Heat Shock Protein 70 Neutralization Exerts Potent Antitumor Effects in Animal Models of Colon Cancer and Melanoma

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Abstract
When overexpressed, the stress protein heat shock protein 70 (HSP70) increases the oncogenic potential of cancer cells in rodent models. HSP70 also prevents apoptosis, thereby increasing the survival of cells exposed to a wide range of otherwise lethal stimuli. These protective functions of HSP70 involve its interaction with and neutralization of the adaptor molecule apoptotic protease activation factor-1, implicated in caspase activation, and the flavoprotein apoptosis-inducing factor (AIF), involved in caspase-independent cell death. We have shown previously that a peptide containing the AIF sequence involved in its interaction with HSP70 (ADD70, amino acids 150-228) binds to and neutralizes HSP70 in the cytosol, thereby sensitizing cancer cells to apoptosis induced by a variety of death stimuli. Here, we show that expression of ADD70 in tumor cells decreases their tumorigenicity in syngeneic animals without affecting their growth in immunodeficient animals. ADD70 antitumorigenic effects are associated with an increase in tumor-infiltrating cytotoxic CD8+ T cells. In addition, ADD70 sensitizes rat colon cancer cells (ROb) and mouse melanoma cells (B16F10) to the chemotherapeutic agent cisplatin. ADD70 also shows an additive effect with HSP90 inhibition by 17-allylamino-17-demethoxygeldanamycin in vitro. Altogether, these data indicate the potential interest of targeting the HSP70 interaction with AIF for cancer therapy. (Cancer Res 2006; 66: 4191–7)

Introduction
Apoptosis is a cell death process frequently activated by anticancer drugs. Almost universally, apoptosis involves mitochondrial proteins, such as cytochrome c and apoptosis-inducing factor (AIF; ref. 1). These molecules, which are confined to the intermembrane space of living cells, can be released into the cytosol of stressed cells. Once in the cytosol, cytochrome c interacts with the adaptor molecule apoptotic protease activation factor-1 (ApaF-1) to trigger its ATP-dependent oligomerization (2, 3) and the formation of the so-called apoptosome complex (4, 5) in which caspase-9 is activated, setting on a caspase cascade that leads to apoptosis (3). In contrast to cytochrome c, AIF migrates to the nucleus where it induces DNA fragmentation and apoptosis in a caspase-independent way (6, 7). Replacement of endogenous cytochrome c by a mutant that lacks Apaf-1 binding site reduces caspase activation in response to stress yet has no major incidence on mouse development and cell-autonomous cell death regulation (8). Genetic inactivation of AIF abolishes the first wave of apoptosis that occurs during cavitation and is required for early embryonic morphogenesis (9), although it is not clear whether this effect is related to the lethal function of AIF or its contribution to reduct homeostasis and oxidative phosphorylation (10, 11).

Heat shock proteins (HSP) contribute to the tight regulation of the apoptotic process. The small stress protein HSP27 associates with cytochrome c when released from the mitochondria to the cytosol, thus preventing the formation of the apoptosome and subsequent activation of caspases (12, 13). HSP90 shows a similar antiapoptotic effect by associating with Apaf-1 to prevent the caspase cascade activation (14). The stress-inducible HSP70 neutralizes both the Apaf-1-mediated caspase-dependent and the AIF-mediated caspase-independent apoptotic pathways because it binds to Apaf-1 to prevent the recruitment of procaspase-9 to the apoptosome (15) and binds to AIF to prevent its nuclear redistribution and proapoptotic function (16, 17).

Under normal conditions, HSP70 functions as an ATP-dependent chaperone and assists the folding of newly synthesized proteins and polypeptides, the assembly of multiprotein complexes, and the transport of proteins across cellular membranes (18, 19). While hardly or no expressed in normal tissues and basal conditions, HSP70 is constitutively expressed in human tumor samples from various origins, and its expression may further increase after chemotherapy (20). HSP70 up-regulation, as a consequence of either oncogenic transformation or cellular stress, may inhibit apoptosis induced by a wide range of cellular insults, as suggested by transfection experiments in vitro (16, 21). HSP70 overexpression in cancer cells also increases their tumorigenicity in rodent models (22), and high HSP70 expression in human breast cancer, glioblastoma, endometrial, or renal tumors has been associated with metastasis, poor prognosis, and resistance to chemotherapy or radiation therapy (23–28). The down-regulation of HSP70 can be sufficient to kill tumor cells or to sensitize them to cytotoxic drug-induced apoptosis in vitro and to decrease their tumorigenicity in vivo (29, 30). For all these reasons, HSP70 seems as an interesting molecular target for sensitizing tumor cell to cancer therapy.

