Hepatocyte Growth Factor/Scatter Factor (HGF/SF) Blocks the Mitochondrial Pathway of Apoptosis Signaling in Breast Cancer Cells

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ABSTRACT

The cytokine hepatocyte growth factor/scatter factor (HGF/SF) has been found to protect a variety of epithelial and cancer cell types against cytotoxicity and apoptosis induced by DNA damage, but the specific apoptotic signaling events and the levels at which they are blocked by HGF/SF have not been identified. We found that treatment of MDA-MB-453 human breast cancer cells with adriamycin (aka. doxorubicin, a DNA topoisomerase IIα inhibitor) induced a series of time-dependent events, including the mitochondrial release of cytochrome c and apoptosis-inducing factor (AIF), mitochondrial membrane depolarization, activation of a set of caspases (-9, -3, -7, -2, and -8), cleavage of PARP [poly (ADP-ribose) polymerase], and up-regulation of expression of Fas ligand. All of these events were blocked by pre-incubation of the cells with HGF/SF. In contrast, the pan-caspase inhibitor z-VAD-fmk blocked some of these events (eg., caspase-3 activation and PARP cleavage), but did not block cytochrome c release or mitochondrial depolarization. These findings suggest that HGF/SF functions, in part, upstream of the mitochondria, to block mitochondrial apoptosis signaling, prevent activation of multiple caspases, and protect breast cancer cells against apoptosis.
INTRODUCTION

The cytokine hepatocyte growth factor/scatter factor [HGF/SF] stimulates cell motility, invasion, and proliferation, and induces epithelial morphogenesis and angiogenesis, in different cellular contexts (1-6). These biologic actions are mediated by the HGF/SF receptor, c-Met, a transmembrane tyrosine kinase (7). Previous studies indicate that the expression of HGF/SF and its receptor (c-Met) increase significantly during progression of breast cancer, gliomas, and other tumor types, and that high levels of HGF/SF are associated with invasion, angiogenesis, and poor prognosis (8-12). We found that in addition to stimulating cell invasion and angiogenesis, HGF/SF protects various epithelial and carcinoma cell types against apoptosis and cytotoxicity induced by DNA damaging agents (13). Pre-incubation with HGF/SF (50-100 ng/ml) strongly protected Madin-Darby canine kidney (MDCK) epithelial cells and breast cancer cell lines (MDA-MB-453 and EMT6) against toxicity and apoptosis caused by adriamycin (ADR, a DNA topoisomerase IIα inhibitor), X-rays, and UV light. Cell protection was demonstrated in multiple assay systems reflecting different end-points: MTT dye conversion (mitochondrial integrity), trypan blue dye exclusion (membrane integrity), colony formation (reproductive viability), and DNA ladder formation (apoptosis).

Maximal protection of MDA-MB-453 human breast cancer cells against ADR required pre-incubation with HGF/SF for ≥ 48 hr (13). When HGF/SF was added during exposure to ADR and thereafter, there was no cell protection. A clue to HGF/SF-mediated protection was the finding that ADR caused the disappearance of the survival-promoting protein Bcl-XL, while pre-incubation with SF blocked the ADR-induced loss of Bcl-XL. Interestingly, the alterations of Bcl-XL occurred at the protein level, since subsequent studies revealed that neither ADR, nor HGF/SF caused any changes in Bcl-XL mRNA expression. But like HGF/SF cell protection, the ability of HGF/SF to prevent the ADR-induced loss of Bcl-XL protein required a prolonged pre-incubation with HGF/SF. Thus, HGF/SF-induced transcriptional alterations during the 48 hr pre-incubation may contribute to stabilization of Bcl-XL protein and cell protection.

Similar to epithelial and carcinoma cells, HGF/SF protected glioma cell lines against apoptosis induced by ionizing radiation, cis-platinum (a DNA cross-linking agent), camptothecin (a topoisomerase I inhibitor), and etoposide (a topoisomerase IIα inhibitor) (14). The protection of epithelial, breast cancer, prostate cancer, and glioma cell lines against DNA damage-induced apoptosis was due, at least in part, to activation of a cell survival pathway involving phosphatidylinositol-3-kinase (PI3 kinase), c-Akt (protein kinase B), and signaling down-stream of c-Akt (14-16). This HGF/SF induced c-Met → PI3K → c-Akt pathway also resulted in an increased rate of DNA repair (ie., strand break rejoining); and both the HGF/SF mediated cell protection and the HGF/SF enhancement of DNA repair were abrogated or significantly attenuated by the expression of a dominant negative (kinase inactive) c-Akt expression vector (15).

These studies have not directly addressed the mechanism(s) of HGF/SF-mediated cell protection at the level of the specific component(s) of the apoptotic cascade that are activated by DNA damaging agents like ADR. Nor have they identified the apoptosis signaling pathways that are blocked by HGF/SF. The goal of the present study was to identify the specific pathway(s) of ADR-induced cytotoxicity and apoptosis in a human breast cancer cell line that is protected by HGF/SF (MDA-MB-453) and to determine which component(s) of the apoptosis pathway are blocked by
HGF/SF.
MATERIALS AND METHODS

Sources of Reagents and Antibodies. Recombinant human two-chain HGF/SF was generously provided by Dr. Ralph Schwall, Department of Endocrine Research, Genentech, Inc. (South San Francisco, CA). Adriamycin (doxorubicin hydrochloride) and MTT dye (thioazyl blue) were purchased from Sigma Chemical Co. (St. Louis, MO). The primary antibodies utilized in this study for Western blotting are listed in the Table below.

