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Research Article **Oncology**

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Serine protease HtrA1 modulates chemotherapy-induced cytotoxicity

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Resistance to chemotherapy presents a serious challenge in the successful treatment of various cancers and is mainly responsible for mortality associated with disseminated cancers. Here we show that expression of HtrA1, which is frequently downregulated in ovarian cancer, influences tumor response to chemotherapy by modulating chemotherapy-induced cytotoxicity. Downregulation of HtrA1 attenuated cisplatin- and paclitaxel-induced cytotoxicity, while forced expression of HtrA1 enhanced cisplatin- and paclitaxel-induced cytotoxicity. HtrA1 expression was upregulated by both cisplatin and paclitaxel treatment. This upregulation resulted in limited autoproteolysis and activation of HtrA1. Active HtrA1 induces cell death in a serine protease–dependent manner. The potential role of HtrA1 as a predictive factor of clinical response to chemotherapy was assessed in both ovarian and gastric cancer patients receiving cisplatin-based regimens. Patients with ovarian or gastric tumors expressing higher levels of HtrA1 showed a higher response rate compared with those with lower levels of HtrA1 expression. These findings uncover what we believe to be a novel pathway by which serine protease HtrA1 mediates paclitaxel- and cisplatin-induced cytotoxicity and suggest that loss of HtrA1 in ovarian and gastric cancers may contribute to in vivo chemoresistance.

Introduction

Cisplatin and paclitaxel are 2 antitumor agents that are widely used in the treatment of solid tumors (1–3). While the majority of patients initially have a favorable response to these chemotherapeutic agents, resistance usually develops. Platinum compounds, such as cis-platinum-(II)-diammine dichloride (cisplatin), act as DNA and protein cross-linkers. The mechanisms leading to cisplatin resistance include extracellular and intracellular changes that (a) promote drug metabolism, (b) decrease cellular drug accumulation, (c) alter expression of several key apoptotic regulators, and (d) increase repair of DNA adducts (4, 5). In contrast, paclitaxel alters microtubule (MT) assembly and arrests cells in M phase by inhibiting MT depolymerization and suppressing MT dynamics (3). Resistance to paclitaxel has been attributed to increased drug metabolism, overexpression of P-glycoprotein (6), changes in mitotic checkpoint regulation, alterations in MT-associated proteins or tubulin isotype expression, and mutations or posttranslational modifications of tubulins (3). These observations demonstrate the multifactorial nature of chemoresistance. However, the effectiveness of current chemotherapy regimens also depends on how well anticancer drugs can induce cell death in cancer cells. Accordingly, alterations in programmed cell death or survival pathways have been reported to influence cancer cell response to chemotherapy as well (2, 4, 7–14). Thus chemoresistance is viewed by some as the result of changes in the finely tuned balance between survival and cell death pathways (10, 12, 15).

We have previously reported that the serine protease HtrA1 is downregulated in a majority of ovarian cancers (16). HtrA1 belongs to a widely conserved family of serine proteases initially identified in prokaryotes as essential chaperones/proteases required for survival at elevated temperatures (hence named high temperature requirement A [HtrA]) (17). Human HtrA1 was originally isolated as a gene repressed in SV40-transformed fibroblasts (18). It is frequently downregulated in ovarian cancer and metastatic melanoma (16, 19). We have previously shown that forced expression of WT HtrA1 induced cell death, whereas protease-inactive mutant (S328A) HtrA1 failed to induce cell death, indicating that serine protease activity in HtrA1 is important for its proapoptotic property (16). Similarly, several reports have previously demonstrated that a closely related protease, HtrA2, also induced cell death mediated by its serine protease activity (20–26). In addition, HtrA2 is reported to mediate cisplatin-induced cell death in renal cells (27). These data, therefore, suggest that HtrA proteins may modulate chemotherapy-induced cytotoxicity and their expression may predict response to chemotherapy. Based on these results, we have investigated whether HtrA1 modulates chemotherapy-induced cytotoxicity and evaluated the mechanism of this modulation. We also investigated whether HtrA1 expression could be of clinical relevance as a predictive factor of response to chemotherapy in 2 different histological types of tumor (ovarian and gastric cancers) in which chemotherapy with paclitaxel and/or cisplatin is commonly used.

Nonstandard abbreviations used: AEBSF, aminoethyl benzenesulfonyl fluoride; CR, complete response; dnCasp9, dominant-negative caspase-9; HtrA, high temperature requirement A; LDH, lactate dehydrogenase; ΔMac25, Mac25-deleted; SAΔMac, ΔMac25 catalytically inactive HtrA1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PR, partial response; SA, serine-to-alanine mutant at catalytic site, position 328; TMA, tissue microarray; WTΔMac, ΔMac25 WT HtrA1.

Conflict of interest: The authors have declared that no conflict of interest exists.

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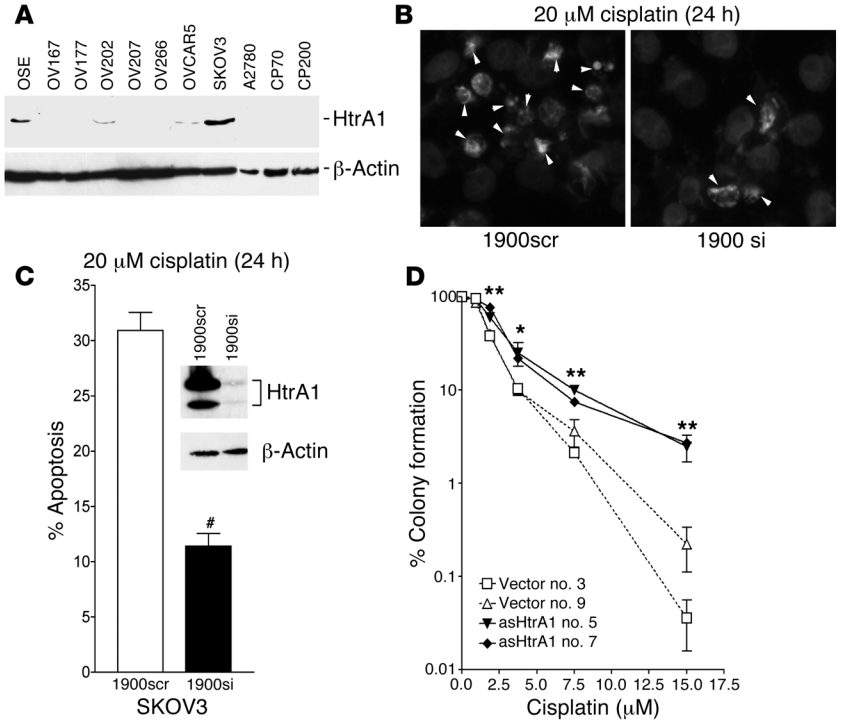

Figure 1

Suppression of HtrA1 by siRNA attenuates cisplatin cytotoxicity. Sensitivity to drugs was assessed by apoptosis and clonogenic survival assay in SKOV3 cells transfected with siRNAs or antisense RNA. (**A**) Immunoblot analysis of HtrA1 expression in ovarian cell lines. (**B**) SKOV3 cells were transiently transfected with siRNAs for 48 hours and subsequently treated with 20 μM cisplatin, and apoptotic cells were visualized by Hoechst staining and counted. Apoptotic cells showing chromatin condensation and fragmentation are indicated by arrowheads. (**C**) Analysis of apoptosis by Hoechst staining showed a significant attenuation of apoptosis in cells transfected with HtrA1 siRNA (1900si) compared with those transfected with scrambled siRNA (1900scr). Immunoblot analysis of siRNA-transfected cells indicated efficient downregulation of HtrA1 in 1900si-transfected cells (inset). (**D**) SKOV3 clonal lines stably expressing antisense RNA or empty vector were treated with various concentrations of cisplatin for 24 hours, washed and grown in fresh drug-free medium for 2 weeks, stained, and counted. Stable suppression of HtrA1 promoted clonogenic survival of antisense-expressing clones (asHtrA1 no. 5 and no. 7) compared with vector-expressing clones (Vector no. 3 and 9). Data are expressed as mean ± SEM and represent 3 independent trials performed at least in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{\#}P < 0.001$ or as indicated; $\alpha = 0.05$, unpaired 2-tailed Student's *t* test for 2 groups, and ANOVA followed by Newman-Keuls test for multiple comparison.

