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Raloxifene-Induced Myeloma Cell Apoptosis: A Study of Nuclear Factor-κB Inhibition and Gene Expression Signature

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ABSTRACT

Because multiple myeloma remains associated with a poor prognosis, novel drugs targeting specific signaling pathways are needed. The efficacy of selective estrogen receptor modulators for the treatment of multiple myeloma is not well documented. In the present report, we studied the antitumor activity of raloxifene, a selective estrogen receptor modulator, on multiple myeloma cell lines. Raloxifene effects were assessed by tetrazolium salt reduction assay, cell cycle analysis, and Western blotting. Mobility shift assay, immunoprecipitation, chromatin immunoprecipitation assay, and gene expression profiling were performed to characterize the mechanisms of raloxifene-induced activity. Indeed, raloxifene, as well as tamoxifen, decreased JJN-3 and U266 myeloma cell viability and induced caspase-dependent apoptosis. Raloxifene and tamoxifen also increased the cytotoxic response to vincristine and arsenic trioxide. Moreover, raloxifene inhibited constitutive nuclear factor-κB (NF-κB) activity in myeloma cells by removing p65 from its binding sites through estrogen receptor α interaction with p65. It is noteworthy that microarray analysis showed that raloxifene treatment decreased the expression of known NF-κB-regulated genes involved in myeloma cell survival and myeloma-induced bone lesions (e.g., *c-myc*, *mip-1α*, *hgf*, *pac1*,. . .) and induced the expression of a subset of genes regulating cellular cycle (e.g., *p21*, *gadd34*, *cyclin G2*,. . .). In conclusion, raloxifene induces myeloma cell cycle arrest and apoptosis partly through NF-κB-dependent mechanisms. These findings also provide a transcriptional profile of raloxifene treatment on multiple myeloma cells, offering the framework for future studies of selective estrogen receptor modulators therapy in multiple myeloma.

Multiple myeloma is a bone marrow disease characterized by uncontrolled plasma cell proliferation and by various clinical manifestations such as hyperproteinemia, renal insufficiency, anemia, and skeletal destruction. Osteolysis is a major clinical complication of multiple myeloma and is associated with bone pain and pathological fractures (Roodman, 2004). Multiple myeloma treatment remains unsatisfactory, and new drugs targeting key signaling pathways required for myeloma growth or survival are needed.

Raloxifene is a selective estrogen receptor modulator (SERM) registered for the treatment of osteoporosis (Delmas et al., 2002). SERMs have both estrogen-agonistic and antagonistic properties depending on the tissue, the cell type, and even the target gene. Raloxifene thus has in vitro antiestrogen activities on breast tumor cells, and raloxifene treatment is associated with a decreased incidence of invasive breast cancer (Martino et al., 2004). However, raloxifene properties on the proliferation and survival of other cancer cells, including multiple myeloma cells, have not been explored extensively.

Many studies have reported the crucial role of the transcription factor NF-κB in the growth and survival of various

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ABBREVIATIONS: SERM, selective estrogen receptor modulator; NF-κB, nuclear factor-κB; PS-341, bortezomib; ER, estrogen receptor; IL, interleukin; DMSO, dimethyl sulfoxide; BAY 11-7085, (*E*)3-[(4-*t*-butylphenyl)sulfonyl]-2-propenenitrile; MG-132, *N*-benzoyloxycarbonyl (*Z*)-Leu-Leu-leucinal; PARP, poly(ADP-ribose) polymerase; IKK, IκB kinase complex; HA, hemagglutinin; GST, glutathione *S*-transferase; PBS, phosphate-buffered saline; ChIP, chromatin immunoprecipitation; RT, reverse transcriptase; PCR, polymerase chain reaction; CT, control; RAL, raloxifene; TAM, tamoxifen; Z-VAD-FMK, *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone.

tumor cells (Panwalkar et al., 2004). The typical heterodimeric p50/p65 NF-κB complex is sequestered in the cytoplasm through its association with IκB family inhibitors. Activation by various stimuli leads to IκB protein phosphorylation and degradation, allowing for NF-κB translocation into the nucleus, where it specifically binds DNA and stimulates target gene transcription (Greten and Karin, 2004). In contrast to normal cells, NF-κB is constitutively activated in various cancer cells, including myeloma cells. This constitutive activity favors cell proliferation, inhibits apoptosis, and enhances resistance to cytotoxic agents (Hideshima et al., 2002; Bharti et al., 2003). Moreover, elevated levels of NF-κB activity were found in relapsing and refractory multiple myeloma (Feinman et al., 1999). Several established antimyeloma therapies, (e.g., dexamethasone, histone deacetylase inhibitors, and thalidomide) inhibit NF-κB activity (Mitsiades et al., 2002b, 2004). It has been demonstrated that bortezomib (PS-341), a proteasome inhibitor, is highly efficient for the treatment of refractory multiple myeloma (Richardson et al., 2005), and it is believed that PS-341 efficacy against multiple myeloma occurs mainly as a result of the inhibition of NF-κB activity (Mitsiades et al., 2002a).

The rationale for the investigation of raloxifene activity against multiple myeloma cells was based on the following scientific arguments: 1) some myeloma cells express estrogen receptors (ER) α and β (Otsuki et al., 2000); 2) tamoxifen has been shown to induce apoptosis in multiple myeloma cells and is currently tested in clinical trials (Treon et al., 1998; Fassas et al., 2001; Gauduchon et al., 2005); 3) ERs can inhibit NF-κB activity in the presence of their ligands (Galien and Garcia, 1997; Harnish et al., 2000; Valentine et al., 2000); 4) raloxifene decreases osteoclastic bone resorption through modulation of cytokines such as IL-6 and the RANKL/OPG system (Cheung et al., 2003); and 5) raloxifene is a well tolerated drug largely used for the treatment of postmenopausal osteoporosis.

The aim of our study was thus to investigate the response of myeloma cells to raloxifene. We also characterized the signaling pathways involved in this effect and performed a microarray analysis to determine the transcriptional profile of raloxifene-treated multiple myeloma cells. These findings provide the framework for future studies of raloxifene activity in multiple myeloma.

Materials and Methods

Cell Culture and Reagents. JJN-3 and RPMI 8226 myeloma cells were kindly provided by Dr. B. Van Camp (Free University Brussels, Brussels, Belgium). U266 myeloma cells were obtained from the European Collection of Cell Cultures (Salisbury, Wiltshire, UK).

Cells were grown in RPMI 1640 medium supplemented with 5 or 10% fetal calf serum, penicillin (100 IU/ml), and streptomycin (100 μg/ml). For all experiments, myeloma cells were plated in phenol red-free RPMI 1640 supplemented with 1% charcoal-treated fetal calf serum, penicillin (100 IU/ml), and streptomycin (100 μg/ml).