| Primary Antibody | Antibody Type | Manufacturer | Catalog # | Dilution |
|------------------|---------------|--------------|-----------|----------|
| α-Actin (I-19)   | goat polyclonal IgG | Santa Cruz Biotechnology, Inc., Santa Cruz, CA | Sc-1616 | 1:1000 |
| AIF (N-19)       | goat polyclonal IgG | "            | Sc-9417   | 1: 200   |
| Apaf-1 (N-19)    | goat polyclonal IgG | "            | Sc-7232   | 1: 400   |
| Bid (C-20)       | goat polyclonal IgG | "            | Sc-6538   | 1: 200   |
| Caspase-3 P20 (N-19) | goat polyclonal IgG | "            | Sc-1226   | 1: 400   |
| Caspase-8 (T-16) | goat polyclonal IgG | "            | Sc-6134   | 1: 400   |
| Caspase-9 (H-83) | rabbit polyclonal IgG | "            | Sc-7885   | 1: 400   |
| Caspase-7        | rabbit polyclonal IgG | R & D Systems, Minneapolis, MN | AF823 | 1: 600 |
| Caspase-10       | rabbit polyclonal IgG | "            | AF834     | 1: 900   |
| Cox 4            | mouse monoclonal IgG | Molecular Probes, Eugene, OR | A6431 | 1:1000 |
| Cytochrome c     | mouse monoclonal IgG | Pharmingen, San Diego, CA | 556433   | 1:1000   |
| Fas (N-18)       | rabbit polyclonal IgG | "            | Sc-714    | 1: 200   |
| FasL (C-20)      | rabbit polyclonal IgG | "            | Sc-957    | 1: 200   |
| PARP (Ab-2)      | mouse monoclonal IgG | Oncogene Research Products, Boston, MA | AM30-100UG | 1:1000 |

The caspase and other inhibitors used in this study are listed in the Table below.

| Inhibitor Name | Specificity | Manufacturer | Catalog # | Concentration (ìM) |
|----------------|-------------|--------------|-----------|-------------------|
| z-VAD-fmk      | pancaspase  | R & D Systems, Minneapolis, MN | FMKC01 | 150   |
| ALLN           | calpain, other proteases | Sigma, St. Louis, MO | A-6060 | 100  |
| DEVD-CHO       | caspase-3 | CalBiochem, La Jolla, CA | 235423 | 150  |
| IETD-CHO       | caspase-8 | "            | 218773    | 150  |
| LEHD-CHO       | caspase-9 | "            | 218776    | 150  |

Cell Lines and Culture. MDA-MB-453 human breast cancer cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with fetal calf serum (5% v/v), non-essential amino acids (100 mM), L-glutamine (5 mM), streptomycin (100 ìg/ml), and penicillin (100 units/ml) (all from BioWhittaker, Walkersville, MD) (13,15). Cells were grown at 37°C in a humidified atmosphere of 95% air and 5% CO2.
Adriamycin [ADR] Treatment. Subconfluent proliferating cells in 100 mm plastic dishes or 96-well plates were pre-incubated ± HGF/SF (100 ng/ml x 48 hr) in serum-free DMEM and then sham-treated (control) or treated with ADR (10 µM x 2 hr, at 37°C) in complete culture medium (DMEM plus 5% fetal calf serum). Cultures were then washed twice to remove the ADR, post-incubated in fresh drug-free complete culture medium at 37°C for up to 72 hr. Cells were harvested at different time points for Western blot analyses or at T=72 hr for MTT assays of cell viability (see below).

MTT Cell Viability Assay. This assay is based on the ability of viable mitochondria to convert MTT, a soluble tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide] into an insoluble formazan precipitate, which is dissolved in dimethyl sulfoxide and quantitated by spectrophotometry (17). To test the effects of HGF/SF or of other agents on ADR-induced cytotoxicity, subconfluent proliferating cells in 96-well dishes were pre-incubated with HGF/SF (100 ng/ml X 48 hr), exposed to ADR (10 µM x 2 hr, at 37°C), and post-incubated for 72 hr in fresh ADR-free medium (13,15). The cells were then assayed for MTT dye conversion, a measure of mitochondrial function. Cell viability was calculated as the amount of MTT dye conversion relative to that of sham-treated control cells.

Western Blotting

Mitochondrial Separation. Cells were harvested using trypsin and washed two times with phosphate-buffered saline (PBS). Cell fractionations were performed essentially as described before (18). The cell pellet was resuspended in digitonin lysis buffer (75 mM NaCl, 1 mM NaH$_2$PO$_4$, 8 mM Na$_2$HPO$_4$, 250 mM sucrose, 190 µg/ml digitonin; 150 µl per 100 mm dish) and incubated on ice for 5 min. The cells were spun for 5 min at 14,000 rpm at 4°C, and 1.5 µl of protease inhibitor (Protease Inhibitor Cocktail Set I, CalBiochem) was added. The pellet was re-suspended in 80 µl of RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, in PBS) with protease inhibitor (1:100 dilution), incubated on ice for 30 min, and then spun for 15 min at 14,000 rpm at 4°C. The supernatant was designated as the "cytosolic fraction", while the pellet, which contains the mitochondria, was designated the "pellet fraction".

Whole Cell Lysates. Cells were collected using trypsin, washed two times with PBS, and then pelleted by centrifugation. The pellet was re-suspended in RIPA buffer, incubated on ice for 30 min, and spun for 15 min at 14,000 rpm at 4°C to remove the insoluble material.

