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Growth Factor-Antagonized Rexinoid Apoptosis Involves Permissive PPARγ/RXR Heterodimers to Activate the Intrinsic Death Pathway by NO

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SUMMARY

Growth factor (GF) deprivation and/or blocking of cognate signaling can induce apoptosis and is the basis of several cancer treatment paradigms. We observed that RXR agonists (rexinoids) induce apoptosis of tumor cells when GF support is abrogated. This "rexinoid apoptosis" involves activation of both iNOS and eNOS by RXR-PPARγ and results in production of apoptogenic NO. IGF/EGF-induced IGF receptor 1-mediated MAP kinase blocks rexinoid apoptosis by RXR phosphorylation. Combining rexinoids with the MAPK inhibitor U0126 induced apoptosis in human cancer cells in vitro and ex vivo and blocked xenograft growth in vivo. Our results suggest a regulatory mechanism in which GF signaling antagonizes RXR-PPARγ-mediated default apoptosis to sustain cell life.

INTRODUCTION

Retinoids bind to heterodimers composed of retinoic acid (RARα, β, or γ) and retinoid X receptors (RXRα, β, or γ) and initiate the sequence of events leading to transcription activation by inducing allosteric alterations in a network of energetically coupled residues that ultimately alter the structure of interaction surfaces with secondary effectors, such as coregulators (de Lera et al., 2007; Shulman et al., 2004). In the absence of ligand, RXR-RAR heterodimers interact with corepressor complexes, which are released upon RAR agonist binding, thus allowing recruitment of coactivator complexes and the transcriptional machinery. Depending on RAR isotype, cell, and target gene contexts, RXR-selective agonists ("rexinoids") alone are generally unable to dissociate corepressor and activate transcription, a phenomenon referred to as "RXR subordination," but a rexinoid can synergize with a retinoid to enhance the RXR-RAR-mediated transcription through cooperative recruitment of coactivators (Germain et al., 2002). RXRs can additionally heterodimerize with a variety of other nuclear receptors, such as PPARs, VDR, TR, or orphan receptors. In RXR-VDR or RXR-TR, RXR is subordinated to its partner, but in permissive heterodimers, such as PPAR-RXR, transcription can be activated by rexinoids autonomously (for a recent review and references, see de Lera et al., 2007).

Data from cellular and animal models and clinical trials reveal a potent antitumor activity of rexinoids, which are less toxic than retinoids, thus favoring their use in the clinic (for a recent review and references, see Altucci et al., 2007). Indeed, the selective RXR agonist LG1069 (targretin, bexarotene) is currently used as treatment for persistent or refractory cutaneous T cell lymphoma. Moreover, recently completed phase 3 clinical trials

SIGNIFICANCE

In animals, tissue homeostasis is attained by the balance between cellular proliferation, survival, and death. Cancer results from deregulated growth, often caused by (epi)genetic alterations of GF signaling networks and/or pathways that ensure apoptogenic eradication of aberrant growth. We describe here in mechanistic detail that growth factor (IGFs and EGF) signaling is necessary for survival of leukemic and solid cancer cells in culture, if RXR-PPARγ signaling is switched on. Indeed, the combination of rexinoids with compounds that block GF action induces cell death in vitro and dramatically inhibits growth of xenografts in vivo. The GF-rexinoid antagonism is likely of relevance for organogenesis and tissue regeneration and may be exploited for cancer therapeutic and preventive strategies.

combining bexarotene with chemotherapy revealed that a subgroup of patients with non-small-cell lung cancer may benefit from the addition of bexarotene to the therapeutic scheme (Blumenschein et al., 2008; Ramlau et al., 2008). In addition, studies with transgenic MMTV-erbB2 and A/J mouse carcinogenesis models have revealed the potential of rexinoids to prevent cancer in both lung and mammary gland (Li et al., 2007; Liby et al., 2007a; Liby et al., 2007b). Finally, RXR is a therapeutic target for acute promyelocytic leukemias because of the suppression of RARα fusion-mediated transformation, possibly as consequence of rexinoid-induced apoptosis (Zeisig et al., 2007).

We have previously studied signaling pathways by which rexinoids can act despite the subordination of its receptor and have uncovered two distinct mechanisms. One involves the induction of differentiation and postmaturation apoptosis of acute promyelocytic leukemia (APL) cells in the presence of elevated cAMP levels (Benoit et al., 1999). This rexinoid-cAMP synergy was operative even in all-*trans* retinoic acid (ATRA)-resistant APL cells, indicating a mechanism of action distinct from that of retinoids. Indeed, increased cAMP levels result in "desubordination" of RXR in the RXR-RAR heterodimer, thus activating gene programs that synergize with cAMP-dependent programs to induce acute myeloid leukemia (AML) blast differentiation and apoptosis. Interestingly, apoptosis is induced in cells unresponsive to ATRA and involves the simultaneous induction of the tumor-selective TNF-related apoptosis-inducing ligand (TRAIL) and its cognate receptor (Altucci et al., 2005).

The second signaling option was discovered when growth factor support of AML cells growing in the presence of a rexinoid was lowered by decreasing the serum concentration. Under such conditions, the rexinoid alone induced rapid cell death, referred to as "autonomous rexinoid apoptosis," because it did not involve coactivation of other pathways and resulted in apoptosis without differentiation (Benoit et al., 2001). Notably, this signaling paradigm also is active in non-APL AML patient blasts.

Although there are divergent reports from mouse genetic analyses on the possibility that PPARγ may act in some tissues as tumor suppressor (McAlpine et al., 2006; Saez et al., 2003), there is no controversy regarding the antiproliferative capacity of the activated RXR-PPARγ heterodimer against a great number of transformed cells and the potential of cognate agonists for adjuvant and chemopreventive cancer therapy (Elrod and Sun, 2008). The present work was initiated to decipher the mechanistic basis of autonomous rexinoid apoptosis (Benoit et al., 2001).

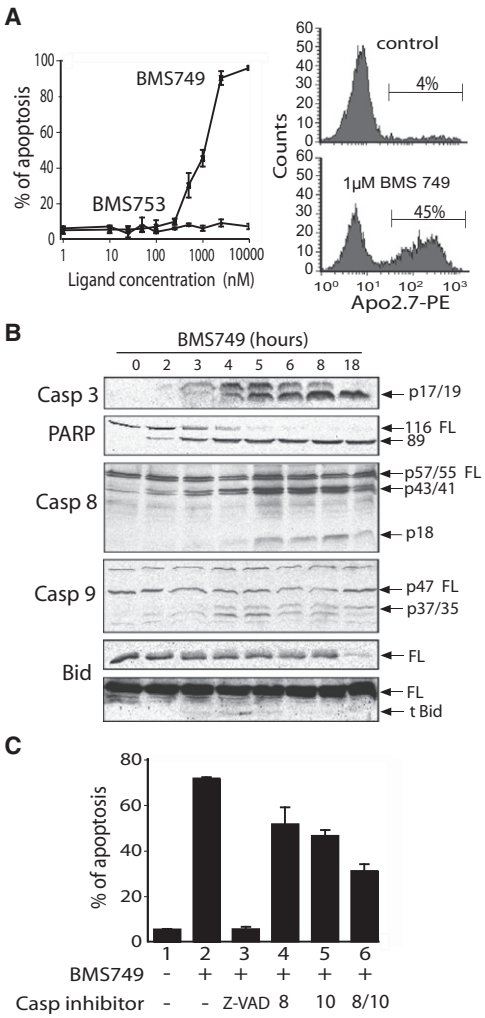

Figure 1. The Rexinoid BMS749 Induces Caspase-Dependent PLB985 Cell Apoptosis

(A) BMS749 induces a dose-dependent apoptosis, whereas the RARα agonist BMS753 has no effect. Cells were treated for 24 hr as indicated, apoptosis was revealed by Apo2.7 staining. Right panel, FACScan of Apo2.7 staining.

(B) Rexinoid treatment induces the cleavage of casp-3, −9, and 8; PARP; and Bid as shown by western blotting. Full length (FL) and cleaved fragments are indicated.

(C) Caspase inhibitors reduce BMS749-induced apoptosis. PLB985 cells were pretreated for 2 hr with 100 μM Z-VAD-FMK, Z-IETD-FMK, and Z-AEVD-FMK or combinations prior to addition of BMS749. After 48 hr, the sub-G1 fractions representing apoptotic bodies were analyzed by PI staining and FACS analysis. Bars represent the mean ± SD.

