Epigenetic Modulation of Retinoic Acid Receptor β2 by the Histone Deacetylase Inhibitor MS-275 in Human Renal Cell Carcinoma

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Abstract

Purpose: Histone deacetylase (HDAC) inhibitors have been shown to reverse epigenetic repression of certain genes, including retinoic acid receptor β2 (*RARβ2*). In this study, we examined whether *RARβ2* expression is repressed in human renal cell carcinoma (RCC) and whether the HDAC inhibitor MS-275 may revert its epigenetic repression.

Experimental Design: Six human tumor RCC cell lines were analyzed for *RARβ2* gene expression and for methylation and acetylation status at the promoter level. Modulation of *RARβ2* expression and correlation with antitumor activity by combination of MS-275 with 13-*cis*-retinoic acid (CRA) was assessed in a *RARβ2*-negative RCC cell line.

Results: *RARβ2* expression was either strongly present, weakly expressed, or absent in the RCC cell lines analyzed. Methylation-specific PCR indicated that the *RARβ2* promoter was partially methylated in three of the cell lines. CRA treatment did not inhibit clonogenic growth in the *RARβ2*-negative cell line RCC1.18, whereas MS-275 induced a dose-dependent inhibitory effect. A greater inhibitory effect was observed with combination treatment (MS-275 + CRA). Treatment with MS-275 was associated with histone acetylation at the promoter level and synergistic gene reexpression of *RARβ2* in combination with CRA. *RARβ2* reexpression was associated with synergistic induction of the retinoid-responsive gene *HOXA5*. *In vivo*, single-agent CRA treatment showed no significant effect, whereas MS-275 and the combination induced a regression of RCC1.18 tumor xenografts. Discontinuation of treatment produced tumor recurrence in MS-275-treated mice, whereas animals treated with the combination remained tumor free.

Conclusion: The HDAC inhibitor MS-275 seems to revert retinoid resistance due to epigenetic silencing of *RARβ2* in a human RCC model and has greater antitumor activity in combination with CRA compared with single agents. Thus, the combination of HDAC inhibitors and retinoids may represent a novel therapeutic approach in patients with RCC.

It is estimated that renal cell carcinoma (RCC) has been diagnosed in >35,000 patients and has caused the death of >12,000 people in the United States during 2004 (1). Metastatic RCC is characterized by a high level of resistance to systemic treatment, including immunotherapy and chemotherapy. Thus, novel therapeutic approaches are needed to control this disease.

Retinol (vitamin A) and its active metabolites and derivatives, such as retinoids [i.e., 13-*cis*-retinoic acid (CRA) and all-*trans*-retinoic acid], have been shown to have some chemopreventive and therapeutic activity in cancer (2, 3). However, retinoid resistance represents a major hurdle in cancer treatment, including for RCC patients (3, 4). RA exerts its effects mainly via members of the nuclear receptor superfamily, the retinoic acid receptors (RAR) and the retinoid X receptors, which form heterodimers (5–7). The human *RARβ* gene is expressed as three different isoforms: *β1*, *β2*, and *β4* (8). The biologically active *RARβ2* isoform is under the regulation of the P2 promoter containing a high-affinity retinoic acid response element (*RARE*), which is associated with the transcriptional activation of *RARβ2* by RA in a variety of cells (7).

Pharmacologic doses of RA induce cell differentiation and cell cycle arrest in some epithelial tumor cell lines but not in others (9). Retinoid resistance has been associated with loss or down-regulation of *RARβ2* expression in breast, prostate, colon, lung, and kidney cancers (3). Hoffman et al. have reported that *RARβ2* was not expressed by retinoid-resistant RCC cell lines but was present in a retinoid-sensitive RCC cell line and increased following incubation with RA (10). Accumulated evidence has shown *RARβ2* to be the principal mediator of the differentiation and antiproliferative effects of retinoids in epithelial tumor cells (11, 12). Exogenous *RARβ2* restores RA-induced inhibition in *RARβ2*-negative

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cells and *RARβ* antagonist or antisense mRNA block the effect of all-*trans*-retinoic acid. A possible cause of *RARβ2* gene expression modulation has been associated with the aberrant methylation of CpG islands in the promoter region and histone deacetylation of associated chromatin (13, 14). The methyl CpG binding protein MeCP2 binds to the methylated CpG islands and induces recruitment of chromatin-associated factors, including Sin3a and histone deacetylase (HDAC) corepressor complexes (15, 16).

Nucleosomes, the repeating units of the human genome, consist of DNA wrapping around a histone octamer formed by one H3-H4 tetramer and two H2A-H2B dimers. The acetylation status of histones alters chromatin structure, which in turn is involved in gene expression. Two enzyme classes can affect the acetylation of histones—histone acetyltransferases and HDACs (17). HDACs are involved in oncogenic transformation by mediating the transcriptional regulation of genes that are involved in cell cycle progression, proliferation, and apoptosis. Thus, HDACs represent a rational target for therapeutic interventions. Several HDAC inhibitors have been characterized that inhibit tumor growth *in vitro* and *in vivo* and are in clinical trials (18). A series of synthetic benzamide derivatives with HDAC-inhibitory activity have been originally generated by Mitsui Pharmaceuticals. One of these, MS-275, has shown induction of chromatin hyperacetylation and antitumor activity by inhibition of HDAC enzyme activity (19). MS-275 has also shown inhibition of tumor cell growth in nude mice that was comparable or superior to conventional cytotoxic agents, such as 5-fluorouracil (19). This orally active synthetic benzamide is currently in phase I clinical trial. Our group and others have reported that treatment with HDAC inhibitors, including MS-275, may reverse epigenetic repression of *RARβ2* in epithelial tumors, including prostate and breast (20–22).