Western Analysis. Western blot analyses were performed essentially as described earlier (13,15). Equal aliquots of whole cell lysate protein (50 µg per lane) or cytosolic vs pellet fractions (20 µg of each per lane) were electrophoresed on 4-13% SDS-polyacrylamide gradient gels or 15% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Millipore). The membranes were blotted using the primary antibodies listed above and with the appropriate secondary antibodies. The blotted proteins were visualized using the enhanced chemiluminescence detection system (Amersham), with colored markers (BioRad) as size standards.

Mitochondrial Membrane Potential. Mitochondrial membrane potential was assessed using DePsipher Assay kits, as per the manufacturer’s instructions (R & D Systems, Minneapolis, MN). measured Briefly, cells were harvested, counted, and washed twice with PBS. The cell pellets corresponding to 1 x 10$^6$ cells per assay were then incubated with 1.0 ml of DePsipher solution at 37°C for 15 min and...
re-suspended in 0.5 ml of reaction buffer. The cells were observed immediately by fluorescence microscopy, using a Nikon UFX-II microscope with band-pass filters for fluorescein and rhodamine. In healthy cells, the mitochondria appear red following aggregation of the DePsipher within the mitochondria. The red aggregates emit visible light at 590 nm. In dying cells or cells with disrupted mitochondrial membrane potential, the dye remains in its monomeric form in the cytoplasm and will appear green with an emission wavelength of 530 nm.
RESULTS

Role of mitochondria in ADR-induced toxicity. The basic experimental design for these studies is illustrated below:

| MDA-MB-453 cells (Subconfluent, Proliferating) | ± HGF/SF (100 ng/ml) | ADR (10 µM) | - HGF/SF (100 ng/ml) | Post-Incubate 2-72 hr | Assay End-Points: |
|-----------------------------------------------|-----------------------|-------------|-----------------------|-----------------------|------------------|
| Pre-Incubate x 48 hr                          |                        | Post-Incubate 2-72 hr |                        | - cytochrome release, caspase activation |
|                                               |                        | x 2 hr       |                        | - cell viability (MTT) |

To assess the function of the mitochondria in ADR-induced apoptosis and in HGF/SF-mediated protection against ADR, MDA-MB-453 human breast cancer cells were pre-incubated without (-) or with (+) HGF/SF, exposed to ADR, washed twice to remove the drug, and post-incubated for up to 72 hr in fresh drug-free medium, without or with HGF/SF, respectively. Using this protocol, cell viability at T=72 hr post exposure to ADR, as determined by MTT assays, is about 20% in cells treated with ADR alone and about 90% in cells pre-incubated with HGF/SF for 48 hr prior to treatment with ADR. After the 72 hr post-ADR incubation, the cells were harvested and processed to separate the cytosol from the particulate fraction (pellet), which includes the mitochondria.

Cytochrome c is normally localized in the space between the outer and inner mitochondrial membranes, and is only released into the cytosol in the setting of apoptosis (19). As demonstrated in Fig. 1, cytochrome c could be detected in the cytosolic fraction (supernatant) from ADR-treated cells at T=72 hr after exposure to ADR. However, pre-treatment with HGF/SF fully blocked the release of cytochrome c into the cytosol. Similar to cytochrome c, the 55 kDa apoptosis-inducing factor (AIF), an oxidoreductase also implicated as a potential mediator of nuclear apoptosis (20), was detected in supernatants from ADR-treated cells, but was not found in supernatants from [HGF/SF+ADR]-treated cells. As a control for the integrity of the fractionation procedure, Cox 4 - which is localized in the inner mitochondrial membrane - was not detected in the supernatant under any conditions. On the other hand, α-actin was detected in both the pellet and the supernatant; and the quantities of α-actin protein were not affected by any of the cell treatments.

Fig. 2 shows the time course for the release of cytochrome c and AIF from the mitochondria of ADR-treated MDA-MB-453 cells. Neither protein was detected in the cytosol at T=2 hr or 8 hr post ADR treatment, but both proteins were detectable in the cytosol at T=24 hr. A faint cytochrome c band in the cytosolic fraction could be detected at T=16 hr (panel C), suggesting that this is the earliest time by which significant amounts of cytochrome c are released from the mitochondria. The cytosolic levels of cytochrome c and AIF both increased, roughly in parallel, at T=48 hr and T=72 hr. On the other hand, the cytosolic levels of heat shock protein 27 (HSP27) - which has been reported to inhibit the activation of procaspase-9 (21), were unaffected by cell treatments with ADR and/or HGF/SF.

Caspase activation in cells treated with ADR alone or [HGF/SF+ADR]. To assess the potential role of caspases in ADR-induced apoptosis, cells were treated as above, and they were harvested at T=72 hr post ADR treatment for preparation of cytosolic fractions (Fig. 3) or whole cell lysates (Fig. 4). Caspase activation was assessed by Western blotting, utilizing antibodies that detect both the pro-caspase as well as one of the cleaved caspase fragments. For caspases activated in response to ADR, it is expected that the levels of the pro-caspase would decrease, with the concomitant appearance of a cleaved caspase fragment. Based on these criteria, Fig. 3 shows evidence for ADR-induced activation of the initiator caspase (-9), effector caspases (-3 and -7), and a caspase frequently
linked to death receptor signaling (-8). The cytosolic levels of the 130 kDa apoptosis activating factor-1 (Apaf-1) (22) were unaffected by treatment with ADR or HGF/SF. The presence of several higher molecular weight bands was noted in the Apaf-1 Western blot of cells treated with ADR alone. These could represent polymerized forms of Apaf-1 (23) or else contaminating bands.