Results

HtrA1 downregulation attenuates cisplatin cytotoxicity. To investigate the effect of HtrA1 downregulation on cisplatin-induced cytotoxicity, we first analyzed HtrA1 expression in ovarian cancer cell lines by immunoblot analyses. Because HtrA1 is expressed in SKOV3 (Figure 1A), we selected this line for subsequent studies. SKOV3 cells were first transfected with scrambled siRNA (1900scr) or HtrA1 siRNA (1900si) for 48 hours prior to 24 hours of cisplatin treatment. Cells were then stained with Hoechst 33258 and examined for apoptotic morphological changes (Figure 1B). Immunoblot analysis confirmed efficient downregulation of HtrA1 by the siRNA (1900si) (Figure 1C, inset). Fluorescence microscopy indicated that downregulation of HtrA1 attenuated cisplatin-induced cell death in SKOV3 cells (Figure 1, B and C).

To test whether reduced cytotoxicity also translated into better clonogenic survival, we assessed colony formation of previously-described SKOV3 clones (16) expressing antisense HtrA1 RNA. Downregulation of HtrA1 (antisense HtrA1 clones 5 and 7) promoted clonogenic survival of these clonal lines compared with empty vector controls (vectors 3 and 9; Figure 1D). Similar results were observed when SKOV3 cells were treated with paclitaxel (Supplemental Figure 1, A and B; supplemental material available online with this article; doi:10.1172/JCI27698DS1). These results suggest that downregulation of HtrA1 attenuates cisplatin and paclitaxel cytotoxicity in SKOV3 cells.

Reexpression of HtrA1 promotes cisplatin cytotoxicity. To investigate whether HtrA1 reexpression affects cisplatin cytotoxicity, HtrA1-deficient OV167 cells (Figure 1A) were transfected with empty vector or WT HtrA1, treated with 20 μM cisplatin for 24 hours, and analyzed for apoptotic morphological changes. OV167 cells transfected with WT HtrA1 (Figure 2A) showed increased cisplatin cytotoxicity compared with controls transfected with empty vector (Figure 2A). Consistent with these results, colony-forming assays indicated that stable expression of HtrA1 in OV167 reduced clonogenic survival in the presence of cisplatin (Figure 2B). To confirm that the observed differences in colony formation were reflective of differences in cell killing, we also examined the sensitivities of the lines to cisplatin by directly assessing apoptosis. Hoechst staining demonstrated that HtrA1 transfectants were more sensitive to cisplatin-induced apoptosis (Figure 2C). These results suggest that reexpression of HtrA1 in the deficient OV167 cell line promotes cisplatin sensitivity. Similarly, reexpression of HtrA1 also sensitized OV167 cells to paclitaxel (Supplemental Figure 2, A and B).

Finally, to test whether increased cisplatin cytotoxicity following HtrA1 expression was cell line specific, A2780 cells were transfected with empty vector or WT HtrA1, treated with 20 μM cisplatin for 24 hours, and analyzed for apoptotic morphological changes. A2780 cells transfected with WT HtrA1 (Figure 2D) showed increased cisplatin cytotoxicity compared with controls transfected with empty vector (Figure 2D). Consistent with these results, colony-forming assays indicated that stable expression of HtrA1 in A2780 cells (Figure 2E) reduced clonogenic survival in the presence of cisplatin (Figure 2E). These results suggest that enhanced cisplatin cytotoxicity following HtrA1 expression is not limited to 1 cell line.

HtrA1 expression is regulated by cisplatin and paclitaxel. To begin to elucidate the mechanism by which HtrA1 modulates cisplatin- and paclitaxel-induced cytotoxicity, we investigated whether HtrA1 expression could be regulated by these drugs. Immunoblot analysis of whole-cell lysates taken from SKOV3 cells treated with 5 μM cisplatin indicated that HtrA1 was upregulated by cisplatin within a few hours of drug treatment (Figure 3A). Treatment of SKOV3 cells with 30 nM paclitaxel also resulted in upregulation