In this study, the hypothesis tested was that retinoid resistance in RCC is associated with loss of *RARβ2* expression due to an epigenetic mechanism, and treatment with the HDAC inhibitor MS-275 may revert *RARβ2* silencing. Results show that MS-275 treatment reinduced *RARβ2* expression in *RARβ2*-negative human RCC cell lines and restores retinoid sensitivity.

Materials and Methods

Cell lines and reagents. Six human RCC cell lines, RCC1.1, RCC1.4, RCC1.11, RCC1.18, RCC1.24, and RCC1.26, were kindly provided by Dr. Elisabeth Jaffee (Johns Hopkins University, Baltimore, MD). These cell lines were established from primary renal cell tumors. Repeated morphologic examination and immunohistochemistry assessment (CD10 and RCC positivity) by a cytopathologist was consistent with RCCs. The cells were cultured in RCC medium [containing RPMI 1640 (Life Technologies, Gaithersburg, MD), 10% tryptose phosphate broth (Sigma, St. Louis, MO), 1% L-glutamine (Life Technologies), 1% nonessential amino acids (Life Technologies), 1% sodium pyruvate (Sigma), and 1% penicillin/streptomycin (Life Technologies)] with 20% fetal bovine serum and kept in an incubator at 37°C in an atmosphere containing 5% CO_2. For the *in vitro* experiments, tumor cells were treated with different concentrations of CRA (Sigma) or MS-275 (kindly provided by Schering AG, Berlin, Germany) or vehicle (DMSO) in RCC medium with 10% fetal bovine serum. For *in vivo* experiments, CRA and MS-275 were suspended in propylene glycol (Sigma) or 0.5% methocel (Fluka, Buchs SG, Switzerland), respectively, and given by gavage.

RNA isolation and RT-PCR. Total RNA was extracted from tumor cells or tumor tissues by TRIzol (Life Technologies), and the first strand was synthesized with oligo(dT) as primer using 1 μg total RNA according to the manufacturer's instructions. Two sets of primers for detecting *RARβ2* were used to ensure reliable data. For the tumor cell line experiments, the product was 256 bp long and covered exons 3 and 4 (sense strand 5′-GACTGTATGGATGTTCGTTCAG-3′ and antisense strand 5′-ATTTGTCCTGGCAGACGAAGCA-3′). Samples were processed in a Perkin-Elmer (Norwalk, CT) 9600 GeneAmp thermocycling system under the following conditions: 2-minute denaturation step at 94°C followed by 35 amplification cycles (30 seconds at 94°C for denaturation, 30 seconds at 60°C for primer annealing, and 45 seconds at 72°C for primer extension) and final extension at 72°C for 10 minutes. For the tumor tissue experiments, the product was 1247 bp long and crossed from exons 3 to 9 (sense strand 5′-GTAGTAGGAAGTGAGCTGTTCA-3′ and antisense strand 5′-GCACTGATGCTACGGAGATCT-3′). The conditions for the long PCR product were 5-minute denaturation step at 94°C followed by 35 amplification cycles (45 seconds at 94°C for denaturation, 1 minute at 56°C for primer annealing, and 2 minutes at 72°C for primer extension) and final extension at 72°C for 10 minutes. Real-time PCR (RT-PCR) with primers encoding for β-actin (638 bp product, sense strand 5′-ATGATGATATCGCCGCGC-3′ and antisense strand 5′-CTCCTTAATGTCACGCACGATTTC-3′) was used as an internal RNA control.

Quantitative real-time RT-PCR analysis. Quantitative RT-PCR for specific genes was done to confirm the differences in genes identified by RT-PCR. Single-strand cDNA was synthesized from RCC total RNA (1 μg) by reverse transcription using oligo(dT) as the primer. According to the manufacturer's protocol, quantitative RT-PCR was done using an ABI PRISM 7700 sequence detector system (PE-Applied Biosystems, Foster City, CA) with a 2× SYBR Green PCR Master Mix (PE-Applied Biosystems), reverse-transcribed cDNA, and gene specific primers. To quantify the amount of target mRNA in the samples, a standard curve of *RARβ2* was prepared for each run using the plasmid containing the target gene as well as a standard curve for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as internal control. This enabled standardization of the initial mRNA content of cells relative to the amount of *GAPDH*. The sequences of the specific primers were as follows: *RARβ2* primers are the same as in RT-PCR (256 bp), and the sense sequence was 5′-TGAACGGGAAGCTCACTGG-3′ and the antisense sequence was 5′-TCCACCACCCTGTTGCTGTA-3′ for *GAPDH*. Other primers were used in this experiment, including *RARα* (23), *RARγ* (24), and *HOXA5* (25). The relative expression of target gene was determined by the difference of the threshold cycle (Ct) between target gene and *GAPDH* (relative expression = $2^{\Delta CT}$, $\Delta Ct = Ct_{GAPDH} - Ct_{target\ gene}$; ABI User Bulletins).

DNA extraction and methylation-specific PCR. Genomic DNA was isolated from cell lines and primary tissues following the instruction of the DNeasy Tissue kit (Qiagen, Valencia, CA). DNA (~1 μg) was modified by bisulfite treatment and subjected to methylation-specific PCR (MSP; ref. 26). The first MSP primers were designed from 80 to 284 bp (upstream 5′-TATGYGAGTTGTTTGAGGATTGGGA-3′ and downstream 5′-AATAATCATTTACCATTTTCCAAACTTA-3′). The next MSP primer sequences that specifically recognized methylated *RARβ2* sequence (105-254 bp) were 5′-TGTCGAGAACGCGAGCGATTC-3′ (upstream or sense) and 5′-CGACCAATCCAACCGAAACGA-3′ (downstream) and the unmethylated *RARβ2* sequence (100-261 bp) were 5′-TTGGGATGTTGAGAATGTGAGTGATTT-3′ (upstream) and 5′-CCTACTCAACCAATCCAACCAAAACAA-3′ (downstream or antisense).