One of the major substrates for activated caspase-3 is poly [ADP (ribose)] polymerase (PARP). Along with the activation of caspases-9 and -3, cells treated with ADR alone showed nearly complete cleavage of the full-length PARP (112 kDa) to generate the 85 kDa cleaved PARP fragment (Fig. 4). Another down-stream (effector) caspase, caspase-7, was also activated in ADR-treated cells. We also found evidence for the ADR-induced activation of caspase-2 in our cells. Consistent with the finding of ADR-induced caspase-8 activation, the level of cell-associated Fas ligand (FasL), but not Fas itself, was up-regulated in ADR-treated cells. However, there was no evidence for activation of procaspase-10, an upstream death effector domain containing caspase that may associate with activated death receptors in a manner similar to caspase-8 (24).

Recently, it has been demonstrated that the cleavage of Bid, a pro-apoptotic protein, results in the generation of a carboxyl-terminal Bid fragment that can trigger mitochondrial-mediated apoptosis (25). Although the Bid antibody we used detects only full-length (25 kDa) Bid protein and not cleaved Bid fragments, there was no evidence of an ADR-induced decrease in the levels of full-length Bid. However, it should be noted that Bid measurements are usually performed with mitochondrial rather than whole cell lysates. Thus, we cannot conclusively rule out participation of Bid in ADR-induced apoptosis signaling in our experimental system.

All of these ADR-induced alterations - including activation of caspases-9, -3, -7, -8, and -2, cleavage of PARP, and up-regulation of FasL protein levels - were blocked in MDA-MB-453 cells pre-incubated with HGF/SF. Thus, HGF/SF appears to completely block the ADR-induced release of cytochrome c and AIF from mitochondria as well as the ADR-induced caspase activation. These findings suggest that HGF/SF acts, in part, upstream of the mitochondria to prevent mitochondrial damage or dysfunction. However, they do not rule out the possibility that HGF/SF can also act independently to block apoptotic signaling down-stream of the mitochondria.

**Time course for ADR-induced caspase activation.** In an attempt to order the apoptosis signaling events induced by ADR, we determined a time course for the activation (cleavage) of several caspases following exposure of MDA-MB-453 cells to ADR. As shown in Fig. 5, no apoptosis related events were detected at T=2 or 8 hr. However, at T=24 hr post ADR exposure, activation of caspases-9 and -7 were easily detectable, as was PARP cleavage. Cleavage of caspases-3 and -8 were detectable at T=48 hr, and up-regulation of FasL was detected at T=72 hr. These time course assays are somewhat limited by differences in the efficacy of detection of different cleaved protein fragments, and by the possibility that some of these fragments may be labile. Nevertheless, they do suggest that caspase activation follows or is detectable simultaneously with cytochrome c release, which was detected at T=16 and 24 hr, but not at T=2 or 8 hr. The pro-apoptotic Bcl-2 related protein Bad was not detected in MDA-MB-453 cells, utilizing several different commercially available Bad antibodies.

**Effects of caspase inhibitors on ADR-induced cytotoxicity.** We utilized MTT assays of cell viability to determine the extent to which inhibitors could protect cells against ADR-induced toxicity. The MTT assay measures the ability of functional mitochondria to convert (reduce) a tetrazolium dye into formazan (17). Consistent with previously published findings (13,15), pre-treatment with HGF/SF fully blocked the loss of cell viability caused by exposure to ADR (Fig. 6). Treatment with the pan-caspase inhibitor z-VAD-fmk (26) at concentrations of 100-150 µM gave strong, but not complete,
protection against ADR in the cell viability assay. Thus, the protection by z-VAD-fmk was about two-thirds of that provided by HGF/SF. A caspase-3 inhibitor gave a lesser degree of protection than did z-VAD-fmk, and we were unable to detect any protection in cells treated with caspase-9 or caspase-8 inhibitors. It is noted here that z-VAD-fmk is a generally more effective caspase inhibitor than the others, so failure of the caspase-9 and -8 inhibitors to protect cells could have been due, in part, to their weak inhibitory activity. ALLN (N-acetyl-leucyl-leucyl-norleucinal), which is a strong inhibitor of calpain and a weaker inhibitor of other proteases (including the 26S proteasome) (27) also gave little or no protection of ADR-treated cells.

**Caspase inhibition does not block ADR-induced released of cytochrome c and AIF.** Caspases may function both up-stream and down-stream of the mitochondria in apoptosis signaling pathways. We utilized the pan-caspase inhibitor z-VAD-fmk to determine if caspase activity is required for the release of cytochrome c or AIF caused by ADR. Thus, cells were treated with ADR without or with z-VAD-fmk (150 µM) and assayed at T=72 hr post-ADR treatment. As shown in Fig. 7A, the levels of cytochrome c and AIF in supernatants from cells treated with [ADR+z-VAD-fmk] were similar to those in cells treated with ADR alone. As a control, the inner mitochondrial marker Cox 4 was found only in the mitochondrial (pellet) fraction. However, the cleavage of PARP (Fig. 7A) and procaspase-3 (Fig. 7B) were strongly inhibited by z-VAD-fmk, indicating that the z-VAD-fmk concentration was sufficient to inactivate effector caspases, such as caspase-3. Note that PARP is localized primarily in the nucleus and was, thus, observed in the pellet rather than the supernatant fraction. These findings suggest that the ADR-induced release of cytochrome c and AIF from mitochondria is not dependent upon caspases, or at least is not dependent upon caspases that are inhibitable by z-VAD-fmk.