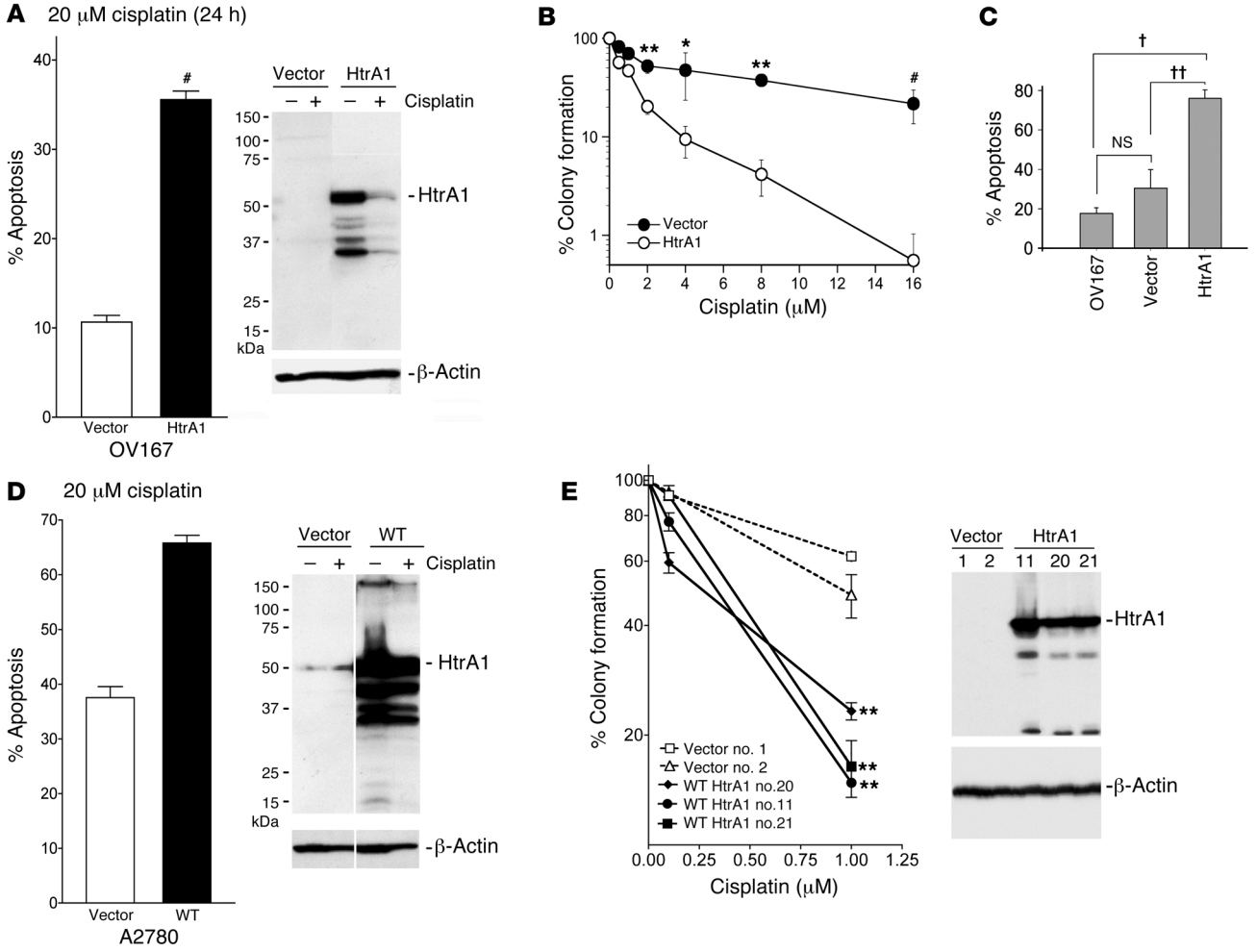

Figure 2

Reexpression of HtrA1 promotes cisplatin toxicity. (**A**) HtrA1-transfected OV167 cells showed a significant increase in cisplatin-induced cell death compared with cells transfected with vector. Immunoblot analyses indicated expression of HtrA1 in cells transfected with HtrA1. β-Actin immunoblots represent loading controls. (**B**) OV167 cells stably expressing HtrA1 also showed a significant decrease in clonogenic survival under cisplatin treatment compared with vector-transfected cells. (**C**) Increased cytotoxicity following cisplatin treatment observed in OV167 cells stably expressing HtrA1 was due to an increase in apoptosis, as these cells showed significantly higher apoptosis compared with vector-transfected or parental OV167 cell lines. (**D**) To test whether HtrA1 expression promotes cisplatin cytotoxicity in another HtrA1-deficient cell line, A2780 cells were transiently transfected with HtrA1 and treated with cisplatin for 24 hours. HtrA1-transfected A2780 cells showed a significant increase in cisplatin-induced cell death compared with cells transfected with vector. Immunoblot analyses indicated expression of HtrA1 in cells transfected with HtrA1. (**E**) A2780 clonal lines stably expressing HtrA1 (HtrA1 nos. 11, 20, and 21) showed a decrease in clonogenic survival compared to vector-transfected clones (Vector nos. 1 and 2) when treated with various concentrations of cisplatin for 24 hours. Immunoblot analyses of these stable clones indicated HtrA1 expression in A2780 clones transfected with HtrA1 (nos. 11, 20, and 21). Data are expressed as mean ± SEM and represent 3 independent trials performed at least in triplicate. $^{*}P < 0.05$, $^{**}P < 0.001$, $^{\#}P < 0.0001$, $^{\dagger}P < 0.0005$, $^{\dagger\dagger}P < 0.005$; $\alpha = 0.05$, unpaired 2-tailed Student's *t* test for 2 groups, and ANOVA followed by Newman-Keuls test for multiple comparison.

of HtrA1 within 2 hours; and the upregulation was sustained for at least 8 hours (Figure 3B).

HtrA1 upregulation results in catalytic activation of HtrA1. After 24 hours of paclitaxel treatment, endogenous HtrA1 was not only upregulated (Figure 3C, indicated by the dashed oval) but also underwent limited proteolysis resulting in a 35-kDa product (Figure 3C, lane 2 indicated by an asterisk). This proteolysis was inhibited in cells expressing catalytically inactive mutant HtrA1 (serine-to-alanine mutant at catalytic site, position 328 [SA]), suggesting that generation of the 35-kDa product requires the protease activity of HtrA1 (Figure 3C, lane 4). Consistent with this observation, forced expression of WT HtrA1 in SKOV3 cells alone was sufficient to produce the 35-kDa product (Figure 3C, lane 5). In addition, levels of the 35-kDa product produced during chemotherapeutic drug treatment were comparable to those achieved by transfection. To rule out the possibility that HtrA1 proteolysis following transfection or paclitaxel treatment was mediated by caspases, we examined HtrA1 proteolysis following forced expression in cells pretreated with the broad-spectrum caspase inhibitor Z-VAD-FMK or cotransfected with dominant-negative caspase 9 (dnCasp9) (28) to inhibit caspase activation downstream of mitochondrial cytochrome *c* release. We utilized OV202 cells for these