Raloxifene was a kind gift from Eli Lilly (Indianapolis, IN). Tamoxifen and 17β-estradiol were purchased from Sigma-Aldrich (Schnelldorf, Germany). Raloxifene, tamoxifen, and 17β-estradiol were dissolved in ethanol at a concentration of 1 mM. Vincristin sulfate was obtained from Teva Pharma (Wilrijk, Belgium). The broad-spectrum caspase inhibitor (Z-VAD-FMK) (Alexis Corporation, San Diego, CA) was dissolved in 10 mM DMSO. The NF-κB inhibitor BAY 11-7085 and the proteasome inhibitor MG-132 (Alexis Corporation) were dissolved in 20 mM ethanol and in 20 mM DMSO, respectively.

MTS Reduction Assay. Myeloma cells were seeded at 2×10^4 cells/well in flat bottomed 96-well plates (VWR, Leuven, Belgium). Cell viability was assessed by reduction of the tetrazolium salt (MTS) to the formazan product in viable cells ("Cell Titer 96 Aqueous"; Promega, Leiden, The Netherlands) as described previously (Olivier et al., 2005). The data were expressed as a percentage of absorbance observed in untreated cells.

Cell Cycle Analysis. The JJN-3 and U266 cells were synchronized at S phase by incubation with an inhibitor of DNA synthesis, thymidine (2.5 mM), for 24 h. The cells were then washed and seeded at 10^6 cells/well in six-well plates. The cells were treated with raloxifene or tamoxifen for 16 or 24 h, washed twice in PBS, and fixed with 2 ml of ice-cold 70% ethanol before being centrifuged and incubated (at 37°C for 30 min) in PBS containing RNase (5 μg/ml) and propidium iodide (50 μg/ml). The cells were analyzed with a flow cytometer equipped with an argon laser emitting at 488 nm (Epics XL; Beckman Coulter Inc., Fullerton, CA). Red fluorescence intensity was measured using a 620 ± 15-nm band pass filter. Graphical and cell cycle analyses were performed with FlowJo version 6.01 software (Tree Star, Ashland, OR).

Western Blotting Assays. Total protein expression was estimated by Western blotting as described previously (Olivier et al., 2005). Anti-ERα, anti-ERβ (sc-543 and sc-8974; Santa Cruz Biotechnology, Santa Cruz, CA), anti-PARP, anti-caspase 8, anti-caspase 9 (all from BD Biosciences, Erembodegem, Belgium), anti-Bid (sc-6538; Santa Cruz Biotechnology), anti-caspase 3 (Alexis Corporation), anti-Myc (sc-40; Santa Cruz Biotechnology), anti-$p21^{WAF1}$ (Oncogene, Boston, MA), or anti-actin (Sigma-Aldrich) antibodies were used as primary antibodies. Horseradish peroxidase-linked anti-rabbit, anti-goat, or anti-mouse antibodies (DakoCytomation, Glostrup, Denmark) were used as secondary antibodies. The reaction was revealed with the enhanced chemiluminescence detection reagent (ECL kit; GE Healthcare, Buckinghamshire, UK).

Kinase Assay. For endogenous IKK activity, JJN-3 cells (5×10^6 cells) were treated with raloxifene for the indicated times and subsequently lysed with 1% Triton X-100 lysis buffer. Cellular extracts were incubated with 5 μl of anti-IKKγ antibody (sc-8330) or 5 μl of the control anti-HA antibody (sc-805) (both from Santa Cruz Biotechnology) for 2 h at 4°C. Then, 100 μl of protein A-coupled Sepharose beads were added and incubated under gentle agitation overnight at 4°C. After three washes in lysis buffer, the kinase activity was assayed by using 1 μg of GST-IκBα as substrate.

Electrophoretic Mobility Shift Assay. Electrophoretic mobility shift assays were performed as described previously (Bureau et al., 2002). The κB probe was 5′-TTGGCAACGGCAGGGGAATTCCCCTCTCCTTAGGTT-3′. Nuclear extract samples containing 5 μg of protein were mixed with 5 μl of binding buffer (20 mM HEPES, pH 7.6, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 20% glycerol, 0.1% Nonidet P-40, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride), 2 μl of bovine serum albumin, 3 μl of polydIdC, and the ^{32}P-labeled probe. The complexes were separated on a polyacrylamide gel. To confirm specificity, competition assays were performed with a 50-fold excess of unlabeled wild-type and nonspecific probes. For supershifting experiments, 1 μg of each antibody (p50, p65, RelB, and c-Rel, all from Santa Cruz Biotechnology, and p52 from Cell Signaling (Leiden, The Netherlands) was incubated with the extracts for 30 min on ice before the addition of the ^{32}P-labeled probe.

Immunofluorescence. Myeloma cells (10^6 cells/well in six-well plates) were cytocentrifuged and fixed in 4% formaldehyde. The slides were blocked with PBS containing 1.5% dry milk before incubation with a goat anti-p65 antibody (1/100 dilution; Santa Cruz Biotechnology) for 45 min at 37°C. Slides were then washed in PBS and incubated with a secondary anti-goat antibody conjugated to fluorescein isothiocyanate (1/50 dilution; DakoCytomation) for 45 min at 37°C. Slides were washed in PBS and analyzed under a

fluorescence microscope (magnification, 40×; Nikon Eclipse E800 with Sony DXC-9100P).

Chromatin Immunoprecipitation. Chromatin immunoprecipitation (ChIP) assays were performed with a ChIP assay kit (Upstate Biotechnology, Dundee, UK). Anti-p65 antibody (5 μl), 5 μl of antiestrogen receptor antibody, or 5 μl of the control anti-HA antibody (all from Santa Cruz Biotechnology) was used for the immunoprecipitation. The *mip-1α* promoter sequence was detected with the sense 5′-CTCTTCACACTCACAGGAGA-3′ primer and the antisense 5′-TAGGCAGCCCTGGCGGAT-3′ primer. PCR was performed using the AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA) according to the following protocol: 99°C for 3 min followed by 30 amplification cycles (95°C for 1 min, 50°C for 1 min, and 72°C for 1 min).

Immunoprecipitation. Nuclear and cytoplasmic protein extracts of JJN-3 cells (5×10^6 cells/dish) were prepared as described previously (Delhalle et al., 2002). For immunoprecipitation, nuclear extracts were incubated with 5 μl of anti-ERα antibody or 5 μl of the control anti-HA antibody (sc-805) (both from Santa Cruz Biotechnology) for 2 h at 4°C. Then, 100 μl of protein A-coupled Sepharose beads were added and incubated under gentle agitation overnight at 4°C. After three washes in lysis buffer, bound proteins were resolved by SDS-polyacrylamide gel electrophoresis and analyzed by Western blotting.

Affymetrix Microarray Analysis. Total RNA was extracted from control or treated JJN-3 cells using RNeasy columns from QIAGEN (Valencia, CA). The integrity of the RNA was checked with the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). RNAs from three independent experiments were pooled and used as a template to generate double-stranded cDNAs using the Superscript II RT kit (Invitrogen, Merelbeke, Belgium). Thereafter, biotin-labeled cRNA was generated using the Bioarray High Yield RNA Transcript labeling kit (Enzo Life Science, New York, NY). The cRNAs were hybridized with the Human Genome U-133A array (Affymetrix, Santa Clara, CA). Data were processed using Gene Chip Operating Software (Affymetrix).

