Drug-induced Modulation of Heat Shock Protein HSPB1 in an Ovarian Cancer Cell Model

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Abstract. *Background: Heat-shock protein HSPB1 (alternative name HSP27) plays a pivotal role in cell survival pathways, apoptosis, metastasis and has been frequently linked to treatment resistance in ovarian cancer (OC) and other malignancies. Characteristic HSPB1 induction in different solid tumors is often caused by cytotoxic agents. Materials and Methods: An in vitro OC cell model system was established to characterize resistance mechanisms during chemotherapy. Human OC cell lines OVCAR-3, SK-OV-3 and TOV-21G were treated with paclitaxel or carboplatin. Cellular growth was analyzed by cell counting. Intra- and extracellular HSPB1 concentrations were assessed by western blot and enzyme-linked immunosorbent assays. Results: Incubation with paclitaxel, and with carboplatin significantly reduced cell growth without a definitive increase of intracellular HSPB1 expression. HSPB1 demonstrated drug-inducible secretion into the extracellular compartment. Conclusion: Despite its current lack of analysis in patient samples, serum soluble HSPB1 may function as a specific biomarker for monitoring response to chemotherapy in patients with OC.*

Besides the development of new-generation anticancer drugs with significantly enhanced efficacy, the establishment of tools to facilitate and accelerate early-diagnosis of cancer and follow-up of therapy success has increasingly become the focus of modern clinical oncology. Due to its central function in cell-cycle regulation, apoptosis and general stress response, the small heat-shock protein HSPB1 (alternative name HSP27) was revealed to be an important molecular factor behind genesis, progression and treatment resistance of various cancer entities, including ovarian cancer (OC) (1-6). By protecting the cell under hostile conditions, overexpression of HSPB1 has been linked with resistance to anticancer treatment. HSPB1 induction caused by cytostatic agents has been shown in different types of solid tumors, however, there exist conflicting data on HSPB1 properties and functionality in OC using different chemotherapeutics. In several studies, HSPB1 was correlated with poor clinical outcome (7). In OC, the amount of circulating HSPB1 has been suggested as a predictive and prognostic biomarker (8-10).

Although OC currently is the third most common female malignancy, globally, it is the leading cause of deaths associated with gynecological cancer (11). With a mean age-standardized 5-year survival of 37.6% in European women between 2000 and 2007, the prognosis of OC remains poor (12). Treatment with paclitaxel and carboplatin exclusively constitute the international standard-care for patients suffering from early-stage OC with poor prognosis, however, recent guidelines recommend adding bevacizumab to current first-line therapies for advanced OC (13).

In this study, a model system of OC chemotherapy was established utilizing paclitaxel-, and carboplatin-treated OC cell lines for analysis of HSPB1-dependent resistance and the investigation of drug-dependent intracellular and extracellular HSPB1 expression patterns. Thereby, we aimed to further our understanding of the potential of HSPB1 in monitoring cytostatic-associated therapy progress.

Materials and Methods

Chemicals and antibodies. Paclitaxel was purchased from Biomol (Hamburg, Germany) and was used at final concentrations of 2 nM for OVCAR-3 cells, 3 nM for SK-OV-3 cells and 4 nM for TOV-21G cells, with dimethyl sulfoxide (DMSO; Carl Roth, Karlsruhe, Germany) as solvent. Carboplatin was purchased from Abcam Biochemicals (Cambridge, UK) and was used at final concentrations of 50 μM for OVCAR-3 and TOV-21G cells, and 125 μM for SK-OV-3 cells. Antibodies directed against HSPB1 (mouse monoclonal antibody clone G31; #2402) and β-actin (ACTB; rabbit monoclonal

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antibody clone 13E5; #4970) were purchased from Cell Signaling Technology (Danvers, MA, USA). Dye-conjugated secondary antibodies directed against mouse (IRDye 800CW goat anti-mouse IgG; no. 926-32210) and rabbit (IRDye 680 goat anti-rabbit IgG; no. 926-32221) were purchased from LI-COR Biotechnology (Lincoln, NE, USA).