Sodium bisulfite DNA sequencing. Modified DNA was amplified by PCR with primer 1 (5′-GTATAGAGGAATTTAAAGTGTGGGTTGGG-3′, upstream, nucleotides −415 to −386, Genbank accession no. X56849) and primer 2 (5′-CCTATAATTAATCCAAATAATCATTTACC-3′, downstream, sequence position from +269 to +298). The conditions were as follows: 5 minutes at 95°C and 6 minutes at 80°C followed by 37 cycles (20 seconds at 95°C, 45 seconds at 55°C, and 45 seconds at 72°C) and final extension for 5 minutes at 72°C. PCR products were

cloned into the TA vector pCR2.1-TOPO and transformed into bacteria according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Plasmid DNA from isolated clones containing modified *RARβ2* sequence was purified using Wizard Plus Minipres (Promega, Madison, WI) and subjected to automated DNA sequence analysis (ABI automated sequencing).

Chromatin immunoprecipitation assay. The histone acetylation status of the *RARβ2* promoter was examined using the chromatin immunoprecipitation assay. An antibody specific for acetylated histone H4 was used to immunoprecipitate formaldehyde-cross-linked, sonicated chromatin from cells treated with MS-275 or the combination. Semiquantitative PCR analysis of DNA bound to immunocomplexes was done to detect a 192-bp fragment of the *RARβ2* core promoter region (−165 to +27, sense strand primer 5′-CTCTGGCTGTCTGCTTTTGC-3′; antisense strand primer 5′-CAGCTCACTTCCTACTACTTC-3′), which included *βRAREs* and TATA sequences (27). Hs578t served as a positive control because its *RARβ2* promoter is unmethylated and related histone H3 and H4 are acetylated (20).

Western blotting. The protein was obtained according to the kit manual from cell or tissues treated by different drugs. After electrophoresis, the proteins were blotted onto a nitrocellulose membrane. The membrane were blocked with blocking solution containing 5% nonfat milk for overnight and then incubated with primary antibody (anti-acetylated H3 1:2,000). Incubation with the secondary antibodies was done at room temperature for 1 hour. Strict washing (6 of 10-minute washing with PBS + Tween 20) was done after antibody incubation.

Colony formation assay. Exponentially growing tumor cells were seeded (200 cells/well) in six-well plates (Costar, Corning, NY) or 500 cells in 100 × 20 dish (Corning, Corning, NY) and allowed to attach for 48 hours. RCC1.11 cells were treated with CRA (1-10 μmol/L) or MS-275 (0.5 μmol/L) or the combination in complete medium containing DMSO (<0.1%). Cells were rinsed after 72 hours and fresh medium was added. Cultures were observed for 7 to 10 days and then fixed and stained with crystal violet. Colonies containing >30 cells were scored as survivors. Each condition was counted in triplicate (10 fields per well) on an inverted microscope. Results are expressed as mean ± SE number of colonies.

***Tumor growth* in vivo.** Male 4- to 6-week-old severe combined immunodeficient or athymic nude mice (NCI) were kept in a temperature-controlled room on a 12:12-hour light/dark schedule with food and water *ad libitum*. Animals were injected s.c. in the flank region with 2×10^6 tumor cells (RCC1.18) resuspended in Hank's solution and mixed with Matrigel (1:1, Collaborative Biomedical Products, Bedford, MA) in a final volume of 0.2 mL. As the tumor volume reached a measurable size (50-100 mm^3), 20 animals for each tumor were randomly placed in four groups (five animals per group): control, CRA, MS-275, and combination. Animals in the control group were treated with a daily administration (5 days/wk) of vehicle (polyethylene glycol) by gavage. CRA (30 mg/kg/d) and MS-275 (20 mg/kg/mL) were given by gavage. Tumor volume was measured with a caliper twice weekly and calculated according to the following formula: A (length) × B (width) × C (height) × 0.5236 and reported as mean ± SE. The animals were treated for ~4 weeks and then sacrificed.

Statistical analysis. Differences between means of unpaired samples were evaluated by Student's t test using the Sigmaplot program. $P < 0.05$ was taken to indicate statistical significance.

Results

Epigenetic repression of RARβ2 in human renal cell carcinoma cell lines. To determine the frequency of retinoid receptor expression and associated retinoid resistance in human RCC, six different cell lines were used that had been recently isolated from patients with RCC. Semiquantitative RT-PCR was done to detect *RARβ2* gene expression. The results showed that two of six cell lines (RCC1.1 and RCC1.11) strongly expressed *RARβ2*. Two other cell lines (RCC1.24 and RCC1.26) faintly expressed *RARβ2*, whereas two others (RCC1.4 and RCC1.18) were negative for *RARβ2* (Fig. 1A).

