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Nitric Oxide Inhibits the Transcription Repressor Yin-Yang 1 Binding Activity at the Silencer Region of the Fas Promoter: A Pivotal Role for Nitric Oxide in the Up-Regulation of Fas Gene Expression in Human Tumor Cells

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NO has been increasingly implicated in control of the transcriptional machinery and serves as an intracellular second messenger to modify gene expression. We have demonstrated that NO up-regulated Fas receptor expression in ovarian carcinoma cell lines, albeit the mechanism involved is not known. Thus, we hypothesized that NO, directly or indirectly, may modify the transcriptional machinery that is responsible for the increased expression of the Fas gene. We examined the effect of NO on Fas gene expression using a Fas promoter-driven luciferase reporter system. Transient transfection of AD10 cells with pGL.3-FasP demonstrated that the IFN-γ-dependent NO generation increases the trans-activation of the Fas promoter, and this increase was blocked by the NOS inhibitor (N\textsuperscript{G}-monomethyl-L-arginine), but could be restored by the addition of the NO donor S-nitroso-N-acetylpenicillamine. Systematic deletion of the Fas promoter revealed that the functional region responsible for the NO-mediated effect was located at the silencer region, suggesting that NO may be responsible for the disruption of a repressor mechanism. We demonstrate that NO up-regulates the expression of the Fas receptor on AD10 cells via the specific inactivation of the transcription repressor yin-yang 1 DNA binding activity to the silencer region of the Fas promoter. These findings reveal a new mechanism of NO-mediated gene regulation by interfering with a repressor transcription factor at the silencer region of the Fas promoter. *The Journal of Immunology*, 2001, 167: 75–81.

The Fas receptor (CD95 or APO-1) is a classic type I transmembrane surface molecule and a member of the TNF receptor superfamily widely expressed in mammalian cells (1). The signal from Fas seems to be restricted to apoptosis upon trimerization by the interaction with its natural ligand Fas ligand or by agonistic Abs (2, 3). It has been shown that expression of the Fas gene is enhanced by IFN-γ and TNF-α and by the activation of lymphocytes (2, 4, 5). We have demonstrated that one of the cellular mechanisms by which IFN-γ sensitizes the human ovarian carcinoma cell line AD10 to Fas-mediated apoptosis is mediated by the induction of inducible NO synthase (iNOS) and subsequent generation of NO in the tumor cells. The generation of NO correlated with an increased expression of the Fas gene at both the transcriptional level and the surface protein level (6). It has been shown that NO increases expression of the Fas receptor in aortic vascular smooth muscle cells and correlates with increased sensitivity to Fas-mediated apoptosis. Further, up-regulation of Fas expression by NO was shown to be a cGMP-independent mechanism (7). However, the molecular mechanism of the interaction of NO with the Fas transcriptional machinery remains elusive. Thus, we hypothesized that NO, directly or indirectly, might modify the transcriptional machinery toward increased expression of the Fas gene.

Functional analysis of the 5′-flanking region of the Fas gene has revealed three major regions within the ~2000 bp upstream of the translational initiation site. The Fas promoter exhibits silencer activity residing between nucleotide positions −1781 and −1007 and a strong enhancer region between −1007 and −425. A basal promoter activity resided in the region between −425 and −1 (8). Several putative transcription factor binding sites have been identified by sequence comparison (8–10), but the functional analysis of the Fas promoter is not completely understood.

In this study we aimed to determine 1) whether NO is implicated in the molecular mechanism of the IFN-γ-mediated up-regulation of Fas expression on AD10 cells, 2) the specific functional region of the Fas promoter that is responsible for the stimulatory effect of NO, and 3) the identity of the putative NO-sensitive transcription factor(s) and its mechanism(s) of action toward the regulation of Fas expression in AD10 cells.

