Disruption of HSP90 Function Reverts Tumor Necrosis Factor-induced Necrosis to Apoptosis*

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Triggering tumor necrosis factor receptor-1 (TNFR1) induces apoptosis in various cell lines. In contrast, stimulation of TNFR1 in L929sA cells leads to necrosis. Inhibition of HSP90, a chaperone for many kinases, by geldanamycin or radicicol shifted the response of L929sA cells to TNF from necrosis to apoptosis. This shift was blocked by CrmA but not by BCL-2 overexpression, suggesting that it occurred through activation of procaspase-8. Geldanamycin pretreatment led to a proteasome-dependent decrease in the levels of several TNFR1-interacting proteins including the kinases receptor-interacting protein, inhibitor of κB kinase-α, inhibitor of κB kinase-β, and to a lesser extent the adaptors NF-κB essential modulator and tumor necrosis factor receptor-associated factor 2. As a consequence, NF-κB, p38MAPK, and JNK activation were abolished. No significant decrease in the levels of mitogen-activated protein kinases, adaptor proteins TNFR-associated death domain and Fas-associated death domain, or caspase-3, -8, and -9 could be detected. These results suggest that HSP90 client proteins play a crucial role in necrotic signaling. We conclude that inhibition of HSP90 may alter the composition of the TNFR1 complex, favoring the caspase-8-dependent apoptotic pathway. In the absence of geldanamycin, certain HSP90 client proteins may be preferentially recruited to the TNFR1 complex, promoting necrosis. Thus, the availability of proteins such as receptor-interacting protein, Fas-associated death domain, and caspase-8 can determine whether TNFR1 activation will lead to apoptosis or to necrosis.

The primary paradigm of natural programmed cell death (PCD) is observed during normal embryogenesis. Schweichel and Merker (1, 2) identified three pathways of programmed cell death. The first pathway was characterized by the condensation of nucleus and cytoplasm and corresponds to apoptosis. The second pathway was characterized by abundant autophagic vacuoles and no or minimal nuclear changes (as often seen in cells dying by necrosis), whereas the third pathway was a more rare variant of necrotic cell death. Apoptosis is morphologically characterized by membrane blebbing, shrinking of the cell and its organelles, internucleosomal degradation of DNA, and disintegration of the cell after which the fragments are phagocytosed by neighboring cells (3, 4). Necrosis is characterized by swelling of the cell and the organelles and results in disintegration of the cell membrane and in lysis (5). One of the cell lines intensively studied in our laboratory is the L929 fibrosarcoma cell line. In these cells apoptotic as well as necrotic cell death can be induced. Stimulation of TNF in L929sA cells leads to necrotic cell death. This process is strongly sensitized by pretreatment with the pancaspase inhibitor benzoylxylo-carbonyl-Val-Ala-Asp(OMe)-fluoromethylketone (zVAD-fmk)1 or overexpression of CrmA (6). In L929sAhFas cells transfected with human Fas, the apoptotic cell death pathway can be induced by clustering of FAS with agonistic anti-FAS antibodies. This apoptotic cell death process can be reverted to necrosis by inhibiting caspase-activity (7).

Holler and colleagues (8) demonstrated that pretreatment of human Jurkat T-cell lymphoma cells with geldanamycin (GA) protects these cells from FASL-mediated caspase-independent cell death in the presence of zVAD-fmk. The specific target of GA has been identified as the 90-kDa heat shock protein (HSP90) (9–12). Heat shock proteins are a group of chaperone proteins that help to maintain protein stability, to renature unfolded proteins, or to target them for degradation when cells are subjected to heat shock or other types of stress (13). Geldanamycin prevents the ATP-dependent release of the client protein undergoing refolding from HSP90 (14). The stabilized complex is then ubiquitinated and degraded (15, 16). Unlike the better characterized HSP70 and HSP60 chaperones, HSP90 displays considerable specificity for its client proteins, including steroid hormone receptors and kinases (reviewed in Refs. 13, 17, and 18). Lewis and colleagues reported (19) that one of the HSP90 client proteins is the death domain kinase receptor-interacting protein (RIP) (19). They showed that disruption of HSP90 function by the addition of GA results in the degradation of RIP and blockade of TNF-induced NF-κB activation. In the present study, we analyzed whether GA-induced inhibition of HSP90 function would block TNFRI1-induced