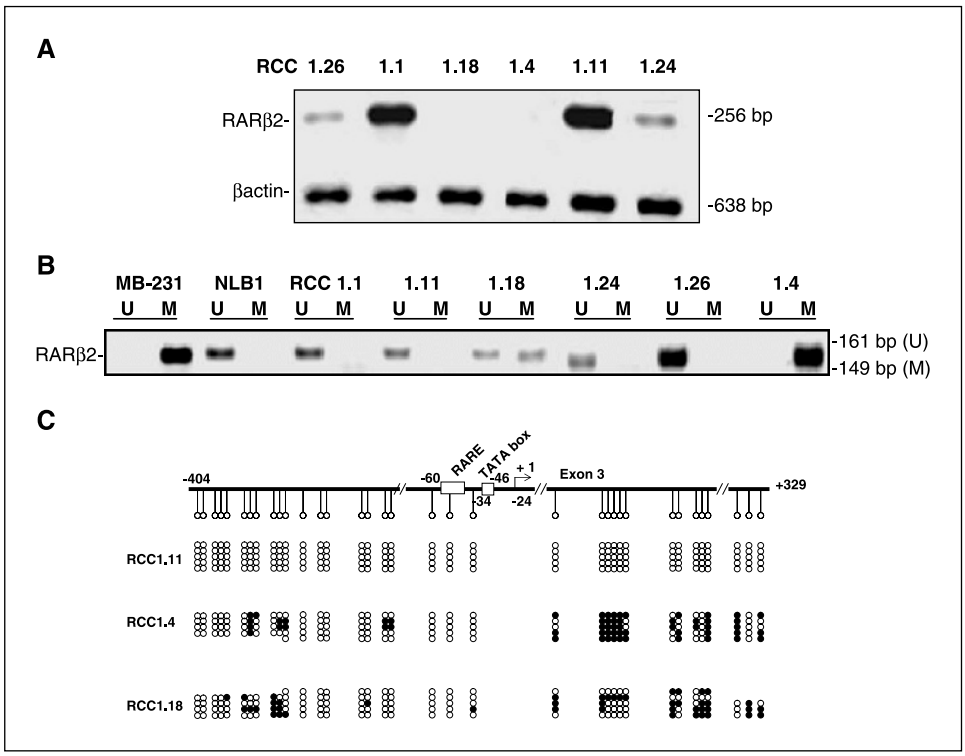

Fig. 1. *RARβ2* gene expression and RARβ2 promoter methylation status in human RCC cell lines. *A*, *RARβ2* expression in six cell lines of RCC was detected by qualitative RT-PCR. *B*, MSP analysis of *RARβ2* in six human RCC cell lines was done. DNA from MDA-MB-231 (*RARβ2* methylation; *M*) and NLB1 (*RARβ2* unmethylation; *U*) cell lines were used as controls. *C*, bisulfite-modified DNA extracted from RCC1.11, RCC1.18, and RCC1.4 cell lines was sequenced and assessed for CpG island methylation status. *Filled circles*, 5-methylcytosines (methylated CpG islands) in five clones for each cell line; empty circles, unmethylation of CpG islands.