**Time course of ADR-induced mitochondrial depolarization and effect of HGF/SF.** We used DePsipher dye to determine the time course of depolarization of the inner mitochondrial membrane (loss of Δψ) in ADR-treated cells, and to correlate these findings with the observed time course for the release of cytochrome c and AIF. Punctate extranuclear red staining indicates mitochondria with normal membrane potential, while cells with depolarized mitochondria show loss of the red fluorescence. The green fluorescence shows the cellular morphology. At T=8 hr post treatment with ADR alone, there was no evidence for loss of mitochondrial membrane potential (see Fig. 8). However, at T=24-72 hr post ADR, there was a time-dependent increase in the percentage of cells showing mitochondrial depolarization, from about 10-70%. Thus, there was no evidence for the recovery of Δψ in cells treated with ADR alone during the time interval studied.

The time course for mitochondrial depolarization appeared to be similar to that for the release of cytochrome c and AIF (Fig. 2). Furthermore, pre-treatment with HGF/SF nearly fully blocked mitochondrial depolarization, consistent with the high degree of inhibition of the release of cytochrome c and AIF and cell protection. In contrast to HGF/SF, the caspase inhibitor z-VAD-fmk, which partially protected cells against ADR in the MTT assay, had little or no effect on the extent or time course of mitochondrial depolarization (Fig. 9). This finding suggests that like mitochondrial release of cytochrome c and AIF, mitochondrial depolarization is not dependent upon a z-VAD-fmk inhibitable caspase. Further implications of the findings shown in Fig. 9 are considered in the Discussion.
DISCUSSION

These findings suggest that HGF/SF inhibits ADR-induced mitochondrial apoptosis signaling at the level of or upstream of the mitochondria, by blocking the release of apoptosis promoting mitochondrial factors, including cytochrome c and AIF. However, our findings do not rule out the possibility that ADR also inhibits post-mitochondrial apoptosis signaling, since the latter events would not be detectable if the mitochondria are prevented by HGF/SF from releasing their cytochrome c. In our experimental assay system, the earliest detectable ADR-induced release of cytochrome c occurred at T=16 hr after a 2 hr exposure to ADR.

The skeletal events involved in the mitochondrial apoptosis pathways in mammalian cells are well established. These events include: release of cytochrome c from the inter-mitochondrial membrane space to the cytosol, the formation of the "aposome" (a high molecular weight complex of cytochrome c/Apaf-1/procaspase-9), caspase 9 activation, caspase-3 activation, and cleavage of key cellular proteins (e.g., PARP, lamins, etc.) (22,23,28-30). Recent studies suggest that following an apoptogenic stimulus, individual cells release all of their mitochondrial cytochrome c content very rapidly, over a 5-10 minute interval. However, different cells within the same cell population initiate cytochrome c release at different times (31). Although the latent period before cytochrome c release varies from cell to cell, there is a minimal time interval before which no cells release cytochrome c.

Our time course studies suggests a latent period of about T=16 hr before ADR-induced cytochrome c release. Of course, this time interval could vary according to the cell type, ADR dose, and other factors. However, our findings do suggest that as yet unidentified pre-mitochondrial molecular events must be completed before any cytochrome c release can be initiated. Our findings further indicate that the amount of cytochrome c released into the cytosol increases progressively between 24 and 72 hr. If it is true that once cytochrome c release is initiated, a cell releases its entire cytochrome c content, then the findings suggest that between 24 and 72 hr, an increasing proportion of cells undergo cytochrome c release.

Recent studies also suggest that mitochondrial depolarization (i.e., loss of mitochondrial membrane potential, Δψ) is not the cause of cytochrome c release but a reflection of cytochrome c release or a temporally independent event (32). Studies utilizing the DePsipher dye suggest that ADR-induced mitochondrial membrane depolarization follows a time course similar to that for the release of cytochrome c and AIF. Thus, no loss of Δψ was observed at T=8 hr post ADR exposure, while the proportion of depolarized cells increased progressively from T=24 hr to 72 hr. Again, pre-treatment with HGF/SF completely blocked the ADR-induced depolarization, suggesting that HGF/SF may inhibit a common upstream event leading to both cytochrome c release and loss of mitochondrial membrane potential. The early pre-mitochondrial signaling events that lead to mitochondrial dysfunction have not been identified. However, in some contexts, these events may include pro-apoptotic sphingolipid signaling (i.e., ceramide production) (33), the translocation of pro-apoptotic Bcl-2 family proteins (e.g., Bax, Bim, tBid) to the mitochondrial membrane, and the accumulation of other, partially characterized, apoptogenic factors within the cytoplasm (25,34-36).

ADR induced the activation of various caspases, including caspases-9, -3, -7, -8, and -2. The earliest caspase activation (caspases-9 and -7), along with evidence of PARP cleavage, occurred at 24 hr post exposure to ADR, suggesting that caspase activation occurs simultaneously with or later
than cytochrome c release. Caspase-8 activation and up-regulation of Fas ligand were observed at T=48 to 72 hr, suggesting that death receptor signaling induced by ADR is a relatively late event. However, these studies must be interpreted with the caution that the sensitivity of detection of the cleaved protein fragments may vary depending upon the antibody used and other factors. In any event, all detectable ADR-induced events, including the activation of multiple caspases (-9, -7, -3, -8, and -2) and the up-regulation of Fas ligand, were blocked by pre-incubation with HGF/SF.