Materials and Methods

Cell culture and reagents

The AD10 cell line is an adriamycin-resistant, multidrug resistant phenotype-expressing, subline derived from the human ovarian carcinoma cell line A2780 and was obtained from Dr. R. Ozols (Fox Chase Cancer Center, Philadelphia, PA). The PC-3 cell line is a metastatic bone-derived human prostatic adenocarcinoma obtained from American Type Culture Collection (Manassas, VA), CRL-1435. Cell cultures were maintained as previously described (6). For every experimental condition cells were cultured in 1% FBS 24 h before treatments. S-nitroso-N-acetylpenicillamine (SNAP) was provided and synthesized by Dr. J. Fukuto (University of California, Los Angeles, CA). DEA-NONOate (diethylamine NONOate)
and DETA-NONOate (diethylenetriamine NONOate) were purchased from Cayman Chemical Co. (Ann Arbor, MI). For iNOS induction, cultured cells were stimulated 18 h with 100 U human recombinant IFN-γ (PeproTech, Rocky Hill, NJ). NOS activity was blocked by incubation with 1 mM N^6- monomethyl-l-arginine (l-NMA; Alexis, San Diego, CA) 18 h before IFN-γ treatment. DTT (Life Technologies, Gaithersburg, MD) was added directly to the nuclear extracts where indicated.

**Flow cytometry**

Surface Fas Ag expression on tumor cells was determined by flow cytometry as previously reported (6).

**Plasmids constructs and reporter system**

The human Fas promoter containing 1781 bp upstream of the translation initiation site was amplified by PCR using the forward primer AAG CTT TTT TGG CTA CAT TTT T and the reverse primer GGT TGT TGA GCA ATC TCT CGA AA. The genomic DNA extracted from cultured AD10 cells was used as a template. PCR were conducted using the Advantage-HF 2 PCR kit (Clontech, Palo Alto, CA) following the manufacturer’s recommendations. The PCR product was gel-purified and incubated 30 min with Taq polymerase (Life Technologies) in the presence of 50 μM dATP for adenosine tailing. The resulting fragment was ligated to pCR2.1 TA vector (Invitrogen, Carlsbad, CA), resulting in generation of the pCR-FasP construct. The cloned Fas promoter was released from the pCR-FasP construct by digestion with SpeI and XhoI and ligated to pGL3Basic (Promega, Madison, WI) that had been digested with Nhel and BglII, yielding the pGL3-FasP construct. We further generated two constructs deleting two functional region upstream of the Fas gene core promoter, pGL3-FasEC (silencer deleted) and pGL3-FasC (silencer and enhancer deleted). Two forward primers with the sequences pAGT AAT GAT GTC ATT ATC G (flank-1007) and pCTG CAG GAA CGC CCC GGG ACA GGA ATG C (flank-420) and a universal reverse primer with the sequence pACG CGT AAG AGC TGA GTC ATC C (located in the pGL3 vector) were used in PCR as described above. The resulting amplified products were circularized and replicated after being transformed into competent TOP10-F’ Escherichia coli strain. The truncations were confirmed by automated sequencing and restriction digestion.

**Transcriptional elements search**

Specific DNA sequences from GenBank comprising the FasCD95 5’-flanking region (accession no. X87652 and D31968) and functional regions derived from them (8) were analyzed for the presence of potential trans-acting elements using Transcription Element Search software on the Internet (Computational Biology and Informatics Laboratory, University of Pennsylvania School of Medicine, Philadelphia, PA; http://www.cbil.upenn.edu/tess/).

**RT-PCR**

Total RNA was extracted and purified from ~1 × 10^6 cells for each experimental condition by a single-step monophasic solution of phenol and guanidine isothiocyanate-chloroform using TRIzol reagent (Life Technologies). One microgram of total RNA was reverse transcribed to first-strand cDNA for 1 h at 42°C with 200 U SuperScript II reverse transcriptase and 20 μM random hexamer primers (Life Technologies). Amplification of 1/10th of these cDNA by PCR was performed using the gene-specific primers, yin-yang 1 (YY1) forward 3’-GGG CAC CAC CAC CAC CAC CA-3’ and YY1 reverse 3’-TCT TTG TTG CCC CCC TGG TG-3’; 407-bp expected product. Internal control for equal cDNA loading in each reaction was assessed using the following gene-specific GAPDH primers: GAPDH sense, 5’-GAA CAT ACC TCC TGC TAC CTC TG-3’; and GAPDH antisense, 5’-GTT GGT GTA GCC AAA TTA GTT G-3’ (355-bp expected product). PCR amplifications of each specific DNA sequence were conducted using the hot start method with Platinum Taq polymerase (Life Technologies), followed by a two-step thermal cycling incubation (95°C for 15 s and 60°C for 30 s for 25 cycles, and a final extension at 72°C for 10 min). The numbers of PCR cycles were established based on preliminary titration of the relative amount of amplified product for each gene representing the linear phase of the amplification process. The amplified products were resolved on 1.5% agarose gel electrophoresis, and their relative concentrations were assessed by densitometric analysis of the digitized ethidium bromide-stained image, performed on a Macintosh computer (Apple Computer, Cupertino, CA) using the public domain National Institutes of Health Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