RESULTS

Rexinoid Apoptosis Involves Activation of Intrinsic Death Pathway

Although RXR agonists did not affect growth or differentiation of myeloid PLB985 cells in normal culture conditions, cells adapted to growth in low serum undergo rapid and massive apoptosis upon exposure to the rexinoid BMS749; under identical conditions, the RARα-selective agonist BMS753 was completely inactive (Figure 1A). To assess the contributions of the extrinsic and intrinsic death pathways (Debatin, 2004) to rexinoid apoptosis, the activation of caspases and the effect of caspase inhibitors were assessed. Under apoptogenic (low serum) conditions, rexinoids rapidly activated pro-caspase 9, accompanied by activation of caspase 3 and PARP cleavage (Figure 1B). Pro-caspase 8 activation was observed with similar kinetics. Selective inhibitors of caspase 8 and 10 decreased cell death by about 20% each but did not block it completely (Figure 1C). Cleavage of proapoptotic Bid indicated that the two apoptotic pathways are connected. Together, these data revealed a contribution of both the intrinsic and extrinsic pathways to rexinoid-induced cell death.

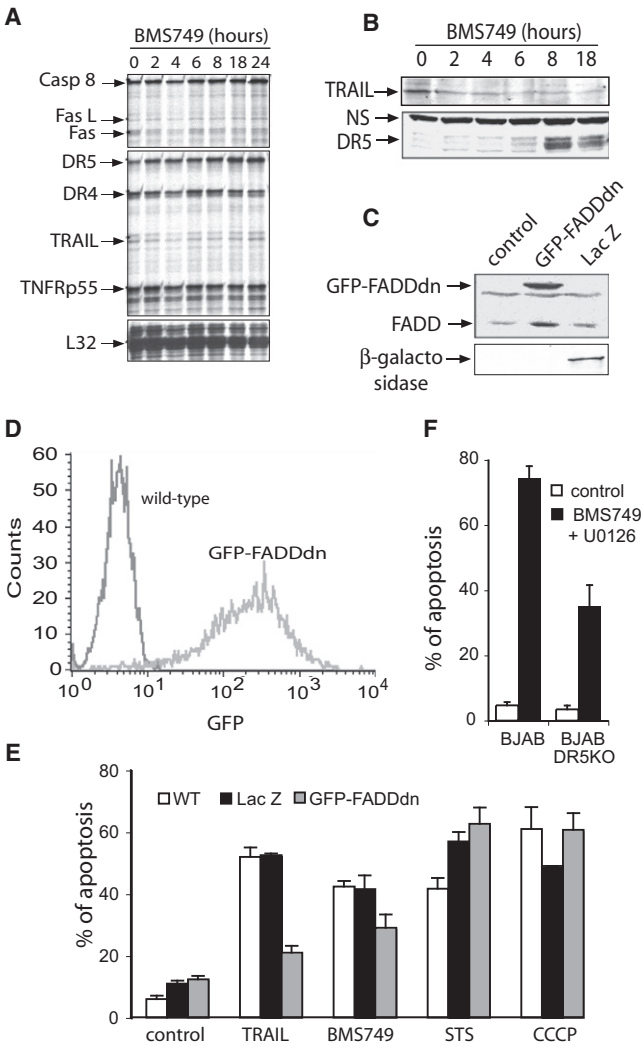

Figure 2. The Death Receptor Pathway Contributes to Rexinoid-Induced Apoptosis

(A) RPA analysis of PLB985 treated with BMS749 is depicted.

(B) Western blot analysis of TRAIL and DR5 protein expression. PLB985 cells were treated as indicated. The nonspecific band (NS) confirms equal loading.

(C and D) PLB985 lines stably expressing a GFP-tagged dominant-negative FADD (GFP-FADDdn) or a β-galactosidase (LacZ) were generated by lentiviral tranduction. Expression of the fusion protein was verified by western blot (C) or FACS analysis (D).

(E) PLB985 cells expressing the GFP-FADDdn protein are less sensitive to rexinoid-induced apoptosis. WT, Lac Z, or GFP-FADDdn PLB985 were treated for 24 hr with BMS749. TRAIL (20 ng/ml) was used to validate the FADD dominant-negative effect. Staurosporine (STS, 100 nM) and CCCP (2 μM) target the mitochondrial apoptotic pathway and were used as negative control.

(F) Deletion of DR5 reduces rexinoid apoptosis. BJAB and DR5-deficient BJAB cells were subjected to rexinoid apoptosis (BMS749 and U0126, see text), and apoptosis was measured. Bars represent the mean ± SD of percentage of 7A6-positive cells.

RNase protection assays showed a moderate induction of DR5 but no regulation of FasL, TNFα, or their cognate receptors by rexinoids (Figure 2A). Although TRAIL expression decreased upon rexinoid treatment, its cognate receptor DR5 was strongly overexpressed (Figure 2B). To assess whether DR5 signaling contributes to rexinoid apoptosis, we transduced a lentiviral dominant-negative FADD fused to GFP (GFP-FADDdn) or β-galactosidase (LacZ) into PLB985. Immunoblotting (Figure 2C) confirmed transgene expression, and FACS analysis of GFP fluorescence revealed that 98% of the cells were infected (Figure 2D). Sensitivity to apoptosis induced by exogenous TRAIL was strongly reduced and close to control levels in GFP-FADDdn but not in wild-type (WT) or LacZ cells, thus demonstrating that the death receptor pathway is effectively inhibited in these cells (Figure 2E). As expected, staurosporine and CCCP, two drugs that target the mitochondrial death pathway, induced apoptosis with the same efficiency in the various cell lines (Figure 2E). That rexinoid BMS749-induced cell death was about 20% to 25% lower in GFP-FADDdn than in WT or LacZ cells indicates a contribution, albeit not a critical one, of the death receptor signaling to rexinoid-induced apoptosis. In support of this conclusion, DR5-deficient BJAB cells (Thomas et al., 2004) exhibited reduced rexinoid apoptosis, compared with parental BJAB, but still retained significant susceptibility (Figure 2F).

Activation of the intrinsic pathway is initiated by permeabilization of the outer mitochondrial membrane, resulting in a loss of transmembrane potential ($\Delta\Psi m$) (Green and Kroemer, 2004). Indeed, rexinoids induce a loss of $\Delta\Psi m$, as demonstrated by DiOC6 staining (Figure 3A) and the release of proapoptotic mitochondrial cytochrome c and Smac/Diablo in the cytoplasm 2–3 hr after rexinoid exposure (Figure 3B). To validate the critical involvement of the intrinsic death pathway in rexinoid-induced apoptosis, we generated cells stably expressing Bcl-2 and GFP or only GFP from bicistronic vectors. Immunoblotting (Figure 3C) and FACS analysis of GFP fluorescence (Figure 3D) confirmed transgene expression. Notably, the sensitivity to rexinoids ("BMS749") as well as staurosporine ("STS") is dramatically reduced in Bcl-2-overexpressing cells, compared with WT or vector (pIRES)-nucleofected cells, demonstrating a dominant contribution of mitochondrial-dependent apoptosis in rexinoid-induced cell death (Figure 3E).

Rexinoid-Induced Nitric Oxide Production Is Required for Apoptosis

Death by mitochondrial damage and concomitant loss of membrane potential can be caused by multiple intracellular events, including the generation of reactive oxygen species (ROS) or nitric oxide (NO). In assaying multiple pathways, we detected the production of NO in BMS749-treated PLB985 cells (Figure 4A, left panel) and confirmed by DAF-FM staining (Figure 4A, right panel) the presence of increased levels of cytoplasmic NO in the treated cells. NO production was similarly observed in BJAB and DR5-deficient BJAB cells (data not shown). This induction was blocked by L-NMMA, a pan-NO synthase inhibitor (Figures 4B and 4C). Interestingly, the kinetics of NO production correlated with that of cytochrome c release (Figure 3B). In keeping with the observation that NO can activate the mitochondrial apoptosis pathway (Brune, 2003), L-NMMA decreased rexinoid apoptosis by 60% (Figure 4C) and abrogated cytochrome c release (Figure 4D). Inhibitors of ROS, L-NAC, and GSH failed to inhibit rexinoid apoptosis, indicating that only NO is involved in this process (Figures 4C and 4D).