Real Time PCR. Real time PCR was carried out using the TaqMan platform with the SYBR Green master mix (Applied Biosystems), as described previously (Heid et al., 1996). Primers, whose sequences were available upon request, were designed using the Primer Express software (Applied Biosystems).

Statistical Analysis. Data are presented as mean values ± S.E.M. (standard error of the mean). Data were analyzed by the

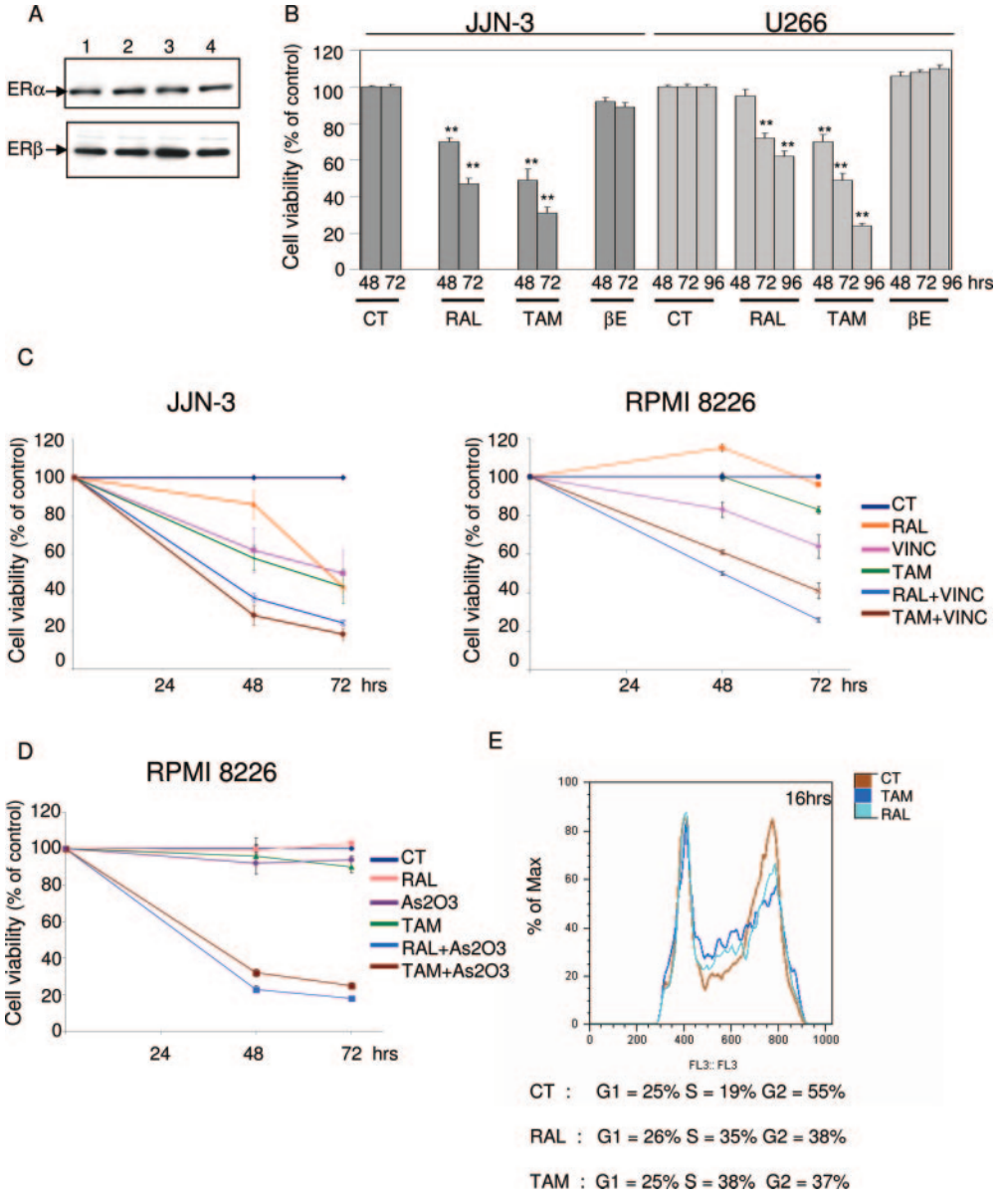

Fig. 1. Levels of ERα and ERβ in myeloma cells and SERM effect on multiple myeloma cell viability and cell cycle. A, estrogen receptor expression. Cellular extracts from JJN-3 (1), U266 (2), RPMI 8226 (3), and human breast cancer MCF7-AZ (4, positive control) cells were analyzed by Western blotting for ERα (top) and ERβ (bottom) expression. B, SERMs reduce myeloma cell viability. JJN-3 cells or U266 cells were treated with control media (CT), raloxifene (RAL), tamoxifen (TAM), or 17β-estradiol (βE) at 5 μM for 48, 72, or 96 h. Cell viability was measured with the MTS reduction assay. Data are expressed as mean percentage of control levels ± S.E.M. in 12 samples pooled from three independent experiments. **, $p < 0.01$ significantly different from untreated control. C and D, SERM effect on vincristine or arsenic trioxide cytotoxicity. C, JJN-3 cells were treated with either CT, RAL (5 μM), TAM (5 μM), vincristine (VINC, 0.1 μg/ml), or vincristine + raloxifene (RAL+VINC) or vincristine + tamoxifen (TAM+VINC) for 48 or 72 h. RPMI 8226 cells were treated as JJN-3 cells but with vincristine at 0.001 μg/ml. D, RPMI 8226 cells were treated with either CT, RAL (5 μM), TAM (5 μM), arsenic trioxide (As203, 5 μM), or arsenic trioxide + raloxifene (RAL+As203) or arsenic trioxide + tamoxifen (TAM+As203) for 48 or 72 h. Cell viability was measured with the MTS reduction assay. Data are expressed as mean percentage of control levels ± S.E.M. in four samples from one representative experiment. E, cell cycle analysis after raloxifene or tamoxifen treatment. JJN-3 cells were treated with thymidine at 2.5 mM for 24 h. Synchronized cells were replated in six wells and treated with CT, RAL, or TAM at 5 μM for 16 h. After treatment, cell cycles were analyzed by fluorescence-activated cell sorting. The data are representative of at least three distinct experiments.

Mann-Whitney test. In all analyses, $p < 0.05$ was considered statistically significant.

Results

ERα and ERβ Expression in Multiple Myeloma Cell Lines. Before studying the effects of raloxifene on multiple myeloma cell growth and survival, we verified the expression of the two ER isoforms in JJN-3, U266, and RPMI 8226 cells. All cell lines expressed both ERα and ERβ, as previously shown (Fig. 1A) (Treon et al., 1998).

Raloxifene and Tamoxifen Inhibit Myeloma Cell Proliferation and Induce Cell Cycle Arrest. Raloxifene and tamoxifen (both at 5 μM) decreased JJN-3 cell viability after 48 or 72 h of treatment, whereas the same concentration of 17β-estradiol did not (Fig. 1B). Both raloxifene and tamoxifen also decreased U266 cell viability (Fig. 1B), but an equivalent cytotoxicity required a longer incubation in these cells. A weak effect of raloxifene and tamoxifen was also observed on RPMI 8226 cells after 72 h of treatment (cell viability was decreased by 23 and 17%, respectively) (data not shown).