1 The abbreviations used are: zVAD-fmk, benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone; Bcl-2, B-cell lymphoma; CrmA, cyto-kine response modifier A; ERK, extracellular signal-regulated kinase; FADD, Fas-associated death domain; GA, geldanamycin; HSP90, 90-kDa heat shock protein; IKK, inhibitory κB kinase; JNK, c-Jun NH2-terminal kinase; MAPK, mitogen-activated protein kinase; NEMO, nuclear factor-κB essential modulator; NF-κB, nuclear factor-κB; PI, propidium iodide; RAF-1, Ras-associated factor 1; RIP, receptor interacting protein; TNF, tumor necrosis factor; TNFR1, 55-kDa tumor necrosis factor receptor; TRADD, TNFR-associated death domain; TRAP2, TNF receptor-associated factor 2; dsRNA, double-stranded RNA; hTNF, human tumor necrosis factor; IL, interleukin; Ac-DEVD-amc, acetyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-aminomethylcoumarin.
necrosis in L929 fibrosarcoma cells. We show that inhibition of the function of HSP90 leads to a switch from TNFR1 necrotic cell death to apoptotic cell death. This correlates with the preferential decrease of the expression levels of several TNFR1 signaling-associated proteins, resulting in the abolishment of TNF-mediated activation of NF-κB, JNK, p38MAPK, and necrosis. Moreover, GA pretreatment does not affect the expression levels of proteins that constitute the apoptotic signaling axis, such as TRADD, FADD, and procaspase-8, and thus promotes a TNFR1-induced apoptotic response.

EXPERIMENTAL PROCEDURES

Cells—L929aA is a murine fibrosarcoma cell line, derived from L929, which was selected for its sensitivity to the cytotoxic activity of TNF (5). L929aA was transfected with the human FAS receptor cDNA with or without human BCL-2 cDNA and with cDNA encoding CrmA from cowpox virus, resulting in L929aAhFas, L929aAhFasBCL2, and L929aACrmA clones, as described previously (5-7). L929aA and derivatives were cultured in Dulbecco’s modified Eagle’s medium supplemented with 5% newborn bovine serum, 5% fetal calf serum, penicillin (100 units/ml), streptomycin (0.1 mg/ml), and 1-glutamine (0.03%).

Antibodies, Cytokines, and Reagents—Recombinant hTNF was produced in Escherichia coli and purified to at least 99% homogeneity. The specific biological activity was 2.3 × 10³ units/mg as determined in a standardized cytoxicity assay on L929aA cells. Secreted IL-6 in the supernatant was quantified using a bioassay, viz. IL-6-dependent growth of TT71 cells (20). Anti-human Fas antibody (clone 2R2) was purchased from Cell Diagnostica (Munster, Germany). Ga and MG132 were obtained from Sigma (St. Louis, MO) and used at 25 μM, respectively. Propidium iodide (PI, BD Biosciences) was dissolved at 3 mM in phosphate-buffered saline and was used at 30 μM. The caspase peptide inhibitor zVAD-fmk was added by Bachem (Bubendorf, Switzerland) and used at 25 μM. The caspase fluorogenic substrate acetyl-Asp(Ac)-Glu-Val-Asp(Ac)-aminomethylcoumarin (Ac-DEVD-amc) was obtained from Peptide Institute (Osaka, Japan) and used at 50 μM. Anti-murine RIP antibodies and polyclonal antibodies against JNK, p38MAPK, ERK, IKK-α, and IKK-β were obtained from New England Biolabs (Beverly, MA). Antibodies against TRADD, TRAF2, RAP-1, and NEMO were from Santa Cruz Biotechnology (Santa Cruz, CA), antibodies against mouse Bid were from R&D Systems (Minneopolis, MN), and antibodies against cytochrome c were from Amersham Biosciences. Rabbit polyclonal antibodies against recombinant murine caspase-3 and -8 were prepared at the Centre d’Economie Rurale (Laboratoire d’Héronologie Animale, Marloie, Belgium). Anti-caspase-2 antibody was kindly provided by Professor Dr. Kumar Sharad (The Hanson Centre for Cancer Research, Institute of Medical and Veterinary Science, Adelaide, Australia) (21). Anti-murine RIP antibodies were obtained from Transduction Laboratories (Lexington, KY).