Two main types of synthases are involved in the production of NO in normal cells: inducible NO synthase (iNOS) and endothelial

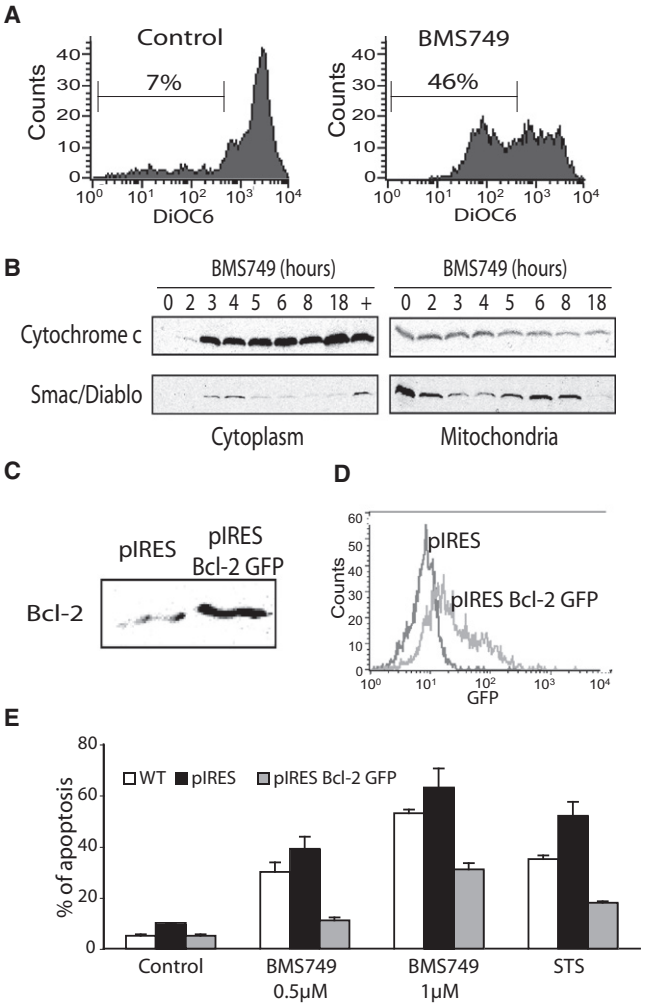

Figure 3. Involvement of the Mitochondrial Death Pathway in Rexinoid-Induced Apoptosis

(A) BMS749 induces loss of mitochondrial transmembrane potential. PLB985 cells were treated for 24 hr with 1 μM BMS749 and analyzed for DiOC6 uptake.

(B) Western blot analysis of cytochrome c and Smac/Diablo release. Cells were treated with BMS749, as indicated, and mitochondrial and cytoplasmic proteins were analyzed.

(C and D) PLB985 cells were stably transfected with pIRES EGFP or pIRES Bcl-2 EGFP vectors. Western blot for Bcl-2 (C) and FACS analysis for GFP (D) confirmed expression of the transgenes.

(E) Bcl-2 overexpression decreases rexinoid-induced apoptosis. WT, pIRES, or pIRES Bcl-2 GFP expressing cells were treated as indicated. Bars represented percentage of 7A6-positive cells (mean ± SD).

NO synthase (eNOS). iNOS generally produces a rapid and large burst of NO, whereas eNOS, a constitutive enzyme, is involved in maintenance of NO levels. Western blot analysis revealed strong iNOS induction within 1 hr of BMS749 exposure, while eNOS levels remained unchanged (Figure 4E). However, phosphorylation of specific residues of eNOS has been shown to modulate the activity of the enzyme; in particular, Ser_{1177} phosphorylation has been demonstrated to be an important positive regulator. Indeed, BMS749 induced within 2 hr the levels of $pSer_{1177}$ eNOS, and RNAi-mediated knockdown confirmed the critical involvement of both NOSs in rexinoid apoptosis, because depletion of either NOS significantly decreased the apoptosis (Figure 4F; note that a combination of both siRNAs proved to be lethal and could not be tested). Together, these data suggest that a synergy between the two NOSs results in a large and sustained increase of NO levels.

Because DR5 is a target of p53, we wondered whether its induction by the rexinoid was mediated by NO-activated p53, thereby linking the intrinsic with the extrinsic death pathway. Indeed, upon rexinoid exposure, a strong increase of p53-activating phophorylation at serine 15 was observed (Figure 4G) in PLB985 and BJAB cells, and this phosphorylation was inhibited by L-NMMA, revealing the involvement of NO. As expected from these results, retroviral transduction of p53 shRNA reduced rexinoid apoptosis in BJAB cells (data not shown). Together, these results demonstrate that both the extrinsic and intrinsic pathways execute rexinoid apoptosis.

IGF Receptor I Activation Antagonizes Rexinoid-Apoptosis

That PLB985 or NB4 cells maintained under normal serum conditions do not exhibit any apoptotic response to rexinoids and that progressive increase in the serum concentration inhibited rexinoid apoptosis (Figure 5A) (Benoit et al., 2001) prompted us to investigate the possible contribution of growth factor signaling to this antagonism. Of a battery of growth factors and cytokines, only IGF1, IGFII, and EGF antagonized rexinoid apoptosis under low serum conditions (Figure 5B; data not shown). Importantly, the addition of IGFs (1 and II) also inhibited NO induction, whereas depletion of residual IGFs in the medium by neutralizing antibodies significantly enhanced this process (Figure 5C) and subsequent apoptosis (data not shown). Moreover, IGFs also blocked cytochrome c release (Figure 5D). Note that apoptosis induced by TRAIL (extrinsic death pathway) or etoposide (intrinsic death pathway) was not rescued by these growth factors (see Figure S1A available with this article online). These results confirm the ability of growth factors to selectively counteract apoptosis proceeding along the rexinoid-NO signaling axis.

Analysis of the signaling cascades triggered by these growth factors identified MAP kinase activation as a cause of the block of rexinoid apoptosis. Indeed, the MEK1/2 inhibitor U0126 completely blocked EGF- or IGF-mediated survival, whereas p38 MAP kinase (SB) or PI3 kinase (LY) inhibitors were ineffective (Figure 5E). In keeping with these results, the addition of EGF to the culture medium induced a rapid and sustained phosphorylation of the MEK1/2 substrate p42/44 MAP kinase, as shown by western blot analysis (Figure 5F). The induction of p42/44 activity by IGF1, EGF, and FCS was confirmed by in vitro kinase assays using Elk-1 as substrate (Figure 5G). No significant changes were observed in the phosphorylation status of p38 or Akt (Figure S1B).

Although IGF receptor 1 (IGFR1) is well expressed, in agreement with previous observations (Chen et al., 1993; Stegmaier et al., 2005), no EGF receptor could be detected in PLB985. Notably, both IGF1 and EGF, as well as FCS, stimulated IGFR1 autophosphorylation (Figure 6A) and Elk1 phosphorylation (Figure 6B). Moreover, the IGFR1 tyrosine kinase inhibitor AG1024 antagonized IGF1 as well as EGF-induced survival, whereas