Targeting a HSP is an emerging concept in cancer therapy. For example, the HSP90 inhibitor 17-allylamino-17-demethoxygeldanamycin (17AAG) is currently tested for its chemosensitizing effects in phase II clinical trials, with encouraging results in melanoma cancers as a chemosensitizing agent (31). This derivative of geldanamycin, an ansamycin antibiotic isolated from Streptomyces hygroscopicus, associates with HSP90, thus affecting the function of signaling proteins whose structure depends on HSP90 (32). Concerning HSP70, we have recently shown the feasibility of a “positive” HSP70-targeting, chemosensitizing strategy, as opposed to “negative” strategies based on siRNA or antisense constructs.
Based on the before-mentioned demonstration that HSP70 specifically binds AIF to sequester it in the cytosol (33), we prepared a construct encoding the minimal AIF region required for HSP70 binding. This AIF derivative, which interacts with and thereby captures endogenous HSP70, was not cytotoxic yet displayed chemosensitizing properties in vitro (34).

In the present study, we explored the consequences of HSP70 neutralization by ADD70 in a rat colon carcinoma and a mouse melanoma in vivo. Our results indicate that ADD70 expression delays tumor growth and reduces the metastatic potential of these tumors in syngeneic animals. The peptide also sensitizes tumors to the commonly used anticancer drug cisplatin, which correlates with tumor infiltration with CD8+ T cells. In addition, the HSP90-targeting drug 17AAG was found to induce compensatory HSP70 expression in tumor cells, which could explain the additive chemosensitizing effect of ADD70 and 17AAG.

Materials and Methods

Cells, plasmids, and transfections. The rat PROb colorectal carcinoma cell line (35) and the mouse B16F10 melanoma cell line (36) were grown as monolayers in a controlled atmosphere (37°C, 5% CO2) using RPMI 1640 (Bio Whittaker, Fontenay-sous-Bois, France) supplemented with 10% (v/v) FCS. Cells were stably transfected with a cDNA coding for GFP-ADD70 or a green fluorescent protein (GFP) control construction. Transfections were done by using the Superfect reagent (Qiagen GmbH, Berlin, Germany). Potential pools of G418 (Sigma-Aldrich, Saint Quentin Fallavier, France)–resistant cells were analyzed for GFP expression by fluorescence-activated cell sorting analysis.

Immunoblotting. Whole-cell extracts were prepared by lysing the cells with 2% SDS, 137 mmol/L NaCl, 2.7 mmol/L KCL, 8 mmol/L Na2HPO4, and NaCl/Pi (pH 7.4; ref. 37). Protein concentration was measured in the supernatant by using the micro bichinic acid protein assay (Pierce, Anseries, France). Proteins were separated in a 8% to 12% SDS-polyacrylamide gel and electroblotted to polyvinylidene difluoride membranes (Bio-Rad, Ivory sur Seine, France). After blocking nonspecific binding sites with 5% nonfat milk in T-PBS (PBS, 0.1% Tween 20), blots were incubated with specific antibodies, washed in T-PBS, incubated 30 minutes at room temperature with horseradish peroxidase–conjugated goat anti-mouse antibody (Jackson Immunoresearch Laboratories, West Grove, PE), and revealed following the enhanced chemiluminescence Western blotting analysis procedure (Amersham, Les Ulis, France). All the Western analyses were repeated thrice.