Induction of Apoptosis or Necrosis for Fluorescence-activated Cell Sorter Analysis and Western Blotting—The cells were kept in suspension by seeding 10⁵ cells in uncoated 24-well tissue culture plates. The cells were kept in suspension with TNF Occurs at a Premitochondrial Level—The fluorogenic substrate Assay for Caspase Activity—The fluorogenic substrate assay for caspase activity was carried out as described previously (7). Cells were transferred to Eppendorf tubes, washed in cold phosphate buffer, and lysed in 150 μl of caspase buffer system. Cell debris was removed by centrifugation, and caspase activity was determined by incubating 15 μl of the soluble fraction with 50 μM Ac-DEVD-amc in 150 μl of cell-free system buffer containing 10 μM Hepes, pH 7.4, 220 mM mannitol, 68 mM sucrose, 2 mM NaCl, 2.5 mM KH₂PO₄, 0.5 mM EGTA, 2 mM MgCl₂, 0.5 mM sodium pyruvate, 0.5 mM l-glutamine, and 10 mM dithiothreitol. The release of fluorescent aminomethylcoumarin was measured for 1 h at 2-min intervals by fluorometry (excitation at 360 nm and emission at 480 nm) (Cytofluor; PerSeptive Biosystems, Cambridge, MA); the maximal rate of increase in fluorescence was calculated (ΔF/min).

Light Microscopic Analyses—The cells were seeded in 6-well adherent plates at a density of 2 × 10⁵ cells per well. 1 μg GA was added 16 h before stimulation. After preincubation for 1 h with or without 25 μM zVAD-fmk, hTNF (10,000 units/ml) was added to the cells for different time intervals. Light microscopic pictures were taken on different time points (Integrated Modulation Contrast, ×400).

RESULTS

Disruption of HSP90 Function in L929aA Cells Reverts TNFR1-induced Necrosis to Apoptosis—A previous report demonstrated that pretreatment of human Jurkat T-cell lymphoma cells with the pancaspase inhibitor zVAD-fmk shifts their response to FASL from apoptosis to necrosis. However, pretreating these cells with the combination of zVAD-fmk and the HSP90 inhibitor geldanamycin protects them from FAS-mediated necrosis (8). L929aA cells respond to TNF by caspase-independent necrosis even without the addition of caspase inhibitors (6). Therefore, we analyzed the effect of GA on TNFR1-induced necrosis in these cells. Preincubation of the cells with GA during 16 h strongly sensitized the cells to the cytotoxic effect of TNF. However, analysis of the cell morphology by light microscopy revealed that the cells did not die by necrosis but instead responded to apoptosis characterized by membrane blebbing and nuclear condensation (Fig. 1A). The morphological assessment of apoptotic cell death after pretreatment with GA was confirmed using biochemical parameters such as (in order of appearance) caspase activation, tBid generation, cytochrome c release, DNA hypoploidy, and cell membrane permeabilization (Fig. 1, B and C). None of these typically apoptosis-related events was detected in TNF-induced necrosis in the absence of GA (Fig. 1, B and C). To exclude the possibility that the effect described may not arise from a side effect of GA, we analyzed the effect of a structurally unrelated inhibitor of HSP90, viz. radicicol. Pretreatment of the cells with radicicol also caused a shift from necrosis to apoptosis in response to TNF (data not shown). These results confirm that inhibition of HSP90 in L929aA cells reverts TNFR1-induced necrotic cell death to apoptosis.