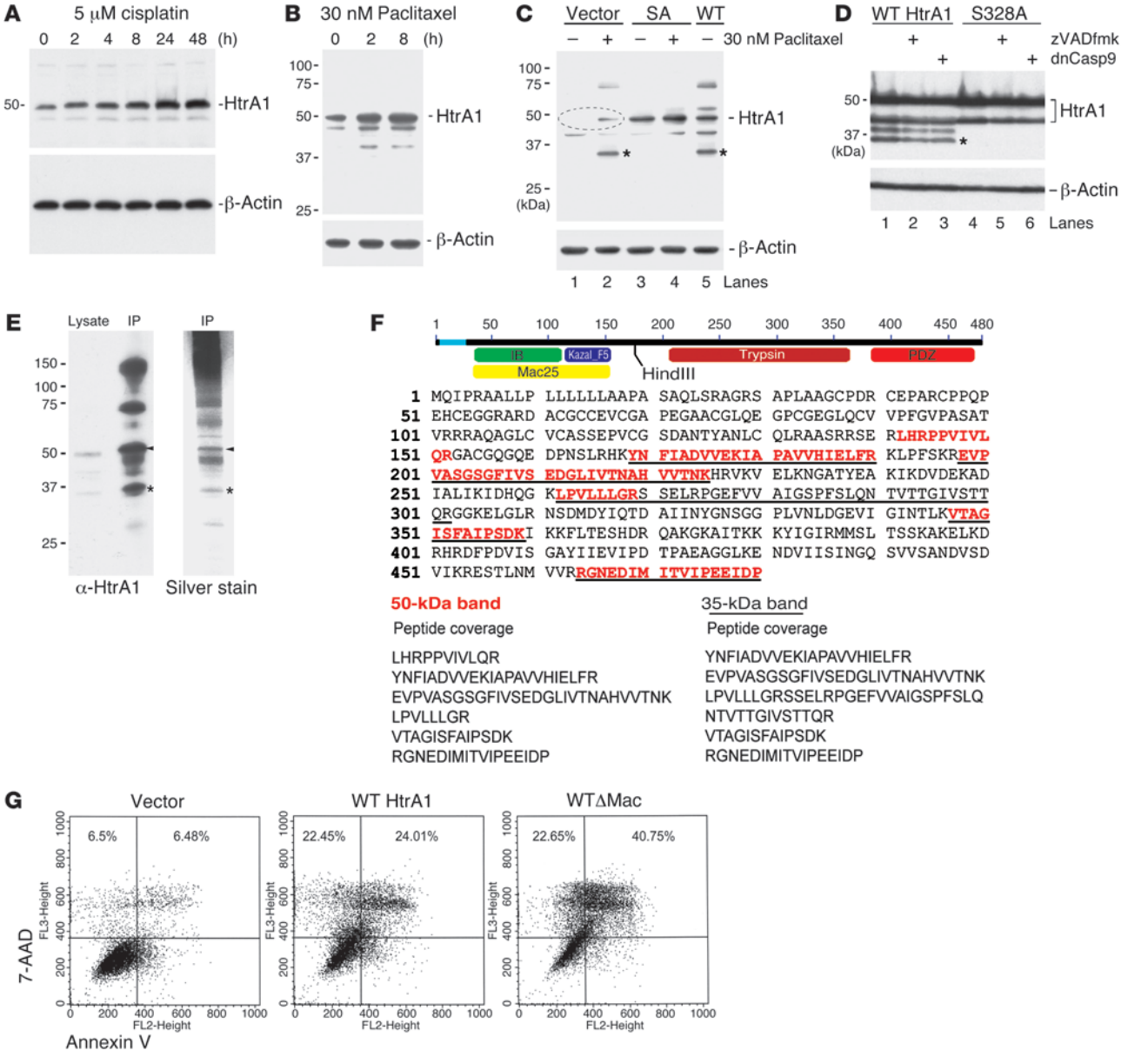

Figure 3

HtrA1 is upregulated and activated during chemotherapeutic drug treatment. (**A** and **B**) SKOV3 cells were treated with cisplatin or paclitaxel, and lysates were taken at various time points. Immunoblot analysis of HtrA1 expression in these lysates (30 μg/lane) indicated upregulation of HtrA1 by cisplatin and paclitaxel. β-Actin immunoblots in the lower panels represent loading controls. (**C**) Immunoblot analysis of cells treated with paclitaxel for 24 hours indicated upregulation of HtrA1 (indicated by the dashed oval) and proteolysis of HtrA1 (35-kDa product in lane 2 indicated by an asterisk) that is dependent on HtrA1 protease activity, since protease mutant SA transfectants did not produce the smaller fragment in the presence of paclitaxel (lane 4). Forced expression of WT HtrA1 also produced a similar 35-kDa product (lane 5). (**D**) Pretreatment with 10 μM Z-VAD-FMK or cotransfection with dnCasp9 did not prevent proteolytic processing of HtrA1 (lanes 2 and 3). However, catalytic inactivation of HtrA1 (S328A) inhibited proteolysis of HtrA1 (lanes 4–6). Autocatalytic products are indicated by an asterisk. (**E**) To determine the domain composition of the 35-kDa product, it was immunoprecipitated (IP) with anti-HtrA1. Immunoblot and silver stain analyses detected 35-kDa and 50-kDa bands (indicated by asterisks and arrowheads, respectively). (**F**) LC-MS/MS analysis of the 35-kDa band showed peptide coverage missing in the Mac25 domain (underlined) but present in the 50-kDa band (red). (**G**) To compare the activities of 35-kDa and 50-kDa HtrA1, plasmid constructs (full-length and ΔMac25) were transfected into OV202 cells, and apoptotic activity was analyzed by annexin V labeling. Transfection of WTΔMac induced higher cell death compared with full-length HtrA1 (WT HtrA1).

analyses due to their high transfection efficiency and relatively low levels of endogenous HtrA1 expression (see Figure 1A). In lysates from OV202 cells transiently transfected with WT HtrA1 for 24 hours, SDS-PAGE and immunoblotting with anti-HtrA1 antibody revealed limited proteolysis of HtrA1 that was not inhibited by Z-VAD-FMK nor dnCasp9, suggesting that the proteolysis is not due to activation of caspases (Figure 3D, lanes 2 and 3). In contrast, expression of protease-inactive mutant (S328A) HtrA1 inhib-

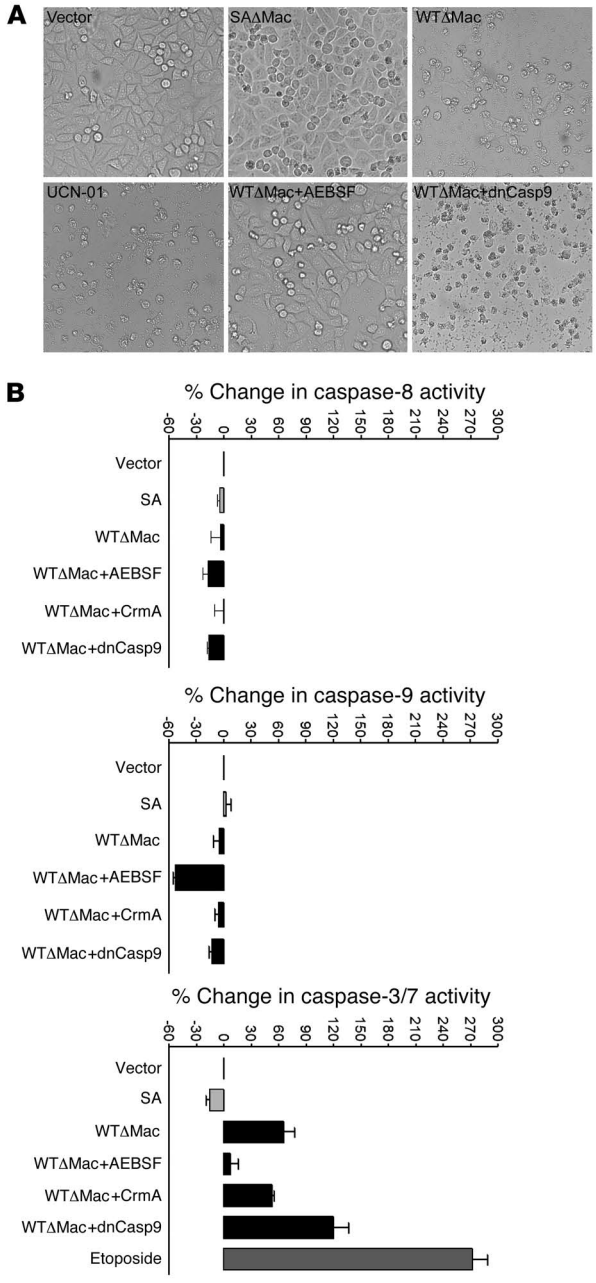

Figure 4
HtrA1-induced cell death is dependent on serine protease activity. OV202 cells were transiently transfected with various plasmids, and cell death was analyzed by morphologic examination, MTT reduction, and LDH release assays. (**A**) WTΔMac-transfected cells showed extensive cell death, which could be prevented by pretreatment with 50 μg/ml serine protease inhibitor AEBSF but not by cotransfection with dnCasp9. Vector- and SAΔMac-transfected cells did not show extensive cell death. 20 μM UCN-01–treated cells were used as positive controls. (**B**) OV202 cells transfected with various HtrA1 constructs for 8 hours were assayed for caspase activities using specific caspase substrates provided in Promega's Caspase-Glo assay kits. In some groups, cells were cotransfected with the caspase inhibitor CrmA (cytokine response modifier A) or dnCasp9 or were preincubated with AEBSF (50 μg/ml). No significant increase in caspase-8 or -9 activity (relative to vector control) was observed following WTΔMac transfection. However, a significant increase in caspase-3/7 activity was observed with WTΔMac transfection. Caspase-3/7 activation was not blocked by CrmA or dnCasp9 but was blocked by AEBSF. 20 μM UCN-01 treatment was used as a positive control for the caspase-3/7 activity assay. Data are from experiments performed in triplicate, and error bars represent SEM.

ited the limited proteolysis of HtrA1, suggesting that the limited proteolysis is the result of autocatalysis.