The in vitro concentrations of raloxifene and tamoxifen required to induce apoptosis of breast cancer cells or glioma cells vary from 1 to 40 μM (Frasor et al., 2004; Hui et al., 2004). Based on these studies and on our dose-response data (not shown), all our experiments were carried out using a 5 μM concentration, which inhibited JJN-3 cell growth by 50%.

Next, we determined whether the exposure of multiple myeloma cells to these SERMs potentiates the cytotoxic effects of other chemotherapeutic drugs currently used in the treatment of multiple myeloma. Raloxifene or tamoxifen enhanced the cytotoxic effects of vincristine against JJN-3 and RPMI 8226 cells (Fig. 1C). After 72 h of treatment, vincristine (at 0.1 μg/ml), raloxifene, or tamoxifen (at 5 μM) alone decreased JJN-3 cell viability by ~50%, whereas the addition of raloxifene or tamoxifen to vincristine induced, respectively, a 76 and 82% decrease in JJN-3 cell viability (Fig. 1C). RPMI 8226 cells showed a similar response to vincristine alone, and both raloxifene and tamoxifen again enhanced vincristine-induced cell death (Fig. 1C). A synergistic effect between SERM and arsenic trioxide (at 5 μM) was also observed in RPMI 8226 myeloma cells (Fig. 1D), but such an effect could not be investigated in JJN-3 cells, because these cells are extremely sensitive to arsenic trioxide (data not shown). A weaker but reproducible additive cytotoxic effect was also observed when U266 cells were treated simultaneously with arsenic trioxide and SERMs (data not shown).

Fluorescence-activated cell sorting analysis showed that both raloxifene and tamoxifen (both at 5 μM) blocked the cell cycle before G_2/M in JJN-3 cells, as shown by an increased proportion of cells in the S phase and a decrease of the

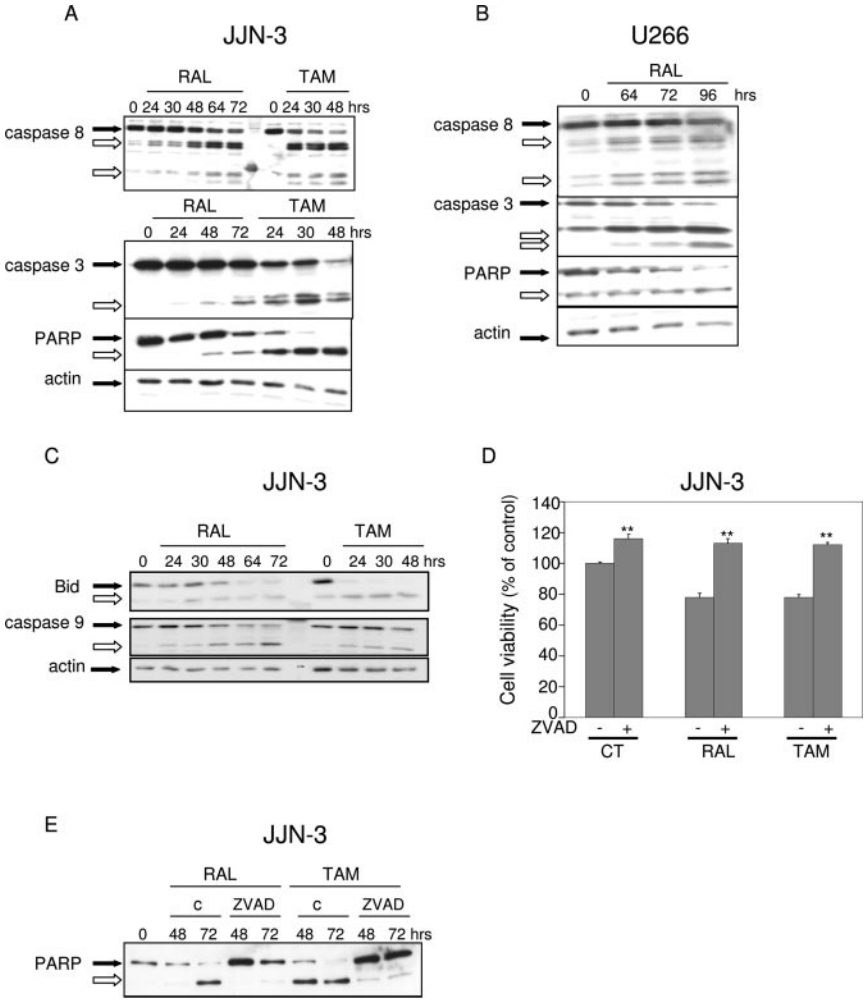

Fig. 2. SERMs induce myeloma cell apoptosis through caspase activation. A and B, JJN-3 (A) and U266 (B) cells were treated with raloxifene or tamoxifen at 5 μM for the indicated times. Total protein extracts were analyzed by Western blotting with anti-caspase 8, anti-caspase 3, anti-PARP, or anti-actin antibodies. C, JJN-3 cells were treated as in A. Total protein extracts were analyzed by Western blotting with anti-Bid, anti-caspase 9, or anti-actin antibodies. Black arrows indicate full-length proteins, whereas white arrows show cleaved proteins. D and E, caspase inhibitor effect on SERM-induced myeloma cell apoptosis. D, JJN-3 cells were pretreated with the caspase inhibitor Z-VAD-FMK at 50 μM for 1 h before treatment with CT, RAL, or TAM at 5 μM for 48 or 72 h. Cell viability was measured with the MTS reduction assay. Data are expressed as mean percentage of control levels ± S.E.M. in 14 samples pooled from three independent experiments. **, $p < 0.01$ significantly different from untreated control. E, JJN-3 cells were treated as in D. Total protein extracts were analyzed by Western blotting with anti-PARP antibody. Black arrows indicate full-length proteins, whereas white arrows show cleaved proteins.

number of cells in G_2/M (Fig. 1E). In U266 cells, raloxifene and tamoxifen also blocked cell cycle progression but in the G_2/M phase (data not shown).

Raloxifene and Tamoxifen Induce Myeloma Cell Apoptosis. To verify that raloxifene and tamoxifen induced cell death by apoptosis, we studied the activation of caspases and the cleavage of PARP in JJN-3 and U266 cells. Figure 2A shows that tamoxifen and raloxifene induced a proteolytic cleavage of caspase-8, caspase-3, and PARP (Fig. 2, A and B). The involvement of the mitochondrial apoptotic pathway was also shown by the cleavage of Bid and caspase-9 in JJN-3 cells after raloxifene and tamoxifen treatment (Fig. 2C). To confirm that raloxifene and tamoxifen induced caspase-dependent apoptosis, we tested the effect of the caspase inhibitor Z-VAD-FMK on cell death and PARP cleavage induced by raloxifene or tamoxifen. Pretreatment of JJN-3 cells with Z-VAD-FMK (at 50 μM) for 1 h before the addition of raloxifene or tamoxifen (both at 5 μM) had a cytoprotective effect on myeloma cells (Fig. 2D). In the same experimental conditions, the presence of the caspase inhibitor induced a marked decrease of raloxifene or tamoxifen-induced PARP cleavage in JJN-3 cells (Fig. 2E).