Cell culture. The OC cell lines OVCAR-3 and SK-OV-3 (Cell Lines Service, Heidelberg, Germany) were propagated in RPMI-1640 medium (Biochrom, Berlin, Germany) supplemented with 10% fetal bovine serum (Biochrom) and 0.125% gentamicin (Ratiopharm, Ulm, Germany), and in DMEM F12 (Invitrogen, Darmstadt, Germany) supplemented with 5% fetal bovine serum (Biochrom) and 0.125% gentamicin (Ratiopharm), respectively. TOV-21G cells (American Type Culture Collection, Manassas, VA, USA) were propagated in Medium 199 (Biochrom) and MCDB105 (tebu-Bio, Offenbach, Germany) 1:1 media mixture containing 15% fetal bovine serum (Biochrom) and 0.125% gentamicin (Ratiopharm) in 5% CO2 atmosphere and 37˚C. Cells were passaged twice per week using a trypsin/ethylenediaminetetra-acetic acid solution (0.05%, 0.02%, Biochrom). For experiments, cells were plated in 6-well cell-culture plates.

Proliferation assay. The cellular growth of paclitaxel-, and carboplatin-treated cells was examined by cell counting with a CASY Cell Counter and Analyzer Model TT (Roche Applied Science, Mannheim, Germany). Therefore, 300,000 OVCAR-3, 150,000 SK-OV-3, or 250,000 TOV-21G cells per well were seeded in 6-well cell culture plates and incubated in the presence of paclitaxel (OVCAR-3: 2 nM; SK-OV-3: 3 nM; TOV-21G: 4 nM) or carboplatin (OVCAR-3 and TOV-21G: 50 μM; SK-OV-3: 125 μM) for 120 h. The number of living cells was determined by trypsin/ethylenediaminetetra-acetic acid detachment of adherent cells and subsequent analysis: 100 μl of cell suspension was diluted in 10,000 μl CASYton (Roche Applied Science) and analysis of 400 μl dilution was performed in triplicates using a capillary of 150 μm in diameter. Drug-treated cells were compared to vehicle-treated controls. Gate settings of 9.00 μm/15.75 μm, 7.00 μm/15.13 μm, and 5.25 μm/10.13 μm for OVCAR-3, SK-OV-3 and TOV-21G cells, respectively, were used to ensure discrimination between living and dead cells, as well as cellular debris.

Western blot analysis. After incubation with paclitaxel or carboplatin on a 6-well cell culture plate, cells were harvested and lysed in buffer containing 50 mM Tris-HCl (pH 6.8), 10% glycerol, 5% β-mercaptoethanol, 2% sodium dodecyl sulfate and 0.01% bromophenol blue. Proteins were transfered onto a polyvinylidene difluoride Immobilon-FL Transfer Membrane (Millipore, Darmstadt, Germany) and blocked for 1 h in blocking buffer containing 5% milk powder (Carl Roth, Karlsruhe, Germany) in TBS-T (50 mM Tris-HCl, 150 mM NaCl and 0,01% Tween 20). HSPB1 and ACTB were detected by incubation with target specific primary antibodies followed by species-specific secondary antibodies. Protein signals were visualized by using the LiCor Odyssey Fluorescence System (LI-COR Biotechnology) and were quantified by Image Studio Lite 5.0.21 (LI-COR Biotechnology) normalized to ACTB as internal reference.

Enzyme linked immuno-sorbent assay (ELISA). HSPB1 concentrations of cell culture supernatants were determined by use of the DuoSet ELISA kit for human HSPB1 (DY1580) (R&D, Minneapolis, MN, USA) according to the manufacturer's instructions using an Asys Atlantis Microplate Washer (Biochrom). Cell-culture supernatant was centrifuged (1,300 × *g*, 5 min), incubated with ELISA capture antibody (overnight at 4˚C), and assayed in duplicates using R&D Substrate Reagent Pack containing stabilized hydrogen peroxide and stabilized tetramethylbenzidine in a BMG FLUOstar OPTIMA Microplate Reader (BMG Labtech, Offenbach, Germany) with excitation filter A-450 and determined by OPTIMA software 2.10 (BMG Labtech).