The GA-induced Shift from Necrosis to Apoptosis after Stimulation with TNF Occurs at a Premitochondrial Level—In a previous report we demonstrated that the antioxidant butylated hydroxyanisole can shift the response of L929aAhFas cells to treatment with the combination of interferon and dsRNA from necrosis to apoptosis. This shift occurred at the mitochondrial level and was completely blocked by overexpression of BCL-2 (5, 22). Because tBid generation and cytochrome c release were observed also in cells treated with the combination of GA and TNF (Fig. 1C), we investigated whether or not TNFR1-induced apoptosis in the presence of GA is dependent on the mitochondrial apoptotic pathway. We analyzed the effect of GA on TNF-induced necrosis in L929aAhFasBCL2 cells stably transfected with Bcl-2. Signaling through the intrinsic pathway, originating at the mitochondria, is attenuated in these cells (5, 22). In comparison with parental L929aAhFas, a substantial delay in apoptotic cell death induced by TNF in the presence of GA was observed in L929aAhFasBCL2 cells, as revealed by uptake of propidium iodide and hypoploidy (Fig. 2A, left). Although overexpression of BCL-2 had a partial inhibitory effect on apoptosis induced by TNF and GA, as
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Demonstrated by the decrease in caspase-3 activity (Fig. 2, A and B, left), the cells still responded by apoptosis. A similar effect on FAS-mediated apoptosis was observed before (5, 22), suggesting that the mitochondrial apoptotic pathway amplifies the apoptotic death process, and in its absence the process is delayed. Moreover, tBid generation during TNF/GA treatment clearly preceded cytochrome c release (Fig. 1C), suggesting that the shift to apoptosis occurred upstream to the mitochondria. Caspase-8 is required for death receptor-induced apoptosis and is a known Bid-cleaving protease (23, 24). Therefore, we tested whether the apoptotic process was initiated by caspase-8 by analyzing the effect of GA on TNFR1-induced cell death response in L929sACrmA cells. CrmA, the viral serpin-like inhibitor of caspase-8, should block death receptor-induced apoptosis in these cells. Contrary to their parental L929sA cells, the L929sACrmA cells still died by necrosis when treated with TNF after pretreatment with GA. Indeed, no hypoploidy, caspase-3 activity, or caspase-3 cleavage was detected (Fig. 2, A and B, right). Inhibition of caspase activation by pretreatment with zVAD-fmk yielded similar results in L929sA cells (Fig. 2C). In both cases, cells responded to TNF by necrosis even after pretreatment with GA. However, cell death was delayed in comparison with cells treated with TNF in the absence of GA (Fig. 2C). Thus, inhibition of caspase-8 blocks TNFR1-induced apoptosis in the presence of GA. This finding suggests that the apoptotic process induced by TNF in the presence of GA is mediated through activation of caspase-8, a receptor-mediated event.

Decrease of the Expression Levels of RIP, IKKα, IKKβ, NEMO, and TRAF2 Results in the Abolishment of TNF-mediated Activation of NF-κB and the MAPKs in the Presence of GA—The above results show that an efficient necrotic response requires a functional HSP90 and suggest that one or more of the chaperone’s client proteins are involved in the TNFR1 complex signaling to necrosis or in the prevention of apoptosis. Therefore, we investigated the effect of HSP90 inhibition by GA on the expression levels of different TNFR1 complex-associated adaptor proteins, kinases, and caspases. Western blot analysis of different kinases revealed that the detected levels of RIP, RAP-1, IKK-α, and IKK-β are decreased in the presence of GA, whereas those of ERK, p38MAPK, JNK, and dsRNA-activated protein kinase are not affected (Fig. 3A). No, or a minor effect on the expression levels of adaptors recruited to the TNFR1 complex, such as TRADD and FADD, was detected (Fig. 3A). However, a decrease in the expression levels of two RIP-interacting proteins, TRAF2 and NEMO, was clearly apparent in cells treated with GA (Fig. 3A). TRAF2 is an adaptor protein known also to interact with TRADD and TNFR2 (25), whereas NEMO is a scaffold protein playing a central role in the assembly of the IKK complex (26–29). We could not detect any change in the levels of caspase-3, -8, and -9. Surprisingly, caspase-2 levels clearly dropped in the presence of GA. Several reports demonstrated that the decrease in expression levels of HSP90 client proteins is caused by degradation by the proteasome (15, 16). Indeed, 1 h of treatment with the proteasome inhibitor MG132 was sufficient to restore approximately half of the expression level of RIP that was lost because of 15 h of pretreatment with GA (Fig. 3A). RIP was also shown to be involved in the activation of NF-κB (30, 31). RIP was also shown to be involved in the activation of JNK and p38MAPK (32, 33). Moreover, disruption of HSP90 function was reported to result in blockage of NF-κB activation (19). Therefore, we investigated whether degradation of RIP, IKK-α, IKK-β and, to a lesser extent, NEMO and TRAF2 in the presence of GA abrogated the activation of NF-κB, JNK, and p38MAPK in L929sA cells. First, we analyzed the effect of GA on the TNF-induced secretion of IL-6, one of the major NF-κB-regulated cytokines. In the presence of
GA, TNF-induced IL-6 production was blocked completely; also, the basal level of IL-6 was decreased (Fig. 4A). Next we analyzed the activation of JNK and p38MAPK by Western blotting using phosphospecific antibodies. In the absence of GA, TNF treatment led to an early transient activation of JNK and p38MAPK (Fig. 4B). The activation of JNK was more prolonged than that of p38MAPK. The primary transient activation of p38MAPK was followed by a secondary activation (Fig. 4B). Such a biphasic activation pattern for MAPKs in response to a combined treatment with TNF and cycloheximide was reported previously (34). The activation of both MAPKs by TNF was prevented by pretreatment with GA. In conclusion, these results show that the expression levels of TNFR1 adaptor protein FADD and the initiator caspase-8 required to initiate apoptosis are not affected by the pretreatment of GA. However, the expression levels of RIP, IKK-α, IKK-β, NEMO, and TRAF2 are reduced by pretreatment with GA. As a consequence, NF-κB, p38MAPK, and JNK activation are abolished. The restoration of the expression level of RIP by inhibiting the proteasome suggests that treatment with GA leads to degradation of HSP90 client proteins.