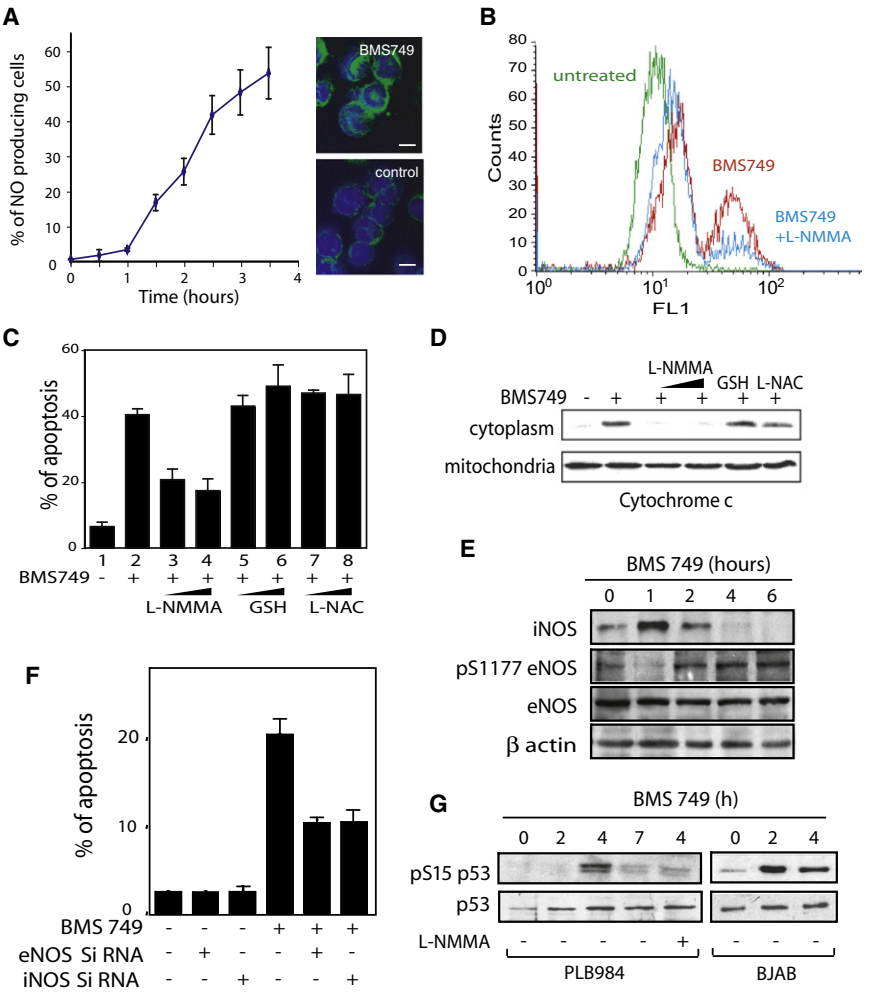

Figure 4. Nitric Oxide (NO) Production Is Required for Rexinoid Apoptosis

(A) Time course of NO production in PLB985 cells treated with BMS749, depicted as mean ± SD of percentage of DAF-positive cells. Right hand panel shows staining of cells with DAF-FM; fluorescence is produced as a result of DAF-FM interaction with NO. Scale bar, 10 μm.

(B) FACScan analysis reveals NO production. Cells were pretreated or not with 500 μM of NOS inhibitor L-NMMA, exposed for 2 hr to BMS749, and prepared for flow cytometry.

(C) NOS inhibitor decreases rexinoid apoptosis. PLB985 cells were pretreated for 2 hr with L-NMMA (200 and 500 μM), GSH, or L-NAC (1 and 5 mM) before BMS749 treatment for 24 hr.

(D) NO production induces cytochrome c release. PLB985 cells were pretreated for 2 hr with L-NMMA (200 and 500 μM), or 2 mM GSH, or L-NAC. BMS749 was added for 4 hr before harvesting the cells.

(E) BMS749 induces iNOS and the phosphorylation of eNOS.

(F) PLB985 transfected with eNOS- and iNOS-specific siRNA were exposed to BMS749. Apoptosis in (C) and (F) was assessed by FACScan detection of Apo2.7 staining.

(G) BMS749 activates p53 in PLB985 and BJAB cells as detected by western blot of Ser_{15} phosphorylation, which is blocked by treatment with L-NMMA. Bars represent the mean ± SD of percentage of 7A6-positive cells.

the EGFR inhibitor AG1478 had no effect (Figures 6C and 6D). Thus, as it was observed for hepatocytes (Hallak et al., 2002), EGF operates in PLB958 cells through IGFR1. The MEK/p42/44 MAPK pathway thus acts upstream of the NO production step to mediate the antiapoptotic action of IGFs and EGF against rexinoid-induced cell death. Note that, although the rexinoid was ineffective in presence of 10% FCS, addition of the MEK1/2 inhibitor U0126 turned it into a powerful death inducer (Figure 5H); this permits the study of rexinoid apoptosis under culture conditions without the need to adapt cells to low serum.

Rexinoid-Induced Apoptosis Is Mediated by the RXR-PPARγ Heterodimer and Is Blocked by Growth Factor-Mediated $RXR\alpha_{S260}$ Phosphorylation

By screening a panel of ligands for multiple permissive and nonpermissive RXR complexes, we observed that the PPARγ agonist troglitazone (TZD) induced PLB985 apoptosis. Importantly, apoptosis occurred under the exact conditions of rexinoid apoptosis—that is, when serum concentration is limiting (Figure 7A). Indeed, the pattern of caspase activation, NO production, and cytochrome c release was indistinguishable from that seen with rexinoids (Figures S2A–S2C). PPARγ agonists troglitazone and 15-deoxy-Δ-12,14-prostaglandin J2 (15d-PGJ2) synergized with various rexinoids for apoptosis induction, whereas no effect was seen with PPARα (WY) or PPARβ (BMS990) agonists (Figures 7A and 7B), thus revealing the specific involvement of RXR-PPARγ heterodimers. As expected, the addition of rexinoids enhanced TZD-induced apoptosis, which was attenuated by IGF1, EGF, and FCS (Figure S2D). Finally, siRNA-mediated depletion of either PPARγ or RXR demonstrated their critical implication in both NO production (Figure 7C) and apoptosis (Figure 7D). RNA interference confirmed that RXR−PPARγ heterodimers are required for eNOS phosphorylation (Figure 7E), and chromatin immunoprecipitation revealed PPARγ and RXR binding to a region 1 kb upstream of the transcriptional start site (TSS) of *NOS2A*, leading to RNA polymerase II recruitment at the TSS (Figure 7F). No such binding or polymerase recruitment was seen to a region 6 kb upstream. Taken together, these data fully support a model in which the permissive RXR-PPARγ heterodimer mediates the rexinoid signal, which activates the intrinsic death pathway though NO production.

A number of RXR and PPARγ phosphorylation sites have been identified previously, but only $PPAR\gamma_{S112}$ and $RXR\alpha_{S260}$ are substrates for ERK1/2. Most importantly, phosphorylation of $RXR\alpha_{S260}$ has been linked to attenuated ligand responsiveness to several nuclear receptor heterodimers in *ras*-transformed cells as a result of impaired coactivator recruitment and repression of the transactivation capacity (Macoritto et al., 2008); in colorectal

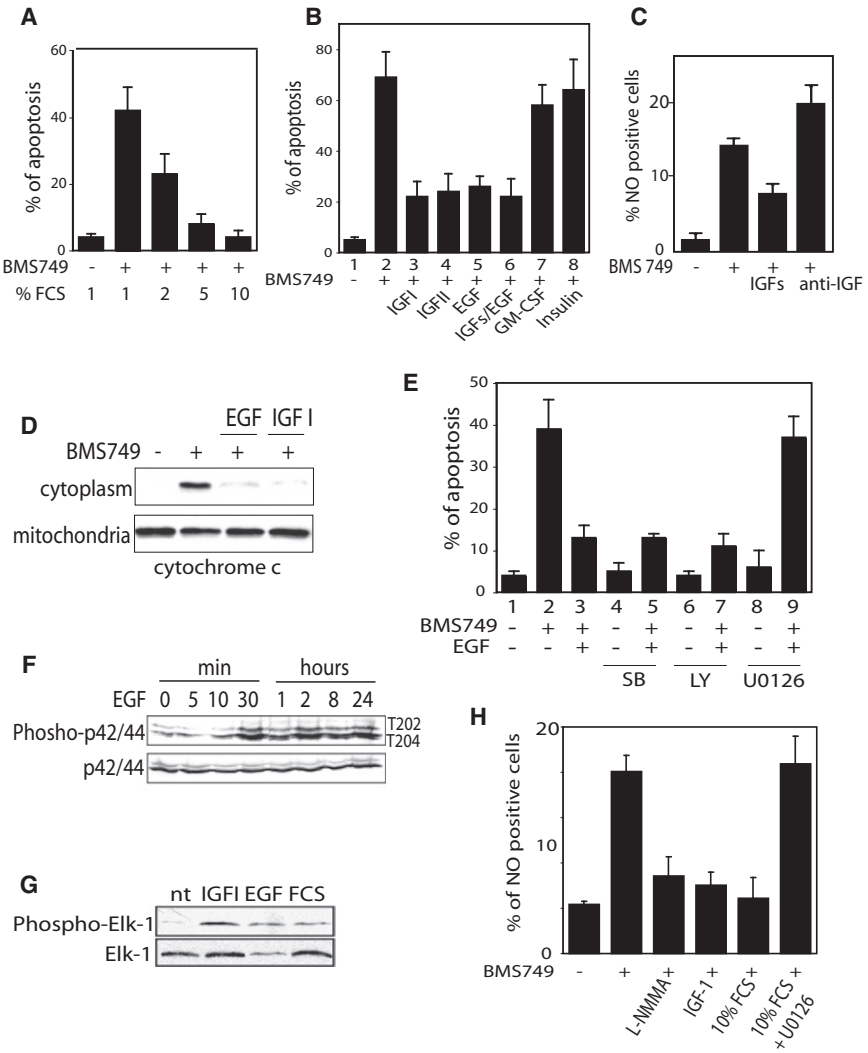

Figure 5. IGFI, IGFII, and EGF Rescue PLB985 from Rexinoid Apoptosis

(A) Serum factors inhibit rexinoid-induced death. Cells treated for 24 hr with BMS749 and increasing amounts of serum were assayed for apoptosis.

