligonucleotides.

Drug sensitivity of the sensitive cell line was not influenced by any of the antisense compounds applied either alone or in combination. Control oligonucleotides for hsp90β and Pgp did not influence drug sensitivity (Figure 6). As expected from previous results the Pgp antisense oligonucleotide reduced chemoresistance up to 50% in the doxorubicin-resistant LoVo Dx^R cell line (Figure 6, rf 25 instead of 50 without oligonucleotide).[20,21] In combination with a hsp90β antisensense oligonucleotide

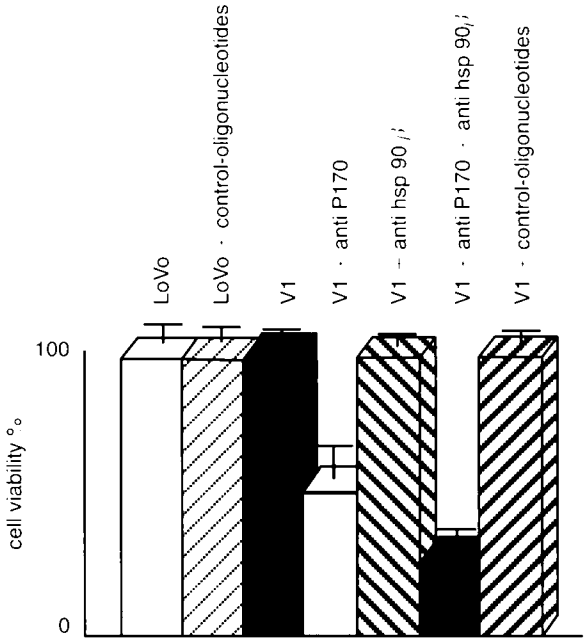

Figure 6. Reduction of cell viability (MTT assay) after incubation of doxorubicin-sensitive or -resistant LoVo and LoVo Dx^R cells with antisense oligonucleotides against Pgp and hsp90β alone or in combination and the respective controls (controls for LoVo: hsp90β and Pgp oligonucleotides and the respective random sequences; controls for LoVo Dx^R: random oligonucleotides for hsp90β and Pgp). Data are expressed as mean ± SD ($n = 6$).

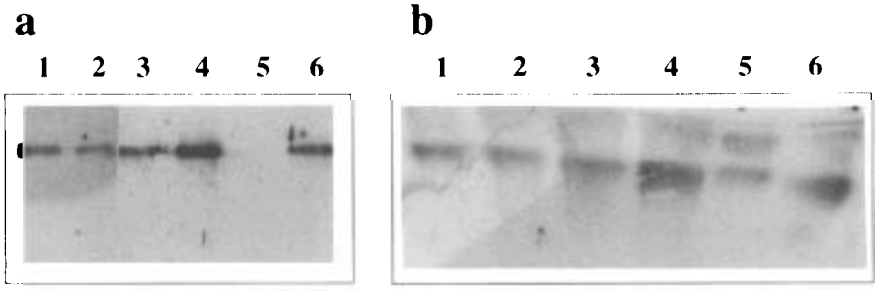

Figure 5. Agarose gel electrophoresis under non-denaturating conditions after immunoprecipitation with an anti-Pgp antibody. After electrophoresis and blotting of the gel the membrane was cut into two pieces (a and b, respectively) with the same sequence of probes. Detection was done with an anti-hsp90 antibody (SPA 830) (a: lanes 1–6) or with an anti-Pgp antibody (C219) (b: lanes 1–6), respectively. For both (a) and (b): lane 1, KB; lane 2, $KBCh^R8$-5; lane 3, S180; lane 4, S180 Dx^R; lane 5, LoVo; lane 6, LoVo Dx^R. The doxorubicin-sensitive KB cell line was only positive for the Pgp after the immunoprecipitation procedure but not in total protein extracts.

(5′-CCT CCT CTC CAT GGT GCA-3′, control sequence: 5′-CAC GTG GTA CCT CTC CTC-3′) deduced from the hsp90β sequence, the doxorubicin resistance was further reduced 2-fold to a rf of 12.5 as shown in Figure 6.[22] In Northern blot experiments a time-dependent reduction of the hsp90β mRNA could be detected after exposure to an anti-hsp90β antisense oligonucleotide (data not shown). However, hsp90β reduction alone does not influence doxorubicin resistance in the LoVo Dx^R cell line, nor did the application of the respective control oligonucleotides alone or in combination (Figure 6).

The influence of a reduction of hsp90β on the half-life of Pgp was investigated by Western blot analysis of doxorubicin-resistant LoVo Dx^R cells after an incubation of the cells either in the presence of the above-mentioned Pgp antisense oligonucleotide alone or in combination with the hsp90β antisense oligonucleotide (Figure 7). The combination of both oligonucleotides resulted in a reduced half-life of the Pgp which may contribute to the observed increase of drug sensitivity when the cells were subjected to the drug sensitivity assay (Figure 6).