To determine the domain composition of the 35-kDa HtrA1 fragment, HtrA1 products were immunoprecipitated following expression in OV202 and resolved on duplicate SDS-polyacrylamide gels. One gel was transferred to PVDF and probed with anti-HtrA1 antibodies. This analysis indicated the presence of 50-kDa and 35-kDa bands in lysate (Figure 3E, left lane of left panel) and enrichment of both bands in immunoprecipitates (Figure 3E, right lane of left panel). The duplicate gel was silver stained so that the 35-kDa and 50-kDa bands could be excised for trypsin digestion and LC-MS/MS analysis. Peptide coverage analyses indicated the presence of intact C-terminal polypeptide in both 50-kDa and 35-kDa HtrA1 species (Figure 3F, highlighted in gray for 50-kDa band and underlined for 35-kDa band). Furthermore, both species contained peptides mapping to trypsin and PDZ domains. In contrast, only the 50-kDa species contained a peptide mapping to the Mac25 domain (Figure 3F, highlighted in gray between 141 and 152 residues), suggesting that proteolytic removal of the N-terminal Mac25 domain has occurred during generation of the 35-kDa HtrA1 species.

Because most proteases exist as inactive zymogens that require proteolytic cleavage for activation, it was possible that limited proteolysis of full-length HtrA1 into the Mac25-deleted (ΔMac25) 35-kDa product represented HtrA1 activation. To test this possibility, we cloned a WTΔMac using the HindIII restriction site present between Mac25 and PDZ domains (Figure 3F, schematics of HtrA1 domain composition). To determine whether the 35-kDa HtrA1 product is an active protease capable of inducing cell death, OV202 cells were transfected with empty vector, full-length HtrA1, or WTΔMac for 24 hours, and cell death was analyzed by annexin V labeling followed by flow microfluorimetry. Results shown in Figure 3G indicate that ΔMac25 induced more cell death (63%) than full-length HtrA1 (46%). These results suggest that limited proteolysis of HtrA1 into a 35-kDa product may represent activation of HtrA1 protease activity.

Active HtrA1 induces cell death and activates caspase-3/7 activity. To determine whether serine protease activity is required for cell death, OV202 cells were transiently transfected with empty vector, ΔMac25 WT HtrA1 (WTΔMac), or ΔMac25 catalytically inactive HtrA1 (SAΔMac) in the presence or absence of dnCasp9 or the serine protease inhibitor aminoethyl benzenesulfonyl fluoride (AEBSF). Twenty-four hours later, cells were examined by light microscopy. The staurosporine analog UCN-01, which is routinely used to induce apoptosis in a variety of cells (29, 30), was used as a positive control. Extensive cell shrinkage, indicative of cell death, was observed in WTΔMac-transfected cells compared with SAΔMac- or vector-transfected cells (Figure 4A). This WTΔMac-induced cell death was blocked by AEBSF (50 μg/ml) but not by dnCasp9 (Figure 4A). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and lactate dehydrogenase release assays (Promega) also indicated that WTΔMac-transfected cells exhibit significantly lower viability than vector- or SAΔMac-trans-

Table 1
Ovarian cancer patient characteristics

No. of subjects	60
Median age at diagnosis, yr (range)	61 (38–80)
No. alive at time of analysis	16 (26.66%)
Average length of follow-up,[A] mo	43.7
Chemotherapy, *n*	
Cisplatin/Cytoxan	13 (21.67%)
Cisplatin/Taxol	47 (78.33%)
Primary surgery, *n*	
Optimal debulking	53 (88.33)
Suboptimal	7 (11.67)
Stage, *n*	
3	46 (76.66%)
4	14 (23.34%)
Grade, *n*	
2	1 (1.67%)
3	30 (50%)
4	29 (48.33%)
Histology, *n*	
Mixed	1 (1.67%)
Endometrioid	8 (13.33%)
Serous	51 (85%)
Pre-CA125, median (range)	830 (8.2–86,000)
Post-CA125, median (range)	12.05 (4.7–947)

[A]Follow-up refers to time from date of initial surgery to death or to most recent follow-up visit. Pre-CA125, ovarian carcinoma antigen CA125 levels before surgery; Post-CA125, ovarian carcinoma antigen CA125 levels after chemotherapy, measured in IU.

fected cells (Supplemental Figure 3, A and B). Finally, to determine whether expression of active HtrA1 (WTΔMac) results in caspase activation, we performed caspase activity assays using luminogenic substrates that are preferred by caspase-8, caspase-9, or caspase-3 and -7. For these experiments, cells were harvested 8 hours after transfection rather than 24 hours later because preliminary data indicated that cells harvested 24 hours after transfection exhibit less caspase activity due to loss of cytoplasmic contents during the cell death process. There was no significant increase in activities of caspases 8 and 9 following WTΔMac transfection compared with SAΔMac transfection (Figure 4B). However, increased caspase-3/7 activity was observed following WTΔMac transfection. This activation of caspase-3 and/or -7 was blocked by preincubation of cells with 50 μg/ml AEBSF but not by cotransfection of cytokine response modifier A (CrmA) or dnCasp9. These results collectively suggest that HtrA1 induces cell death through its serine protease activity and results in activation of caspase-3 and/or -7 during the cell death process.

Relationship between HtrA1 expression and clinical chemosensitivity. Since expression of HtrA1 contributes to chemosensitivity in vitro, we next assessed whether HtrA1 expression in primary ovarian tumors also correlates with chemosensitivity in vivo. HtrA1 expression was determined by immunohistochemistry using anti-HtrA1 antibodies on a tissue microarray (TMA) containing 60 primary epithelial ovarian tumors. Patient characteristics are described in Table 1. Levels of HtrA1 expression were categorized as low (weak staining, 0), moderate (1+), and high (intense staining, 2+). Representative sections showing these intensities are shown in Figure 5A. Independently, tumor response to chemotherapy was defined as complete response (CR), partial response (PR), and no response (NR) according to standard criteria described in Methods. As shown in Figure 5B, tumors with high levels of HtrA1 showed a 90% (27/30) response rate compared with 62% (8/13) and 65% (11/17) response rates in tumors with low and moderate levels of HtrA1, respectively. Response to chemotherapy was significantly different between low and high ($P = 0.0276$) or moderate and high ($P = 0.0342$) staining groups. There was no statistically significant difference between low versus moderate staining groups. Therefore, the low and moderate groups were combined and reanalyzed against the high group by the χ^2 test. Results shown in Figure 5B indicate a statistically significant difference between the low/moderate and high groups ($P = 0.0146$). The odds ratio for not responding to therapy in the cohort with low levels of HtrA1 was 5.21 (95% confidence interval, 1.278–21.24).

To assess whether these results were unique to ovarian cancer, HtrA1 expression was also assessed in 51 gastric cancer specimens. In general, HtrA1 immunostaining in gastric cancers is weaker than in ovarian cancers, and no intense (2+) staining was observed. Representative staining intensities are shown in Figure 5C. Characteristics of patients included in the analysis are shown in Table 2. In this population, 28 patients (55%) achieved an objective response (8 CRs, 20 PRs) to a platinum-based chemotherapy as defined in Methods. Twenty-eight patients showed moderate levels (1+) of HtrA1 staining, whereas 23 patients displayed undetectable (0) HtrA1 staining (Figure 5D). Thirty-five percent of patients (8/23) with undetectable HtrA1 expression had a response to chemotherapy compared with 71% of patients (20/28) whose tumors expressed moderate levels of HtrA1 (Figure 5D). Univariate analysis of HtrA1 expression and chemoresponse in gastric cancer indicated a statistically significant association between undetectable HtrA1 expression and poor response to chemotherapy ($P = 0.0089$; χ^2 test). The odds ratio for not responding to therapy in the cohort with undetectable HtrA1 was 4.688 (95% confidence interval, 1.430–15.36). These results suggest that tumor cell HtrA1 expression is associated with a better response to chemotherapy.