(B) PLB985 were cotreated with BMS749 and 1 μg/ml of the indicated growth factors; 48 hr later, apoptosis was measured.

(C) Blocking IGF signaling by IGF neutralizing antibodies (anti-IGF) increases NO production.

(D) EGF and IGF1 inhibit apoptosis by blocking cytochrome c release. PLB985 cells were pretreated for 2 hr with EGF and IGFI and exposed for 4 hr to BMS749.

(E) MEK1/2 inhibition reverses EGF-induced survival. PLB985 cells were pretreated for 2 hr with 10 μM SB203580, 10 μM LY294002, and 5 μM U0126. The cells were then exposed to a combination of BMS749 and EGF or IGF, and 24 hr later, apoptosis was measured.

(F) Kinetics of p42/44 phosphorylation under EGF exposure assessed by western blotting.

(G) In vitro kinase assay of p42/44 activity with extracts of PLB985 treated or not for 1 hr with IGFI, EGF, or 10% FCS. Elk-1 was used as p42/44 target.

(H) NO production is blocked by growth factor signaling. PLB985 cells were treated with IGF1, 10% FCS, or 10% FCS+U0126. Bars represent the mean ± SD.

cancer, RXRα_{S260} phosphorylation impairs the antiproliferative response to thiazolidinediones and RXR ligands (Yamazaki et al., 2007), and differential ubiquitin/proteasome-dependent degradation in normal hepatocytes and hepatocellular carcinoma was reported to originate from differential RXRα_{S260} phosphorylation (Adachi et al., 2002). In the case of PPARγ2, phosphorylation of S_{112} (S_{84} in PPARγ1) leads to a predisposition to sumoylation of K_{107} and confers strong repression activity on the receptor (Adams et al., 1997; Yamashita et al., 2004). To assess whether the antagonistic affect of growth factor/serum-stimulation on rexinoid apoptosis could be mediated by MAPK phosphorylation of (one of) these residues, the impact of the corresponding mutation on NO production was determined. Although transient overexpression of PPAR$\gamma 2_{S112A}$ and PPAR$\gamma 2_{K107R}$ mutants increased the basal level of number of NO-positive cells, none of the mutants prevented serum from inhibiting NO production (Figure 7G). In striking contrast, the expression of RXRα_{S260A} completely abrogated the serum effect, revealing that serum-induced MAPK activation targets this residue to prevent the production of NO and, thus, rexinoid apoptosis.

Growth Factor-Antagonized RXR Apoptosis Is a General Phenomenon in Different Cell Types

It is tempting to speculate that rexinoid apoptosis may correspond to a general phenomenon that allows the eradication of cells that are not growth factor-supported. Indeed, analysis of distinct cell types supported this hypothesis. Within the hematopoietic lineage, several cell lines displayed apoptogenic sensitivity toward rexinoids exclusively in low serum (Figure 8A; data not shown). In cases where adaptation to low serum was possible, primary blast cultures obtained from patients with acute myeloid leukemia showed dramatic apoptosis when rexinoid, low serum, and MAP kinase inhibitors were combined (Figure 8B). Note that the extent of death was similar to that seen with HDAC inhibitors MS275 and SAHA. Also, nonhematopoietic cell lines, including SKBR3, MCF7, and ZR75 breast cancer and HCT116 and HT29 colon cancer cell lines, displayed rexinoid apoptosis (Figures 8C and 8D; data not shown). In the case of SKBR3, additional blocking of HER2 signaling by gefinitib (IRESSA) strongly enhanced apoptosis, which was sensitive to the NOS inhibitor L-NMMA, thus supporting the implication of NO signaling (Figure 8C; data not shown).

Anticancer Action of Rexinoid Apoptosis In Vivo

To assess antitumor efficacy of rexinoid apoptosis in vivo, we treated nude mice bearing HCT116 tumor xenografts with BMS749 alone and in combination with U0126 for 2 weeks.

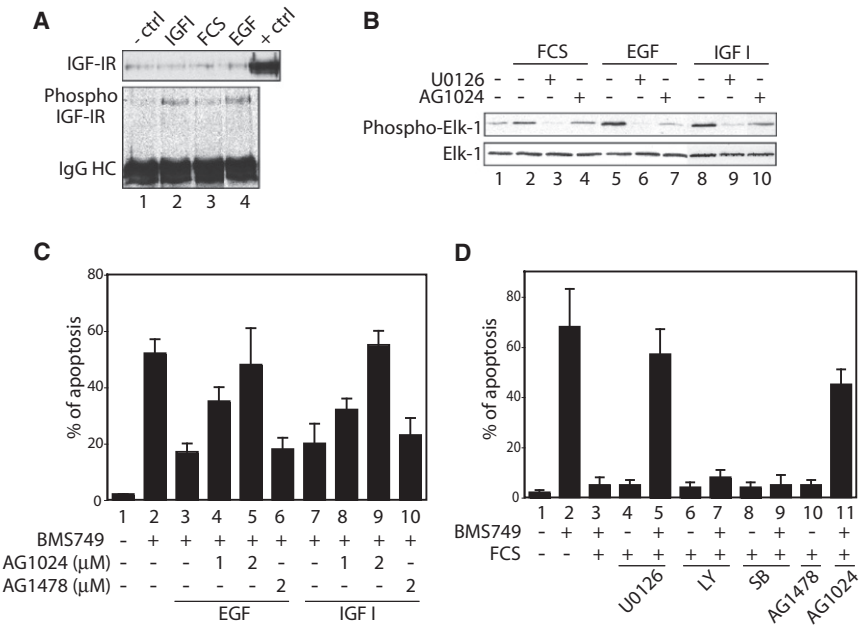

Figure 6. p42/44 MAP Kinase Pathway Mediates the Survival Signal Counteracting Rexinoid Apoptosis Through IGFRI Activation

(A) Growth factors enhance autophosphorylation of IGF-1R. Extracts of PLB985 cells treated for 1 hr with IGF1, EGF, or FCS were immunoprecipitated with anti-phosphoIGF1R antibody. Nonphosphorylated IGF1R is depicted above.

(B) p42/44 kinase assays with protein extracts of PLB985 treated as indicated (U0126, 5 μM; AG1024, 2 μM). Elk1 was used as p42/44 substrate.

(C) PLB985 pretreated with the tyrosine kinase inhibitors AG1024 or AG1478 were exposed to BMS749 in combination or not with EGF or IGFI and subjected to apoptosis analysis.

(D) FCS-mediated survival involves IGFRI and p42/44 activation. PLB985 pretreated for 2 hr with 10 μM U0126, 10 μM LY294002 (LY), 10 μM SB203580 (SB), or 2 μM AG1024 or AG1478 were exposed for 48 hr to BMS749 combined or not with 10% FCS, and apoptosis was measured. Bars represent the mean ± SD.

Using 20 mpk BMS749 and 30 mpk U0126, we did not observe any toxicity, as supported by the normalized weight profile (Figure S3). Although BMS749 and U0126 alone had a moderate effect of tumor growth (21% ± 13% and 44% ± 10% growth inhibition, respectively), their combination dramatically impaired tumor cell expansion in vivo (83% ± 7%; see Figures 8E and 8F). Using luciferase-expressing HCT116, the combined treatment halted tumor growth in the first week of treatment nearly quantitatively (Figure 8G). These results indicate that the combination of rexinoids with agents that interfere with growth factor signaling may correspond to an efficient cancer-therapeutic paradigm.

DISCUSSION

The anticancer activity of retinoids is well established, and the induction of differentiation and/or apoptosis is a hallmark of this therapeutic potential (Altucci and Gronemeyer, 2001; Altucci et al., 2007). Clinical studies and experiments with the Ptch1$^{+/-}$ mouse model indicate that retinoids can also act in a cancer-preventive manner in the cases of basal cell carcinoma (Orlandi et al., 2004; So et al., 2004) and oral cancers (Lippman et al., 2005). Retinoids act through binding to RAR in RAR-RXR heterodimers, but RXR is a promiscuous heterodimerization partner for multiple nuclear receptors.

