S-Nitrosylation of the Death Receptor Fas Promotes Fas Ligand–Mediated Apoptosis in Cancer Cells

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BACKGROUND & AIMS: Fas belongs to the family of tumor necrosis factor receptors which induce apoptosis. Many cancer cells express Fas but do not undergo Fas-mediated apoptosis. Nitric oxide reverses this resistance by increasing levels of Fas at the plasma membrane. We studied the mechanisms by which NO affects Fas function. **METHODS:** Colon and mammary cancer cell lines were incubated with the NO donor glyceryl trinitrate or lipid A; S-nitrosylation of Fas was monitored using the biotin switch assay. Fas constructs that contained mutations at cysteine residues that prevent S-nitrosylation were used to investigate the involvement of S-nitrosylation in Fas-mediated cell death. Apoptosis was monitored according to morphologic criteria. **RESULTS:** NO induced S-nitrosylation of cysteine residues 199 and 304 in the cytoplasmic part of Fas. In cancer cells that overexpressed wild-type Fas, S-nitrosylation induced Fas recruitment to lipid rafts and sensitized the cells to Fas ligand. In cells that expressed a mutant form of Fas in which cysteine 304 was replaced by valine residue, NO-mediated translocation of Fas to lipid rafts was affected and the death-inducing signal complex and synergistic effect of glyceryl trinitrate–Fas ligand were inhibited significantly. These effects were not observed in cells that expressed Fas with a mutation at cysteine 199. **CONCLUSIONS: We identified post-translational modifications (S-nitrosylation of cysteine residues 199 and 304) in the cytoplasmic domain of Fas. S-nitrosylation at cysteine 304 promotes redistribution of Fas to lipid rafts, formation of the death-inducing signal complex, and induction of cell death.**

Keywords: Colon Cancer; Tumor; Signaling; Localization.

Fas (APO-1/CD95) is a transmembrane receptor that belongs to the tumor necrosis factor–receptor superfamily. Fas plays a physiological role in immune response control (eg, through mediating activation-induced T-cell death).[1,2] Interaction of the Fas receptor with its ligand (FasL) initiates assembly of an intracellular death-inducing signaling complex (DISC)[3] that contains the Fas-associated death domain (FADD) adaptor protein and either procaspase-8 or procaspase-10.[4,5] Autoprocessing of these proenzymes within the DISC generates proteolytically active enzymes.[6] In type I cells, in which Fas is localized mainly in plasma membrane lipid rafts,[7–10] active caspase-8 directly activates a cascade of effector caspases in a mitochondria-independent manner. In type II cells, whose rafts do not contain Fas, limited amounts of caspase-8 are activated at the DISC and cleave the proapoptotic Bcl-2 family member Bid. In turn, truncated Bid moves to the mitochondria to stimulate the release of soluble factors that activate the caspase cascade in the cytosol.[11,12]

Engagement of Fas can trigger apoptosis of some cancer cells[13,14] but not all cancer cells respond to FasL. Changes of Fas expression frequently are found in cancer mutations in the Fas gene,[15,16] down-regulation of Fas expression at the cell surface,[17] and high levels of anti-apoptotic proteins such as FADD-like interleukin-1-beta-converting enzyme (FLICE)-like inhibitory protein; Bcl-2 and inhibitors of apoptosis[18–21] can decrease the potency of the Fas-mediated apoptotic signal. A series of post-translational modifications also modulate Fas-dependent cell death. The membrane-proximal region of the cytoplasmic domain of Fas can be phosphorylated by an unidentified kinase.[22,23] The receptor also may be palmitoylated at cysteine 199 (Cys199) in human beings and Cys194 in mice, and this facilitates Fas redistribution into lipid rafts and its association with ezrin and the actin cytoskeleton.[24,25] Cys294 of murine Fas can be S-glutathionylated after FasL engagement, which involves caspase-dependent degradation of glutaredoxin 1, and this promotes aggregation of the receptor, its recruitment into lipid rafts, and propagation of Fas-dependent

Abbreviations used in this paper: ALPS, autoimmune lymphoproliferative syndrome; Cys, cysteine; DISC, death-inducing signaling complex; FADD, Fas-associated death domain; FasL, Fas-ligand; FLICE, FADD-like interleukin-1-beta-converting enzyme; GFP, green fluorescent protein; GTN, glyceryl trinitrate; NO, nitric oxide; NOS, nitric oxide synthase; SNO, nitrosothiol; SNO-proteins, S-nitrosylated proteins; 3D, 3-dimensional; WT, wild-type.

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apoptosis.[26,27] Less Fas accumulates at the surface of cancer cells, which have a reduced apoptotic response to FasL.[28–30] Fas expression is positively regulated by the transcription factor nuclear factor-κB,[31] whereas other transcription factors such as activator protein-1, signal transducer and activator of transcription 3,[32] Yin Yang 1,[33] and the Fas-associated phosphatase-1[34,35] have the opposite effect.

The down-regulation or mutation of Fas has been proposed as a mechanism by which cancer cells avoid destruction by the immune system through reduced apoptosis sensitivity.[36] Breaking such resistance was rendered possible with some anticancer drugs that enhance Fas-receptor expression and aggregation at the surface of tumor cells, thereby increasing the apoptotic response to FasL.[36–38] We have shown that nitric oxide also has a sensitizing effect by increasing Fas expression on cancer cells,[39] which raises the possibility that Fas-receptor expression or function could be regulated by S-nitrosylation, especially because Fas harbors a 3-dimensional (3D)