**Nuclear extracts preparation**

Cultured cells (1 × 10^6) treated under different experimental conditions were washed twice with ice-cold Dulbecco’s PBS (MediaTech, Herndon, VA). P-40 lysis buffer (10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 3 mM MgCl₂, 0.5% Nonidet P-40, and 0.1 mM EDTA) was added to the top of the washed cells and incubated on ice for 5 min. Lysed cells were collected by gentle pipetting three or four times and transferred to a microcentrifuge tube. Nuclear pellets for each experimental condition were generated by two consecutive centrifugation and washing steps at 1200 rpm. Nuclear pellets were lysed in buffer C (20 mM HEPES (pH 7.9), 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 0.1 mM EDTA, and 0.5 mM PMSF). Total nuclear protein concentrations were determined using the method of Bradford (11).

**EMSA**

Nuclear protein extracts (2 μg) were assayed for DNA interaction by EMSA as described previously with modifications (12, 13). The double-stranded YY1 consensus binding sequence 5’-GGG CAT CAG GGT CT C CAT TTT G AA GGC GGA TTA CTC CCC-3’ (Geneva, Montreal, Canada) oligonucleotide was radiolabeled with [γ-32P]ATP (ICN Pharmaceuticals, Costa Mesa, CA) by incubation with 10 U T4 polynucleotide kinase (New England Biolabs, Beverly, MA) and further purified using the QIAquick gel extraction kit (Qiagen, Valencia, CA). After the DNA-binding reaction the samples were resolved on 4–15% Tris-HCl-polyacrylamide minigels (Bio-Rad, Richmond, CA), and the gels were dried and autoradiographed. The specificity of the DNA-binding reaction was determined by competition assays performed with a 100-fold excess of unlabeled YY1 oligonucleotide and by supershifting using a rabbit YY1 polyclonal Ab (Geneika Biotechnology, Montreal, Canada). Relative concentrations of specific YY1-shifted bands were assessed by densitometric analysis of the digitized autoradiographic images using the National Institutes of Health Image program described above.

**Transfections and reporter gene system**

AD10 cells (7 × 10^4/vector) were transfected with 10 μg DNA using 60 μL Lipofectamine reagent (Life Technologies) according to the manufacturer’s recommendations. Transfected cells were then distributed onto a six-well culture plate and incubated under different experimental conditions. After incubation for each experimental condition, the cells were harvested, and luciferase activity was assessed using the enhanced luciferase assay kit (Analytical Luminescence Laboratory, Ann Arbor, MI) and reading in an automatic luminometer (Analytical Luminescence Laboratory) following the manufacturer’s recommendations. For internal transfection efficiency assessment cells were cotransfected with a reporter vector containing the β-galactosidase gene driven by the CMV promoter (Clontech, Palo Alto, CA).

**Statistical analysis**

The experimental values were expressed as the mean ± SEM for the number of separate experiments indicated in each case. One-way ANOVA was used to compare variances within groups and among them. Bartlett’s tests were used to establish the homogeneity of variance on the basis of the differences among SD values. Whenever needed, post hoc unpaired multiple comparison tests (Bonferroni’s test) and Student’s t test were used for comparison between two groups. Significant differences were considered for probabilities <5% (p < 0.05).