This finding is consistent with a scheme in which a caspase cascade triggered by the release of cytochrome c is blocked by HGF/SF at a pre-mitochondrial and/or mitochondrial level. Again, we cannot rule out the possibility that HGF/SF independently inhibits apoptosis signaling down-stream of the mitochondria. Thus, a recent study suggests that c-Akt - which we previously showed to be activated by HGF/SF in various cell types (14-16) - can mediate apoptosis inhibition distal to the mitochondria (37). Studies utilizing the pan-caspase inhibitor z-VAD-fmk yielded interesting findings. Based on the MTT assay, z-VAD-fmk partially protected the cells against ADR, giving about two-thirds of the protection afforded by HGF/SF. And z-VAD-fmk blocked some apoptosis signaling events (eg., caspase-3 activation and PARP cleavage). However, z-VAD-fmk failed to inhibit the release of cytochrome c or AIF, nor did it block mitochondrial membrane depolarization. Thus, z-VAD-fmk appears to inhibit post-mitochondrial events in our experimental system.

While the MTT assays revealed a viability level of at least 65% in cells treated with [z-VAD-fmk + ADR], the DePsipher staining showed that about 80% of the cells treated with [z-VAD-fmk + ADR] exhibited mitochondrial depolarization at T=72 hr after exposure to ADR. The finding that z-VAD-fmk protects MDA-MB-453 cells against ADR (based on MTT assays) but does not block mitochondrial dysfunction suggests the existence of a caspase-dependent cell death check-point down-stream of the mitochondria. However, MTT assays do not measure cell survival per se, but assess mitochondrial function (ie., the ability to reduce a tetrazolium dye). Thus, increased MTT dye conversion in [z-VAD-fmk + ADR]-treated cells could represent more surviving cells or the same number of surviving cells with more active mitochondria per cell.

We previously established the role of a PI3 kinase → c-Akt signaling pathway and signaling down-stream of c-Akt in HGF/SF-mediated cell protection in several different cell types, including MDA-MB-453 cells, Madin-Darby canine kidney (MDCK) epithelial cells, and human glioma cell lines (14-16). In these studies, HGF/SF induced the phosphorylation and activation of c-Akt, and selective pharmacologic inhibitors of PI3 kinase enzymatic activity significantly inhibited the HGF/SF-mediated cell protection. Furthermore, expression vectors encoding dominant negative inhibitory forms of p85 (the regulatory subunit of PI3 kinase) and c-Akt blocked or substantially attenuated the HGF/SF-mediated cell protection, while wild-type and constitutively active forms of c-Akt afforded some cellular protection in the absence of HGF/SF. Although the molecular pathways through which c-Akt mediates the protection by HGF/SF are unclear at this time, we did show that in MDCK cells, HGF/SF causes the phosphorylation of a forkhead family transcription factor (FKHR). FKHR is a pro-apoptotic transcription factor for which phosphorylation by c-Akt within the forkhead domain causes nuclear exclusion and, therefore, inactivation (38,39).

Some of the pathways through which c-Akt can stimulate cell survival involve direct phosphorylation of components of the apoptosis machinery, and thus, by-pass the need for alterations of cellular transcriptional pathways. For example, c-Akt can induce the phosphorylation and
inactivation of the pro-apoptotic Bcl-2 family member Bad and of the initiator caspase, caspase-9 (37,40,41). Since we were unable to detect Bad protein in MDA-MB-453 or MDCK cells using several different antibodies, it is unlikely that the inactivation of Bad is required for HGF/SF-mediated cell protection. c-Akt has other phosphorylation targets that may participate in cell signaling related to apoptosis/survival, such as the apoptosis signaling kinase ASK-1 and glycogen synthase kinase-3 (GSK-3) (42,43). The role of these substrates, if any, in HGF/SF-mediated cell protection remains to be determined.

Finally, recent studies have established an additional level of regulation of apoptosis signaling, through members of the inhibitor of apoptosis (IAP) family of proteins (44-47). These are endogenous cellular proteins that bind to various caspases and either prevent their activation or inhibit their enzymatic activity. In addition, the mitochondrial protein SMAC (aka. DIABLO) is released into the cytosol in the setting of apoptosis and disrupts the IAP-mediated inhibition of caspases, thereby promoting apoptosis (48-50). The roles of the IAPs and SMAC in DNA-damage induced apoptosis and in HGF/SF-mediated cell protection are unclear at present. However, it is interesting that over-expression of XIAP protected ovarian epithelial cancer cells against cis-platinum-induced apoptosis, in part, by enhancing c-Akt activity and inhibiting caspase-3 activity (51). Further study is required to determine if HGF/SF either blocks the expression or release of SMAC or enhances the function of IAP proteins as a means of cell protection.
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FIGURE LEGENDS

Fig. 1. Adriamycin (ADR) induces and HGF/SF blocks the mitochondrial release of cytochrome c and apoptosis inducing factor (AIF) in MDA-MB-453 human breast cancer cells. Subconfluent proliferating cells were pre-incubated ± HGF/SF (100 ng/ml x 48 hr), exposed to ADR (10 µM x 2 hr), washed, and post-incubated in fresh drug-free medium for 72 hr. The cells were harvested for separation of the mitochondrial fraction (pellet) from the cytosolic fraction (supernatant) and Western blotting, as described in the Materials and Methods section.