Cell death and cell growth analysis in vitro. Adherent cells (10^6) were plated onto six-well culture plates in complete medium. Cells were treated with ciplatin (CDDP, 25 mmol/L) or staurosporin (100 nmol/L) for 24 hours. Cell death was measured by the crystal violet colorimetric assay (Pierce, Annies, France). Proteins were separated in a 8% to 12% SDS-polyacrylamide gel and electroblotted to polyvinylidene difluoride membranes (Bio-Rad, Ivory sur Seine, France). After blocking nonspecific binding sites with 5% nonfat milk in T-PBS (PBS, 0.1% Tween 20), blots were incubated with specific antibodies, washed in T-PBS, incubated 30 minutes at room temperature with horseradish peroxidase–conjugated goat anti-mouse antibody (Jackson Immunoresearch Laboratories, West Grove, PE), and revealed following the enhanced chemiluminescence Western blotting analysis procedure (Amersham, Les Ulis, France). All the Western analyses were repeated thrice.

Histologic study of the tumor cell injection site. Animals were killed 15 days after cell injection. The site of tumor cell injection was resected and snap-frozen in methylbutane that had been cooled in liquid nitrogen. The presence of tumor cells was confirmed by immunolabeling the slides with 12C, a mAb raised against PROb cells. An immunohistochemical study of tumor-infiltrating inflammatory cells was done on acetone-fixed 5-μm cryostat sections. Mouse mAbs to rat monocyes (ED2), dendritics cells (ED1), CD8+ cells (OX8), CD4+ cells (W3/25), NK cells (3-2-3), and TCR cells (R7/3) were obtained from Serotec (Oxford, United Kingdom). Sections were incubated with mAb and biotinylated sheep antibody to mouse IgG (Amersharm), subsequently incubated with streptavidin-peroxidase, and stained with aminoethylcarbazole. Two independent experiments were done in which five rats were injected with the different cells (therefore, results are in total from 10 rats for each group), and two observers have analyzed the different sections. Results are expressed with a semiquantitative score.

Results and Discussion

ADD70 reduces the tumorigenicity of rat colon cancer PROb and mouse melanoma B16F10 cells. Rat colon carcinoma PROb cells and mouse melanoma B16F10 cells were stably transfected with a pcDNA3 plasmid encoding ADD70-GFP or GFP alone before selection of GFP-positive cells by cell sorting (Fig. 1A). ADD70 did not significantly influence tumor cell growth in culture (Fig. 1B). We already reported that ADD70, by its ability to sequester HSP70, sensitizes cancer cells to anticancer drugs in vitro (34). In accordance with these results, ADD70 increased the sensitivity of PROb and B16F10 cells to apoptosis induced by exposure to the cytotoxic agent cisplatin (CDDP) and staurosporin (Fig. 1C). We then studied whether ADD70 could influence cancer cell growth in vivo. Parental (PROb), control-transfected (PROb-Control), and ADD70-transfected (PROb-ADD70) cells were injected s.c. into syngeneic BDIX rats (1 × 10^5 cells per rat). The two control populations yielded tumors that continuously progressed, whereas tumors generated by injection of PROb-ADD70 cells progressed for about 2 weeks and then stopped growing or partially regressed. An important delay was also observed in the growth of ADD70-expressing B16F10 cells (B16F10-ADD70) when injected s.c. (2 × 10^5 cells per mouse) to syngeneic C57/B16 mice compared with parental (B16F10) and control-transfected (B16F10-Control) cells injected in the same conditions induced tumorigenic tumors (Fig. 2). The three distinct populations of B16F10 (5 × 10^5 cells per mouse) cells were also injected i.v. to syngeneic mice, as B16F10 cells are known to generate a number of lung metastasis in this model. At day 21 after i.v. injection of B16F10 cells, the number of lung metastasis was significantly lower with B16F10-ADD70 cells (8 ± 1.5, n = 6) than with B16F10 and B16F10-Control cells (30 ± 7 and 50 ± 5, respectively, n = 6). Altogether, these data indicate that ADD70 exerts antitumorigenic and antimetastatic properties.