**Results**

NO mediates the IFN-γ-dependent activation of the human Fas promoter in AD10 cells

We have reported that the IFN-γ-induced sensitization of the human ovarian carcinoma AD10 cell line to Fas-mediated apoptosis is due in part to the generation of NO by the induction of iNOS in these cells and subsequently by the up-regulation of Fas gene expression (Fig. 1A) (6). We examined whether IFN-γ-dependent induction of iNOS and subsequent generation of NO mediated activation of the human Fas promoter in AD10 cells. Transient transfection of AD10 cells with the luciferase-based reporter vector pGL3-FasP and further treatment with IFN-γ (100 U/ml) for 18 h revealed a significant increase in functional activation of the Fas promoter, as determined by an increase in luciferase activity (Fig. 1B, lane 2). In contrast, functional activation of the Fas promoter...
was completely abrogated when pGL3-FasP-transfected AD10 cells were treated with IFN-γ in the presence of 1 mM of the NOS inhibitor L-NMA (Fig. 1B, lane 3). To confirm the specific role of NO in the control of human Fas gene expression, we assessed Fas promoter activity in the presence of the NO donor SNAP. Treatment with SNAP (100 μM) of pGL3-FasP-transfected AD10 cells that were incubated in the presence of 1 mM L-NMA and IFN-γ completely restored the activity of the Fas promoter (Fig. 1B, lane 4). These results strongly suggest that the IFN-γ-mediated up-regulation of Fas gene expression in AD10 cells could be due in part to generation of NO. Furthermore, using the prostate adenocarcinoma cell line PC-3, we confirmed the same phenomenon of sensitization by IFN-γ and its correlation with the induction of iNOS and subsequent generation of NO by these tumor cells, including the up-regulation of Fas gene expression (data not shown). These findings suggest the broad distribution of this type of mechanism in solid tumor systems.

**FIGURE 1.** NO mediates the IFN-γ-dependent activation of the human Fas promoter and up-regulates Fas gene expression. IFN-γ-stimulated AD10 cells were incubated in the presence (lanes 3 and 4) or absence (lane 2) of 1 mM of the NOS inhibitor L-NMA. The NO donor SNAP (100 μM) was added to the IFN-γ-treated cells after inhibition of NOS activity by 1 mM L-NMA (lane 4). Determination of the Fas receptor surface expression on AD10 cell by FACS (A) or luciferase activity after transient transfection with the Fas-promoter driven luciferase reporter vector (B) revealed a direct effect of NO in the activation of Fas gene expression. n = 3. **p < 0.005; ***p < 0.001.

**NO up-regulates Fas gene expression by disruption of transcriptional control at the silencer region of the Fas promoter**

In an effort to determine which region of the human Fas promoter is responsible for the responsiveness to NO, we performed a systematic deletion of the Fas promoter based on previous functional analysis of the human Fas promoter (8). pGL3-FasP was used to generate two other reporter constructs, pGL3-FasEC and pGL3-C, by selective PCR amplification; these constructs contain −21007 and −2425 bp upstream of the translational initiation of the Fas gene, respectively (Fig. 2A). pGL3-FasEC comprises the previously identified enhancer and core promoter region in which the silencer region has been removed. pGL3-FasC consists of only the core promoter region in which the whole silencer and enhancer regions have been deleted. Transient transfection of these three Fas promoter-driven reporter vectors in AD10 revealed that the Fas promoter responds to NO, but lacks its responsiveness to NO upon

**FIGURE 2.** NO disrupts transcriptional control of the Fas promoter at the silencer region. Systematic deletions of functional regions on the Fas promoter (A) were transiently transfected in AD10 cells and incubated in the presence or the absence of 200 μM of SNAP. Luciferase activity (B) revealed a significant NO-responsive segment located in the silencer region of the Fas promoter. n = 3. ***p < 0.001.
deletion of the silencer region. Although removal of the silencer region resulted in an expected increase in luciferase activity compared with that of the full promoter, the sensitivity to NO was minimal and not significant. Removal of both silencer and enhancer regions resulted in a basal promoter activity that was not responsive to SNAP treatment (Fig. 2B). Therefore, these results suggest that NO, or a derivative of it, is likely to act in the disruption of a repressor mechanism located at the silencer region of the Fas promoter.