SERMs Inhibit NF-κB Activity in Myeloma Cells. Because NF-κB is constitutively active in multiple myeloma cells and promotes myeloma cell growth, survival, and drug resistance (Feinman et al., 1999; Bharti et al., 2003), we investigated the effects of the NF-κB inhibitor BAY 11-7085 or the proteasome inhibitor MG-132 on JJN-3 cell viability. Increasing concentrations of BAY 11-7085 (1–4 μM) or MG-132 (0.1–0.4 μM) decreased JJN-3 viability after 24 h, as determined by the MTS reduction assay (Fig. 3A).

We next investigated whether the SERMs inhibited the IKK activity and NF-κB-binding activity in myeloma cells. As shown by kinase assay performed with a GST-IκBα substrate, a marked IKK activity was observed in untreated JJN-3 cells, and raloxifene only weakly decreased this activity after 16 and 24 h of treatment (Fig. 3B). Consistent with these data, mobility shift assays also showed that NF-κB was constitutively active in the multiple myeloma cell lines. Raloxifene decreased NF-κB DNA-binding activity in JJN-3 or U266 cells (Fig. 3, C and D). In JJN-3 cells, the inhibition was already observed after 30 min and was complete after 16 h of treatment. A similar but less pronounced inhibition was observed after treatment of JJN-3 or U266 cells with the

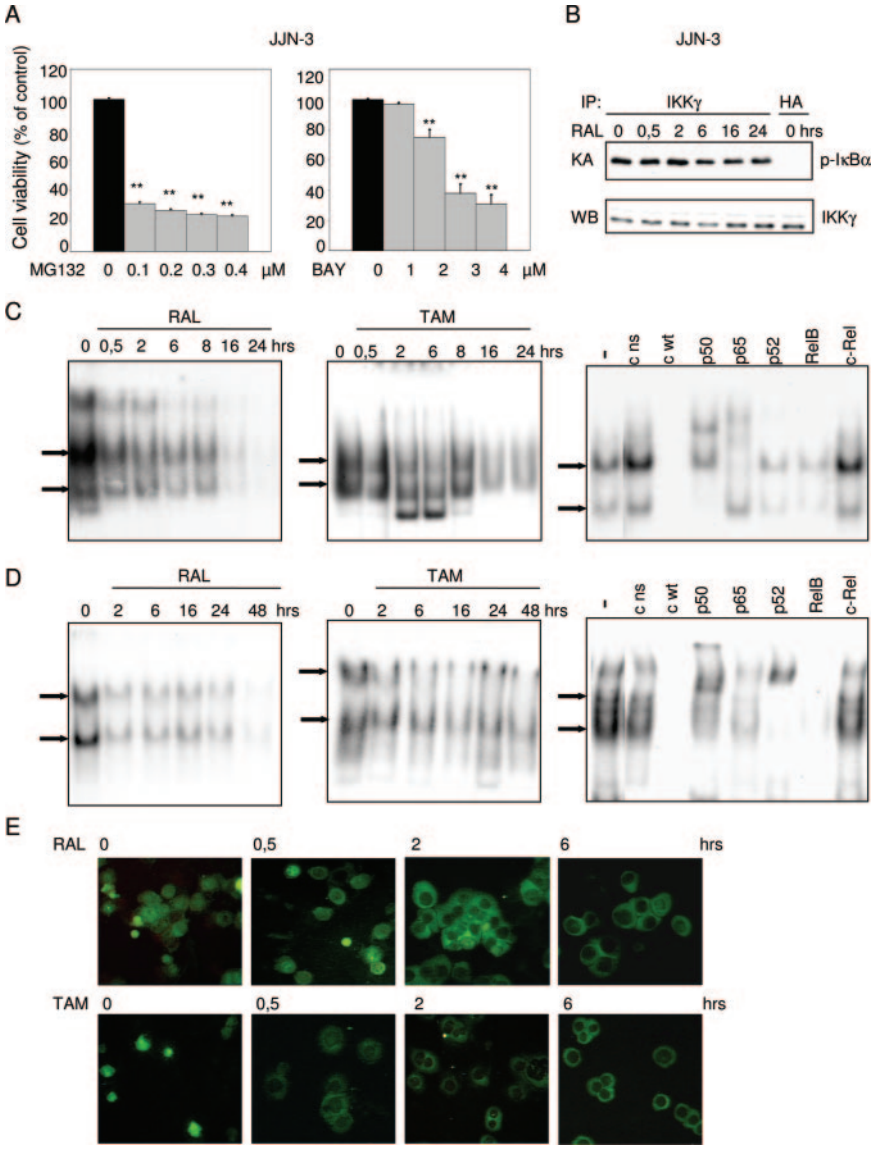

Fig. 3. NF-κB inhibition induces myeloma cell death and raloxifene inhibits NF-κB activity in myeloma cells. A, JJN-3 cells were treated with BAY 11-7085 (BAY) or MG-132 at the indicated concentrations for 24 h. Cell viability was measured with the MTS reduction assay. Data are expressed as mean percentage of control levels ± S.E.M. in 16 samples pooled from three independent experiments. **, $p < 0.01$ significantly different from untreated control. B, IKK activity after raloxifene treatment. JJN-3 cells were untreated or treated with RAL (5 μM) for the indicated times. Whole extracts were immunoprecipitated (IP) with anti-IKKγ or anti-HA (negative control) antibodies and kinase assay was performed with a GST-IκBα substrate. The presence of IKKγ in the extracts before the immunoprecipitation is illustrated by Western blotting. C and D, NF-κB-binding activity after raloxifene or tamoxifen treatment. JJN-3 (C) and U266 (D) cells were untreated or treated with RAL or TAM at 5 μM for the indicated times, and nuclear extracts were analyzed by electrophoretic mobility shift assays for NF-κB DNA-binding activity. Competition experiments were performed with a 50-fold excess of an unlabeled nonspecific (c ns) or specific (c wt) probe. Supershift experiments were performed with antibodies against p50, p65, p52, RelB, and c-Rel. Arrows indicate the NF-κB complexes. E, subcellular distribution of p65 in response to raloxifene or tamoxifen treatment. Immunofluorescence staining of p65 was performed on JJN-3 cells either untreated (0) or treated with RAL (5 μM) or TAM (5 μM) for the indicated times.

same concentration of tamoxifen (Fig. 3, C and D). NF-κB DNA-binding complexes contained various homo- or heterodimers of p50, p52, p65, and RelB subunits in JJN-3 cells, whereas the NF-κB complex was mainly constituted by p52/RelB subunits in U266 cells as demonstrated by supershift experiments (Fig. 3, C and D). Competition experiments performed with a 50-fold excess of an unlabeled palindromic κb probe or a nonspecific probe confirmed the specificity of NF-κB-binding activity in JJN-3 or U266 cells (Fig. 3, C and D).