ADD70 enhances tumor cell immunogenicity. We next studied whether the immune system contributed to the antitumoral effect of ADD70. When injected s.c. into immunodecient nude rats, PROb, PROb-Control, and PROb-ADD70 yielded tumors of similar size (Fig. 3A). Similarly, B16F10, B16F10-Control, and B16F10-ADD70 cells proliferated indistinguishably when injected
into athymic nude (nu/nu) mice (Fig. 3B). This observation suggested that the decreased tumorigenicity of ADD70-expressing tumor cells could be related to the induction of an antitumor immune response. To evaluate this hypothesis, BDIX rats injected s.c. with PROb, PROb-Control, or PROb-ADD70 cells were rechallenged 21 days later with PROb cells, which were injected into the opposite flank. Although a second tumor seemed in every animal bearing a PROb or a PROb-Control tumor, none of the rats, even those initially injected with PROb-ADD70 cells, developed a second tumor (Fig. 3C). Conversely, when rechallenged with GV1A1 glioma cells instead of PROb cells, all the BDIX rats developed a second progressive tumor, including those initially injected with PROb-ADD70 cells (Fig. 3C). Altogether, these results indicate that ADD70 facilitated the induction of a tumor cell–specific immune response.

ADD70 induces an increase in the number and cytotoxic activity of CD8+ tumor-infiltrating T cells. We then did an extensive immunohistochemical analysis of tumor sections obtained at day 15 after PROb cells injection in BDIX rats. Tumor cells were identified by using the 12C mAb. When compared with

Figure 1. ADD70 sensitizes rat colon carcinoma PROb and mouse melanoma B16F10 cells to apoptosis. A, GFP expression in PROb and B16F10 cells stably transfected with pcDNA3 vector encoding ADD70-GFP (ADD70) or GFP (Control) as determined by flow cytometry analysis. Full curves, untransfected cells. Empty curves, transfected cell populations. Points, mean of three independent experiments; bars, SD < 3%. B, growth curves of parental (○), GFP-transfected (□), and ADD70-GFP–transfected (●) PROb (left) and B16F10 (right) cell populations. Points, mean of three independent experiments; bars, SD. *, P < 0.05; **, P < 0.005 versus the two control cell populations (PROb and PROb-Control).

Figure 2. ADD70 decreases the size of colon PROb and melanoma B16F10 tumors in syngeneic animals. A, a total of 10^6 wild type PROb cells (□), PROb-Control (○), and PROb-ADD70–transfected cells (●) were injected s.c. on day 0 into BDIX rats (nine rats per group). B, B16F10-wt (□), B16F10-control (○), and B16F10-ADD70–transfected cells (●) were injected s.c. on day 0 into C57/B16 mice (2 × 10^5 cells per mouse; 10 mice per group). The tumor size was measured at the indicated times points. Points, mean (X) tumor volumes; bars, SD. *, P < 0.05; **, P < 0.005 versus the two control cell populations (PROb and PROb-Control, □ and ○).
tumor nodules induced by PROb and PROb-Control cells, those induced by PROb-ADD70 cells exhibited a stronger infiltration by CD8$^+$ T cells (identified as OX8$^+$ cells; Table 1; Fig. 4A). Although there was a slight increase in the number of tumor-infiltrating R7/3$^+$ (TCR) cells in PROb-ADD70 tumors compared with PROb and PROb-Control tumors, there were no difference for tumor infiltration by NK cells (identified by using the 3-2-3 Ab), macrophages (identified as ED2$^+$ cells), monocyctic/dendritic cells (identified as ED1$^+$ cells), and CD4$^+$ T cells (identified as W3/25$^+$ cells; Table 1; Fig. 4A). CD8$^+$ (OX8$^+$) T cells isolated from PROb-ADD70 tumors developed in BDIX rats were observed to be more cytotoxic than those isolated from PROb and PROb-Control tumors grown in the same conditions (Fig. 4B). In contrast, NK (3-2-3$^+$) cells isolated from PROb-ADD70, PROb, and PROb-Control tumors

Table 1. Immunohistochemical analyses of tumor-infiltrating inflammatory cells

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NOTE: Semiquantitative evaluation of antigen expression and location in tumor sections studied 15 days after s.c. injection of 1 × 10$^6$ PROb, PROb-Control, and PROb-ADD70 cells into syngeneic rats. Two independent experiment were done (each in five rats), and results were analyzed by two observers. −, not labeled cells; ±, 0% to 10% labeled cells; +, 10% to 20% labeled cells; ++, 20% to 40% labeled cells; ++++, 40% to 60% labeled cells; ++++, 60% to 80% labeled cells; +++++, 80% to 100% labeled cells.
exhibited an almost comparable cytotoxic activity (Fig. 4B). These results suggested that CD8+ T cells that infiltrate PROb-ADD70 tumors in syngeneic animals may play a role in the decreased tumorigenicity of cells expressing this peptide.