Rexinoids, synthetic RXR-selective ligands (de Lera et al., 2007), display lower toxicity than retinoids do and are increasingly recognized for their cancer therapeutic and preventive potential (Altucci et al., 2007; Gottardis et al., 1996). Rexinoids have caught significant attention as recent molecular and mouse genetic studies continue to provide mechanistic insight. The observations that epidermis-selective RXRα and/or RXRβ ablation generates hyperplasia and hyperkeratinization and that RXRα-PPARγ heterodimers can act as suppressors of chemically induced epidermal tumorigenesis (Indra et al., 2007; Ocadiz-Delgado et al., 2008) emphasize the importance of RXRs as anticancer drug targets. Although RXR is "subordinated" to RAR in the corresponding heterodimer (Germain et al., 2002), rexinoids have several options to exert activity in the absence of retinoids. First, they can cross-talk with other pathways to "activate" RAR-RXR; this option has promise as therapeutic approach for AML. Indeed, the cross-talk between cAMP and rexinoids allows to target the majority of AML cases (Altucci et al., 2005), while retinoic acid-based "differentiation therapy" is limited to APL. Second, rexinoids activate "permissive" RXR heterodimers (Shulman et al., 2004), such as RXR-PPARγ; finally, they interfere with oncogenic transformation by aberrant RAR fusion proteins (Zeisig et al., 2007).

Here, we report a fourth anticancer signaling option for rexinoids, the growth factor-antagonized induction of "rexinoid apoptosis" (Benoit et al., 2001) mediated by the putative tumor suppressor PPARγ-RXR (Indra et al., 2007) through NO-dependent activation of the intrinsic death pathway (Figure 8H), and reveal its therapeutic potential in vitro, in leukemic blasts ex vivo, and in xenograft assays in vivo. Rexinoid apoptosis in absence of growth factor support is entirely different from the rexinoid-cAMP cross-talk mentioned above (Altucci et al., 2005), because it (1) is not inhibited but is increased by RAR antagonists, (2) involves the RXR-PPARγ permissive heterodimer, (3) does not require prior differentiation, and (4) involves intrinsic and extrinsic death pathways.

Conceptually, our results suggest the existence of a regulatory paradigm by which a cell (compartment/tissue) that is dependent on growth factor support, particularly IGFs or EGF (others remain to be validated), will be eliminated by rexinoid apoptosis as soon as GF support ceases. The beauty of this concept is that solely the presence of GF (in cases of constant rexinoid levels) suffices to promote survival, and its absence automatically leads to death. Apart from GF modulation, this model implies the existence of endogenous RXR ligands, which may represent a second trigger, allowing nonsupported cells to survive in absence of (sufficient) rexinoid. The existence of endogenous

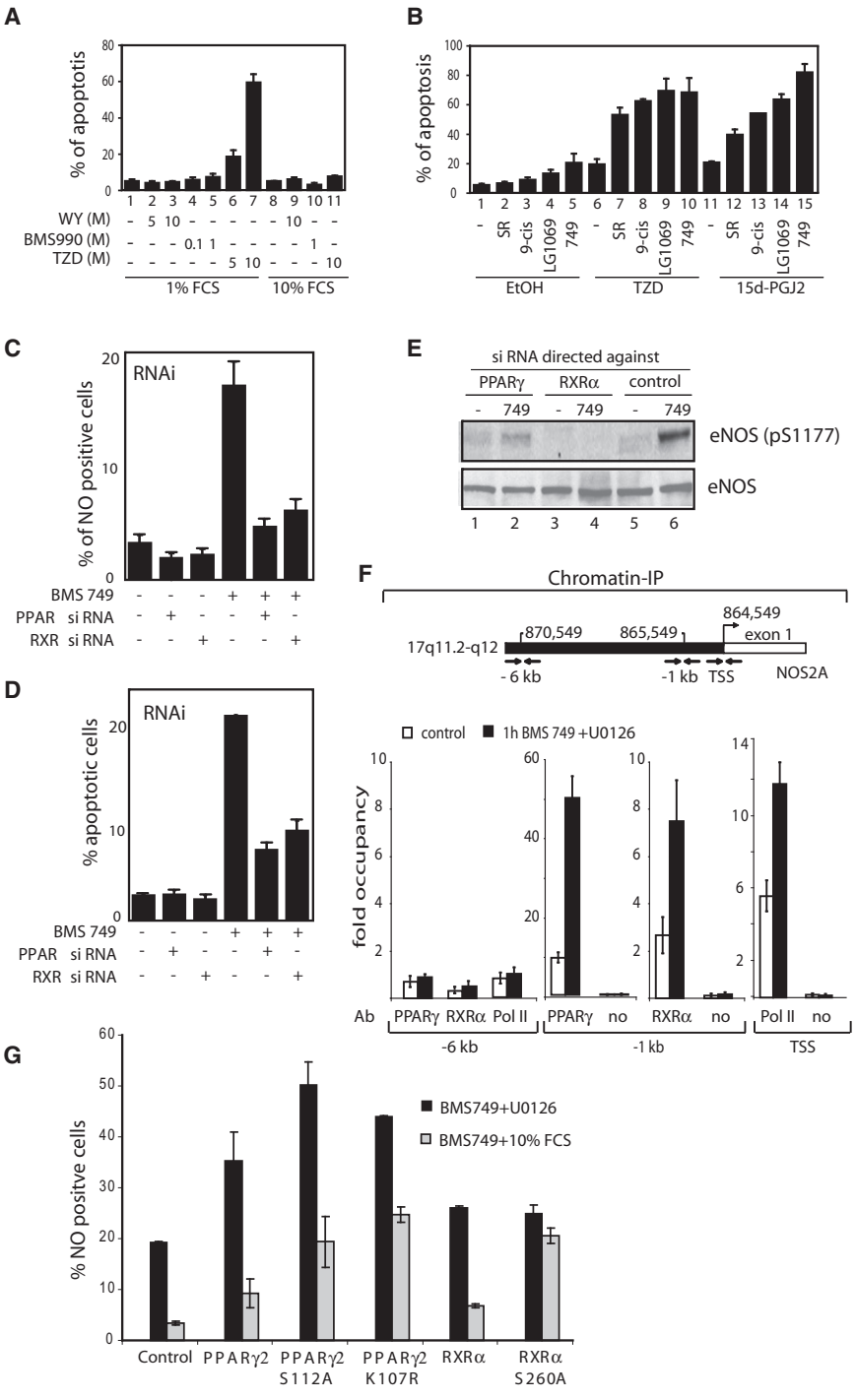

Figure 7. Rexinoid Apoptosis Involves RXRα-PPARγ Heterodimer Binding to the iNOS Promoter and Leads to eNOS Phosphorylation

(A) PLB985 cultured in 1% or 10% FCS were treated for 24 hr with PPAR agonists as indicated, and apoptosis was measured.

(B) PLB985 were exposed to four different rexinoids: SR11237 (SR; 1 μM), 9-*cis* RA (1 μM), LG1069 (1 μM), and BMS749 (0.5 μM), in combination with troglitazone (TZD) or 15-deoxy-D(delta)^{12,14}-prostaglandin J_2 (15d-PGJ2). Apoptosis analyses reveal synergistic action of PPARγ and RXR agonists.

(C and D) Inhibition of iNOS and eNOS by siRNA reduces NO positivity (C) and apoptosis (D) induced by BMS749.

(E) Phosphorylation of eNOS at S_{1177} is induced by BMS749 and abrogated by knocking down RXRα or PPARγ.

(F) ChIP assays reveal ligand-induced binding of PPARγ and RXRα upstream of the iNOS promoter and PolII to the transcription start site (TSS). Assays were performed with HCT116 cells grown in 0.5% serum. Ligands and/or FCS were added for 1 hr. Data represent fold occupancy determined by real-time PCR of ChIPed DNA.

(G) RXR$α_{S260}$ is targeted by growth factors while phosphorylation-deficient PPARγ mutants still support serum-dependent suppression of NO-mediated rexinoid apoptosis. Note that serum does not antagonize NO production in cells expressing RXR$α_{S260A}$. Bars represent the mean ± SD.

rexinoids has been reported elsewhere (de Urquiza et al., 2000; Solomin et al., 1998), and data from RXR mutant mice add credence to such concept. Indeed, interdigital webbing (syndactyly) has been observed in RXR AF1 domain mutants and is an accepted marker for an apoptotic defect leaving the interdigital mesenchyme intact during embryonic development (Mascrez et al., 2001). Therefore, it is possible that rexinoid apoptosis is a general mechanism involved in tissue development, repair, and homeostasis.