To determine the subcellular localization of NF-κB subunits, immunofluorescence staining was performed with an anti-p65 antibody. Although p65 was exclusively observed in the nucleus of untreated cells, raloxifene or tamoxifen treatment rapidly induced p65 relocalization to the cytoplasm of JJN-3 (Fig. 3E) and U266 cells (data not shown).

ERα Interaction with p65. To study the mechanisms of raloxifene-mediated NF-κB inhibition in myeloma cells, we investigated whether ERα directly interacts with p65. Nuclear extracts from untreated or raloxifene-stimulated JJN-3 cells were immunoprecipitated with an anti-ERα antibody, and the resulting precipitates were analyzed by an anti-p65 Western blotting analysis (Fig. 4A). Whereas p65 was weakly associated with ERα in untreated cells, this interaction was strongly enhanced after 5 min of raloxifene treatment and became undetectable after longer stimulation (Fig. 4A). These data suggested that raloxifene may induce a conformation change in ERα, resulting in decreased NF-κB DNA-binding activity and, consequently, in the release and translocation of p65 from the nucleus to the cytoplasm.

To confirm this hypothesis, we evaluated the binding of p65 to NF-κB-regulated promoters in the presence of raloxifene by ChIP assays. We selected the *mip-1α* gene promoter, because *mip-1α* is a known NF-κB-regulated gene whose expression was significantly decreased after raloxifene treatment of JJN-3 cells (see Table 1 and Fig. 5A). Raloxifene treatment led to the dissociation of p65 (Fig. 4B, top left) and ERα (Fig. 4B, middle left) binding to the *mip-1α* gene promoter. These data thus showed that p65 and ERα are released from the NF-κB-binding site on specific gene promoters upon treatment with raloxifene.

Gene Expression after Raloxifene Treatment. Gene expression in JJN-3 cells either untreated or treated with raloxifene for 2 h was compared by microarray analyses. This

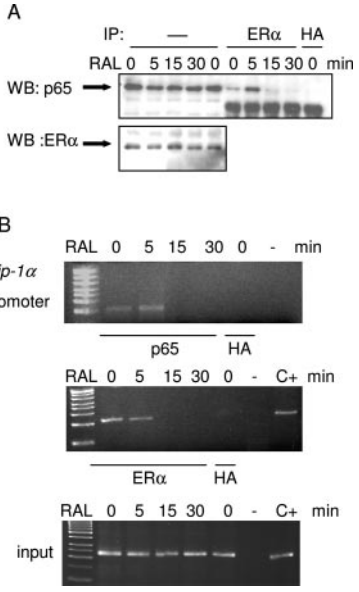

Fig. 4. ERα interaction with p65. A, immunoprecipitation assay. JJN-3 cells were untreated (0) or treated with RAL (5 μM) for the indicated times. Nuclear extracts were immunoprecipitated (IP) with anti-ERα or anti-HA (negative control) antibodies followed by anti-p65 Western blot analysis. The presence of p65 and ERα in the nuclear extracts before the immunoprecipitation is illustrated by Western blotting. B, p65 and ERα are released from the *mip-1α* promoter after raloxifene treatment. JJN-3 cells were untreated (0) or treated with RAL at 5 μM for the indicated times, and ChIP assays were performed. The immunoprecipitation was done with antibodies against p65 (top), ERα (middle), or HA (negative control). The promoter DNA fragments were amplified by PCR using primers flanking the NF-κB sites of *mip-1α* gene promoter. The input panels represent chromatin fragments that were reversed cross-linked and amplified by PCR (bottom). Positive control (C+) lane represents DNA of JJN-3 cells directly amplified by PCR. There was no amplified fragment in the absence of DNA (−).

A

	JJN3 % of control means (± SEM)	U266 % of control means (± SEM)
c-myc	14,7 (3,4)	34,2 (4,6)
mip-1α	37,0 (6,0)	91,4 (20,8)
hgf	69,7 (8,9)	NE
pac 1	38,8 (7,1)	NE
gadd 34	203,8 (16,1)	204,8 (60,1)
p21	209,5 (31,5)	204,8 (60,8)
cyclin G2	373,2 (94,0)	274,1 (73,5)

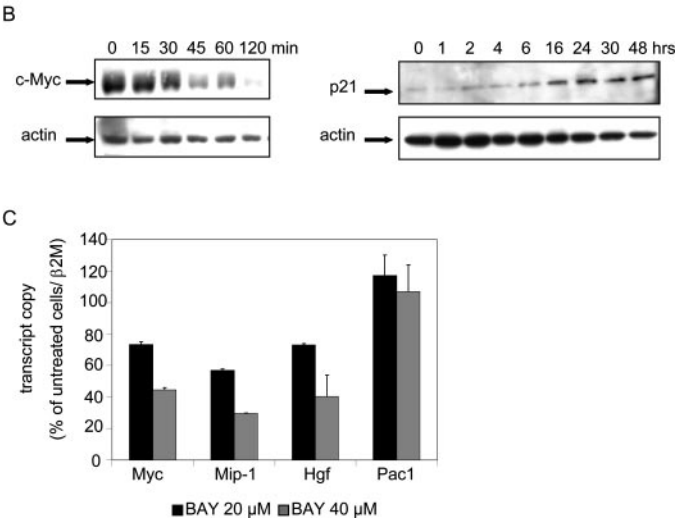

Fig. 5. Raloxifene-modulated genes in myeloma cells. A, expression of selected raloxifene target genes: *c-myc*, *mip-1α*, *hgf*, *pac-1*, *gadd 34*, *p21*, and *cyclin G2*. RNA was extracted from JJN-3 or U266 cells untreated or treated with raloxifene at 5 μM for 2 h. Levels of transcripts were measured by real time RT-PCR. The number of transcript copies for control cells is set to 100%. The data were normalized by quantification of the β_2-microglobulin transcripts (β2M) and are expressed as mean percentage of control levels ± S.E.M. in six samples pooled from three independent experiments. B, c-Myc and p21 protein expression. Total cellular extracts from raloxifene-treated JJN-3 cells were analyzed by Western blotting with anti-p21, anti-c-Myc, or anti-actin antibodies. C, expression of selected target genes *c-myc* (Myc), *mip-1α* (Mip-1), *hgf* (Hgf), and *dusp2/pac-1* (Pac). RNA was extracted from JJN-3 cells untreated or treated with BAY 11-7085 at 20 or 40 μM for 2 h. Levels of transcripts were measured as in A. The data are expressed as mean percentage of control levels ± S.E.M. in four samples pooled from two independent experiments.

TABLE 1

Genes regulated by raloxifene

Microarray analyses were carried out using RNA extracted from JJN-3 cells, either untreated or treated with raloxifene at 5 μM for 2 h. The table shows a list of selected raloxifene-modulated genes with the ratio of gene expression in raloxifene-treated cells versus untreated JJN-3 cells (-fold change over control) from two independent experiments.