Discussion

In this study, we have characterized the role of the serine protease HtrA1 in the cytotoxic action of cisplatin and paclitaxel, 2 drugs that are extensively used to treat a wide range of cancers. While roughly 70% of ovarian cancer patients initially have a favorable response to this drug combination, recurrence with drug resistant disease is common, and the 5-year survival in advanced-stage ovarian cancer is only 15–20% (31). In most patients, gastric cancer is unresectable at diagnosis or a relapse occurs following operation, resulting in a 5-year overall survival of less than 20% (32–34). Platinum-based chemotherapy allows remission rates of approximately 40–60% in selected patients with advanced gastric cancer (35). However, the duration of response and overall survival are generally poor. These observations highlight the need for improved understanding of the mechanisms of drug resistance in cancer.

Numerous reports support the role of proteins involved in programmed cell death and survival pathways in modulating chemoresistance phenotype (12). For example, Schmitt et al. reported that antiapoptotic protein Bcl-2 expression produced multidrug resistance in vivo in a murine Eμ-*Myc* model of B cell lymphoma (36). In the clinical setting, elevated expression of the antiapoptotic protein Mcl-1 is observed at the time of leukemic relapse, suggesting that overexpression of Mcl-1 may be associated with resistance to chemotherapy (37). Conversely, reduced expression

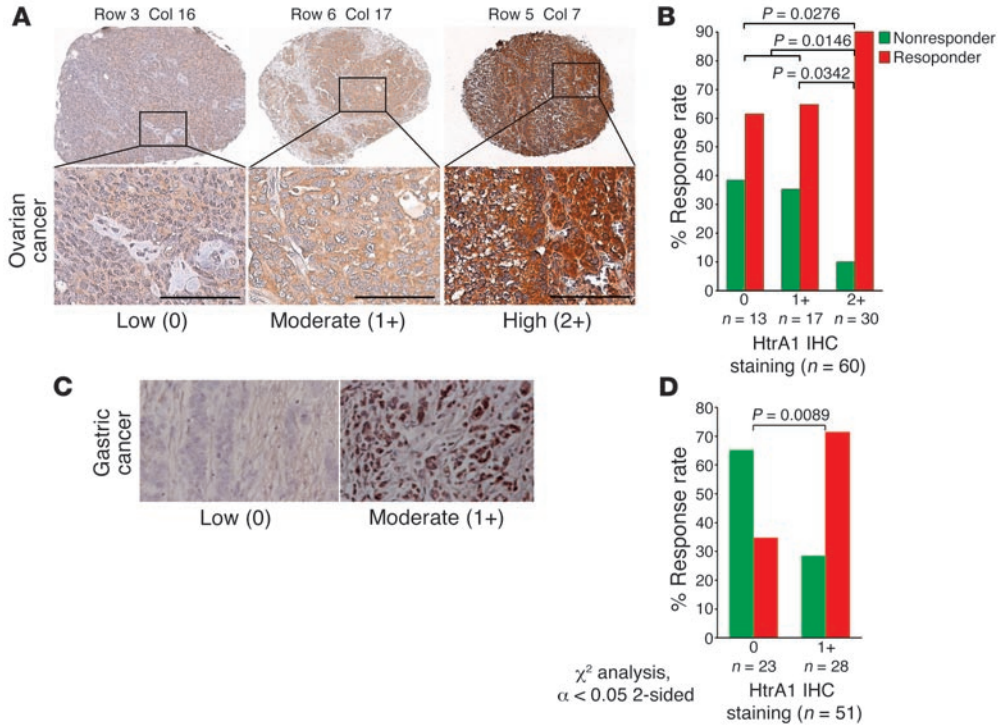

Figure 5

Expression of HtrA1 correlates with patient response to chemotherapy. (**A**) Immunohistochemical analysis of HtrA1 expression in primary ovarian tumors on TMA showing low (0), moderate (1+), and high (2+) levels of HtrA1 staining. A small section of TMA with 3 representative HtrA1 expression levels is shown. Scale bars represent approximately 100 μm. (**B**) Univariate analysis of chemoresponse showing a significant association between HtrA1 expression and chemoresponse in ovarian cancer. (**C**) Immunohistochemical analysis of HtrA1 expression in primary gastric tumors showing low (0) and moderate (1+) levels of HtrA1 staining. Original magnification, ×250. (**D**) Univariate analysis of chemoresponse showing significant association between HtrA1 expression and chemoresponse in gastric cancer; 2-sided χ^2 analysis, $\alpha = 0.05$.

of the proapoptotic protein Bax is associated with poor response rate to chemotherapy in women with metastatic breast cancer (13). These results suggest that alterations in programmed cell death and survival pathways can alter chemosensitivity. Consistent with these results, we showed that downregulation of HtrA1 in ovarian cancer cells produces resistance to 2 disparate agents in vitro. Furthermore, HtrA1 expression in ovarian cancer cell lines promotes drug sensitivity in vitro and is associated with a better response to chemotherapy in vivo. The concentrations of cisplatin and paclitaxel used in our in vitro studies are within the limit of the therapeutic doses achieved in patient serum (38, 39). Therefore, our results suggest that downregulation of HtrA1 in ovarian cancer may represent an additional mechanism of chemoresistance.

Downregulation of HtrA1 was also predictive of lower response rates to platinum-based regimens in patients with gastric cancer. The observation that most biological markers in gastric cancer, although promising, have failed to correctly predict response to cisplatin-based therapy (40) heightens the significance of the current results. In general, HtrA1 expression in gastric specimens was weaker than that in ovarian specimens. This result is consistent with the Northern blot analysis of HtrA1 expression in multiple tissues, which showed higher levels of HtrA1 expression in ovary compared with small intestine, colon, and stomach (16, 41). These results reflect tissue-specific variation in expression of HtrA1. Due to the relatively low levels of HtrA1 expression in gastric cancer, we scored gastric specimens as absent/low (0) and moderate (1+). Accordingly, we compared chemoresponse rate in the absent/low and moderate gastric cancer groups. In gastric cancer, moderate (1+) staining was associated with better response to chemotherapy, whereas in ovarian cancer, intense (2+) staining was associated with better response to chemotherapy. These data highlight the need to take into consideration differences in tissue-specific expression in determining response to chemotherapy in different tumor types and may reflect differences in responsiveness to HtrA1 expression due to intrinsic levels of serine protease inhibitors in these different tissues.