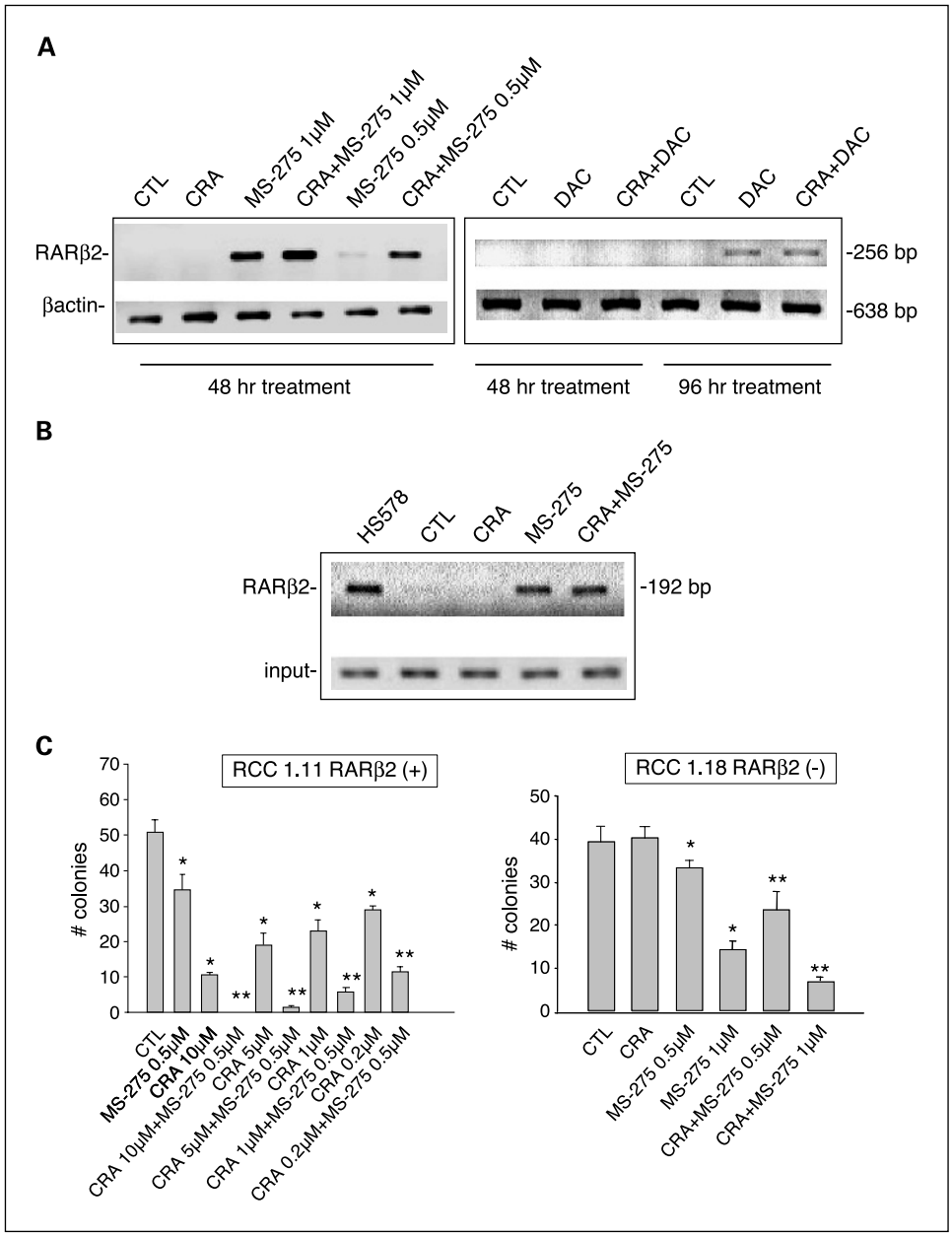

Fig. 2. MS-275 modulates RARβ2 expression and restores retinoid sensitivity in RARβ2-negative RCC1.18 cell lines *in vitro*. *A*, *RARβ2* and β-actin (internal control) expression was analyzed by RT-PCR as described in Materials and Methods. Total RNA samples were prepared from untreated RCC1.18 cells, and RCC1.18 cells were treated (48 and 96 hours) with 0.5 to 1.0 μmol/L MS-275, 1 μmol/L 5-aza-2′-deoxycytidine (*DAC*), and 10 μmol/L CRA. RT-PCR analysis showed a dose-dependent reinduction of *RARβ2* by MS-275 in the presence of CRA at 48 hours and by 5-aza-2′-deoxycytidine at 96 hours. *B*, *RARβ2* promoter acetylation (chromatin immunoprecipitation assay) of *RARβ2*-positive (Hs578t) and *RARβ2*-negative (RCC1.18) cells treated with CRA (10 μmol/L), MS-275 (0.5 μmol/L), or combination. *C*, effect of MS-275 and CRA on *RARβ2*-positive RCC1.11 and *RARβ2*-negative RCC1.18 cell colony formation. Single-agent MS-275 (0.5 μmol/L) induced a 32% inhibition of RCC1.11 colony formation compared with control, whereas a dose-dependent inhibition was observed with CRA. Combination of MS-275 + CRA induced a dose-dependent greater inhibitory effect up to complete inhibition. Single-agent MS-275, 0.5 and 1.0 μmol/L, induced 15.8% and 64.2% inhibition of RCC1.18 colony formation compared with control, whereas treatment with CRA (10 μmol/L) showed no effect. However, the combination of MS-275 + CRA induced an additive inhibitory effect (40.8% and 82.5% inhibition). Similar results were observed with 1 μmol/L CRA. *Columns*, mean colonies; *bars*, SE. *, $P < 0.01$ versus control; **, $P < 0.01$ versus single agents.

These results were confirmed by real-time RT-PCR (data not shown). To determine whether lack of *RARβ2* expression was due to aberrant methylation at the promoter level, the *RARβ2* promoter was analyzed by MSP. The results showed that RCC1.1, RCC1.11, and RCC1.26 cell lines did not present a methylated band, the RCC1.24 cell line exhibited an unmethylated band and weakly positive methylated band, the RCC1.18 cell line presented both unmethylated and methylated bands, whereas RCC1.4 cell line showed only a methylated band (Fig. 1B). To better characterize the *RARβ2* methylation status detected by MSP, bisulfite-modified DNA sequencing was done in the RCC1.11, RCC1.18, and RCC1.4 cell lines (Fig. 1C). The flank region of RARE and TATA box of the *RARβ2* promoter and the first exon in RCC1.4 and RCC1.18 cell lines were methylated (35% and 28% CpG island methylation, respectively). Only one clone of RCC1.18 was found to have one methylated CpG in the RARE area. There were no methylated CpG islands seen in the RCC1.11 cell line. These results were consistent with the MSP results.

***MS-275 induces RARβ2 reexpression and restores retinoid sensitivity in a RARβ2-negative cell line* in vitro.** Based on previous evidence of *RARβ2* induction by HDAC inhibitors in other tumor cell lines, it was hypothesized that the epigenetically repressed *RARβ2* could be restored by treatment with the HDAC inhibitor MS-275. Thus, the *RARβ2*-negative cell line RCC1.18 was treated with MS-275 (0.5 and 1.0 μmol/L) in the presence or absence of CRA (10 μmol/L) for 48 hours. RT-PCR analysis revealed a dose-dependent reinduction of *RARβ2* by MS-275 in the presence of retinoid (Fig. 2A). There was no induction of *RARβ2* in CRA-only-treated cells, and similar results were obtained with the *RARβ2*-negative cell line RCC1.4 (data not shown). To confirm that *RARβ2* was repressed by

methylation RCC1.18 cell line was treated with the demethylating agent 5-aza-2′-deoxycytidine (1 μmol/L). Ninety-six-hour but not 48-hour exposure with 5-aza-2′-deoxycytidine was able to reinduce *RARβ2* expression (Fig. 2A). The next test was to determine whether reexpression of *RARβ2* was due to the direct effect of MS-275 on histone acetylation; this was examined by using the chromatin immunoprecipitation assay. Analysis showed a high constitutive H4 deacetylation at the *RARβ2* promoter in RCC1.18, and treatment with CRA alone did not increase histone acetylation status (Fig. 2B). However, MS-275 induced a significant increase of histone acetylation associated with the *RARβ2* promoter after 24-hour treatment. Then, based on RT-PCR and MSP data, it was investigated whether treatment with MS-275 modulates retinoid response. To determine the antiproliferative effect of CRA and MS-275, these treatments were tested in a clonogenic assay. The *RARβ2*-positive cell line RCC1.11 and the *RARβ2*-negative cell line RCC1.18 were exposed to MS-275 (0.5-1.0 μmol/L) and CRA (10 μmol/L) for 72 hours (Fig. 2C). As expected, RCC1.11 growth was inhibited by CRA in a dose-dependent fashion (Fig. 2C). The lowest dose of CRA (0.2 μmol/L) inhibited 46% clones compared with control ($P < 0.001$). MS-275 treatment induced a 30% inhibition as single agent. However, combination of MS-275 with CRA had a greater inhibitory effect (up to >90% inhibition). In contrast, the *RARβ2*-negative cell line RCC1.18 was resistant to CRA treatment. MS-275 treatment induced 15.8% and 64.2% inhibition as single agent. However, MS-275 restored the sensitivity of RCC1.18 to CRA with a greater inhibitory effect on combination treatment.