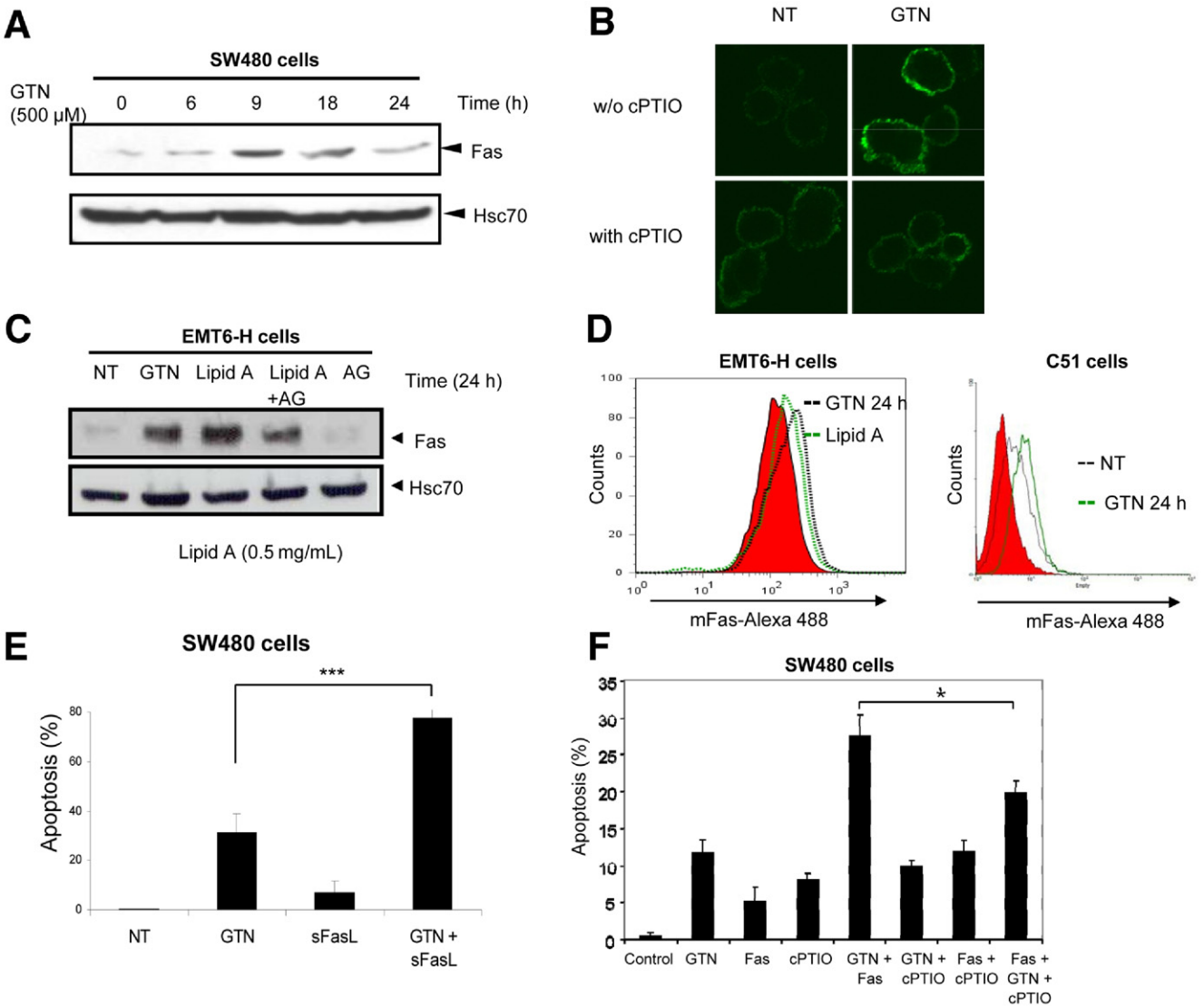

Figure 1. NO increases Fas expression and sensitizes cells to FasL-mediated apoptosis. Colon cancer cells SW480 and C51 and mammary cancer cells EMT6-H were left nontreated (NT) or treated with 500 mmol/L GTN or with 0.5 mg/mL lipid A for the indicated times. (*A* and *C*) For analysis of whole-cell Fas expression, cells were lysed and 50 μg of each lysate was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblot using the anti-Fas antibody. Constitutive heat shock protein 70 (Hsc70) was used as loading control. (*C*) To verify that any increase in Fas expression in lipid A–treated EMT6-H cells involved inducible NOS activity and hence NO, cells were treated with the NOS inhibitor aminoguanidine (AG). Cell surface expression of Fas was evaluated using a mouse anti-Fas antibody by (*B*) confocal microscopy in SW480 cells or by (*D*) flow cytometry in EMT6-H and C51 cells. Data are representative of at least 3 independent experiments. (*E*) Apoptosis was induced in SW480 by treatment with 500 μmol/L GTN for 24 hours then with or without soluble FasL (sFasL) for 24 hours. (*F*) To verify that apoptosis induced by GTN and FasL involved NO, SW480 cells were treated with the NO scavenger 2-(4-Carboxyphenyl)-dihydro-tetramethyl-imidazol-oxide (cPTIO) and GTN for 24 hours then with or without sFasL for 6 hours. This short treatment with sFasL (6 vs 24 h) is owing to the high toxicity of cPTIO. Apoptotic cells were identified by fluorescence microscopy after staining nuclei with Hoechst 33342 and then counted. Data are the ± standard error of the mean of at least 3 independent experiments. *$P < .05$ and ***$P < .001$.

structure of the proposed consensus motif of S-nitrosylation. We show here that both exogenous and endogenous NO induce S-nitrosylation of Cys199 and Cys304 of the Fas receptor, and this correlates with enhanced expression of Fas at the plasma membrane and its translocation into lipid rafts. Expression of a non-nitrosylable mutant Fas receptor prevents NO-mediated sensitization to Fas-induced apoptosis. Thus, S-nitrosylation of Fas receptor is an additional posttranslational mechanism that regulates Fas-mediated cell death.

Materials and Methods

Cell Lines

Human SW480 and murine C51 colon cancer cell lines were obtained from the American Tissue Culture Collection (Manassas, VA). EMT6-H cells are clones selected from the murine mammary EMT6 cancer cell line.[40] All cell lines were cultured in a 1:1 (vol/vol) mixture of Dulbecco's modified Eagle medium and HAMF-F10 (Biowittaker, Fontenay-Sous-Bois, France) supplemented with 10% fetal bovine serum and 2 mmol/L L-glutamine at 37°C in a dry atmosphere. All cell lines were tested and found to be free of *Mycoplasma* species infection.

Biotin Switch Assay

The biotin switch assay was performed as described[41] with some modifications.[42] Briefly, cells (1×10^8) were washed in phosphate-buffered saline (PBS) and homogenized in nondenaturing nitrosothiol (SNO) lysis buffer (50 mmol/L Tris-HCl, pH 7.4, 300 mmol/L NaCl, 5 mmol/L ethylenediaminetetraacetic acid [EDTA], 0.1 mmol/L neocuproine, 1% Triton X-100, and protease inhibitors), then 4 volumes of blocking buffer (225 mmol/L HEPES, pH 7.7, 0.9 mmol/L EDTA, 90 μmol/L neocuproine, and 2.5% sodium dodecyl sulfate) were added. Free thiols were blocked by methylation with 20 mmol/L methyl methane thiosulfonate at 50°C for 20 minutes. After removing excess methyl methane thiosulfonate by precipitating twice with acetone, nitrosothiols were reduced to thiols with 1 mmol/L ascorbate without altering the methylated thiols. The newly formed thiols then were linked with sulfydryl-specific biotinylating reagent biotin-N-[6-(biotinamido)-hexyl]-1′-(2′-pyridyldithio) propionamide (HPDP). Biotinylated proteins were detected by immunoblotting using horseradish peroxidase–conjugated streptavidin or were purified by precipitation with Ultralink Immobilized Neutravidin Protein Plus and eluted from the beads with Laemmli buffer. Samples were analyzed by immunoblotting with anti-Fas or anti–green fluorescent protein (GFP) antibodies.

Statistics

Data are presented as means ± standard deviation of the indicated number of experiments. Significant differences were evaluated by analysis of variance followed by the Student *t* test.

Results

NO-Mediated Increase in Fas Expression Sensitizes Cancer Cells to FasL

When SW480 human colon cancer cells are exposed to the NO donor glyceryl trinitrate (GTN) an

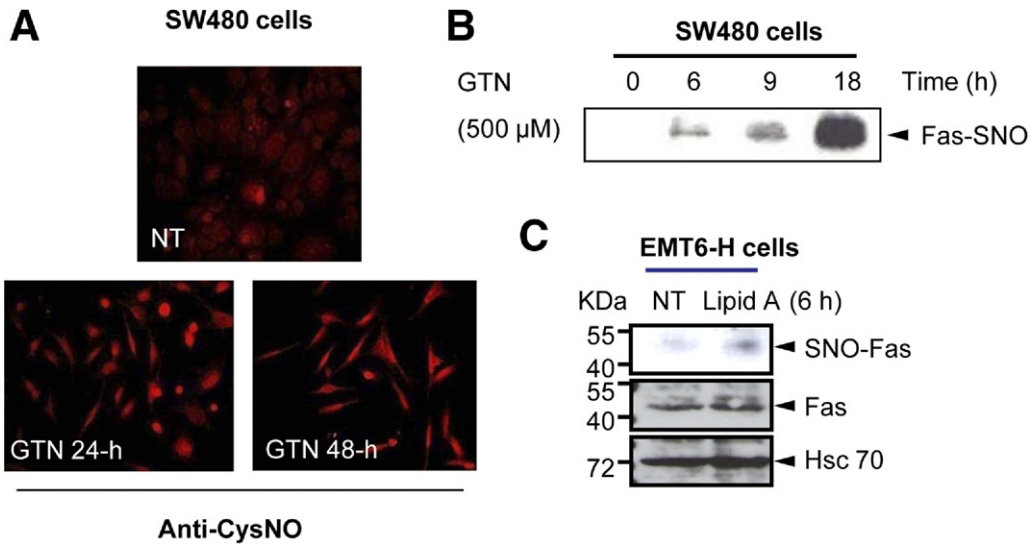

Figure 2. NO induces S-nitrosylation of Fas. SW480 cells were left nontreated (NT) or treated with 500 μmol/L GTN for the indicated times. (*A*) SNO-proteins were detected using an anti-Cys NO antibody by immunofluorescence. Protein extracts were subjected to the biotin switch technique, and the biotinylated proteins were purified with streptavidin-agarose, followed by elution with 2-mercaptoethanol (the SNO-enriched fraction). (*B*) This latter fraction was subjected to immunoblotting for Fas (C-20 antibody). (*C*) EMT6-H cells were treated with 0.5 μg/mL lipid A and Fas S-nitrosylation was detected after purification of biotinylated proteins as indicated before and immunoblotting with an anti-Fas antibody (from Abcam). Constitutive heat shock protein 70 (Hsc70) was used as loading control. Data are representative of at least 3 independent experiments.