Although the present study focuses on cisplatin and paclitaxel, the role of HtrA1 may not be limited to these agents. We have observed upregulation of HtrA1 by a number of anticancer agents, including doxorubicin, gemcitabine, and etoposide, in the breast cancer cell line MCF7 and the ovarian cancer cell line SKOV3, implicating HtrA1 as a stress-induced protein regardless of cell type (data not shown). These results are consistent with previous studies suggesting that both prokaryotic and mammalian HtrAs are stress-related proteins (17). Bacterial HtrA proteins are important in providing a survival advantage to cells under heat stress by clearing deleterious unfolded proteins. Similarly, mammalian HtrA2 provides protein quality control within mitochondria; and loss of HtrA2 function is thought to lead to accumulation of misfolded and damaged proteins in mitochondria, resulting in mitochondrial dysfunction and subsequent cell death (42). In contrast, our results indicate that the presence of HtrA1 confers cisplatin and paclitaxel sensitivity by participating in effector caspase activation and cell

Table 2
Gastric cancer patient characteristics

No. of patients	51
Median age, years (range)	64 (46–79)
Sex, M/F	32/19
Previous surgery, *n*	
Partial gastrectomy	25
Total gastrectomy	26
Site of primary tumor, *n*	
Gastroesophageal junction	11
Body	17
Distal stomach	21
Anastomosis	2
Tumor classification, *n*	
pT2	7
pT3	36
pT4	8
Lymph node status, *n*	
N0	9
N1	24
N2	14
N3	4
Grading, *n*	
1	0
2	8
3	37
NS	6
Histology, *n*	
Adenocarcinoma	38
Signet ring cell	9
Undifferentiated carcinoma	2
Other	2
Lauren classification, *n*	
Intestinal type	26
Diffuse type	25
No. of metastatic sites, *n*	
1	31
2	16
3–4	4
Sites of metastasis, *n*	
Stomach/local relapse	16
Liver	17
Lymph nodes	21
Peritoneum	13
Other	8

death. This apparent paradox can be explained if HtrA1 has 2 distinct functions: (a) under normal physiological conditions it may be required for providing protein quality control similar to human HtrA2 or bacterial HtrAs; and (b) under stressful conditions, when it accumulates in sufficient quantities to autoactivate by N-terminal cleavage of the Kazal inhibitory domain, it digests substrates that lead to caspase activation, thus contributing to chemotherapy-induced cytotoxicity. Such dual roles in normal homeostasis and apoptosis have been suggested for several proteins, such as HtrA2 (42), apoptosis-inducing factor (43), and caspases (44–47). It is possible that chaperone proteases such as HtrA1 and HtrA2 participate not only in the proper removal of damaged proteins but also in the proper removal of damaged cells.

A recent report by Cilenti et al. that the HtrA2 protease also participates in cisplatin-induced cell death in renal cells lends additional support to the dual roles of HtrA serine proteases in stress responses and apoptosis (27). Upregulation of HtrA2 by cisplatin is followed by the release of HtrA2 from mitochondria to the cytoplasm and the degradation of inhibitor of apoptosis proteins. This mechanism still requires some trigger of mitochondrial permeabilization for HtrA2 to participate in cell death. In contrast, our data suggest that HtrA1 is upregulated during drug treatment, undergoes autocatalytic activation, and participates in cell death through its serine protease activity. While the possible substrates of HtrA1 in programmed cell death remain to be identified, these results are consistent with growing evidence suggesting a role of serine proteases in cell death (48–50).

Alterations in cell death pathways have been implicated in drug resistance (2, 10–12), and the results from our studies provide further evidence of the significance of cell death suppression in drug resistance. It should be noted that the increased drug resistance observed with loss of HtrA1 or an increased sensitivity observed with forced expression of HtrA1 do not confer absolute resistance or sensitivity to therapy. Rather, these are relative changes compared with those observed in control cells. However, these in vitro studies indicate that HtrA1 expression may contribute to a better therapeutic index of these agents. Consistent with this observation, analysis of HtrA1 expression in ovarian and gastric tumors indicates that low HtrA1 levels correlate with poor responses of 2 tumor types to chemotherapy. We therefore conclude that downregulation of HtrA1 in ovarian and gastric cancers may modulate sensitivity to various stresses, including chemotherapeutic drugs. The heterogeneity among individual tumors, even those with the same histological type, and individual patients, according to the different metastatic sites of the disease, may also be responsible for the magnitude of response to the same drugs. Therefore, a combination of potential markers of treatment response may contribute to the selection of favorable chemotherapeutic strategies, which could change the target approach from a general to an individual treatment strategy. Analysis of HtrA1 expression warrants further investigation in confirmatory studies and in different tumor histologies.

Methods

Cell culture, transfection, and drug treatment. SKOV3, OVCAR3, and OVCAR5 cells were purchased from ATCC and grown according to the provider's recommendation. OV167, OV177, OV202, OV266, and OSE(tsT) cell lines were established and grown as previously described (51, 52). A2780, Cp70, and C200 cells were grown as previously reported (53). Cells were transfected with plasmids using Lipofectamine Plus (Invitrogen) according to the manufacturer's recommendation. Cisplatin and paclitaxel were purchased from Sigma-Aldrich and dissolved in DMSO prior to use. Z-VAD-FMK was purchased from Calbiochem.

Antibodies. Polyclonal antibodies raised against a polypeptide corresponding to amino acids 161–480 of HtrA1 were affinity purified as previously described (54). Monoclonal antibody against β-actin was purchased from Sigma-Aldrich.

Transfection with siRNA. Scrambled control siRNA (5′-Fluo-UCCUGCUGGAGCCUCAUGUTT-3′) and HtrA1 siRNA targeting the 3′ UTR (5′-Fluo-CGGCCGAAGUUGCCUCUUUTT-3′) were purchased from Sigma-Aldrich and transfected at a final concentration of 0.25 μM in OptiMEM using Oligofectamine (Invitrogen). SKOV3 cells were plated at 20,000 cells/well in a 24-well plate or at 100,000 cells/well in a 6-well plate for transfection with siRNA. Effectiveness of RNA interference was determined by immunoblot after 2 days of transfection. Using standard conditions provided by the

manufacturer, we typically achieved greater than 90% reduction in HtrA1 expression after 2 days of transfection.

Immunoblotting. Cells were lysed in Laemmli buffer containing freshly added 2.5% β-mercaptoethanol, sonicated with Ultrasonic Processor (Cole-Palmer) at 40% output for 10 seconds, and boiled for 5 minutes. Whole-cell lysates were analyzed by Western blotting with anti-HtrA1 antibody (dilution 1:1,000) and anti–β-actin antibody (1:5,000).

Annexin V labeling and Hoechst staining. Labeling was performed as previously described (16). Hoechst staining was performed as previously described (55). After treatment with drugs for 24 hours, cells displaying apoptotic morphological changes (nuclear fragmentation and chromatin condensation) were counted. Percent apoptosis was expressed as the percentage of apoptotic cells in drug treatment groups minus the percentage of apoptotic cells in corresponding untreated control groups.

MTT survival assays. Cells were transiently transfected with various HtrA1 constructs for 24 hours. Vector GFP transfection indicated greater than 80% transfection efficiency using Lipofectamine Plus reagent. To quantify viable cell mass, cells were incubated for 1 hour with MTT by adding 20 μl of 5 mg/ml MTT into each well containing 100 μl culture medium. Cells were then washed with PBS and solubilized with DMSO. Absorbance at 570 nm was read; and survival was calculated as a percentage of untransfected controls.

Lactate dehydrogenase release assays. The release of lactate dehydrogenase (LDH) to culture medium from cells with damaged membranes was determined by measuring LDH activity in the medium in a 10-minute coupled enzymatic assay that results in the conversion of resazurin into fluorescent resorufin (CytoTox-ONE; Promega). LDH activity in the medium without any cells was measured and subtracted from each experimental value. Relative fluorescence units were obtained from each reaction performed in triplicate with a fluorometer and expressed as percent of UCN-01–treated cells.