Statistical analysis. Results of at least five independent experiments were statistically analyzed. Analysis was performed using the Student's *t*-test with $p \leq 0.05$ as the accepted level of significance. Data are expressed as the mean±SD.

Results

Effect of cytostatic treatment on cellular growth of OC cell lines OVCAR-3, SK-OV-3 and TOV-21G. Paclitaxel and carboplatin are currently international standard adjuvant therapy for women with advanced and early-stage but poor-prognosis OC (14). To establish a suitable cell culture model for variable stage OC, we investigated cellular growth of OVCAR-3, SK-OV-3 and TOV-21G cells incubated with paclitaxel or carboplatin using a proliferation assay. We found that both cytostatics strongly diminished cellular growth at intermediate dosages of 2 nM (OVCAR-3), 3 nM (SK-OV-3) and 4 mM (TOV-21G) paclitaxel and 30 μM (OVCAR-3 and TOV-21G) and 125 μM (SK-OV-3) carboplatin over a period of 120 h (Figure 1). DMSO (paclitaxel) and water (carboplatin) served as vehicle control.

Effect of cytostatic treatment on intracellular HSPB1 expression of OC cell lines OVCAR-3, SK-OV-3 and TOV-21G. Increased intracellular HSPB1 expression following cytostatic treatment was repeatedly observed in other cancer entities (15). To determine whether incubation with paclitaxel and carboplatin may be critical for the expression of cytoprotective HSPB1, we carried out western blot analysis of HSPB1 expression levels over a period of 120 h of drug incubation (exemplary western blots are shown in Figure 2). Our experiments on OVCAR-3, SK-OV-3 and TOV-21G cells revealed heterogeneous results between the different cell lines. There was a trend for a non-significant intracellular HSPB1 modulation in OVCAR-3 and TOV-21G cells over 120 h (Figure 3A, B, E and F). In contrast, we detected significantly diminished HSPB1 levels in SK-OV-3 cells when treated with both cytostatics (Figure 3C and D).

Effect of cytostatic treatment of OC cell lines OVCAR-3, SK-OV-3 and TOV-21G on extracellular HSPB1 levels. It was recently hypothesized that cytostatic treatment increases extracellular HSPB1 concentration (16). In order to shed further light on this observation, that might be the result of cytostatically triggered active HSPB1 secretion into the extracellular space, we assessed alterations of HSPB1