MS-275 induction of RARβ2 reexpression is associated with increase of the retinoic acid–responsive gene HOXA5. To determine the specificity of the effect of combination treatment with MS-275 and CRA on RCC1.18 cell line, the gene expression of other *RARs* was assessed by real-time RT-PCR and compared with *RARβ2*. In contrast to *RARβ2*, *RARα* and *RARγ* were expressed in RCC1.18 cell line and were not significantly modulated by 24-hour treatment with MS-275 and/or CRA (Fig. 3A-C). The synergistic induction of *RARβ2* gene expression by MS-275 plus CRA was evident after 12-hour treatment. The gene expression of *HOXA5*, another gene containing *βRARE*, was also assessed in RCC1.18 cell line. As reported in Fig. 3D, *HOXA5* gene expression, which is present at baseline, was synergistically induced by the combination of CRA and MS-275 compared with single agents. The time course by quantitative PCR also showed that *RARβ2* gene expression preceded *HOXA5* gene expression.

***MS-275 restores retinoid sensitivity in a RARβ2-negative renal cell carcinoma cell line* in vivo.** To determine the effect

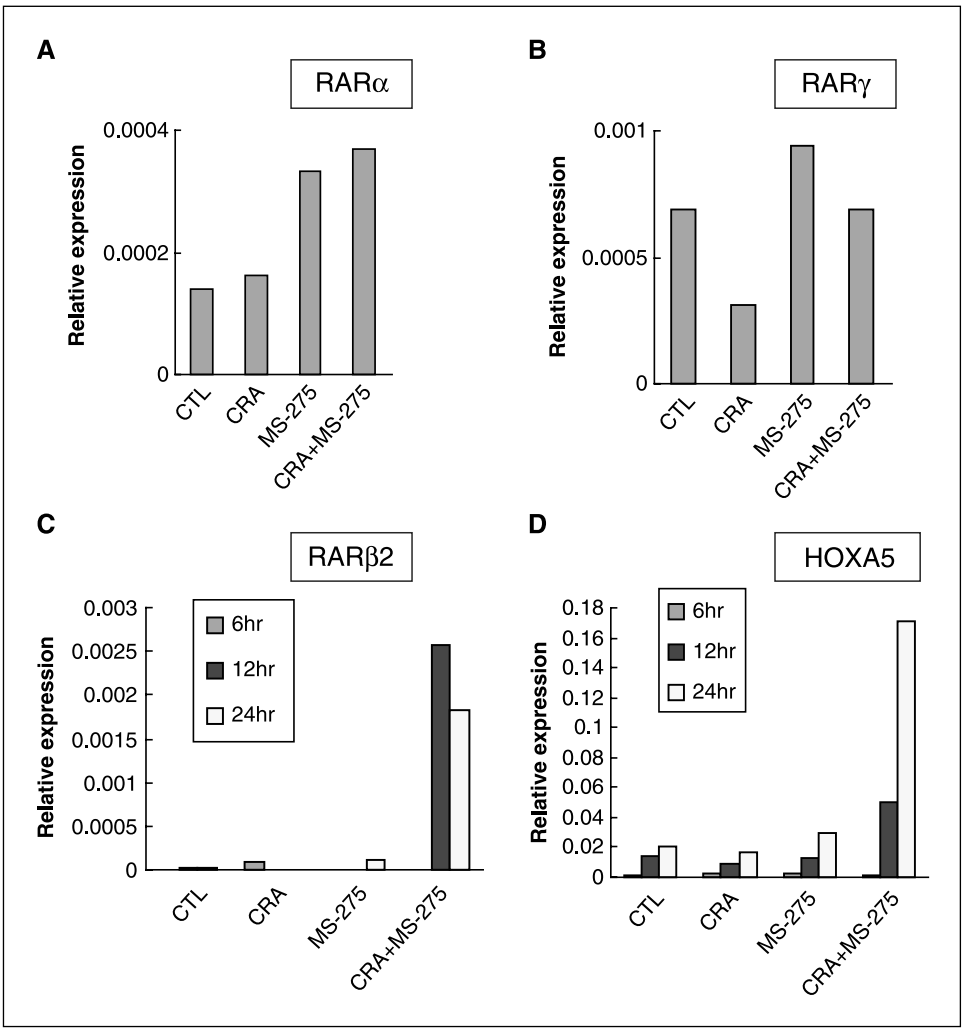

Fig. 3. MS-275 induction of RARβ2 reexpression is associated with increase of the RA-responsive gene *HOXA5* by real-time RT-PCR. *A* and *B*, *RARα* and *RARγ* basal gene expression and following 24-hour treatment with MS-275 (0.5 μmol/L), CRA (1 μmol/L), or combination was assessed in RCC1.18 cells. No significant modulation was observed. *C* and *D*, *RARβ2* and *HOXA5* gene expression following same treatment was assessed at different time points (6, 12, and 24 hours). A synergistic induction of gene expression was observed following combination treatment with CRA and MS-275. Similar results were obtained from two independent experiments.