Identification of a putative cis-acting element cluster for the transcriptional repressor YY1 in the silencer region of the Fas promoter

The finding that the main region that retained responsiveness to NO on the Fas promoter was located at the silencer suggested the repressive nature of a possible trans-acting element. This trans-acting element could be inactivated in the presence of NO, resulting in release of the Fas promoter transcriptional machinery. To identify the presence of a putative repressor trans-acting element in the silencer region of the Fas promoter (−1781 to −1007 bp from the translational initiation site), we analyzed this region using the Transcription Element Search software, searching for potential recognized repressor trans-acting elements. This analysis revealed a noticeable stretch of sequences that matched the consensus transcription element (CCWNTTNNNW) for the transcription factor YY1, a recognized transcriptional repressor (14). Three potential YY1-responsive elements were found to cluster in a very narrow segment within the Fas promoter silencer region, between −1619 and −1533 bp from the translational initiation site (Fig. 3). Repeated IFN-β silencer B motifs also colocalized within this same region, and some of them overlapped with the YY1 silencer cluster. However, no functional relevance of the IFN-β silencer B motifs has been shown in the control of Fas gene expression in several cell types (15). YY1 is a DNA-binding zinc finger transcription repressor that is highly conserved in mammalian cells. Moreover, zinc finger proteins have been suggested to be primary targets of NO-induced disruption of their functional structure (16). Thus, we hypothesized that YY1 is a potential candidate to function as a repressor of Fas gene expression and can be affected by NO.

FIGURE 4. NO does not affect YY1 mRNA expression. Semiquantitative RT-PCR was used to determine the relative expression of YY1 mRNA in AD10 cells treated in the presence of 100 and 500 μM SNAP (lanes 2 and 3, respectively). Relative expression was assessed by densitometric analysis of the specific amplified band corresponding to relative expression of the transcription factor YY1.

NO disrupts the DNA binding activity of the transcriptional repressor YY1 with no effect on its mRNA expression

To investigate the specific role of NO in the activity of the transcription factor YY1, we first examined the relative mRNA expression of YY1 in quiescent AD10 cells cultured for 18 h in the presence of 100 and 500 μM SNAP. Semiquantitative RT-PCR reflected no significant changes in levels of YY1 mRNA expression upon treatment with SNAP (Fig. 4). Similarly, treatment of these cells with NO donors had no effect on the constitutive levels of YY1 protein levels (data not shown). Further, we analyzed by EMSA the DNA binding activity of YY1 in AD10 cells using the double-stranded YY1 consensus binding oligonucleotide. As shown in Fig. 5, nuclear extracts from AD10 cells treated for 6 h in the presence of 100 and 500 μM SNAP exhibited a negligible DNA binding activity of YY1 (Fig. 5, lanes 5 and 6) compared with the untreated control (Fig. 5, lane 1). As a control for potential secondary metabolites other than NO derived from SNAP, we used 500 μM of a SNAP solution allowed to fully decompose for 1 wk (Fig. 5, lane 4) with no significant effect on the YY1 DNA binding activity. The impaired YY1 binding activity caused by NO was completely restored upon addition 1 h before the DNA-binding reaction of 1 mM of the reducing agent DTT to the SNAP-treated nuclear extract (Fig. 5, lane 7). The specificity of the DNA-binding reaction was determined by competition assays performed with a 100-fold excess of unlabeled YY1 oligonucleotide and by supershifting using a rabbit YY1 polyclonal Ab (Fig. 5, lane 2). These results suggest that a potential mechanism by which YY1 binding activity is inhibited is via redox regulation by NO and occurs through thiol modification with consequent disruption of the YY1 activity. Additional experiments performed in the presence of the short half-life (~<2.5 min) NO donor DEA-NONOate in a cell-free EMSA (treatment of the nuclear extracts) and the long half-life (~<20 h) DETA-NONOate on cultured cells revealed results similar to those of the SNAP-treated groups (data not shown).

Identification of the specific YY1 binding activity to its responsive element cluster at the silencer region of the Fas promoter

To determine whether the transcription factor YY1 binds specifically to its putative cluster at the silencer region of the Fas promoter, the YY1 cluster identified above was PCR-amplified and
used as a probe in the EMSA. Using the forward primer 5'-ATT TTG TCA ATT GTC CTT TC-3' and the reverse primer 5'-AGT TCA TTT AAA TAA AAA AA-3' flanking the putative YY1 cluster at the Fas promoter silencer region (Fig. 3, underlined sequence), we amplified using the pGL3-FasP vector as a template. The PCR product was gel-purified and radiolabeled as described in the previous section. Specific YY1 DNA binding activity was observed in untreated AD10 cells. Although other shifted complexes were formed that reflect the multiplicity of overlapping binding sites that can be present in the YY1 cluster used as a probe, the prominent shifted band (Fig. 6, lane 1) was supershifted in the presence of the rabbit YY1 polyclonal Ab (lane 3). Relative YY1 DNA binding activity was determined by densitometric analysis of the YY1 shifted band (lower panel). *** p < 0.001.