**DISCUSSION**

Our results demonstrate that pretreatment with the HSP90 inhibitors GA or radicicol shifts the response to TNF from necrosis to apoptosis in L929sA cells. Several lines of evidence suggest that the shift occurs at the receptor level. First, cleavage of Bid preceded cytochrome c release. Second, overexpression of the caspase-8 inhibitor CrmA abolished the shift from necrosis to apoptosis. Third, BCL-2 overexpression decreased the apoptotic response but did not block it, although BCL-2 overexpression in these cells was able to block the conversion of

**FIG. 2.** TNFR1-induced shift from necrosis to apoptosis is blocked in L929sA CrmA cells but not in L929s-AhFasBcl2 cells. A, cell lines were treated for 16 h with 1 μM GA followed by treatment with 10^4 units/ml hTNF for 1, 2, 4, 6, and 8 h. Cells were analyzed by flow cytometry for loss of membrane impermeability (% PI-positive cells), DNA degradation (% hypoploidy), and caspase activity (relative DEVDase activity). B, caspase-3 cleavage was analyzed by Western blotting on cytosolic lysates (pro-caspase-3, black triangle; activated form of caspase-3, white triangle). The disappearance of procaspase-3 at 4, 6, and 8 h in L929sA CrmA cells is caused by leakage of the cellular contents by the strong necrotic response. C, fluorocytometric analysis for loss of membrane integrity by PI. After treatment for 16 h of L929sA cells with or without 1 μM GA, cells were pretreated for 1 h with 25 μM zVAD-fmk, followed by treatment with 10^4 units/ml hTNF (left). L929sA CrmA cells were treated for 16 h with or without 1 μM GA, followed by treatment with 10^4 units/ml hTNF (right).
necrotic to apoptotic signaling induced by dsRNA and interferon in the presence of the antioxidant butylated hydroxyanisole (22). Taken together, these observations indicate that in L929sA cells the shift from necrotic to apoptotic cell death in the case of TNF/GA occurs at the receptor level, whereas the shift induced on dsRNA/interferon/butylated hydroxyanisole treatment occurs at the mitochondrial level. Inhibition of necrotic cell death by GA has been reported in

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**Fig. 3. Expression levels of RIP, IKK-α, IKK-β, NEMO, and TRAF2 are lower in L929sA cells treated with GA.**

A. Cells were treated with and without 1 μM GA for 16 h. Equal amounts of cytosolic lysates were analyzed by Western blotting with antibodies specific for different DISC-related proteins, kinases, and caspases, viz. RIP, IKK-α, IKK-β, ERK, JNK, p38MAPK, RAF-1, dsRNA-activated protein kinase, TRAF-2, FADD, TRADD, NEMO, pro-caspase-2, -3, -8, and -9. As a control for equal loading, lysates were analyzed with antibodies against actin. Immunoreactive signals were quantified by densitometry.

B. Cells were treated with and without 1 μM GA for 15 h and incubated for 1 h in the presence or absence of 20 μM of the proteasome inhibitor MG132.
Jurkat cells treated with FasL in the presence of zVAD-fmk (8). In addition, GA can protect caspase-8-deficient Jurkat cells from dsRNA-induced necrosis (22). Several reports suggest that the availability of certain proteins such as FADD, caspase-8, RIP, and/or still unknown proteins can determine whether necrosis or apoptosis occurs. FADD or caspase-8 deficiency has been reported to protect Jurkat cells from Fas-mediated apoptosis (35–38), whereas RIP deficiency protects these cells from necrosis (8). This finding implies that death receptors can initiate two alternative cell death pathways, one relying on FADD and caspase-8 and the other dependent on the kinase RIP. Indeed, FADD-deficient Jurkat cells respond to TNF by necrosis (8, 22). Similarly, dsRNA-induced apoptosis shifts to necrosis in FADD- or caspase-8-deficient Jurkats, whereas dsRNA-induced necrosis is blocked in RIP-deficient Jurkats (22). Moreover, accumulating evidence suggests that signaling compounds in necrosis and apoptosis compete and counteract each other. In necrotic cell death, RIP leads to anti-apoptotic signals by the activation of NF-κB and the MAPKs (30, 33). In apoptosis, caspase-8 cleaves RIP and thus may block these anti-apoptotic signals (39–41). These results suggest that TNFR1-induced necrosis and apoptosis use distinct proteins/components in their signaling pathways.