increase in Fas expression is observed,[39] here shown by immunoblotting (Figure 1*A*) and by fluorescent immunostaining (Figure 1*B*, *upper panel*). Similar responses also were observed in the EMT6-H murine mammary cancer and the C51 murine colon cancer cell lines (Figure 1*C* and *D*). When EMT6-H cells were exposed to the lipid A derivative OM-174, which similar to most lipid A molecules induces inducible NO synthase (NOS) expression, Fas expression increased, an effect that was partially reversed by the NOS inhibitor aminoguanidine (Figure 1*C*). This suggests that NO was involved in up-regulating Fas. Cell lines were exposed to 500 μmol/L GTN for 24 hours and then treated with soluble FasL for an additional 24 hours. Apoptotic cell death was induced synergistically ($P < .01$) in SW480 cells (Figure 1*E*) as well as in C51 (Supplementary Materials and Methods, Supplementary Figure 1*A*) and EMT6-H cells (Supplementary Figure 1*B*). GTN-mediated sensitization of FasL-induced apoptosis was dependent of NO because 2-(4-Carboxyphenyl)-dihydro-tetramethyl-imidazol-oxide, a scavenger of NO, significantly affected cell death, suggesting the involvement of NO in this synergistic increase in sensitivity of cells to apoptosis (Figure 1*F*). It was noted that the trimeric recombinant FasL was more efficient to induce apoptosis in C51 cells than the monomeric soluble FasL (Supplementary Figure 1*A*), and the synergistic effect of GTN and FasL was not caused by an increase in NO levels as attested by nitrite accumulation measurement (Supplementary Figure 2).

NO Induces S-Nitrosylation of Fas

To determine whether NO could induce S-nitrosylation of Fas, we first used a biotin switch assay to measure the amount of S-nitrosylated proteins (SNO-proteins) present in cells. The level of SNO-protein in SW480 cells increased significantly when cells were exposed to 500 μmol/L GTN for 18 hours (Supplementary Figure 3*A*) or for 48 hours (Supplementary Figure 3*B*). To verify the biotin labeling of SNO, reactive methyl methane thiosulfonate (which blocks free thiols on cysteine residues) or ascorbate (which converts SNO to thiols and allows labeling of nascent thiols with biotin-HPDP) were

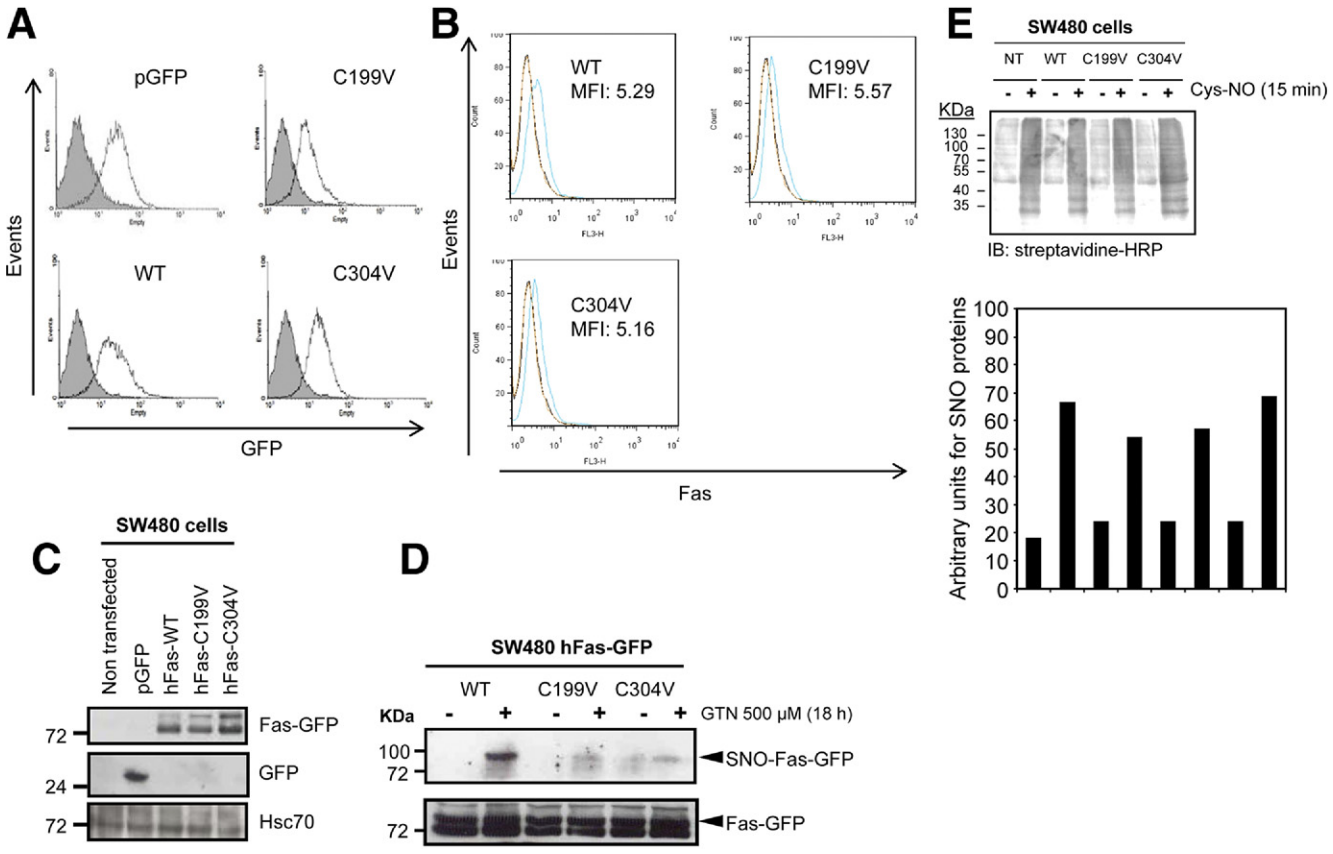

Figure 3. Human Fas is S-nitrosylated at cysteines 199 and 304. SW480 cells were stably transfected with Fas-WT, Fas-C199V, and Fas-C304V. The expression of these different forms of Fas was determined by flow cytometry either at the (*A*) whole Fas level or the (*B*) cell surface Fas level, and by (*C*) immunoblot using an anti-GFP antibody. Cells from these different clones were treated with or without 500 μmol/L GTN for 18 hours before the biotin switch assay. Constitutive heat shock protein 70 (Hsc 70) was used ad loading control. (*D*) Streptavidin-purified proteins were analyzed by immunoblot with anti-GFP antibodies. (*E*) To verify that S-nitrosylation is not affected in cells left nontransfected (NT) or expressing the different mutated forms of Fas, full SNO-protein profiles are assessed by immunoblotting with horseradish peroxidase (HRP)-conjugated streptavidin (streptavidin-HRP) lysates from cells treated with or without the NO donor S-nitroso-cysteine (Cys-NO) at 1 mmol/L for the indicated time (*upper panel*). The histogram shows quantification of SNO-protein levels using densitometric analyses of total protein levels (*lower panel*). Data are representative of at least 3 independent experiments.

omitted. As expected, SNO-proteins were more abundant in the absence of methyl methane thiosulfonate and biotinylated proteins were less abundant in the absence of ascorbate (Supplementary Figure 3*A*). The GTN-induced increase in total SNO-proteins also was detected using immunofluorescence up to 48 hours after the start of the treatment (Figure 2*A*). In cell lysates enriched for SNO-proteins (SNO-purified proteins) (Supplementary Figure 3*B*), Fas was found to accumulate in the SNO-enriched fraction of GTN-treated SW480 cells (Figure 2*B*). A similar partitioning was observed in fractions from EMT6-H cells exposed to the synthetic lipid A, OM-174 (Figure 2*C*). It is noteworthy that the total amount of SNO-protein significantly increased in OM-174–treated cells (Supplementary Figure 3*C*). Thus, both exogenous and endogenous NO triggered S-nitrosylation of the Fas receptor.