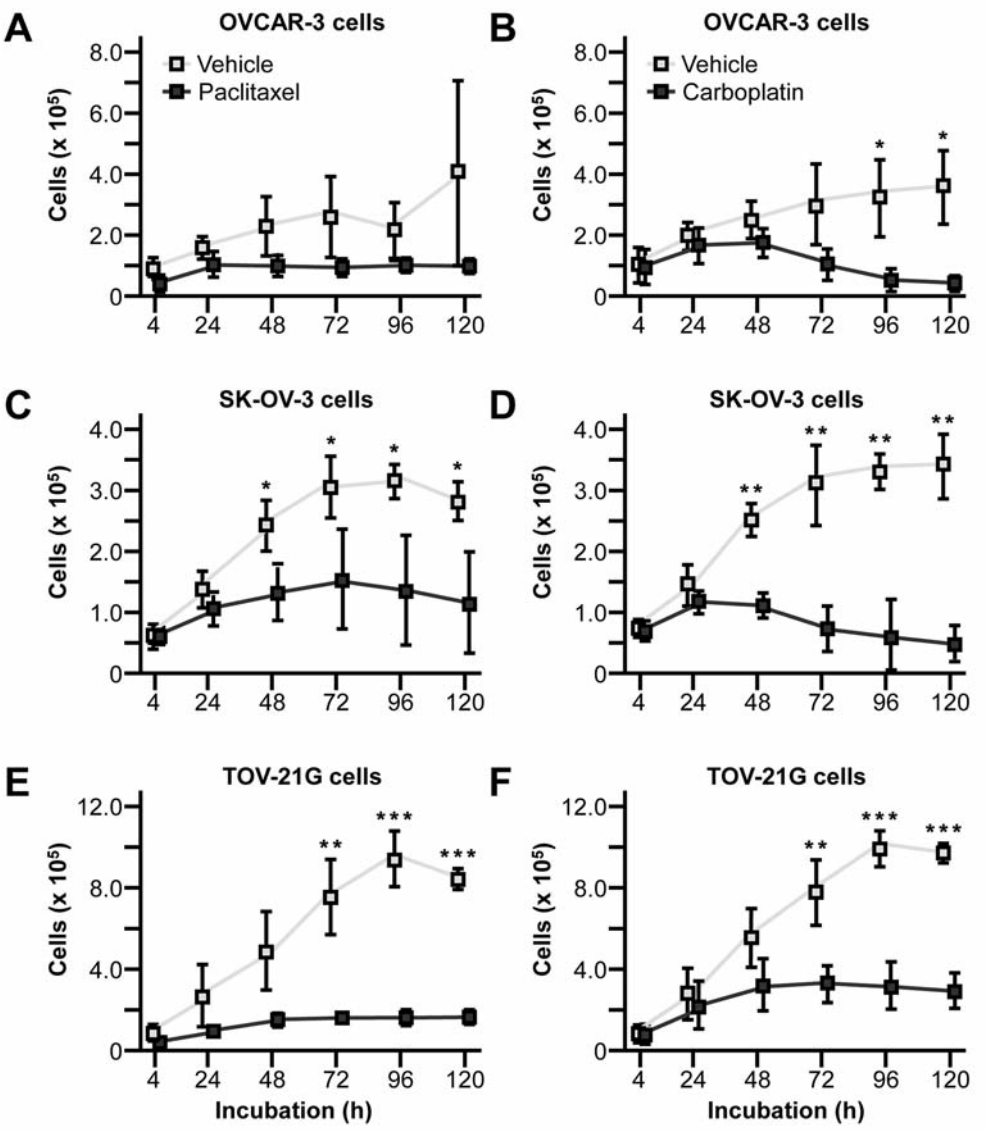

Figure 1. *Incubation of cells with paclitaxel or carboplatin attenuates cell growth in different ovarian cancer cell lines. A-F: OVCAR-3, SK-OV-3 and TOV-21G cells were treated with paclitaxel at 2 nM, 3 nM, and 4 nM, and carboplatin at 30 μM, 125 μM and 30 μM, respectively, over a period of 120 h. Dimethyl sulfoxide and water served as vehicle controls. Proliferation assays were performed after the indicated times of incubation. Data are the mean total number of living cells ±SD; *p≤0.05, **p≤0.01 and ***p≤0.001 as determined by the Student's t-test.*

concentration in cell-culture supernatants utilizing ELISA. As shown in Figure 4, extracellular HSPB1 secreted into the cellular environment was detectable in all incubation experiments utilizing OVCAR-3, SK-OV-3 and TOV-21G cells (Figure 4). Here, no significant differences were observed in cell-culture supernatants of paclitaxel- and carboplatin-treated OVCAR-3 and TOV-21G cells compared to control cells (Figure 4A, B, E and F). Notably, we detected statistically significantly increased extracellular HSPB1 levels in the case of SK-OV-3 cells when treated with both compounds (Figure 4C and D).