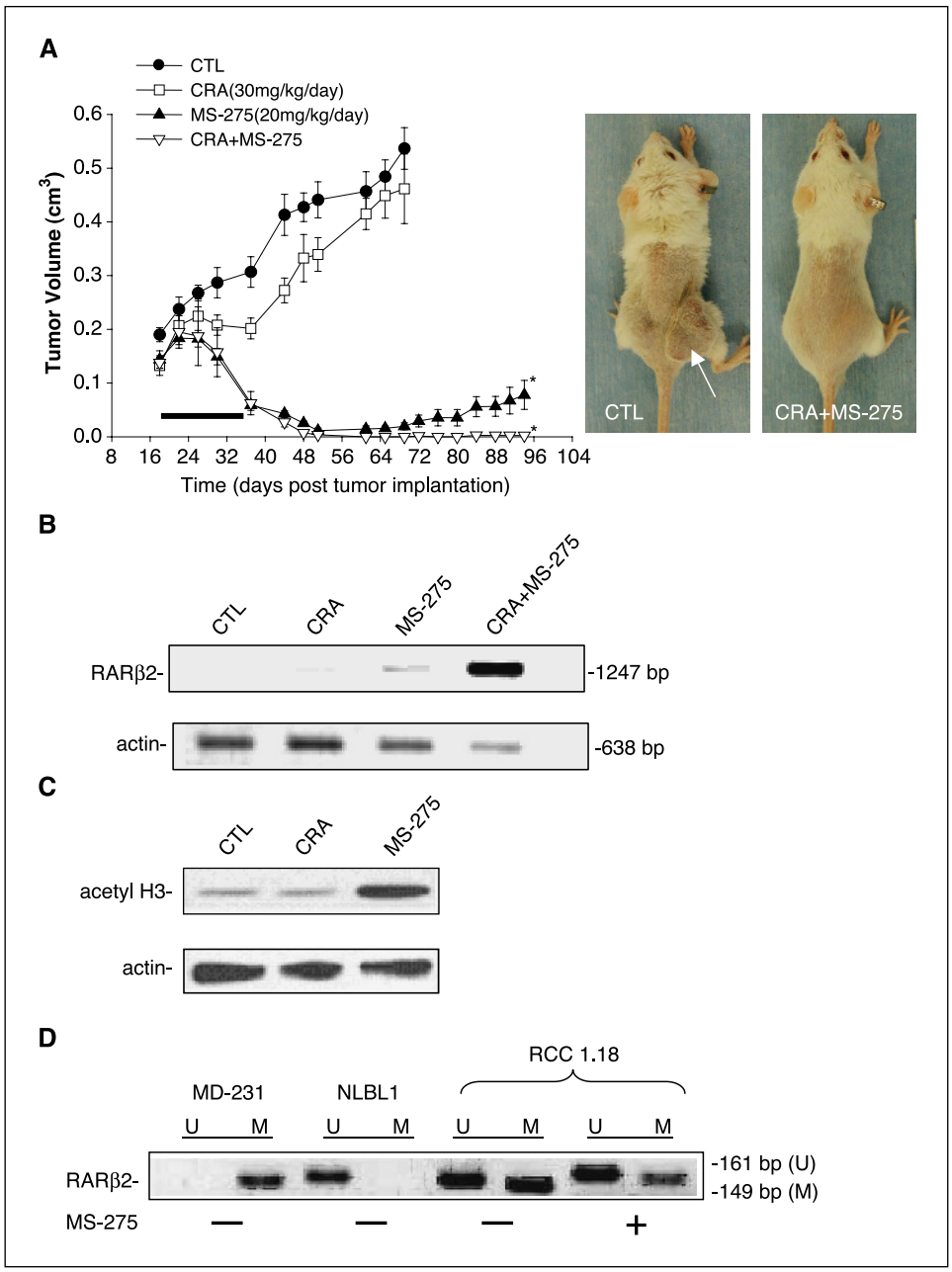

Fig. 4. *In vivo* greater inhibitory effect of MS-275 + CRA on RCC1.18 tumor growth is associated with induction of RARβ2 and persistent methylation status at RARβ2 promoter. *A*, severe combined immunodeficient mice bearing established RCC1.18 tumors were treated with CRA (30 mg/kg/d), MS-275 (20 mg/kg/d), or combination for 3 weeks. *Left,* tumor growth curve showed no significant effect of CRA treatment, whereas MS-275 and MS-275 + CRA induced tumor regression. Following discontinuation of drug treatment, the MS-275 group had tumor recurrence in five of five animals, whereas the MS-275 + CRA group remained tumor free. *Bar,* days of treatment. *, $P < 0.01$ versus control. *Right,* representative mice from control and MS-275 + CRA – treated group. Control mice developed large tumor (*arrow*). Notice the absence of tumor after 3 weeks of treatment with MS-275 + CRA. *B*, qualitative RT-PCR for *RARβ2* was done on RNA isolated from RCC1.18 tumors. Samples from control and CRA-treated nude mice showed persistent loss of *RARβ2* expression. However, samples from MS-275- or MS-275 + CRA – treated animals revealed reexpression of *RARβ2*. Representative PCR analysis from single tumors. *C*, Western blot analysis done on tumor samples revealed induction of acetylated H3 in animals treated with MS-275. *D*, MSP for *RARβ2* done on samples from tumor bearing animals revealed persistent methylation status at the promoter region in both untreated and MS-275 treated tumors.

of MS-275 and CRA on RCC growth *in vivo*, RCC1.18 cells were injected s.c. in severe combined immunodeficient mice. Once the tumors were established, animals received either control vehicle, CRA (30 mg/kg/d), MS-275 (20 mg/kg/d), or the combination of both. The tumor growth curves showed that MS-275 and the combination of MS-275 and CRA had a significant inhibitory effect and induced RCC1.18 tumor regression (Fig. 4A). Following 3 weeks of treatment that was without overt toxicity, most xenografts in the MS-275-treated group and the combination-treated group were not detectable. CRA had no significant effect on RCC1.18 growth. After the discontinuation of treatment, all five animals in the MS-275 group eventually developed tumor recurrence, whereas those animals in the combination group remained tumor free for >2 months. A separate experiment done in nude mice had a similar response but with residual visible tumors (no CRA-induced tumor growth inhibition and 66% and 86% growth inhibition in the MS-275 group and in the combination group, respectively). Thus, mRNA was extracted from tumor tissue and analyzed for *RARβ2* expression by RT-PCR (Fig. 4B). *RARβ2* was not detected in the control and CRA-treated groups. However, some *RARβ2* expression was present in the MS-275-treated group and was significantly induced in the combination-treated animals. The status of H3 acetylation in RCC1.18 tumor was determined by Western blot. MS-275 treatment significantly increased acetylated H3 compared with control and CRA group (Fig. 4C). DNA extraction from the tumor samples and MSP analysis revealed persistent methylation at the promoter region in tumors treated with MS-275 (Fig. 4D).