Our results reveal that the intrinsic death pathway triggered by NO executes rexinoid apoptosis. This finding is supported by the fact that the selective NOS inhibitor L-NMMA dramatically decreases both rexinoid-induced NO production and apoptosis. NO has been described to induce phosphorylation and activation of p53 (Brune, 2003; Schneiderhan et al., 2003), which leads to the induction of DR5 (Wu et al., 1997), as shown in Figure 4G. The role of NO in the modulation of apoptosis is controversial. The two main types of NOS are the inducible (iNOS) and the endothelial (eNOS) forms. iNOS has been shown to rapidly and strongly induce the production of NO during inflammatory responses, whereas eNOS has been assigned the function of producing low amounts of NO for basic cell homeostasis (Li and Wogan, 2005). The role of eNOS as a protective agent has been demonstrated in endothelial cells, where it is able to prevent TNFα, LPS, or ROS-induced apoptosis (Dimmeler et al., 1997). However, this antiapoptotic function seems to be restricted to endothelial cells and a few other cell types. iNOS induction, and the resultant NO increase, plays an important role in septic shock and has an apoptotic role

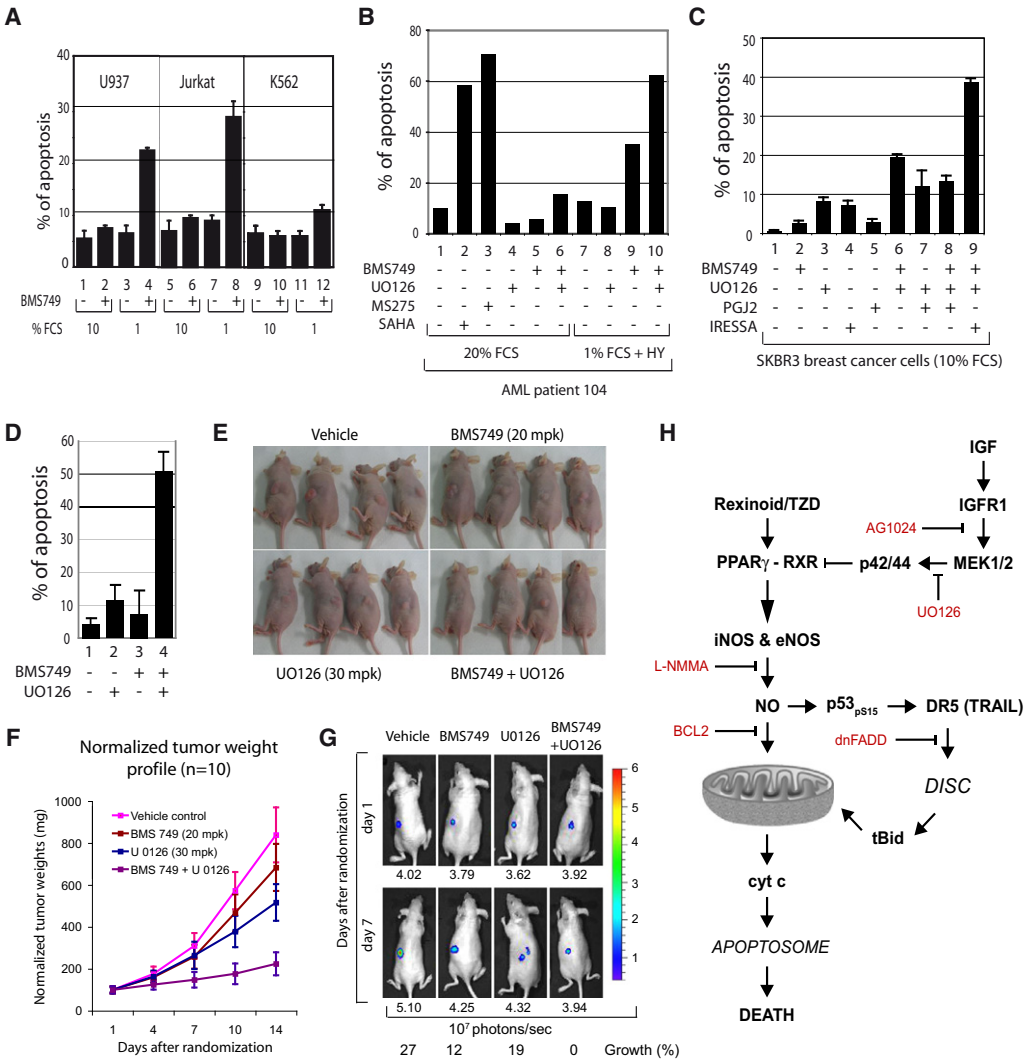

Figure 8. Rexinoid Apoptosis Operates in Different Cell Types In Vitro and In Vivo

(A) Apoptosis induced with BMS749 in hematopoietic cells cultured in 1% or 10% FCS.

(B) Rexinoid apoptosis assays with blasts of AML patient 104 cultured ex vivo. Blasts were cultured in 20% FCS or 1% FCS+HY.

(C) IRESSA potentiates rexinoid apoptosis of SKBR3 cells. Apoptosis of SKBR3 cells grown in 10% FCS and treated with BMS749, U0126, PGJ_2, or IRESSA is shown.

(D) Rexinoid apoptosis induced in HCT116 colon cancer cells.

(E) Efficient in vivo antitumor effect on HCT116 xenografts under conditions of rexinoid apoptosis. Xenografted nude mice were treated every other day with vehicle or the indicated doses of BMS749, U0126, or both for 2 weeks. Photograph of mice at the end of the treatment is shown.

(F) Graphical representation of tumor weights (±SD) in xenografted mice.

(G) In vivo imaging of representative luciferase-tagged HCT116 xenografted mice imaged at the beginning and after 7 days of treatment. Tumor sizes quantified as total amount of light emitted (photons/s) are given below the photos. Note the block of tumor growth by BMS749-U0126 cotreatment.

(H) Schematic representation of RXR-PPARγ-mediated apoptosis in AML cells. Activation of RXR-PPARγ heterodimers by RXR or PPARγ agonists induces NO production by iNOS and eNOS, which, in turn, affects mitochondria integrity and triggers cytochrome c release and apoptosome formation. This activation of the intrinsic death pathway can be inhibited by Bcl-2 overexpression. Additionally, NO release activates p53, which leads to overexpression of DR5 that triggers an amplification loop involving caspase 8 and Bid cleavage. The implicated extrinsic death pathway can be blocked at the DISC by dominant-negative FADD or DR5-deficiency. IGF1-like growth factors activate through binding to IGFRI a MAPK survival pathway which attenuates rexinoid apoptosis by phosphorylating RXRα at S260. In cells grown in serum growth factor, signaling can be blocked by IGFR1kinase inhibitors (AG1024) or downstream by MEK inhibition (U0126), thereby facilitating rexinoid apoptosis in presence of growth factors. Bars indicate mean ± SD.

in most cell types, including macrophages and monocytes. Notably, in eNOS knockout mice, induction in iNOS activity due to LPS was significantly diminished (Connelly et al., 2005). A number of reports have demonstrated a role for PPARγ in the phosphorylation of eNOS at S_{1177} and augmentation of its activity. In fact, NO produced by eNOS has been shown to be essential for enhancement of iNOS activity in macrophages, thereby pointing to a proinflammatory role for eNOS .

We demonstrate here that, under conditions of growth factor or serum limitation, RXR activation generates a strong and sustained NO induction originating from both iNOS and eNOS. Chromatin-IP experiments revealed PPARγ and RXR binding at 1 kb upstream of the iNOS TSS upon BMS749 treatment (Figure 7F). Even though no apparent DR1-type PPRE was found, this region harbors other DRs that could act as response elements; whether these mediate the RXR-PPARγ action in vivo remains to be established. Although transcriptional activation of iNOS is rapid, phosphorylation of eNOS occurs with a delay and leads to a sustained response, suggesting a collaboration between the two types of NOS. A number of mechanisms have been proposed to explain NO-mediated apoptosis through the intrinsic pathway, such as ROS production, formation of peroxynitrite, and protein nitrosylation. Upon the exclusion of ROS, it is possible that protein nitrosylation, peroxynitrite formation, which leads to the opening of the mitochondrial transition pore, or inhibition of cytochrome c oxidase play a critical role in NO-mediated apoptosis (Beltran et al., 2000; Moncada and Erusalimsky, 2002).