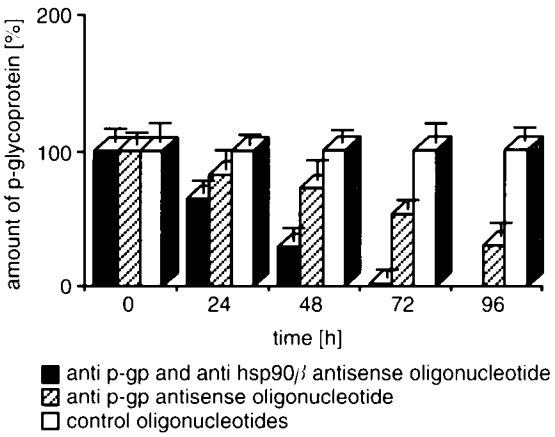

Figure 7. Amount of Pgp in doxorubicin-resistant LoVo Dx^R cells treated with antisense oligonucleotides against Pgp alone or in combination with the anti-hsp90β antisense oligonucleotide and the respective controls (random oligonucleotides for hsp90β and Pgp). Experiments were done in triplicate and data are expressed as mean ± SD. Due to the inhibition of the *de novo* synthesis of the Pgp by the antisense compounds the amount of Pgp is determined by its half-life. A maximal reduction of the Pgp is achieved after 96 h corresponding to the half-life of the protein (about 20 h). Simultaneous inhibition of hsp90β apparently further reduced the half-life of the Pgp; no Pgp is detectable after 96 h in the presence of the Pgp and the hsp90β antisense oligonucleotide, whereas in the presence of the Pgp antisense oligonucleotide alone there is still Pgp left.

Discussion

Predominant mechanisms of cellular drug resistance such as active drug export have been identified in cell culture experiments. However, there is only poor correlation of these cell culture data to clinical observations (e.g. overexpression of Pgp) and to the therapeutic response. These findings tempted us to look for a possible superimposed mechanism of drug resistance interfering with known MDR functions.

Cells express protective so-called heat shock proteins after exposure to heat or other stress factors (chemotherapy, X-radiation). Therefore, we looked for differences in the stress protein pool of two human and a murine sensitive and doxorubicin-resistant cell line. Northern blot experiments looking for a different expression of c-*fos*, h-*ras*, c-*myc*, hsp27, hsp60, hsp70, hsp90α and hsp90β between doxorubicin-sensitive and -resistant cell lines revealed differences in the expression of hsp90β and hsp27. hsp90β was expressed in the resistant LoVo Dx^R cell line but not in the sensitive parental cell line; no differences could be found in the second human cell line (KB and $KBCh^R8$-5, respectively). However, doxorubicin resistance of the $KBCh^R8$-5 cell line is only moderate (3- to 6-fold) and Pgp is hardly detectable, which may explain these findings.[23] In the doxorubicin-resistant murine cell line S180 Dx^R the expression of hsp90β was higher than in the sensitive parental cell line. The two human cell lines differed not only with regard to their hsp90β expression but also in relation to the hsp27 expression. As for hsp90β, no difference in the hsp27 expression between sensitive and resistant KB cells could be detected; however, the resistant LoVo Dx^R cell line expresses hsp27, whereas the sensitive one does not. In the murine cell line S180/S180 Dx^R no hsp27 expression could be detected.

These findings could be confirmed by immunoprecipitation procedures (Figure 5): in the resistant LoVo Dx^R cell line but not in the sensitive parental cell line hsp90 was co-precipitated along with Pgp (using an Pgp antibody for the precipitation) and vice versa. In the sensitive KB cell line as well as in the resistant $KBCh^R8$-5 cell line a co-precipitation of hsp90 with P170 could be detected. However, in the murine cell lines S180/S180 Dx^R the amount of hsp90 which could be co-precipitated along with the Pgp was greater in the resistant cell line than in the sensitive one, corresponding to a higher expression of the hsp90β mRNA in the resistant line.

In antisense experiments the effect of a specific reduction of hsp90β expression on doxorubicin resistance was evaluated in a drug-sensitivity assay

using cell lines LoVo and LoVo Dx^R. Chemosensitivity of the sensitive cell line was not influenced by any of the antisense compounds. Control oligonucleotides for hsp90/β and Pgp either alone or applied in combination did not influence chemosensitivity, nor did the Pgp oligonucleotide or the hsp90/β oligonucleotide alone. As expected from previous results, the Pgp antisense oligonucleotide reduced chemoresistance up to 50% in the doxorubicin-resistant LoVo Dx^R cell line (rf 25 instead of 50 without oligonucleotide).[20] In combination with the hsp90/β antisense oligonucleotide, the reduction of chemoresistance could be increased 2-fold (rf 12.5). The results presented from Northern blots, immunoprecipitation and antisense experiments suggests that there is a cooperation between Pgp and hsp90/β in the resistant LoVo Dx^R cell line, as well as in the doxorubicin-resistant murine cell line S180 Dx^R. In the resistant LoVo Dx^R cell line hsp27 may also be involved in this cooperation. A relationship of hsp27 and the development of drug resistance was also found by Dunn *et al.*[24] However, they found a loss of hsp27 occurring in MCF7 cells made them resistant to novantrone, vincristine and etoposide as well as to doxorubicin. No detectable change was seen in cells made resistant by 5-fluorouracil or X-radiation.

Further experiments with other couples of sensitive and resistant cell lines (which at the moment are established in our laboratory) are necessary to sustain the hypothesis that the cellular stress response may be a superimposed mechanism involved in the development of therapy-induced drug resistance. Another possibility to support our postulated interaction of hsp90 and Pgp and its role for improved drug resistance are co-transfection assays which are ongoing in our laboratory at the moment.

Stress proteins of the hsp90 family are involved in protein stabilization and traffic to the membrane, which could account for an increased stability of the Pgp and a more efficient transport to the membrane, thus confering in part to the resistance of the doxorubicin-resistant cell lines investigated in this study. A possible influence of hsp90/β on the stability and cellular turnover of the Pgp could be deduced from the finding that the reduction of hsp90/β expression by a specific antisense oligonucleotide results in a reduced half-life of the Pgp. A corresponding motif for a role of hsp90 in protein stabilization and traffic could be formed for $pp60^{src}$ encoded by the Rous Sarcoma virus. This kinase is found in a cytosolic complex with two other proteins, p50 and hsp90, and is inactive in this complex. After transport of the complex to the membrane, p50 and hsp90 dissociate from the complex, $pp60^{src}$ is transfered to the membrane and is activated.[25]

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