Human Fas Is S-Nitrosylated at Cysteines 199 and 304

The cytoplasmic part of the Fas receptor includes a cysteine residue close to its transmembrane domain (Cys199) and another one near its death domain (Cys304) (Supplementary Figure 4*A*). Analysis of the 3D structure of the Fas intracellular domain indicated that Cys304 is within a consensus sequence (K/R/H)-C-(D/E) for S-nitrosylation[43] (Supplementary Figure 4*B*). SW480 cells were stably transfected with GFP-tagged Fas constructs mutated, at these cysteine residues (C199V or C304V)[24] and the resultant GFP-positive clones had a high expression of Fas, with comparable intensities either in whole cells (Figure 3*A*) or at the cell surface (Figure 3*B*). Expression of the constructs in cells was confirmed by immunoblotting using an anti-GFP antibody (Figure 3*C*). When SW480 cells were exposed to 500 μmol/L GTN and subjected to the biotin switch assay, a strong increase in SNO-Fas was detected in SW480 cells expressing wild-type Fas (Fas-WT) whereas only a weak increase in SNO-Fas was detected in cells expressing the C199V or C304V Fas mutants (Figure 3*D*). The defective S-nitrosylation of the mutant forms of Fas does not seem to be related to a defect in S-nitrosylation because the profiles of S-nitrosylation in total cell lysates were similar in the different clones (Figure 3*E*).

Cysteine 304 Is Crucial for GTN-Induced Fas Aggregation

The distribution of Fas in cancer cells was monitored more closely by confocal microscopy. Exposure of Fas-WT or Fas-C199V–transfected SW480 cells to 500 μmol/L GTN for 48 hours triggered Fas aggregation, as detected by an anti-Fas antibody on nonpermeabilized cells (supporting cell membrane location of Fas, also well visible in Figure 1*B*), but this response was less pronounced in cells expressing Fas-C304V (Figure 4*A*). We noted that Fas aggregation was more markedly detected

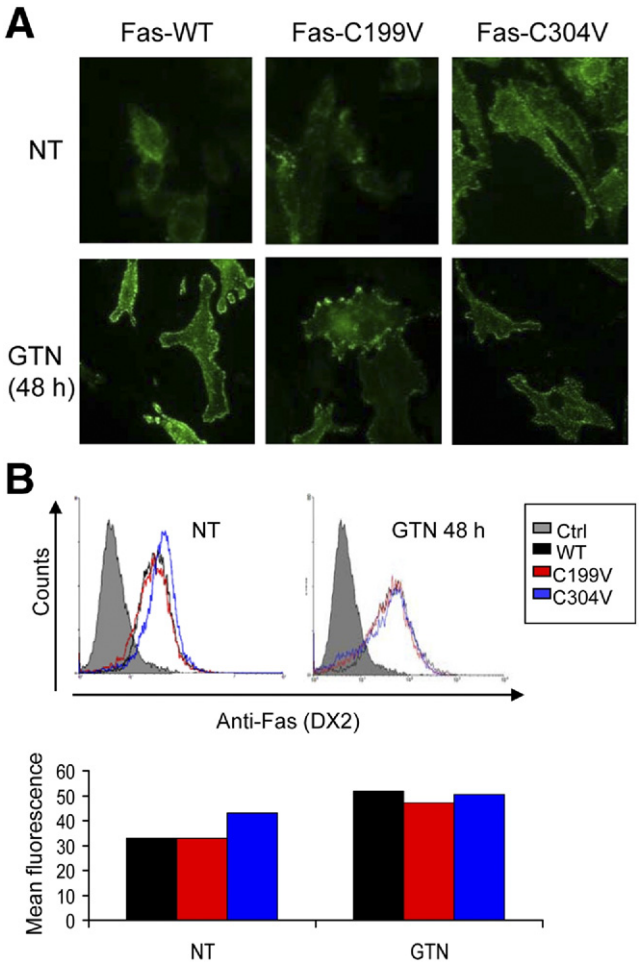

Figure 4. S-nitrosylation is required for Fas aggregation at the cell membrane. SW480 cell lines stably expressing Fas-WT, Fas-C199V, or Fas-C304V were treated with 500 μmol/L GTN for 48 hours or left nontreated (NT). Fas membrane expression was followed by (*A*) confocal microscopy or by (*B*) flow cytometry using an anti-Fas antibody (DX2) and an Alexa Fluor–488– or Alexa Fluor–568–conjugated goat anti-mouse secondary antibody. Data are representative of at least 3 independent experiments.

after 48 hours of treatment with GTN than at 18 hours (data not shown), a time when the best S-nitrosylation was seen, suggesting that aggregation of Fas needed more time to occur and should be a consequence of its S-nitrosylation. Expression of Fas at the plasma membrane increased in GTN-treated SW480 cells expressing Fas-WT, Fas-C199V, and Fas-C304V (Figure 4*B*). These results indicate that S-nitrosylation of both Cys199 and Cys304 determine the membrane localization of Fas and that S-nitrosylated Cys304 in addition favors Fas aggregation. This aggregation previously has been shown to occur in plasma membrane lipid rafts.[7,44,45] Raft fractions were identified by immunoblot detection of caveolin-1 (Figure 5*A*). By using an anti-GFP antibody, Fas was detected in lipid raft fractions (fractions 4–7) from SW480 cells expressing Fas-WT or Fas-C199V but not from cells expressing Fas-C304V. Exposure of cells to