Discussion

Expression of cell survival factor HSPB1 has been detected in various solid tumors. It has repeatedly been described as a pivotal factor for tumor progression and resistance to treatment (15) and was found to be released into the surrounding microenvironment. Extracellular HSPB1, thereby, exerts proangiogenic effects by increased vascular endothelial growth factor (*VEGFA*) transcription and activated secretion of the kinase insert domain receptor (KDR) (17). HSPB1 has been furthermore detected in blood serum and plasma (18, 19).

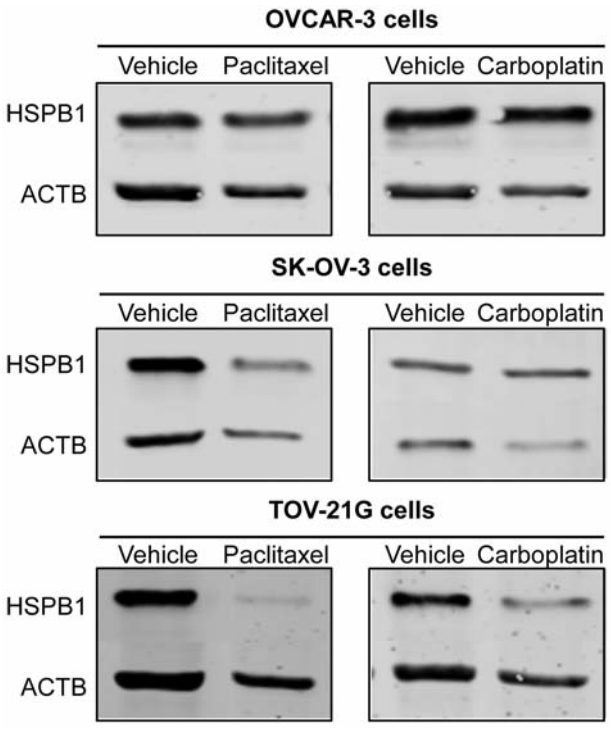

Figure 2. *Western blot analysis of heat-shock protein HSPB1 in cells under cytostatic treatment. OVCAR-3, SK-OV-3 and TOV-21G cells were treated with paclitaxel at 2 nM, 3 nM, and 4 nM, and carboplatin at 30 μM, 125 μM and 30 μM, respectively, over a period of 96 h. Dimethyl sulfoxide and water served as vehicle controls. Total cell extracts were subjected to western blot analyses to quantify HSPB1 protein expression, normalized to β-actin (ACTB) as internal reference.*

Therefore, primarily based on the properties of extracellular HSPB1, the questions that recently arose concerned the probability of using HSPB1 as a novel biomarker for multiple malignancies and structural cell diseases (20). As early as 2000, Cornford *et al*. hypothesized the level of HSPB1 expression to be a reliable predictive biomarker based on samples of 553 patients suffering from aggressive prostate cancer with a poor clinical outcome (shown by immunohistochemistry) (21). These results and the independent prediction of the clinical outcome of prostate cancer by HSPB1 expression analysis were verified by Foster *et al*. (7). In renal cell carcinoma, the level of HSPB1 expression in serum and urine was able to discriminate between low- and high-grade tumors (22). In addition, the diagnostic ability of HSPB1 in pancreatic cancer and cholangiocarcinoma was examined in single promising studies, however, their findings have not yet been verified in reliable meta-analyses (23, 24). HSPB1 also showed promise as a diagnostic marker in gynecological tumors, especially breast cancer and OC. Liebhardt *et al*. showed significant differences concerning HSPB1-positive circulating nanoparticles in patients with metastatic breast cancer compared to women with no history of malignancy (25). Interestingly, Zhao *et al*. demonstrated significantly increased HSPB1 levels in serum samples from 48 patients with epithelial OC by ELISA compared to 59 healthy patients and women with benign ovarian tumors. However, elevated serum levels of HSPB1 were only detected in patients with peritoneal metastases (26). They postulated that HSPB1 might function as indicator for advanced OC progression correlated with peritoneal metastases on the one hand; on the other hand, they highlighted that the HSPB1 level might be useful for measurement of treatment success, due to total serum HSPB1 levels being significantly reduced after combination chemotherapies in patients with peritoneal metastases. This is notable as the present *in vitro* study in different OC cell models showed an increase of extracellular HSPB1 following incubation with paclitaxel or carboplatin in SK-OV-3 cells. Thus, our data may reflect heterogeneity of OC entities, however, the actual function of secreted HSPB1 still remains elusive. Moreover, parallel with the measurement of extracellular HSPB1 by ELISA analysis, we investigated intracellular HSPB1 concentrations by western blot analysis. Interestingly, in SK-OV-3 cells drug-induced decrease of intracellular HSPB1 expression was significantly accompanied by an increase of extracellular HSPB1 levels. Previous studies in prostate cancer cells revealed a significant induction of HSPB1 following cytostatic treatment, which is reasonable due to the cytoprotective and pro-oncogenic properties of HSPB1 (15). Liberation of intracellular HSPB1 from OC cells into serum might either occur through cell damage during the aggressive cell growth or by specific liberation. It could be hypothesized that the intracellular decrease and the extracellular elevation of HSPB1 arise from specific transmembrane transport mechanisms driven by cytostatic treatment.