**ADD70 sensitizes tumor cells to cisplatin in vivo.** To determine whether the chemosensitizing effect of ADD70 observed in vitro (Fig. 1C) was also observed in vivo, BDIX rats bearing PROb-Control and PROb-ADD70 tumors were treated with cisplatin (4 mg/kg) given i.p. as a single dose either at day 21 or at day 35 after tumor cells injection. As shown in Fig. 5A, cisplatin treatment at day 21 inhibited the growth of control tumors (PROb-Control), whereas it provoked a complete regression of PROb-ADD70 tumors. However, this chemosensitizing effect of ADD70 was lost when cisplatin was administered at day 35, indicating a temporal restriction of the therapeutic properties of ADD70. Similar results were obtained in the mouse B16F10 melanoma model. Cisplatin treatment (10 mg/kg, i.p.) could induce a partial regression of B16F10-ADD70 tumors, whereas it only delayed the growth of B16F10-control tumors. Again, this effect was observed only when cisplatin was administered early (at day 4) yet disappeared when the drug was given later (at day 8; Fig. 5B). When immunodeficient nude rats bearing either PROb-ADD70 or PROb-Control tumors were treated with cisplatin (at day 21 after tumor cell injection), we found a similar reduction in the size of all the tumors, expressing or not ADD70 (data not shown). Thus, an immune response is necessary for both the antitumor activity of ADD70 and its chemosensitizing properties in vivo.

ADD70 increases the chemosensitizing effect of the HSP90 inhibitor 17AAG. Although HSP70 inhibition seems as an attractive strategy for treating cancers, the only chemical inhibitor of HSPs currently available for clinical use, 17AAG, targets HSP90. The efficacy of 17AAG could be limited by its ability to induce HSP70 expression (38). Accordingly, we observed that both PROb cells and B16F10 cells exposed to 17AAG exhibited an increase in HSP70 expression (Fig. 6A). As expected, 17AAG did not induce any change in HSP90 cellular content as it binds the protein to neutralize it without inducing its degradation (39), just as ADD70 does with HSP70 (34). The combination of 17AAG and cisplatin

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**Figure 4.** ADD70 induces changes in tumor infiltration. A, tumor sections were done 15 days after s.c. injection of PROb, PROb-Control, or PROb-ADD70 cells into syngeneic BDIX rats. CD8+ lymphocytes, NK, and tumor cells were labeled using OX8, 3-2-3, and 12C antibodies, respectively. B, PROb parental cells were co-cultured at a ratio of 1:5 ratio with CD8+ (OX8 positive) T cells or NK (3-2-3 positive) cells recovered from parental PROb (gray columns), PROb-Control (white columns), or PROb-ADD70 (black columns) tumors (nine tumors per group). After 48 hours, tumor cell viability was measured by the crystal violet colorimetric assay. Columns, mean (n = 4); bars, SD. *, P < 0.05; **, P < 0.005 versus the two control cell populations (PRObs and PROb-Control).

**Figure 5.** ADD70 sensitizes tumor cells to cisplatin treatment. A, PROb-Control (□, ○, and △) and PROb-ADD70 (■, ■, and ▲) were injected s.c. into BDIX rats. The animals were either left untreated (● and □) or treated with cisplatin (4 mg/kg) at day 21 (▲ and △) or at day 35 (■ and ■) after cell injection. Tumors were measured every week (nine rats per group). B, B16-control (□, ○, and △) and B16F10-ADD70 (■, ■, and ▲) transfected cells were injected s.c. into C57/bl6 mice. The mice were either left untreated (● and □) or treated with cisplatin (10 mg/kg) at day 4 (▲ and △) or at day 8 (■ and ■) after cell injection (eight mice per group). *, P < 0.05 versus nontreated animals.
exerted an additive effect in both PROb and B16F10 cell lines, as already described (40). In the presence of ADD70, the cytotoxic effect of the combination was strongly enhanced (Fig. 6B). These results indicate that ADD70 enhances the chemosensitizing effect of 17AAG, suggesting a great interest for simultaneous targeting of both HSP70 and HSP90 in chemosensitizing strategies for anticancer treatment.