GA prevents the ATP-dependent release from HSP90 of a client protein undergoing refolding leading to its degradation (14–16). Here, we analyzed the effect of the inhibition of HSP90 by GA on the expression levels of TNFR1-related adaptor proteins, kinases, and caspases. Our results show that GA induces a decrease in the expression levels of RIP, IKK-α, IKK-β and, to a lesser extent, of NEMO, TRAF-2, and RAP-1, whereas no significant drop in the expression levels of the MAPKs, dsRNA-activated protein kinase, and adaptors of the TNFR1 complex such as TRADD and FADD was observed. The degradation of RIP and RAP-1 in the presence of GA confirms previous reports (15, 19). The strong drop of the expression levels of IKK-α/β in the presence of GA suggests that both kinases are also client proteins of HSP90. Indeed, a recent report demonstrated that the IKK complex, which contains the two catalytic subunits IKK-α and IKK-β and a regulatory subunit NEMO, forms a ~900-kDa heterocomplex with Cdc37 and HSP90 (42). Contrary to our results, the authors could not detect any degradation of the IKK-α/β in HeLa cells after 15 h of GA treatment. Nevertheless, inhibition of HSP90 in the latter cells prevented the recruitment of the IKK complex to TNFR1. The observation that NEMO and TRAF2 are significantly less degraded in the presence of GA than RIP, IKK-α, and IKK-β, suggests that no direct interaction occurs between NEMO and TRAF2 or NEMO. The latter might be degraded because of their recruitment to complexes containing either RIP or IKK-α and IKK-β. Similarly, the decrease in the level of caspase-2 in cells treated with GA may be caused by an indirect interaction of caspase-2 with RIP via RIP-associated Ich-1/
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CED-3 homologous protein with a death domain (43). Holler and colleagues (8) suggested that GA inhibits necrotic cell death because of the degradation of RIP. However, our data show that necrotic cell death can still occur in the presence of zVAD-fmk or CrmA, although RIP is degraded by GA treatment. This finding suggests that other proteins (kinases) may have an important role in signaling to necrosis or that the small amount of RIP still present in the GA-treated cells is sufficient to allow the necrotic process to occur.

RIP, IKK-α, IKK-β, NEMO, and TRAF2 are involved in the signaling to NF-κB, p38MAPK, and JNK (44, 45). NF-κB activation has been implicated in the suppression of apoptosis. For example, RelA-/- mice die at embryonic day 15 as a result of extensive liver apoptosis (44, 46). Other studies indicate that an early but brief activation of JNK and/or p38MAPK can protect cells from TNF-induced apoptosis (34). Indeed, pretreatment with GA blocks TNF-induced activation of NF-κB, p38MAPK, and JNK in our cells, again favoring the apoptotic cell death process.

Our results show that TRADD, FADD, and caspase-3, -8, and -9, which are involved in the apoptotic-signaling pathway, are not affected by the pretreatment with GA. This finding suggests that inhibition of HSP90 alters the composition of the TNFR1 complex, favoring the TRADD-FADD-caspase-8 pathway and thus apoptosis (Fig. 5, right). This fact implies that in the absence of GA one or more HSP90-interacting proteins needed for the induction of necrosis induced by TNF are probably recruited more efficiently to the TNFR1-complex (Fig. 5, left). These proteins may prevent apoptosis by competing with FADD or caspase-8 for recruitment to the TNFR1-complex and by initiating anti-apoptotic mechanisms through NF-κB (30, 31) and activation of JNK and/or p38MAPK (32, 33). Additionally, they may actively contribute to the signaling to and the execution of the necrotic process. In L929A cells we have shown before that butylated hydroxyanisole blocks necrosis induced by TNF (6) or anti-Fas plus zVAD-fmk (7) and shifts the response to TNF to apoptosis (34). Indeed, pretreatment with GA blocks TNF-induced activation of NF-κB, p38MAPK, and JNK in our cells, again favoring the apoptotic cell death process.

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