Affymetrix no.	Gene Name	Gene Symbol	Regulation	
			Exp. 1	Exp. 2
			-fold change	
Myeloma cell death or cell cycle-related				
202431_s_at	v-Myc myelocytomatosis viral oncogene homolog (avian)	*MYC*	−4.3	−3.5
205114_s_at	Chemokine (C–C motif) ligand 3	*CCL3*	−1.9	−2.0
210755_at	Hepatocyte growth factor (hepapoietin A; scatter factor)	*HGF*	−1.6	−1.6
204014_at	Dual specificity phosphatase 4	*DUSP4*	−1.5	−1.7
204794_at	Dual specificity phosphatase 2/pac1	*DUSP2*	−2.8	−7.0
212724_at	Ras homolog gene family, member E	*ARHE*	−1.6	−1.6
202284_s_at	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	*CDKN1A*	1.6	1.5
202769_at	Cyclin G2	*CCNG2*	3.5	4.0
211559_s_at	Cyclin G2	*CCNG2*	3.5	2.8
202770_s_at	Cyclin G2	*CCNG2*	3.7	3.2
201702_s_at	Protein phosphatase 1, regulatory subunit 10	*PPP1R10*	1.6	1.6
201703_s_at	Protein phosphatase 1, regulatory subunit 10	*PPP1R10*	2.0	1.9
202014_at	Protein phosphatase 1, regulatory (inhibitor) subunit 15A	*PPP1R15A*	2.0	2.0
37028_at	Protein phosphatase 1, regulatory (inhibitor) subunit 15A	*PPP1R15A*	1.9	1.5
21152__at	Vascular endothelial growth factor	*VEGF*	1.6	1.5
204912_at	Interleukin 10 receptor, α	*IL10RA*	1.5	1.5
203140_at	B cell CLL/lymphoma 6 (zinc finger protein 51)	*BCL6*	1.6	1.7
207042_at	E2F transcription factor 2	*E2F2*	2.0	1.9
Endoplasmic reticulum stress and translation				
20112__at	Eukaryotic translation initiation factor 5A	*EIF5A*	−2.1	−2.1
20066__at	DnaJ (Hsp40) homolog, subfamily B, member 1	*DNAJB1*	1.5	1.5
21222_at	Putative translation initiation factor	*SUI1*	2.3	2.8
21716__at	Homocysteine-inducible, endoplasmic reticulum stress-inducible	*HERPUD1*	2.1	2.1
20284_s_at	DnaJ (Hsp40) homolog, subfamily B, member 9	*DNAJB9*	2.0	2.1
202843_at	DnaJ (Hsp40) homolog, subfamily B, member 9	*DNAJB9*	2.5	2.5
203810_at	DnaJ (Hsp40) homolog, subfamily B, member 4	*DNAJB4*	1.9	2.0
203811_s_at	DnaJ (Hsp40) homolog, subfamily B, member 4	*DNAJB4*	1.9	2.8
Solute carrier				
200924_s_at	Solute carrier family 3, member 2	*SLC3A2*	1.5	1.5
221881_s_at	Chloride intracellular channel 4	*CLIC4*	1.7	1.9
220924_s_at	Solute carrier family 38, member 2	*SLC38A2*	1.5	1.6
218041_x_at	Solute carrier family 38, member 2	*SLC38A2*	1.6	1.7
203950_s_at	Chloride channel 6	*CLCN6*	1.6	1.5
217678_at	Solute carrier family 7 (cationic amino acid transporter) member 11	*SLC7A11*	1.7	1.5
209921_at	Solute carrier family 7 (cationic amino acid transporter) member 11	*SLC7A11*	1.7	1.9
214963_at	Nucleoporin 160 kDa	*NUP160*	3.2	4.9
Transcription factors				
202672_s_at	Activating transcription factor 3	*ATF3*	2.3	2.6
206175_x_at	Zinc finger protein 222	*ZNF222*	2.1	2.0
210282_at	Zinc finger protein 198	*ZNF198*	1.0	1.7
209102_s_at	HMG-box transcription factor 1	*HBP1*	1.6	1.7
212501_at	CCAAT/enhancer binding protein (C/EBP), β	*CEBPB*	1.5	1.5
215099_s_at	Retinoid X receptor, β	*RXRB*	1.6	1.5
208078_s_at	Transcription factor 8 (represses interleukin 2 expression)	*TCF8*	−1.7	−1.6
Others				
201625_s_at	Insulin-induced gene 1	*INSIG1*	1.6	1.5
202067_s_at	Low-density lipoprotein receptor (familial hypercholesterolemia)	*LDLR*	1.7	1.6
202068_s_at	Low-density lipoprotein receptor (familial hypercholesterolemia)	*LDLR*	2.0	2.1
221638_s_at	Syntaxin 16	*STX16*	1.5	1.5
203439_s_at	Stanniocalcin 2	*STC2*	2.3	1.9
204285_s_at	Phorbol-12-myristate-13-acetate-induced protein 1	*PMAIP1*	1.9	1.9
205417_s_at	Dystroglycan 1 (dystrophin-associated glycoprotein 1)	*DAG1*	1.5	1.6
210836_x_at	Phosphodiesterase 4D, cAMP-specific	*PDE4D*	1.6	1.6
222238_s_at	Polymerase (DNA-directed), μ	*POLM*	1.9	2.3
217221_x_at	RNA-binding motif protein 10	*RBM10*	1.5	1.5
200758_s_at	Nuclear factor (erythroid-derived 2)-like 1	*NFE2L1*	1.7	1.5
202126_at	PRP4 pre-mRNA processing factor 4 homolog B (yeast)	*PRPF4B*	−1.5	−1.7
201129_at	Splicing factor, arginine/serine-rich 7, 35 kDa	*SFRS7*	−1.5	−1.6
213229_at	Dicer1, Dcr-1 homolog (*Drosophila*)	*DICER1*	−2.0	−1.6

study identified 29 genes that were transiently repressed and 75 genes that were induced upon raloxifene treatment. A representative list of raloxifene target genes is shown in Table 1. It is noteworthy that 14 of these raloxifene-regulated genes are known to be associated with cell cycle and apoptosis signaling pathways (Table 1). Indeed, raloxifene down-regulated the transcription of the proto-oncogene *c-myc* and up-regulated the expression of the transcriptional repressor *bcl-6* and of negative cell cycle regulators such as *p21*, *cyclin G2*, and *e2f2* (Horne et al., 1997; Park et al., 2000; Shou et al., 2000; Zhu et al., 2001; Tang et al., 2002). Moreover, raloxifene suppressed the expression of other genes controlling

multiple myeloma cell proliferation and/or survival including *hgf* and *mip-1α* (Anderson et al., 2002). Raloxifene also inhibited the expression of genes coding for the two mitogen-activated protein kinase phosphatases *dusp2* (*pac-1*) and *dusp4*, two mitogen-induced early responsive genes overexpressed in breast cancer (Wang et al., 2003), and of the ras homolog *arhe*.