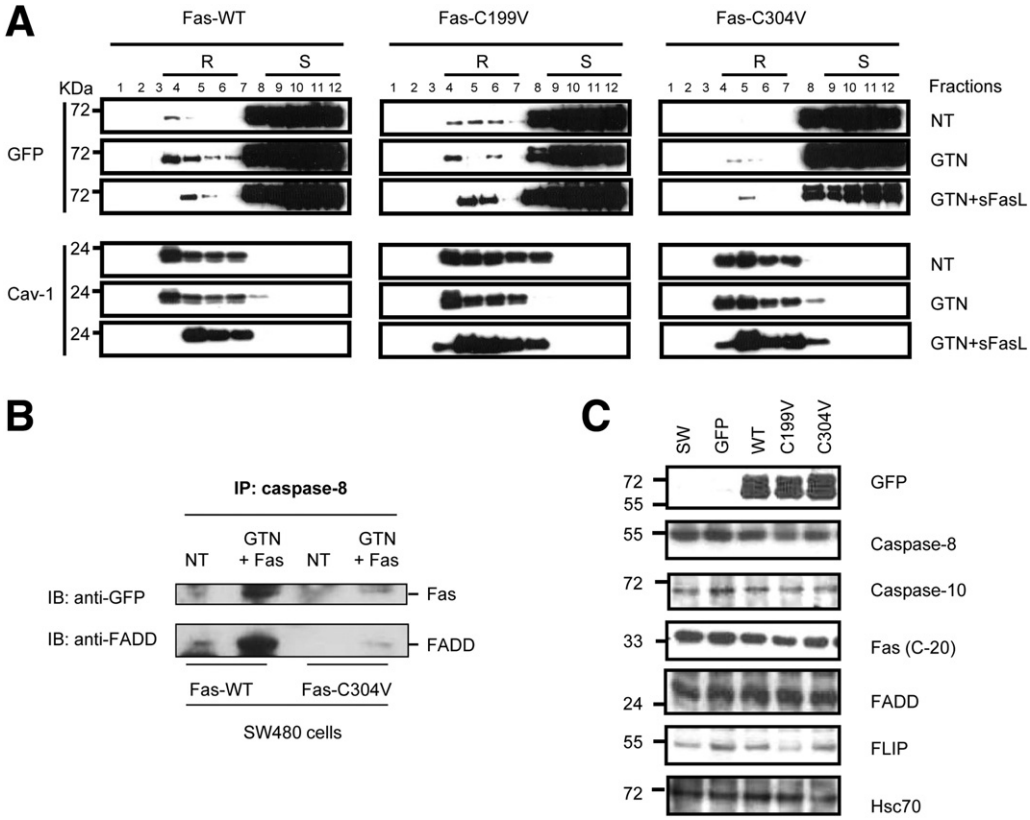

Figure 5. S-nitrosylation is required to target Fas to lipid rafts and facilitated FasL-induced DISC formation. SW480 stably expressing Fas-WT, Fas-C199V, or Fas-C304V were left nontreated (NT) or treated with 500 μmol/L GTN for 24 hours alone or with soluble FasL (sFasL) (0.5 arbitrary unit/mL) for an additional 6 hours. Cells were lysed in Triton X-100 and the lipid raft fraction was isolated using a sucrose gradient centrifugation method. For each harvested fraction, 30 μg of protein was separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. (*A*) Expression of Fas-GFP (~65 kilodaltons) in the low-density microdomains corresponding to lipid rafts (R), shown by the expression of caveolin-1 (Cav-1) (fractions 4–7), and in the soluble nonraft fractions (S) was analyzed by immunoblotting using an anti-GFP antibody. (*B*) SW480 cells expressing Fas-WT or Fas-C304V were treated with 500 μmol/L GTN for 18 hours followed by stimulation with 250 ng/mL recombinant FasL (rFasL) for 1 hour, and DISC formation was analyzed by immunoprecipitation of caspase-8 and immunoblotting of FADD and Fas-GFP. (*C*) Expression in the whole cell of components of the Fas signaling pathway, Fas, FADD, FLICE-like inhibitory protein (c-FLIP), and caspase-8 and caspase-10, are shown for the different clones as indicated. Data are representative of at least 3 independent experiments. Constitutive heat shock protein 70 (Hsc70) was used as loading control.

GTN either alone or with FasL increased the translocation of Fas-WT and Fas-C199V into lipid rafts, but not that of Fas-C304V (Figure 5*A*). Furthermore, FasL induced the recruitment of FADD and caspase-8 to the DISC in SW480 cells expressing Fas-WT and pretreated with GTN, but not in cells expressing Fas-C304V (Figure 5*B*). However, we noted that expression of mutated Fas forms did not affect the expression of Fas signaling molecules such as FADD, caspase-8, caspase-10, and FLICE-like inhibitory protein (Figure 5*C*). Thus, disruption of Cys304 of Fas affects receptor translocation into lipid rafts and DISC formation.

Fas S-Nitrosylation Is Critical for GTN-Induced Cell Sensitization

Treatment of SW480 cells with GTN for 24 hours followed by FasL for 24 hours led to a significant increase in FasL-induced apoptosis in nontransfected cells, and in cells transfected with Fas-WT or Fas-C199V, but not in cells transfected with Fas-C304V ($P < .01$) (Figure 6*A*). Similar observations were made in murine C51 cells that are more resistant to GTN and FasL-induced apoptosis than SW480 cells ($P < .01$) (Figure 6*B*). The failure of GTN to sensitize cells expressing Fas-C304V to FasL could be explained either by defective S-nitrosylation or by the substitution of the cysteine itself. To discriminate between these two possibilities we used EMT6-H cells in which NO can be produced through activation of NOS II (Figure 1*C*). EMT6-H cells are sensitive to FasL-mediated apoptosis, but resistant to GTN-induced cell death. Expression of Fas-WT, Fas-C199V, or Fas-C304V in EMT6-H cells did not modulate their response to FasL but expression of Fas-C304V specifically reduced GTN/FasL-mediated apoptosis ($P < .001$) (Figure 6*C*). To be sure that the mutation of an amino acid residue within or around the death domain is not responsible for the absence of synergy between GTN and FasL, we stably

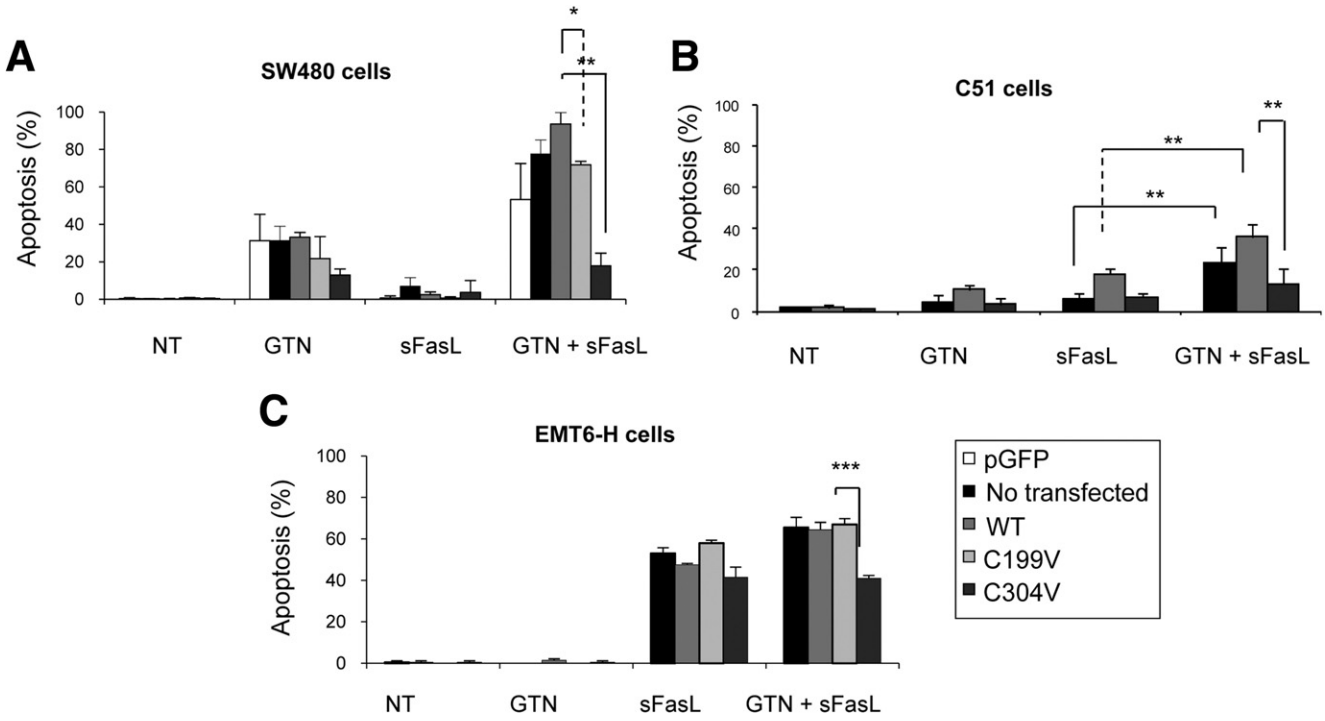

Figure 6. S-nitrosylation is critical for GTN-induced cancer cell sensitization. (*A*) SW480, (*B*) C51, and (*C*) EMT6-H cells expressing GFP vector (pGFP), Fas-GFP (WT), Fas-C199V, or Fas-C304V were nontreated (NT) or treated with 500 μmol/L GTN for 48 hours alone or in combination with 0.5 arbitrary unit/mL soluble FasL (sFasL) or 0.25 μg/mL or recombinant FasL for 24 hours. Apoptotic cells were identified by fluorescence microscopy after staining nuclei with Hoechst 33342. Data are the ± standard error of the mean of at least 3 independent experiments. *$P < .05$, **$P < .01$, and ***$P < .001$.

expressed a Fas-Q257K mutation (described in a patient suffering from autoimmune lymphoproliferative syndrome (ALPS) type Ia or lymphoma)[46] in EMT6-H cells. In the two clones expressing this mutated form of Fas, in terms of the increase in Fas expression as shown by immunoblot (Figure 7*A*, *left panel*) and quantification (Figure 7*A*, *right panel*), the sensitizing effect of GTN to FasL-induced apoptosis remained intact ($P < .01$) (Figure 7*B*). Therefore, apoptosis resistance of cells expressing Fas-C304V to GTN/FasL is not likely to be attributed to the substitution of the cysteine itself but rather to its defective S-nitrosylation.