Discussion
Fas (CD95 or APO-1) is a widely expressed classic type I transmembrane surface receptor of the TNF receptor family (1) that plays a major role in triggering apoptosis in sensitive cells following activation by membrane-bound or soluble Fas ligand. It is involved in mediating nonspecific T cell cytotoxicity and activation-induced cell death in the peripheral immune system (17). Expression of the Fas gene is enhanced by IFN-γ and TNF-α and by activation of lymphocytes (2, 4). Therefore, the expression of the Fas receptor marks a critical point in the decision of the fate of the cell in terms of its survival or death. We have shown that IFN-γ-mediated sensitization to Fas-induced apoptosis of the resistant human carcinoma cell line AD10 is partially due to the induction of iNOS and the subsequent generation of NO. In addition, we have demonstrated the presence of a strong correlation between the generation of NO and the up-regulation of Fas gene expression in AD10 cells (6). In the present study evidence is presented for the first time of a novel molecular mechanism that demonstrates that NO is disrupting a repressor mechanism located primarily at the silencer region of the Fas gene promoter, thereby increasing its expression. Moreover, the NO-mediated effect is due

FIGURE 5. Effect of NO on YY1 DNA binding activity. Nuclear extracts from AD10 cells treated in the presence of 100 and 500 μM SNAP (lanes 5 and 6) were analyzed using EMSA to assess the specific YY1 DNA binding activity. SNAP-treated cells were incubated in the presence of the reducing agent DTT (lane 7). Specific binding of the transcription factor YY1 was assessed by direct competition with a 100-fold excess of cold oligonucleotide (lane 2) or by supershift using the rabbit YY1 polyclonal Ab (lane 3). Relative YY1 DNA binding activity was determined by densitometric analysis of the YY1 shifted band (lower panel). *** p < 0.001.

FIGURE 6. Specific YY1 DNA binding to the putative responsive element cluster identified at the silencer region of the Fas promoter. Nuclear extracts from untreated AD10 cells were analyzed for specific DNA binding activity of the transcription factor YY1 using EMSA. The PCR-amplified YY1 responsive element cluster (Fig. 3, underlined sequence) was used in the EMSA in the presence (lane 2) or the absence of the rabbit YY1 polyclonal Ab to identify the specific binding of the transcription factor YY1 to its putative cluster in the silencer region of the Fas promoter.
specifically to the disruption of the DNA binding activity of the transcription factor YY1, which normally represses Fas expression by binding to a cis-element clustered at the silencer region of the Fas promoter. Hence, NO promotes the removal of YY1 repressor activity and frees the Fas promoter to initiate or increase transcriptional activation of the Fas gene.

Several lines of evidence support the hypothesis that NO regulates the expression of some genes that are implicated in the signal pathway involving regulatory cytokines that modify the cellular response to apoptotic stimuli (18). However, the regulation of apoptosis-related genes by NO is not completely understood. The biological effect of NO has always been shown to be paradoxical. NO appears to inhibit Fas-induced apoptosis in transformed cells derived from the hemopoietic lineage, whereas it mediates sensitization to Fas apoptosis, as observed here in ovarian tumor cells and several other solid tumor cells. This dichotomy represents a crucial point of divergence between the biological natures of these two cell types, and it needs to be considered when making strategies toward the use of NO as an anti-tumor agent. Several reports addressed the question of the inhibition of caspases by NO and further interruption of the programmed cell death. Hence, under the light of recent reports, the final outcome of NO-mediated effects would be determined by many factors, including the local concentration and sources of NO and the presence of reactive molecules that might redirect the redox status in the tumor cell.