Fig. 2. Time course for the mitochondrial release of cytochrome c and AIF in cells treated ± HGF/SF and ± ADR. Subconfluent proliferating cells were pre-incubated ± HGF/SF (100 ng/ml x 48 hr), exposed to ADR (10 µM x 2 hr), washed, and post-incubated in fresh drug-free medium for varying time intervals from T=2 hr to T=72 hr. The cells were harvested for separation of the mitochondrial fraction (pellet) from the cytosolic fraction (supernatant) and Western blotting. The release of cytochrome c and AIF into the cytosol increased in a dose-dependent fashion beginning at 16-24 hr after exposure to ADR.

Fig. 3. Caspase activation in MDA-MB-453 cells treated ± HGF/SF and ± ADR at T=72 hr. Cells were treated as described in Fig. 1 legend, and the cytosolic fractions were assayed for caspase activation by Western blotting at T=72 hr after exposure to ADR. Caspase activation was assessed using antibodies that detect the proform and one of the cleaved fragments of the caspase of interest. Caspase activation is indicated by a decrease in the proform of the caspase associated with the appearance of a band corresponding to the expected Mr of a cleaved fragment. Based on these criteria, ADR induced the activation of procaspases-9, -3, -7, and -8; while pre-incubation with HGF/SF blocked the activation of each of these caspases.

Fig. 4. Apoptotic signaling in MDA-MB-453 cells treated ± HGF/SF and ± ADR at T=72 hr. Cells were treated as described in Fig. 1 legend, and whole cell lysates were assayed by Western blotting at T=72 hr after exposure to ADR. Poly (ADP-ribose) polymerase (PARP), a substrate for caspase-3, was extensively cleaved in ADR-treated cells, while cells exposed to (HGF/SF+ADR) failed to show PARP cleavage. ADR also induced (and HGF/SF blocked) the cleavage of caspases -7 and -2. There was no evidence for caspase-10 activation. ADR induced up-regulation of Fas ligand (FasL) protein levels, which was blocked by HGF/SF. The Bid antibody only detects the full-length 25 kDa Bid protein. However, there was no evidence for a reduction in the levels of full-length Bid in ADR-treated cells.

Fig. 5. Time course for caspase activation in MDA-MB-453 cells treated ± HGF/SF and ± ADR. Cells were treated as described in Fig. 2, and whole cell lysates were assayed at different times from T=2 hr to T=72 hr post exposure to ADR, by Western blotting. PARP cleavage and caspase-9 and-7 activation were observed as early as T=24 hr post ADR treatment, but were not seen at T=2 or T=8 hr. Bad could not be detected in MDA-MB-453 cell lysates. See text for further discussion of these results.

Fig. 6. Effects of caspase inhibitors on ADR-induced cytotoxicity. Subconfluent proliferating cells in 96-well dishes were pre-incubated for 24 hr with each inhibitor, exposed to ADR (10 µM x 2
hr), washed, and post-incubated in the presence of the same inhibitor for T=72 hr. At this time, dishes were processed for MTT dye conversion assays. The cell viability values were expressed as a percentage of the sham-treated control values, mean ± SEMs for ten replicate wells. For comparison, the degree of cell protection by HGF/SF is also shown. The inhibitor concentrations were as follows: z-VAD-fmk (100 µM), caspase-3 inhibitor (150 µM), caspase-9 inhibitor (150 µM), caspase-8 inhibitor (100 µM), and ALLN, a calpain inhibitor (100 µM). See text for description of findings.

Fig 7. Effect of the pancaspase inhibitor z-VAD-fmk on mitochondrial cytochrome c and AIF release. Cells were pre-incubated without or with z-VAD-fmk (100 µM) for 24 hr, exposed to ADR, and post-incubated in fresh ADR-free medium in the absence or presence of z-VAD-fmk for T=72 hr. z-VAD-fmk failed to inhibit the mitochondrial release of cytochrome c or AIF into the cytosol. However, z-VAD-fmk strongly inhibited the cleavage of PARP, a major substrate of the activated caspase-3. Note: PARP is localized within the nucleus, which is contained in the pellet fraction. Panel B shows an assay of whole cell lysates, indicating that z-VAD-fmk blocked or inhibited the ADR-induced activation of caspase-3.

Fig. 8. HGF/SF blocks the mitochondrial depolarization induced by ADR. Subconfluent proliferating cells were pre-incubated ± HGF/SF (100 ng/ml x 48 hr), exposed to ADR (10 µM x 2 hr), washed, and then post-incubated in fresh drug-free culture medium for up to 72 hr. At various times after exposure to ADR, the cells were collected and processed for assessment of mitochondrial membrane potential using DePsipher assays (see Materials and Methods section).

Fig. 9. The pancaspase inhibitor z-VAD-fmk does not block the mitochondrial depolarization induced by ADR. Subconfluent proliferating cells were pre-incubated ± zVAD-fmk (150 µM x 24 hr), exposed to ADR (10 µM x 2 hr), washed, and then post-incubated in fresh drug-free culture medium [again ± z-VAD-fmk] for up to 72 hr. At various times after exposure to ADR, the cells were collected and processed for assessment of mitochondrial membrane potential using DePsipher assays (see Materials and Methods).
**Fig. 1**

| kDa | Pellet | Supernatant | T = 72 hr |
|-----|--------|-------------|-----------|
|     | CON    | HGF/SF      | ADR       |
|     | +ADR   | HGF/SF      | ADR       |
|     | HGF/SF | ADR         | +ADR      |

- **Cyto c (15 kDa)**
- **AIF (55 kDa)**
- **Cox 4 (14 kDa)**
- **α-Actin (43 kDa)**
Fig 2A