Discussion

Posttranslational modification of receptor proteins is a ubiquitous feature of signal transduction. The present study indicates that NO, either exogenously released from GTN or endogenously produced by NOS II activation, S-nitrosylates the death receptor Fas at two cysteine residues in the cytoplasmic region. This domain does not contain the linear consensus sequence (K/R/H)C(D/E) for S-nitrosylation of proteins at cysteine residues,[43] but around Cys304 the 3D conformation of the Fas receptor intracellular domain resembles that of the consensus sequence (Supplementary Figure 2*B*). Similarly, although Cys199 is not in the optimal hydrophobic environment for S-nitrosylation,[47] amino acid residues surrounding Cys199 are to an extent similar to the proposed consensus motif (KRKEVQKT-C199-RKHRKE) and may constitute a favorable environment for S-nitrosylation. Although both Cys199 and Cys304 are S-nitrosylated, Cys304 appears to be the one that mediates GTN-induced aggregation of Fas and its recruitment into lipid rafts and DISC formation, thus leading to an increase in FasL-mediated apoptosis. It has been reported that some antitumor agents act by inducing the translocation of Fas into lipid rafts. For instance, resveratrol, cisplatin, and edelfosin have been reported to induce co-clustering of Fas and rafts and subsequent apoptotic cell death in solid tumors and leukemic cells, but the mechanisms responsible are yet to be defined.[48,49] Likewise GTN-mediated S-nitrosylation of Fas induces it to aggregate and translocate into rafts, and so provides new molecular insight into how death receptor-mediated apoptosis is triggered. Mutation of a residue in the intracellular domain of Fas is not sufficient to explain the results shown here because it does not alter the FasL response of EMT6-H, a partially responsive cell line. Further, a mutation of Fas in an amino acid residue other than cysteine within the death domain is just as sensitive to FasL-induced apoptosis as Fas-WT. Altogether, these results indicate that nitrosylation of Fas at 2 cysteine residues is an additional way its activity can be regulated posttranslationally.

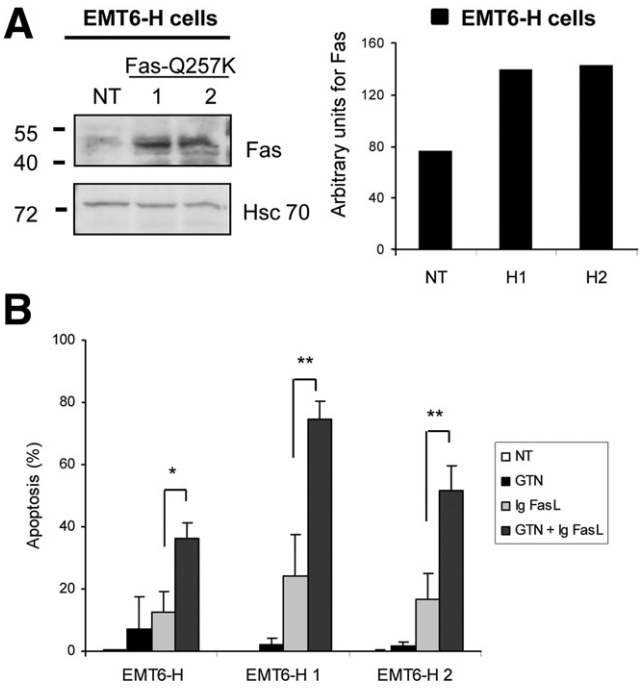

Figure 7. A substitution mutation unrelated to cysteine in the Fas death domain does not affect GTN-mediated sensitization to Fas-induced apoptosis. EMT6-H cells were left nontransfected (NT) or stably transfected with the Fas Q257K plasmid. (*A*) EMT6-H cells and two clones that showed an increase in Fas expression as determined by immunoblot (*left panel*) and densitometric analyses (*right panel*) were treated with 500 μmol/L GTN and/or 0.25 μg/mL recombinant FasL (rFasL) for the indicated times. Constitutive heat shock protein 70 (Hsc70) was used as loading control. (*B*) Apoptotic cells were identified by fluorescence microscopy after staining nuclei with Hoechst 33342. Data are the ± standard error of the mean of at least 3 independent experiments. *$P < .05$, and **$P < .01$.

Mutations in the death domain of Fas were shown to account for the resistance of some immune cells to FasL-mediated cell death, both in mouse models[50] and in patients suffering from lymphoma[51,52] or type Ia ALPS (Fas gene mutation).[1,2,53] Such mutations in the Fas death domain were suggested to interfere with Fas signaling in a dominant-negative way because homotrimers of Fas are crucial for Fas signal transduction (for review see Bidere et al,[54] 2006). Our results may indicate that Fas-C304V also behaves as a dominant-negative receptor for GTN/FasL-induced apoptosis, being partially permissive of apoptosis. This is consistent with the observation that in patients who suffer from ALPS type Ia, heterotrimeric Fas complexes are still capable of recruiting FADD, although to a lesser extent,[16] and that cells expressing heterozygous death domain-mutated Fas are still able to transduce the cell death signal.[55] S-nitrosylation of Fas enhances its aggregation but the molecular mechanism behind this process is largely unknown. We speculate that S-nitrosylation could regulate protein–protein interactions and, similar to phosphorylation, may influence Fas aggregation.

It has been reported that interaction of proteins such as caspase-3 with several other proteins (eg, sphingomyelinase, inducible NOS, and immune response gene) is induced specifically by NO.[56] Our findings show that the failure of GTN to S-nitrosylate Fas dramatically affects the trafficking of Fas to the cell surface (Figure 4*A*). Dynamin-2, a guanosine triphosphatase involved in endocytosis, facilitates Fas protein translocation from the Golgi apparatus via the trans-Golgi network to the cell surface.[57] So we can hypothesize that the loss of Fas S-nitrosylation in cells expressing the Cys304 mutant of the receptor might affect the interaction of dynamin-2, also S-nitrosylated,[58] with Fas and hence expression of the receptor at the cell surface.

Here a new posttranslational modification of Fas has been identified that modulates the receptor response to its ligand, its redistribution into plasma membrane lipid rafts, recruitment of signaling molecules of the DISC, and the downstream apoptotic response. The results indicate that in some circumstances, such as exposure to GTN or activation of endogenous inducible NOS, this posttranslational modification is essential for a synergistic cytotoxic effect with FasL. Because Fas expression is often lower in cancer cells less sensitive to apoptosis, inducers of Fas S-nitrosylation are appropriate tools to restore cell sensitivity to Fas. The present study should not only yield insights into details of the role of NO as a sensitizer of apoptosis but also open perspectives for NO-related therapeutics.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2011.02.053.

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Conflicts of interest

The authors disclose no conflicts.

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Supplementary Materials and Methods

Drugs and Reagents

GTN, a NO donor that requires bioactivation by the enzyme aldehyde dehydrogenase to produce nitrite,[1,2,3] was purchased from Merck (Lyon, France), OM-174 was purchased from OM-Pharma (Meyrin, Switzerland),[4] and Ultralink Immobilized Neutravidin Protein Plus, EZ-Link Biotin-HPDP, and streptavidin-conjugated horseradish peroxidase were purchased from Perbio Science (Brebières, France). The antibodies used included the following: rabbit anti-S-nitroso-cysteine (SNO-Cys) from Sigma-Aldrich (St. Quentin Fallavier, France); rabbit anti-Fas (C-20), mouse anti-GFP, anti–constitutive heat shock protein 70 (Hsc 70) monoclonal antibodies, and horseradish-peroxidase–conjugated monkey anti-goat antibodies from Santa Cruz Biotechnology (Le Perray en Yvelines, France); anti-CD95 (DX2) and anti-FADD from BD Pharmingen; purified mouse anti–caveolin 1 from BD Biosciences (Le Pont de Claix, France); anti–FLICE-like inhibitory protein and anti–caspase-8 monoclonal antibodies from Axxora (Villeurbanne, France); goat anti–caspase-8 from Santa Cruz (San Diego, CA); anti–caspase 10 from Medical and Biological Laboratories (Nagoya, Japan); goat anti-Fas from R&D Systems (Lille, France); and rabbit anti-Fas from Abcam (Paris, France). Bovine serum albumin was from ID Bio (Limoges, France). Protease inhibitor cocktail was from Roche Diagnostics (Meylan, France). Soluble FasL was collected from the supernatant of FasL-transfected Neuro2A cells (provided by Dr Fontana, Lausanne, Switzerland). One arbitrary unit of soluble FasL contained in 100 μL of supernatant of Neuro2A cells that had been confluent for 48 hours.[5] The recombinant immunoglobulin-FasL was kindly provided by Drs Taupin and Legembre (Composantes Innées de la Réponse Immunitaire et Différenciation(CIRID), Bordeaux, France).