The maxim of this study was to establish a cell culture model for investigating cytostatic treatment-dependent alterations of intra- and extracellular HSPB1 expression in OC cells and the ability to use HSPB1 as a specific serum-borne biomarker of cancer occurrence, cancer progression and anticancer therapy success. Surprisingly, the *in vitro* data in the present study are heterogeneous. A cytostatic treatment-dependent increase of HSPB1 expression was not identified, even though we demonstrated an obvious antiproliferative effect of paclitaxel and carboplatin for all cell lines. However, *in vivo* studies from patients with OC also obtained very heterogeneous results concerning the definitive HSPB1 expression pattern (26). Other studies, however, have raised serious concerns on the ability of HSPB1 as a predictive cancer biomarker. Different HSPB1 assays performed by Zimmermann *et al*. showed up to 10-fold difference in serum concentrations of HSPB1 in patients with lung cancer, and correlation coefficients of pairwise assay comparisons ranged from 0.184 to 0.938. They concluded that at least the current commercially available HSPB1 detection methods are not able

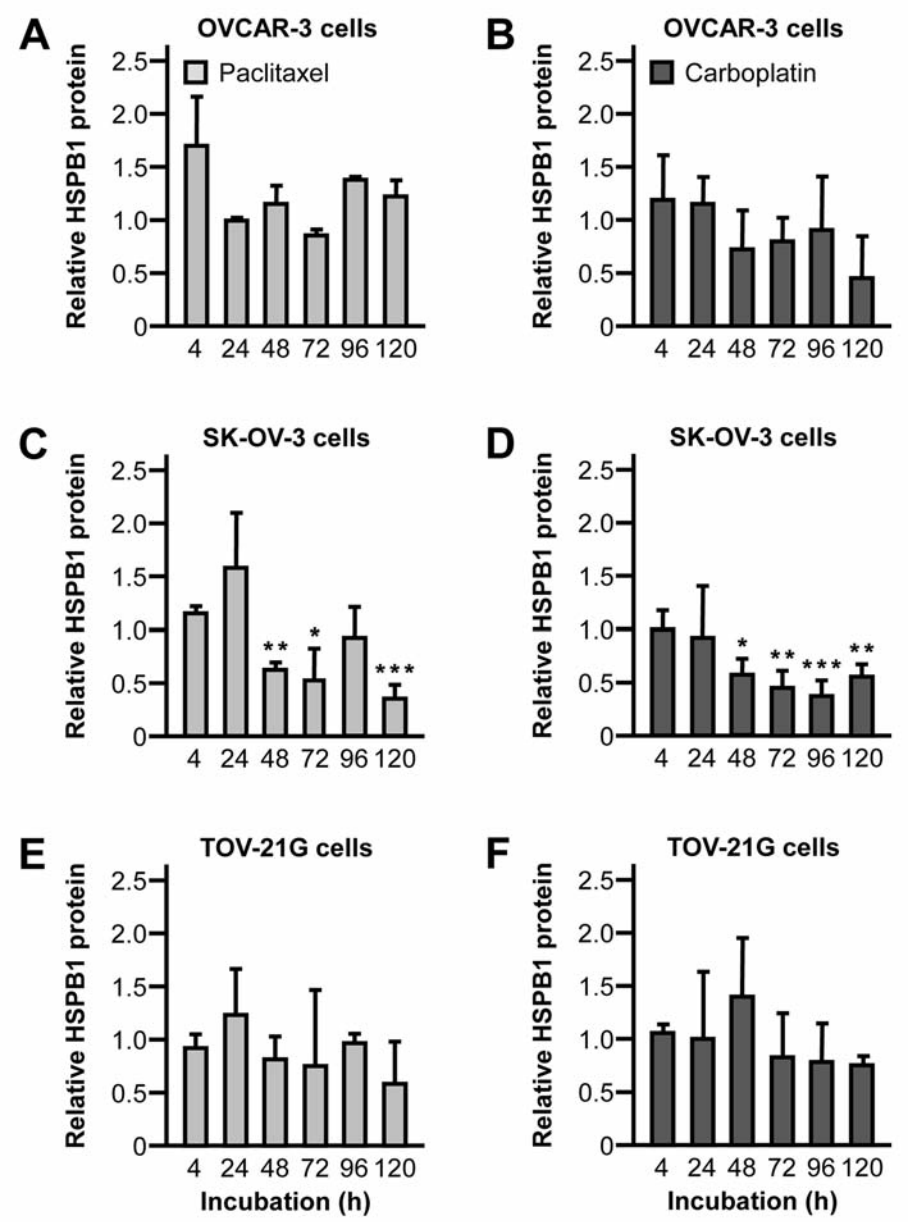