Conclusion

The development of new anticancer drugs requires identification of novel molecular targets. These molecules can either be cytotoxic by themselves or sensitize tumor cells to existing treatment modalities. Because tumor cells exhibit considerable genetic plasticity and may quickly adapt to the cytotoxicity of specific anticancer agents, anticancer strategies must combine several distinct molecules to reach multiple cellular targets. Drugs that target HSPs have recently emerged as potential anticancer agents because several of these proteins are key mediators of the cellular response to damage induced by environmental stressors (41). The only drug thus far available, and used in clinical trials of phase II is 17AAG, a geldanamycin derivative that targets HSP90. Unfortunately, no drugs are available to neutralize HSP70, a HSP that intercepts the main apoptotic pathways that are activated by classic anticancer drugs, either dependent or independent of caspase activation (15, 16). HSP70 has also been reported to display an immunogenic role. Cytosolic HSP70 purified from distinct tumors can elicit tumor-specific immunity by functioning as a vehicle for immunogenic and antiapoptotic properties of HSP70 may predominate on its immunogenic role. Cytosolic HSP70 purified from distinct tumors can elicit tumor-specific immunity by functioning as a vehicle for antigenic peptides (42). The immunogenic and antiapoptotic functions of HSP70 may have opposite effects because tumor cell death has been shown to play a central role in inducing a specific immune response (35). However, we and other groups have shown that whereas overexpression of HSP70 did not have any effect in the cells’ immunogenic properties, depletion of HSP70, by favoring cell death, did induce a specific immune antitumor response (29, 30). These observations, further confirmed by the present work in which HSP70 is neutralized, indicated that the antiapoptotic properties of HSP70 may predominate on its previously reported ability to favor the generation of an antitumor immune response (42).

To validate the potential interest of targeting HSP70, we designed a construct encoding an AIF-derived peptide that binds to and neutralizes HSP70 in the cytosol yet does not exert any toxic effect by itself (33, 34). We showed that ADD70 sensitized immortalized cells to apoptosis in response to many different cytotoxic agents tested in vitro. This sensitizing effect was related to the inhibition of AIF sequestration by HSP70. ADD70 sequesters inducible HSP70 as it has no chemosensitizing effect in cells deficient for the two genes encoding inducible HSP70 (HSP70.1 and HSP70.3; ref. 34). Interaction of ADD70 with HSP70 favors caspase-independent cell death mediated by the nuclear effects of AIF. It could also favor caspase-dependent apoptosis due to the ability of AIF to indirectly facilitate the mitochondrial release of cytochrome c and other apoptogenic molecules. It is also possible that the interaction of HSP70 with ADD70 affects the chaperone interaction of HSP70 with Apaf-1 (34). Interestingly, inducible HSP70 is often constitutively expressed in cancer cells, whereas it is not or hardly expressed in normal cells, suggesting a great specificity of strategies targeting HSP70 towards cancer tissues and limited side effect towards normal tissues.

In the present study, we observed that in two distinct models of tumors developed in syngeneic rodents, ADD70 mediated an antitumor effect. This effect was related to a T cell–mediated, tumor cell–specific immune response, possibly mediated by CD8+ T cells. In addition, expression of ADD70 was observed to enhance tumor sensitivity to the cytotoxic drug cisplatin in vivo and to stimulate the chemosensitizing properties of 17AAG in vitro. Because molecules that selectively inhibit HSP70 have not been identified thus far, we are currently selecting peptide aptamers that mimic the effect of ADD70, to facilitate the screening of small molecules that inhibit HSP70 and could be tested in clinical trials. Whether such agents
would be as efficient as ADD70 to neutralize HSP70 and to chemosensitize cancer cells awaits further investigation.

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References
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