Apoptosis Detection

Cancer cells (3 $\times$ 105 cells/mL of culture medium) were treated at 37°C with the indicated reagents. After treatment, the whole population of cells (attached and loose cells) was collected and apoptosis was assessed by quantifying the percentage of cells with condensed nuclei and fragmented chromatin using Hoechst 33342 staining and fluorescence microscopy as previously described.[1]

Flow Cytometry

The presence of Fas receptor at the plasma membrane or in the cell as a whole was determined by staining cells with phycoerythrin-conjugated anti-Fas monoclonal antibodies (DX2 or goat anti-mFas) and analyzing them by flow cytometry. For plasma membrane staining, 1 $\times$ 106 cells were washed with cold PBS and resuspended in 100 μL of staining buffer (1% bovine serum albumin, 0.1% sodium azide in PBS) before incubation with anti-Fas (1:100 [vol/vol] goat anti-Fas or 1:500 [vol/vol] DX2) or isotype immunoglobulin G control antibody for 1 hour. Cells were washed twice in the buffer, followed by incubation with a fluorescein isothiocyanate or Alexa Fluor–568–conjugated anti-mouse or anti-goat antibody diluted 1:1000 (vol/vol) for 30 minutes. Cells then were washed twice in staining buffer and analyzed using a FACSCalibur system (BD Biosciences) with the CellQuest program (BD Biosciences, Le Pont de Claix, France).

Generation of Cell Clones Stably Expressing Different Forms of Fas-GFP

WT Fas, Fas mutated at Cys^{199} (Fas-C199V), and Fas mutated at Cys^{304} (Fas-C304V) were kindly provided by Dr A. O. Hueber (CNRS UMR 6543, Nice, France). The Fas Q257K mutation was generated using the PBJ1-neo-Fas WT vector.[6] The calcium phosphate technique was used for stable transfection of SW480, C51, and EMT6-H cancer cells with the different plasmids. Briefly, 5 μg of plasmid was mixed with 440 μL of H_20 and 50 μL of 2.5 mol/L $CaCl_2$ and incubated with cells for 6 hours, after which cells were washed with PBS and returned to complete culture medium. Thirty-six hours later, G418 was added to the cultures to a final concentration of 0.8 mg/mL. G418-resistant cells were selected, cloned, and tested for GFP or Fas expression by fluorescence activated cell sorting or immunoblot.

Immunofluorescence

Permeabilized (for intracellular staining) and nonpermeabilized (for plasma membrane staining) cells (3 $\times$ 106) were incubated with primary antibodies (diluted 1:100) in 1% bovine serum albumin in PBS for 45 minutes at room temperature. After washing with PBS, cells were incubated for 30 minutes with appropriate Alexa Fluor–488–conjugated or Texas Red–conjugated antibodies (diluted 1:1000). Samples were imaged by epifluorescence and confocal microscopy. Unrelated isotype-matching antibodies were used as negative controls.

Immunoblot Analysis

Cells were lysed for 30 minutes at 4°C in SNO or immunoprecipitation (IP) lysis buffers. Lysates were centrifuged at 12,000 rpm for 15 minutes and protein concentrations were determined using a Bio-Rad (Marnes-La-Coquette, France) kit according to the manufacturer's protocol. Proteins (50–100 μg) were resolved by 10% sodium dodecyl sulfate (SDS) –polyacrylamide gel electrophoresis and analyzed by immunoblotting. Optimal dilutions of primary antibodies, including a polyclonal anti-Fas/CD95 and a monoclonal anti–Hsc 70, were 1:1000 (vol/vol). The horseradish-peroxidase–conjugated secondary antibodies were used at 1:5000 (vol/vol) dilution and the enhanced chemiluminescence system was used for detection (Santa Cruz Biotechnology).

DISC Immunoprecipitation

Immunoprecipitation was performed as described.[7] Cells ($\times 10^8$) were stimulated and then were lysed on ice in 1 mL lysis buffer (20 mmol/L Tris [ph 7.5], 150 mmol/L NaCl, 1% Nonidet-P40, 10% glycerol, complete protease inhibitor (Roche, Indianapolis, IN) for 30 minutes on ice. After centrifugation at 14,000 × g at 4°C for 30 minutes, supernatants were precleared during 2 hours at 4°C in the presence of protein G sepharose (Dutscher, Brumath, France). After centrifugation at 1000 × g for 3 minutes the supernatant was incubated with anti–caspase-8 antibody (0.2 mg/mL) at 4°C for 20 hours in the presence of 50 μL protein G sepharose. The precipitates were washed 4 times in lysis buffer and resuspended in loading buffer containing 2-mercaptoethanol and subjected to SDS–polyacrylamide gel electrophoresis and immunoblotting.

Lipid Raft Isolation

Lipid rafts were isolated by sucrose density gradient centrifugation as previously described.[8] Cells (1 × 108) were washed in PBS, lysed on ice for 20 minutes in 1 mL of 1% Triton X-100 in 25 mmol/L morpholineethanesulfonic acid, 150 mmol/L NaCl, pH 6.5 buffer with a protease inhibitor cocktail, and homogenized (10 strokes) with a loose-fitting glass Dounce homogenizer (Polylabo, Illkirch, France). Homogenates were mixed with 2 mL of 80% sucrose in 1% Triton X-100 in 25 mmol/L morpholineethanesulfonic acid, 150 mmol/L NaCl, pH 6.5 buffer and placed in a centrifuge tube, then overlaid with 4 mL of 30% sucrose and 4 mL of 5% sucrose and centrifuged at 175,000 × g for 20 hours at 4°C. Fractions (1 mL) were collected from the top of the gradient and fraction 3–7 were precipitated with 2 volumes of acetone and analyzed by immunoblotting.

Detection of NO Production

NO production was determined by measuring the accumulation of nitrites in cell culture media using the Griess microassay as described.[9]

Analysis of the 3D Structure of Fas

The structure of the human death domain of Fas was obtained from the NCBI protein structure database (www.ncbi.nlm.nih.gov). The 3D structure of Fas death domain was resolved experimentally using nuclear magnetic resonance spectroscopy by Huang et al.10 A ribbon representation and consensus motif for S-nitrosylation was obtained using the open-source software package WebLab Viewer Active X version 4.0.