Figure 3. *Long-term cytostatic treatment is followed by heterogeneous alteration of heat-shock protein HSPB1 expression in different ovarian cancer cell lines. OVCAR-3, SK-OV-3 and TOV-21G cells were treated with paclitaxel at 2 nM, 3 nM, and 4 nM, and carboplatin at 30 μM, 125 μM and 30 μM, respectively. Dimethyl sulfoxide and water served as vehicle controls. HSPB1 expression was determined over a period of 120 h. Total cell extracts were subjected to western blot analyses to quantify HSPB1 protein expression normalized to β-actin (ACTB) as internal reference. Data are the mean±SD; *p≤0.05, **p≤0.01 and ***p≤0.001 (compared to vehicle-treated control cells), as determined by the Student's t-test.*

to differentiate patients with lung cancer from healthy controls and are probably also not useful in other diseases (20).

In conclusion, there is a quest to understand the role of extracellular HSPB1 and its expression pattern in OC and other tumor entities. Serum-free HSPB1 detection from blood samples and its usage for tumor stage and tumor progression diagnosis, as well as assessment of treatment response, is still in its infancy. However, the usage of blood-borne HSPB1 as specific biomarker could be a specific, easy and time- and cost-sparing method in clinical oncology. Although further work is required, we believe that the extracellular protein level of HSPB1 has the potential to serve as a biomarker for prognostic stratification of patients with different entities of OC.