In addition, raloxifene up-regulated the expression of genes coding for the growth arrest and DNA damage-induced protein PP1R15A (Gadd34), the transcription factors ATF3 retinoic acid X receptor β (*rxrb*), and CCAAT/enhancer-binding protein β (C/EBPβ), the heat shock family protein 40 (DnaJB), and genes coding for the proteins involved in the response to endoplasmic reticulum stress (DnaJB, HERPUD1) (Table 1) (Hasegawa et al., 2000; Fan et al., 2002). It also repressed the transcription factor 8 (*tcf8*) expression. Consistent with a recent study suggesting that tamoxifen-induced apoptotic response in glioma cells is mediated by an increase in cytosolic calcium (Hui et al., 2004), raloxifene up-regulated the expression of genes controlling intracellular calcium and chloride flux such as *stc2*, *clic4*, and *clcn6* (Table 1).

To validate the microarray experiments, we confirmed by real time RT-PCR that raloxifene inhibited the expression of *mip-1α*, *c-myc*, *pac-1/dusp2*, and less significantly, *hgf*, whereas it induced *gadd 34*, *cyclin G2*, and *p21* gene expression in JJN-3 cells (Fig. 5A). Western blotting analyses also confirmed that raloxifene inhibited c-Myc and induced p21 expression in these cells (Fig. 5B). Likewise, in U266 cells, raloxifene up-regulated the expression of *gadd 34*, *cyclin G2*, and *p21* genes and down-regulated *c-myc* but not *mip-1α* gene expression, whereas the expression of *dusp2*/*pac-1* and *hgf* could not be detected by RT-PCR in these cells (Fig. 5A).

Because raloxifene inhibited constitutive NF-κB activity, and because several of the raloxifene-regulated genes are known NF-κB targets, JJN-3 cells were treated with the NF-κB inhibitor BAY 11-7085 (20 and 40 μM) for 2 h. In these conditions, real time PCR showed a decrease in *mip-1α*, *c-myc*, and *hgf* gene expression, thus indicating that the raloxifene-induced inhibition of these genes is most likely NF-κB-mediated, whereas the drug did not influence *pac1*/*dusp2* expression (Fig. 5C), indicating that raloxifene inhibited its expression through a pathway that is most probably NF-κB-independent.

Discussion

The SERM raloxifene has been approved for the treatment of postmenopausal osteoporosis on the basis of its estrogen-like activity in the bones. However, this drug also displays antiestrogen activities and could therefore be effective for the treatment of ER-expressing breast cancers or multiple myelomas (Delmas et al., 2002; Martino et al., 2004). Indeed, multiple myeloma cells express ER, and previous studies demonstrated that tamoxifen or toremifene could induce multiple myeloma cell death and cell cycle arrest (Treon et al., 1998; Otsuki et al., 2000). We therefore studied the activity of raloxifene on ER-positive multiple myeloma cells, considering that the raloxifene antiresorptive effect on bones and its good clinical tolerance are additional arguments to justify such an investigation.

In our experimental conditions, we indeed observed that raloxifene blocked the cell cycle and induced apoptosis in several ER-positive multiple myeloma cell lines. Moreover, raloxifene also increased vincristine and arsenic trioxide cytotoxic response. To further characterize the mechanisms responsible for raloxifene in vitro activity, we first explored the NF-κB pathway. Indeed, the NF-κB transcription factor is constitutively active in refractory multiple myeloma, and several novel treatments, such as bortezomib, successfully target this factor (Hideshima et al., 2002; Richardson, 2003). Raloxifene could indeed inhibit NF-κB activity, as demonstrated by the study of NF-κB cellular localization, DNA binding, and transcriptional activities. Because ER has been demonstrated to interact with NF-κB (Kalaitzidis and Gilmore, 2005), we further showed that raloxifene treatment led to a transiently increased ER/NF-κB interaction, simultaneously with a loss of NF-κB binding to specifically regulated gene promoters. These data suggest that raloxifene could indeed block the NF-κB biological activity through a modulation of the ER association with p65, whereas raloxifene-induced inhibition of IKK activity was only partial.

We then performed a large scale study of gene expression regulation in multiple myeloma cells treated with raloxifene. This experiment led to the identification of only 104 genes whose expression is modified in response to this treatment. More significantly, only 29 genes showed reduced expression levels in response to raloxifene. However, many of the identified genes could provide us with a new insight into the mechanisms of raloxifene action, because 14 genes are known to regulate cell cycle or apoptosis, and several of them (*c-myc* or *mip-1α*, for instance) have been previously identified as important regulators of multiple myeloma cell survival and disease progression. *Mip-1α* expression by tumor cells in the bone marrow of multiple myeloma patients correlates with an adverse prognosis (Terpos et al., 2003), and this gene is also a key regulator of bone resorption by osteoclasts and could therefore be responsible for the typical multiple myeloma bone lesions (Roodman and Choi, 2004).

It is very tempting to correlate the inhibition of NF-κB with the list of genes obtained from the microarray experiment. Indeed, it is clear that some known NF-κB-regulated genes, such as *c-myc* and *mip-1α*, showed a decreased expression in response to raloxifene. We could also demonstrate that an NF-κB inhibitor had a similar effect on them and that raloxifene treatment was associated with a loss of NF-κB binding to the endogenous *mip-1α* promoter. However, as the number of down-regulated genes is quite low, it is likely that raloxifene inhibits only a subset of NF-κB target genes. Indeed, although raloxifene blocked DNA-binding activities by several distinct NF-κB complexes in the investigated cell lines, this inhibition is quite slow and incomplete. It is thus not surprising that, after 2 h of raloxifene treatment, only a part of the NF-κB-regulated genes is affected. However, gene expression studies performed at later time points would identify many genes that are induced or repressed secondarily in the context of apoptosis or cell cycle arrest.

Raloxifene activity on multiple myeloma cells is clearly not restricted to the inhibition of the NF-κB pathway. Indeed, the induction of cell cycle regulating genes (*p21*, *cyclin G2*) or ion channels as well as the inhibition of *dusp* phosphatase gene expression are likely to be NF-κB-independent. Other specific antimyeloma therapeutic agents also combine NF-

κB-dependent and independent activities. For instance, the proteasome inhibitor bortezomib is highly efficient for the treatment of refractory multiple myeloma (Richardson et al., 2005). This drug blocks IκBα degradation and NF-κB activation, but it also induces p53 and NOXA expression and increases the expression of cyclin-dependent kinase inhibitors including, as raloxifene, p21 (Mitsiades et al., 2002a; Hideshima et al., 2003; Qin et al., 2005).

In conclusion, raloxifene is a very promising drug for the treatment of multiple myeloma as it: 1) blocks cell cycle and induces apoptosis in multiple myeloma cells; 2) potentiates vincristine and arsenic trioxide cytotoxicity; 3) targets NF-κB, a key regulator of multiple myeloma cell survival, associated with the progression of refractory multiple myeloma; 4) decreases IL-6 production by osteoblasts (Cheung et al., 2003); 5) inhibits osteoclast function and could thus limit bone resorption and favor the reparation of lytic lesions; and 6) is a well known and well tolerated compound. Given the poor prognosis of patients with refractory multiple myeloma and the current knowledge on raloxifene pharmacology, we believe that our data provide strong arguments for in vivo tests on animal models and a rapid onset of raloxifene clinical trials in these pathologies.

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