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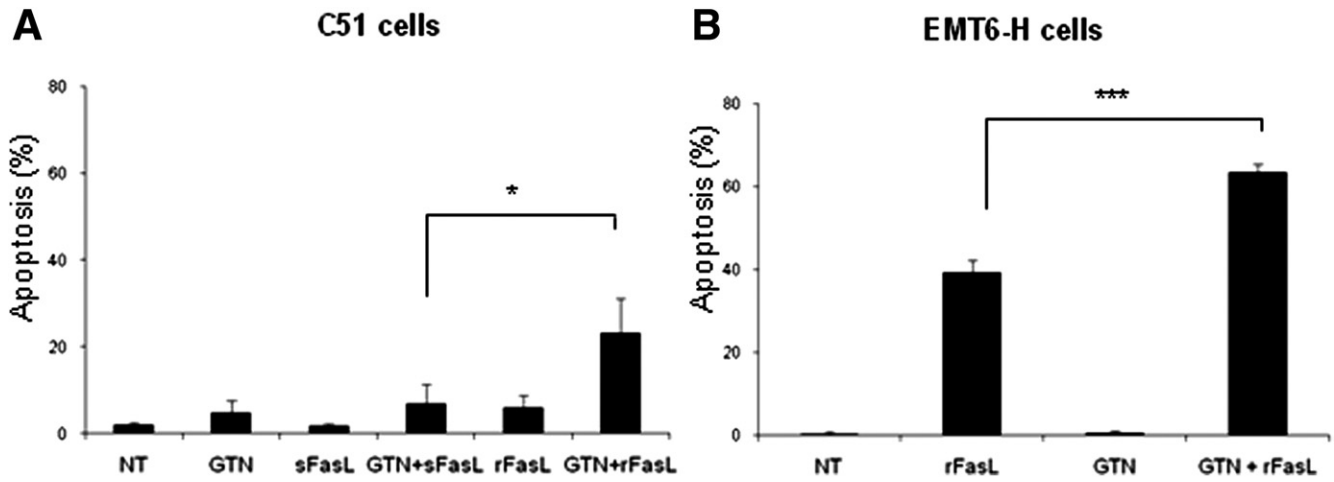
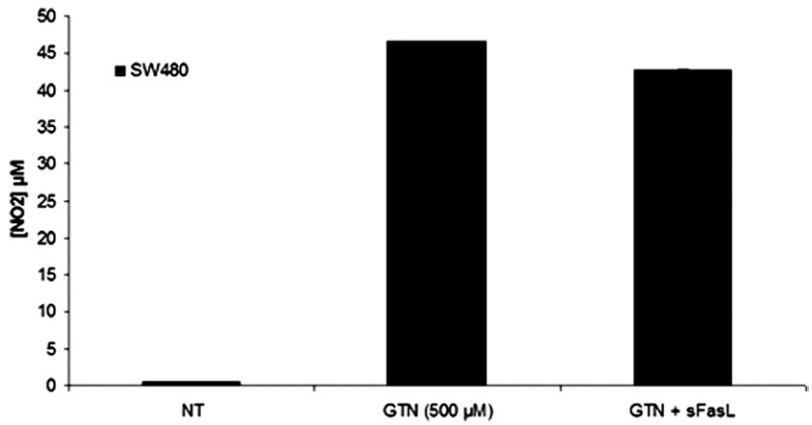
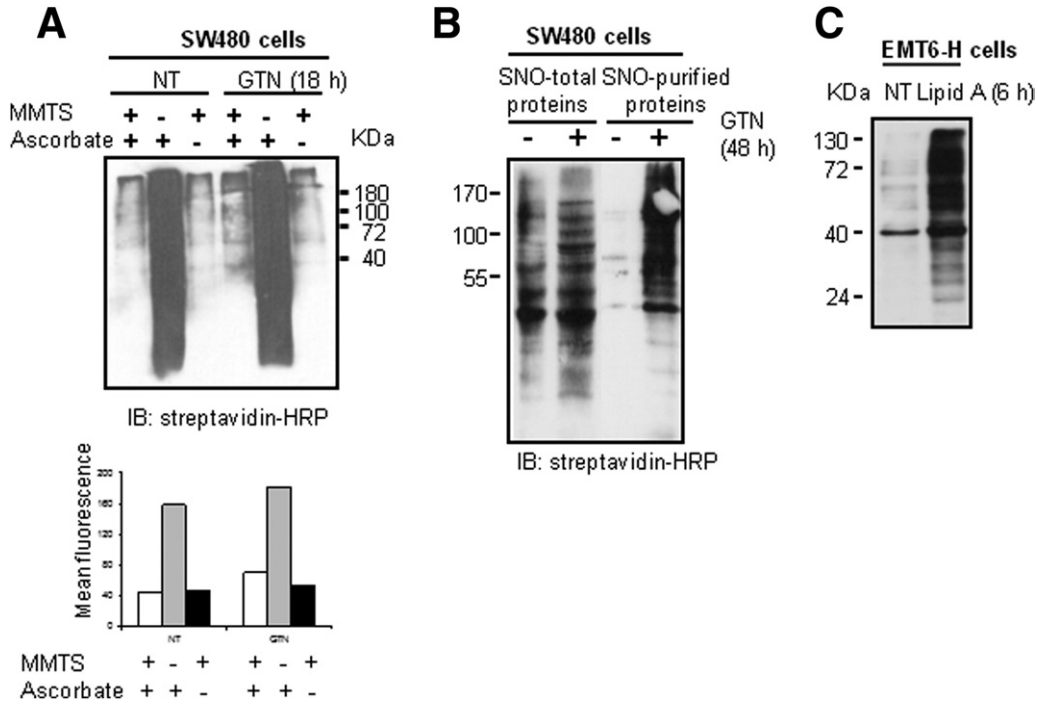


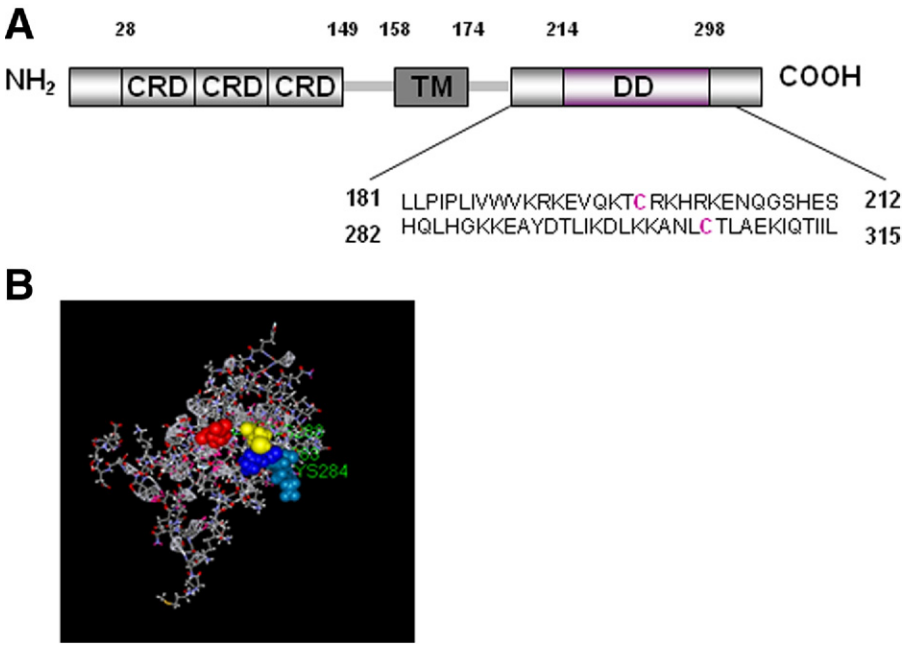
Supplementary Figure 1. Sensitization of cancer cells to apoptosis induced by GTN and soluble FasL (sFasL). Apoptosis was induced in (*A*) colon C51 and (*B*) mammary EMT6-H cancer cells by treatment with 500 μmol/L GTN for 24 hours and then with or without sFasL or recombinant FasL (rFasL) for 24 hours. Apoptotic cells were identified by fluorescence microscopy after staining nuclei with Hoechst 33342 and then counted. Data are the ± standard error of the mean of at least 3 independent experiments.



Supplementary Figure 2. NO production. SW480 cells were treated with 500 μmol/L GTN for 24 hours and then with or without soluble FasL (sFasL) or recombinant FasL (rFasL) for 24 hours, before measuring the accumulation of nitrites (that attests for NO production) in the medium using the standard Griess technique. Data are representative of 3 independent experiments.



Supplementary Figure 3. NO induces SNO. SW480 cells were treated with 500 μmol/L GTN for the indicated times. (*A*) To verify biotin-labeling specificity for SNO, the effect of omitting methyl methane thiosulfonate (reactive thiosulfonate used in blocking free thiols on cysteine by S-methylthiolation) or ascorbate (that converts SNOs to free thiols, which are subjected to biotin-HPDP labeling) in the assay is shown. (*B*) The biotin switch assay was performed, followed by visualization of whole or streptavidin-purified biotinylated proteins using an antibody conjugated to streptavidin- horseradish peroxidase (hrp). (*C*) EMT6-H cells were treated with 0.5 μg/mL lipid A for 6 hours and a biotin switch assay was performed, followed by visualization of streptavidin-purified biotinylated proteins using an antibody conjugated to streptavidin HRP. Data are representative of at least 3 independent experiments.



Supplementary Figure 4. (*A*) Sequence alignment of Fas protein. (*B*) The analysis of the 3D structure of the intracellular domain of Fas. *Red* denotes glutamine residue, *yellow* indicates cysteine residue, and *blue* signifies lysine residue.