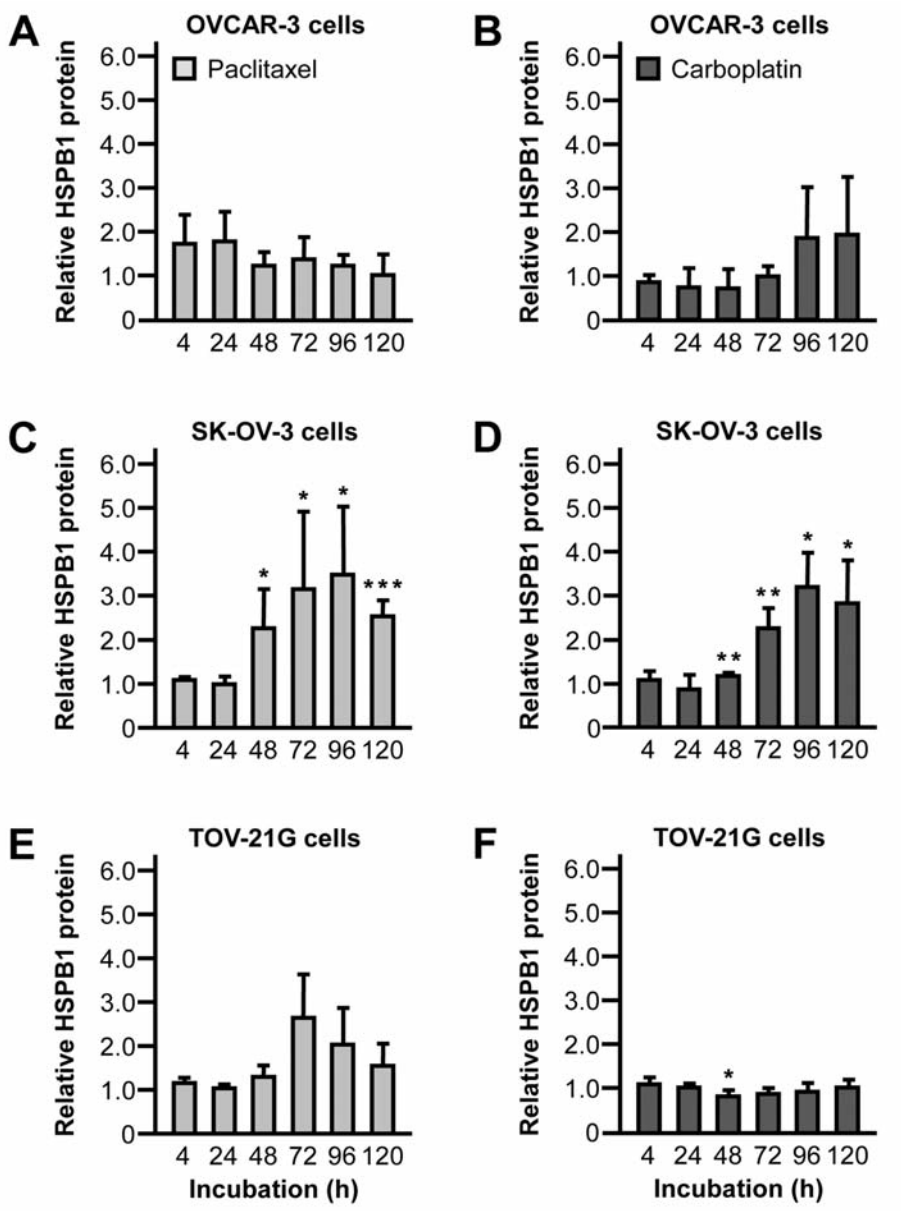

Figure 4. *Cytostatic treatment is followed by a distinct pattern of extracellular heat-shock protein HSPB1 expression. A-F: OVCAR-3, SK-OV-3 and TOV-21G cells were treated with paclitaxel at 2 nM, 3 nM, and 4 nM, and carboplatin at 30 μM, 125 μM and 30 μM, respectively, over a period of 24 h. Dimethyl sulfoxide and water served as vehicle controls. Supernatants were analyzed by enzyme-linked immunosorbent assay to quantify extracellular HSPB1 protein amounts. Data are expressed as the mean±SD (compared and normalized to vehicle-treated control cells). *p<0.05 and ***p<0.001 as determined by the Student's t-test.*

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