Pellet

| CON | HGF/SF | ADR | HGF/SF+ADR | ADR | HGF/SF+ADR |
|-----|-------|-----|------------|-----|------------|
| 17  |      |     |            |     |            |

Supematant

| CON | HGF/SF | ADR | HGF/SF+ADR | ADR | HGF/SF+ADR |
|-----|-------|-----|------------|-----|------------|
| 2 hr|      |     |            |     |            |
| 24 hr|      |     |            |     |            |

- Cyto c (15 kDa)
- AIF (55 kDa)
- Cox 4 (14 kDa)
- HSP27 (27 kDa)
- α-Actin (43 kDa)
Fig. 3

Cytosolic Fractions
T = 72 hr

- Procaspase-9 (50 kDa)
- Cleaved Caspase-9 (35 kDa)
- Procaspase-3 (35 kDa)
- Cleaved Caspase-3 (18 kDa)
- Procaspase-7 (40 kDa)
- Cleaved Caspase-7 (35 kDa)
- Procaspase-8 (50 kDa)
- Cleaved Caspase-8 (20 kDa)
- Apaf 1 (130 kDa)
- α-Actin (43 kDa)
Fig. 4

Whole Cell Lysates
T = 72 hr

| kDa | CON | HGF/SF | ADR | HGF/SF + ADR |
|-----|-----|--------|-----|--------------|
| 110 |     |        |     |              |
| 71  |     |        |     |              |
| 41  |     |        |     |              |
| 31  |     |        |     |              |
| 41  |     |        |     |              |
| 17  |     |        |     |              |
| 41  |     |        |     |              |
| 41  |     |        |     |              |
| 31  |     |        |     |              |
| 17  |     |        |     |              |

- PARP (112 kDa)
- Cleaved PARP (85 kDa)
- Procaspase-7 (40 kDa)
- Cleaved Caspase-7 (35 kDa)
- Procaspase-2 (45 kDa)
- Cleaved Caspase-2 (20 kDa)
- Procaspase-10 (54 kDa)
- Cleaved Caspase-10 (38 kDa)
- FasL (37 kDa)
- Fas (48 kDa)
- Bid (25 kDa)
- α-Actin (43 kDa)
Fig. 5

| kDa | Time after ADR (hr) | Whole Cell Lysates |
|-----|---------------------|-------------------|
|     | CON  | 2  | 8  | 24 | 48 | 72 | CON |
| 110 |      |    |    |    |    |    |     |
| 71  |      |    |    |    |    |    |     |
| 41  |      |    |    |    |    |    |     |
| 31  |      |    |    |    |    |    |     |
| 17  |      |    |    |    |    |    |     |
| 41  |      |    |    |    |    |    |     |
| 31  |      |    |    |    |    |    |     |
| 17  |      |    |    |    |    |    |     |
| 41  |      |    |    |    |    |    |     |
| 31  |      |    |    |    |    |    |     |
| 17  |      |    |    |    |    |    |     |
| 41  |      |    |    |    |    |    |     |
| 31  |      |    |    |    |    |    |     |
| 17  |      |    |    |    |    |    |     |
| 41  |      |    |    |    |    |    |     |

- PARP (112 kDa)
- Cleaved PARP (85 kDa)
- FasL (37 kDa)
- Fas (48 kDa)
- Procaspase-3 (35 kDa)
- Cleaved Caspase-3 (18 kDa)
- Procaspase-7 (40 kDa)
- Cleaved caspase-7 (35 kDa)
- Procaspase-8 (50 kDa)
- Procaspase-9 (50 kDa)
- Cleaved Caspase-9 (35 kDa)
- Bad (27 kDa)
- α-Actin (43 kDa)
Fig. 6

Cell Viability (% of Control)

- z-VAD-fmk
- Caspase-3 Inhibitor
- Caspase-9 Inhibitor
- Caspase-8 Inhibitor
- ALLN

CON, Inhibitor, ADR, HGF/SF, HGF/SF+ADR
**Fig. 7A**

| Pellet | Superнатant |
|--------|-------------|
|        | T=72 hr     |
| kDa    |             |
| 17     | Cyto c (15 kDa) |
| 17     | Cox 4 (14 kDa) |
| 41     | AIF (55 kDa)  |
| 110    | PARP (112 kDa) |
| 71     | Cleaved PARP (85 kDa) |
| 41     | α-Actin (43 kDa) |

**Fig. 7B**

| kDa |
|-----|
| 41  | Whole cell lysates |
|     | T=72 hr             |
| 17  | Procaspase-3 (35 kDa) |
|     | Cleaved Caspase-3 (18 kDa) |
Mitochondria Depolarization

Fig 8

CON 8 hr 24 hr 48 hr 72 hr
ADR Alone

HGF/SF HGF/SF+ADR

% Depolarized Cells

Time after ADR (hr)

CON 8 24 48 72
Fig. 9

Mitochondria Depolarization

CON  ADR  8 hr  24 hr  48 hr  72 hr

z-VAD-fmk  z-VAD-fmk + ADR

% Depolarized Cells

Time after ADR (hr)

- z-VAD-fmk  + z-VAD-fmk
Hepatocyte growth factor/scatter factor (HGF/SF) blocks the mitochondrial pathway of apoptosis signaling in breast cancer cells
Min Gao, Saijun Fan, Itzhak D. Goldberg, John J. Laterra, Richard N. Kitsis and Eliot M. Rosen

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