To identify the point of convergence between growth factor action and rexinoid apoptosis, we studied whether MAPK-dependent phosphorylation of the RXR-PPARγ heterodimer could block apoptosis. Human RXRα can be phosphorylated at S260 by ERK1/2, leading to impaired cofactor recruitment and hormone insensitivity, while phosphorylation of PPARγ$_{S112}$ predisposes it to sumoylation at K107 and leads to strong transrepression that is absent in the K107R mutant. However, the addition of serum still reduced the production of NO in cells transfected with the PPARγ mutants, suggesting that ERK1/2-mediated repression of rexinoid apoptosis does not involve the above PPARγ modification. In contrast, transfection of the nonphosphorylable mutant RXRα$_{S260A}$ abrogated the antiapoptogenic serum effect on NO production, thus revealing that GFs act via phosphorylation of RXRα$_{S260}$ to protect cells from rexinoid apoptosis.

Growth factors play an essential role in cell survival. Serum deprivation in culture media results in apoptosis, but growth factors such as IGF1 and IGFII can support cell survival, at least temporarily (Kurmasheva and Houghton, 2006). IL-3-dependent hematopoietic cells can escape apoptosis in absence of IL-3 upon treatment with IGF, and such an effect has been extended to such diverse cells as cortical neuronal cells, cerebral granule cells, and a variety of malignant cells (Rodriguez-Tarduchy et al., 1992). IGF can activate a number of antiapoptotic genes such as Bcl-2 (Minshall et al., 1997) and induce phosphorylation of BAD, a crucial regulator of mitochondrial apoptosis (Datta et al., 1992). IGF effectively inhibits apoptosis by stress-inducing agents such as ROS and NO in hippocampal neurons, and activation of AKT by IGF-I has been proposed as a central mechanism for the inhibition of NO-induced apoptosis (Matsuzaki et al., 1999). Blocking of IGF1 signaling has been demonstrated in various in vivo models to inhibit tumor growth and enhances the effects of established antitumor therapies (Clemmons, 2007). Thus, it could be intuited that inhibition of growth factor signaling would leave cells particularly susceptible to apoptotic signals. Indeed, sensitization of tumor cells to chemotherapeutic agents by inhibition of growth factors with receptor antibodies or small molecules against downstream signaling is now widely used to treat a variety of tumors (reviewed by Chitnis et al., 2008; Clemmons, 2007; Wanebo et al., 2006). Together, these studies tempted us to hypothesize that RXR, along with PPARγ, could control an endogenous default apoptotic pathway kept in silent state by the action of growth factors.

The link between growth factor support and rexinoid apoptosis prompted us to investigate a possible cancer therapeutic potential of rexinoid apoptosis. It was particularly interesting that a variety of cell types, both hematopoietic and solid cancer cell lines, would undergo rexinoid apoptosis either in low serum or under conditions where MAPK signaling is impaired (see Figure 8). Interestingly, blocking GF support by combining the MAPK inhibitor and IRESSA superactivated rexinoid apoptosis. In one case, we could establish conditions for primary blasts from a patient with AML that allowed us to demonstrate the induction of rexinoid apoptosis, which was similar to that seen with two prototypic HDAC inhibitors. A number of growth factor signaling inhibitors, such as gefinitib and herceptin, have been developed and are in therapeutic use as adjuvants to chemotherapy for the treatment of cancer. Several type 1 insulin-like growth factor receptor (IGF1R) inhibitors (blocking antibodies and tyrosine kinase inhibitors) are available, and at least 12 of them are undergoing clinical trials as combinations with other cancer treatments (Chitnis et al., 2008). Their combination with RXR and/or PPARγ agonists would be an attractive therapeutic strategy. The mouse xenograft experiments described here confirmed the therapeutic potential of rexinoid apoptosis in vivo and revealed the absence of any major toxicity. Interestingly, the experiments demonstrated that even a general growth factor signaling inhibitor such as U0126 can be highly effective in vivo in combination with the RXR ligand BMS749. Recently, it has been shown that combination of the PPARγ agonist rosiglitazone with carboplatin significantly reduces the tumor burden in drug-resistant non-small-cell lung cancer mouse model (Girnun et al., 2007). Such a combination therapy would be particularly attractive in cancers showing resistance to standard treatments. Combination with specific inhibitors such as gefinitib (IRESSA) would augment the selectivity of the treatment and thereby reduce drug toxicity. Further studies in different models of carcinogenesis will be required to establish the full palette and generality of this principle and its therapeutic potential.

EXPERIMENTAL PROCEDURES

Ligands, Chemicals, and Antibodies

Sources and use of antibodies and chemicals are described in Supplemental Data.

Cell Culture, Infection, and Transfection

PLB985, HCT116, MCF7, and HT29 were obtained from the in-house cell culture service, and BJAB wild-type and BJAB DR5 KO cells were a kind gift from Andrew Thorburn. PLB985 were adapted to minimal serum media and were cultured in RPMI, 1% fetal calf serum, complemented with 1% HY (Biosepra), gentamycin, 25 mM HEPES, and 2 mM glutamine. The Virapower lentiviral expression system (Invitrogen) was used to produce lentiviruses. Retroviruses were produced using pCLII ampho helper plasmid in BOSC23 cells. HCT116 cells were transfected with different plasmids using Fugene 6 reagent (Roche). PPARγ2wt plasmid was obtained from C. Ronald Kahn via Addgene (plasmid 11439). Site-directed mutagenesis was performed using the protocol from Quickchange kit (Stratagene). p53 shRNA plasmid was obtained from William Hahn via Addgene (plasmid 10672).

Flow Cytometry

PLB985 cells apoptosis was quantified by detection of the 7A6 mitochondrial antigen (which is selectively exposed in cells undergoing apoptosis) using the Apo2.7 antibody. Mitochondrial membrane potential was assayed by incubating 2.5×10^5 cells with 50 nM $DiOC_6(3)$ for 30 min in the dark and subsequent examination by flow cytometry. NO production was quantified using the selective DAF-FM diacetate probe at 5 μM concentration.

RNase Protection Assay

Total RNA was extracted with Trizol (Invitrogen-GIBCO BRL, Carlsbad, CA), and RNase protection assays were performed according to the supplier's instructions (PharMingen, San Diego, CA).

p42/44 MAP Kinase Assay

p42/44 activity assay was measured in vitro, using the nonradioactive p42/44 MAP Kinase Assay Kit (Cell Signaling Technology) according to the manufacturer's protocol using Elk-1 as substrate.

siRNA Studies

PLB cells were transfected with 50 pmoles of siRNA using Lipofectamine 2000 (Invitrogen) according to manufacturer's protocol. Cells were used for analysis after 36 hr incubation.

Chromatin Immunoprecipitation

Chromatin Immunoprecipitation (ChIP) was done according to standard procedures. In brief, after treatment with ligands, HCT116 cells were cross-linked with 1% paraformaldehyde for 30 min at room temperature. Cells were scraped, sonicated, and then immunoprecipitated with RNA PolII (sc-9001), PPARγ (sc-7196) (Santa Cruz), and RXRα (in-house) antibodies overnight. The recovery of the complexes, washing, de-cross-linking and extraction of DNA was performed according to the Upstate protocol. Quantitative real-time PCR was performed using sybr green mix from QIAGEN and the primers mentioned in Supplemental Data.

In Vivo Experiments

Xenograft study using HCT116 colon carcinoma cell lines were performed in Crl:Nu(Ico)Foxn1Nu mice (Swiss) mice (Charles River); 4×10^6 cells were injected subcutaneously to the left flank. Treatments were started when the tumors were approximately 4 mm. Animals were randomized in the treatment groups, so that each group contains animals of approximately same size of tumors before treatment. BMS749 (20 mg/kg bw) and U0126 (30 mg/kg bw) were administered intraperitoneally for 2 weeks (11 doses, alternate days), and tumor size was determined using Vernier Calliper measurement using the following formula: tumor volume = $(a^2 \times b)/2$, where a is width and b is the length of the tumor. For in vivo imaging experiments, HCT116 cells stably expressing firefly luciferase were used. In vivo imaging was performed after the administration of luciferin (125 mg/kg bw) using a Xenogen IVIS 100 system (Caliper Life Sciences). All animal studies were conducted following protocols approved by the DDSV (Direction Départementale des Services Vétérinaires).

SUPPLEMENTAL DATA

Supplemental data include Supplemental Experimental Procedures and four figures and may be found with this article online at http://www.cell.com/cancer-cell/supplemental/S1535-6108(09)00252-9.

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