Caspase assays. Caspase-Glo assay kits were purchased from Promega. These assays provide proluminogenic substrates selective for caspase-8 (Z-LETD-aminoluciferin), caspase-9 (Z-LEHD-aminoluciferin), and caspase-3/7 (Z-DEVD-aminoluciferin). These substrates are not suitable for luciferase assay until cleaved to release aminoluciferin by specific caspases. Eight hours after transfection or UCN-01 (20 μM) treatment, cells were lysed by adding equal volumes of assay buffer containing specific proluminogenic substrate and luciferase at 25°C for 30 minutes. RLU were obtained from each reaction performed in triplicate with a luminometer and expressed as percent increase or decrease relative to vector-transfected controls.

Clonogenic survival assays. Stable clones expressing WT, mutant HtrA1, control vector, or antisense cDNA were seeded at 2,000 cells/well overnight, treated with various concentrations of cisplatin or paclitaxel for 24 hours, washed, and incubated in drug-free medium for 2 weeks. Colonies were stained with Coomassie blue and counted as previously described (55).

LC-MS/MS analysis of 35-kDa HtrA1 catalytic product. Forty-eight hours after transfection with the WT full-length HtrA1 construct, 5×10^6 OV202 cells were lysed in 0.5 ml RIPA buffer (1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 10 mM Tris, pH 8.0, 0.14 M NaCl). Following sedimentation, the postnuclear supernatant was immunoprecipitated with 4 μg affinity-purified anti-HtrA1 polyclonal antibodies. Samples were resolved by SDS-PAGE and stained with Silver Stain Plus (Bio-Rad). The protein bands with apparent molecular mass of 50 kDa and 35 kDa were excised and digested with trypsin. NanoLC-MS/MS was performed on a Thermo Electron Corporation LCQ Deca ion trap mass spectrometer coupled with an Applied Biosystems 140D pump, with a 1:60 split into a New Objective ProteoPep C18 PicoFrit column (75 μm × 5.0 cm) mounted on the New Objective PicoView source. Peptides were trapped onto a 300 μm × 5 mm C18 trap (Dionex) prior to eluting with a gradient of 4% acetonitrile/0.1% formic acid to 40% acetonitrile/0.1% formic acid in 50 minutes. The LCQ was set to run in data-dependent triple-play mode consisting of full scan (400–1900 amu) and zoom scan, followed by MS/MS mode on the most intense ion. The MS/MS raw data were converted to DTA files using BioWorks 3.0 (Thermo Electron Corp.) and correlated to theoretical fragmentation patterns using the SEQUEST (University of Washington) and Mascot (Matrix Science Ltd.) search algorithms with tryptic peptide sequences from the NCBI nr (nonredundant) protein database.

TMA construction. After approval by the Mayo Clinic Institutional Review Board, archived ovarian epithelial tumor specimens from patients with advanced-stage, high-grade serous, or endometrioid tumors obtained prior to exposure to any chemotherapy were utilized to construct the TMA construction. The array was constructed using a custom-fabricated device that utilizes a 0.6-mm tissue corer and a 240-capacity recipient block. Triplicate cores from each tumor were included, as were cores of liver as fiducial markers and controls for immunohistochemistry reactions. Five-micrometer-thick sections were cut from the TMA blocks.

Immunohistochemistry. Immunohistochemistry for HtrA1 in ovarian and gastric cancer was performed essentially as previously described (56). The expression level of HtrA1 in gastric cancer was calculated and compared in different specimens by 2 separate observers in a double-blind fashion and described as: absent/low (0) or moderate (1+).

Digital imaging system for TMA construction analysis. Digital imaging was performed using a BLISS "Virtual Microscopy" microscope and computer system from Bacus Laboratories Inc. The system consists of a Zeiss Axioplan microscope with computer-interfaced electronic stage controls and a high-resolution 3CCD video camera. The Tracer program included in the BLISS system was used for automated slide scanning to create a virtual microscope slide. A pathologist who was blinded to clinical response absent/low reviewed the images and categorized staining into 3 categories, namely low (weak staining, 0), moderate (1+), and high (intense staining, 2+). A medical oncologist retrieved clinical information and analyzed whether HtrA1 expression influenced patient responses to chemotherapy.

Analysis of response in ovarian cancer. Contingency tables containing levels of HtrA1 staining and tumor responses to chemotherapy were generated, and 2-sided χ^2 analysis was performed using Prism 3.0 statistical software (GraphPad Software). Because of our interest in chemoresponsiveness, we sought to identify a homogeneous group of patients treated with a standard surgical approach. Thus, we identified a group of ovarian cancer patients that met the following criteria. All had advanced-stage disease. Most patients had undergone optimal debulking surgery. All were treated with platinum-based chemotherapy, and all had sufficient follow-up for the assessment of responsiveness to treatment. Cisplatin, Taxol, and Cytoxan were purchased from Bristol-Myers-Squibb. A total of 60 patients who met the above criteria and provided informed consent (see Table 1) were included in this study. Response evaluation was based on the following criteria: a CR was defined as the absence of disease during reassessment laparotomy or negative imaging and ovarian carcinoma antigen CA125 levels less than 35 IU assessed within 2 months after the completion of chemotherapy. PR was defined as presence of microscopic disease during reassessment laparotomy/laparoscopy. Nonresponders consisted of those with progressive or stable disease. Responders consisted of patients showing CR and PR.

Analysis of response in gastric cancer. The study samples consisted of consecutive tissue samples from patients treated from 1995 to 1998 with metastatic or recurrent gastric cancer with available archival tumor samples. The analysis was carried out on tumor tissue from the primary tumors. All patients had undergone a previous curative or palliative gastrectomy and had received a cisplatin-based chemotherapy as the first line of treatment for the advanced disease. The chemotherapy regimen consisted of weekly treatments with 40 mg/m^2 cisplatin, 35 mg/m^2 epirubicin, 250 mg/m^2 6S-leucovorin, 500 mg/m^2 5-fluorouracil, with the support of 5 μg/kg

filgrastim from day 2 to 7, or weekly treatment with 40 mg/m^2 cisplatin, 100 mg/m^2 6S-leucovorin, 500 mg/m^2 5-fluorouracil.

Evaluation of response was performed after 8 weekly cycles. Response evaluation was based on WHO criteria (57). In the case of local relapse, objective response was assessed by combining findings from both CT scan of the abdomen and endoscopy, including a new biopsy of the tumor, if still visible, or a biopsy of the area originally affected by the tumor. PR was defined as the presence of both CT scan evidence of tumor, according to the WHO criteria, and endoscopy showing a 50% reduction of the visible tumor; or complete disappearance of the tumor but positive histology on biopsy of the previously involved area. CR was defined as a complete disappearance of the tumor as seen by CT scan of the abdomen and a complete resolution of the endoscopic findings without histological evidence of neoplastic cells on biopsy of the original site of the tumor. The nonresponder patients were divided into 2 groups: those with stable disease and those with progressive disease.

Statistics. Two-tailed Student's *t* test, ANOVA followed by Newman-Keuls test, and 2-sided χ^2 analyses were performed using Prism 3.0 (GraphPad Software). $P < 0.05$ and $\alpha = 0.05$ were considered statistically significant.

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