Previous studies suggested the regulation of Fas gene expression on normal or tumor cells by NO (6, 7), although the underlying molecular mechanism of this effect is not known. Characterization of the human Fas gene promoter has revealed three major regions within the −2000-bp 5′-flanking region. Functional analysis identified a silencer activity residing between nucleotide position −1781 and −1007 and a strong enhancer region between −1007 and −425 in the human Fas gene. The region between −425 and −1 retained a basal promoter activity (8).

Transiently transfected human ovarian carcinoma cells AD10 with the Fas promoter-driven luciferase expression reporter vector (pGL3-FasP) responded to NO by an increase of luciferase activity. We observed that IFN-γ-mediated up-regulation of luciferase activity was blocked by use of the specific NOS inhibitor l-NMA. Furthermore, treatment with the NO donor SNAP rescued the l-NMA-inhibited luciferase activity in these cells (Fig. 1). These results strongly suggested the participation of NO in the regulation of Fas promoter activity in AD10 cells.

Systematic deletion of the previously identified functional regions on the Fas promoter revealed that the responsiveness to NO observed using the luciferase-based reporter system is present within the silencer region (Fig. 2). This result suggested that one possible mechanism by which NO increases the expression of the Fas gene is by inactivation of a potential repressive transcription factor located on the silencer region of the Fas promoter. Thus, we anticipated that by removing negative regulatory trans-acting signals on the Fas promoter, NO would increase Fas gene expression in AD10 cells. Indeed, our findings concur with this hypothesis.

Silencers have been reported to act synergistically to increase or modify repressor function and to play a definitive role in determining eukaryote gene expression (19). The transcription factor YY1 has been identified as a potential repressor factor in the human IFN-γ gene (20, 21), the IL-3 gene promoter (22), and the GM-CSF gene promoter (23, 24). Although many putative repressor motifs have been localized by sequence comparison in the 5′-flanking region of the Fas gene, no functional implications have been assigned to these trans-regulatory elements (8, 15). Significantly, we have identified a very relevant putative repressor cluster at the silencer region that matched the consensus sequence that binds the transcription factor YY1. Three binding sites for YY1 are located in a very narrow sequence stretch at −1619, −1590, and −1543 bp from the translational initiation site of the Fas gene (Fig. 3). YY1 is a zinc finger transcription factor involved in the negative regulation of many mammalian genes (25). NO is known to interfere in the DNA binding activity of many zinc finger transcription factors via S-nitrosylation of cysteine thiol groups and subsequent S-nitrosothiol formation (16, 26). Exposure of AD10 cells to various concentrations of the NO donor SNAP and other NO donors significantly inhibited the specific DNA binding activity of the transcription factor YY1 (Fig. 5). Furthermore, NO-based inhibition of YY1 DNA binding activity was completely restored by incubation with 1 mM of the reducing agent DTT. It is noteworthy that NO was not able to modify YY1 gene expression in AD10 cells, as assessed by RT-PCR (Fig. 4). Our findings are consistent with the role of NO in blocking YY1 binding activity. Hence, our results suggest that a potential mechanism by which NO inhibits the YY1 DNA binding activity is through redox regulation by NO and is likely to occur through thiol modification with consequent disruption of the YY1 functional structure.

We confirmed the specific binding of the transcription factor YY1 to the silencer region by EMSA and supershift analysis using the sequence comprising the identified putative YY1 binding sites (Fig. 6, underlined sequence) on the Fas silencer region. Multiple shifted complexes were formed using the YY1 cluster probe, which revealed the possible interaction of overlapping transcription factors within this region with a prominent concentration of the specific band corresponding to YY1. It has been shown that YY1 repression activity probably reflects its ability to interfere with the communication between transcription activators and their targets within the general transcription machinery. The simplest mechanism of repression by YY1 is through the transcription activator displacement from their cis-acting element within the promoter region and/or the recruitment of corepressor molecules (14).

In conclusion, our findings demonstrate that the mechanism by which NO up-regulates the expression of the Fas receptor on AD10 cells is probably due to specific inactivation of the transcription repressor YY1 DNA binding activity to the silencer region of the Fas promoter. The identification of NO sensitive trans-regulatory elements provides the molecular basis to explain how such a pleiotropic and reactive molecule could modulate the expression of specific genes involved in the sensitivity of normal and tumor cell to apoptosis. Therefore, a new role for NO-mediated regulation of gene expression is revealed.

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