Discussion

In this study, it is reported that tumor cell lines established from patients with RCC have different levels of *RARβ2* gene expression. Loss of *RARβ2* in RCC cell lines was associated with retinoid resistance and methylated CpG islands in the *RARβ2* promoter and exon region and H4 hypoacetylation at the promoter level. The HDAC inhibitor MS-275 was shown to increase H4 acetylation of the chromatin associated with *RARβ2* promoter and to induce reexpression of *RARβ2* in *RARβ2*-negative RCC cell lines in the presence of CRA. In this preclinical model, MS-275 treatment alone had a significant *in vivo* activity, confirming the results in other tumor models (19). However, restoration of *RARβ2* expression in a RCC cell line was associated with a greater inhibitory effect of the combination of MS-275 with CRA on tumor growth both *in vitro* and *in vivo*. Our data suggest an association between *RARβ2* reexpression and antitumor activity but cannot rule out the possibility that other genes reactivated by CRA and MS-275 may contribute to the anticancer effects observed. Future studies with microarray analysis of MS-275- and CRA-treated RCC cells may provide useful information and implement our understanding of the biological mechanisms responsible for the antitumor activity observed with this drug combination.

Combination of a demethylating agent with a HDAC inhibitor is a rational "epigenetic" therapeutic strategy and is currently being tested in clinical trials. Aberrant DNA methylation is an important mechanism in gene regulation and epithelial tumorigenesis (28). Associated chromatin remodeling also plays a critical role in gene modulation and links between tumorigenesis and altered HDAC activity have been identified (18, 29). Hypermethylation has been reported in pediatric Wilms' tumors and adult RCC and has been associated with inactivation of several genes, such as *VHL*, *RASSF1A*, *P16*, *CASP8*, *MGMT*, *NORE1A*, and *P14* (30, 31). Aberrant methylation at the *RARβ2* promoter and consequent gene silencing has been reported in breast (13, 14), lung (32), prostate (33), esophagus (34), pancreas (35), colon (36), and stomach (37) tumors. There has been a recent report showing *RARβ2* promoter methylation in RCC (38). Our data suggest that the methylation of CpG islands in the promoter and first exon lead to chromatin deacetylation and block the access of transcription factors to the start site of the *RARβ2* gene in RCC. Our findings confirm that methylation is a critical step in tumor suppressor genes silencing and "lock in" function (39). However, it is speculated that chromatin remodeling and HDAC inhibition alone may overcome some degree of methylation-induced repression of certain ligand-inducible genes, such as *RARβ2*, by inducing sufficient acetylation to make the promoter susceptible to RA action without affecting the methylation status. Regardless of the class of HDAC inhibitor used, *RARβ2* reexpression is observed, although there is persistence of DNA methylation (13). Interestingly, our results show that the tumor growth inhibition in severe combined immunodeficient mice is transient with the HDAC inhibitor alone but not in combination with CRA. These data suggest that inhibition of HDACs must be followed by active hyperacetylation induced by CRA via the histone acetyltransferase machinery to achieve the optimal biological results.

In our selection of RCC cell lines, we did not encounter a cell line with fully methylated *RARβ2*. Thus, our data do not rule out the possibility that increases in *RARβ2* expression by the HDAC inhibitor may be attributable to enhancement of transcription from the unmethylated alleles. However, in a previous study, we showed that combination treatment with the HDAC inhibitor trichostatin A and all-*trans*-retinoic acid was able to restore *RARβ2* gene expression in MDA-MB-231 breast carcinoma cell line where both alleles are methylated (20). It remains to be elucidated how the degree of promoter methylation may affect the capability of the HDAC inhibitors of overcoming the epigenetic repression and reinducing *RARβ2* expression in tumor cells.

To determine the specificity of the effect of MS-275 and CRA on *RARβ2* expression, we used real-tme RT-PCR and assessed other *RARs* expression and their modulation by this combination. The result showed that, in contrast to *RARβ2*, *RARα* and *RARγ* are expressed in RCC1.18 cell line and are not significantly modulated by MS-275 and/or CRA (Fig. 3). Several potential RA target genes bear *RAREs*, including Hox genes (40). To determine whether a functional *RARβ2* protein was induced by the combination treatment, we tested the gene expression of one of the Hox genes, *HOXA5*. According to TRANFAC 4.0 transcription element search system, we found three *RARβ* binding sequences in the promoter region, ~2,000 bp upstream from *HOXA5* start site (TGACCT, AGGTCA, and GAGGTCAGGG). *HOXA5* gene expression, which is present at baseline in RCC1.18 cells, was synergistically induced by the combination of CRA and MS-275. The time course by quantitative PCR also showed that *RARβ2* gene expression preceded *HOXA5* gene expression, suggesting that a functional *RARβ2* protein is expressed.

A proposed model suggests that an inactive *RARβ2* promoter may undergo increased HDAC accumulation and associated chromatin acetylation during epithelial cell tumor development (20). The inactive promoter may become silenced due to additional epigenetic mechanisms, such as methylation by HDAC-induced MeCP2 recruitment. *RARβ2* promoter inactivity may result from different mechanisms, including low intracellular levels of retinol and its metabolites. Key enzymes in retinoid metabolism and transport, such as lecitin/retinol acyl transferase and cellular retinol or RA-binding proteins, have been also reported to be reduced in epithelial tumors, including RCC (41, 42). Epigenetic mechanisms may be involved in the reduced expression of these enzymes (43). The methylation status of these enzymes in our RCC cell lines and whether HDAC inhibitor treatment may modulate their expression is currently being investigated.

Clinical trials involving CRA as single agents have shown no significant clinical activity in RCC patients. The combination of CRA with IFN-α has been reported to induce significant antitumor responses in preclinical models but to have only modest clinical activity. Interestingly, Berg et al. have shown that in a small group of renal cancer patients who underwent tumor biopsy before and after treatment, up-regulation of *RARβ* expression—and not baseline expression—correlated with response to CRA and IFN-α (44). These clinical data suggest that *RARβ2* induction, rather than constitutive expression, may predict which tumor will respond to retinoid-based therapy. Reexpression of epigenetically silenced

RARβ2 with consequent restoration of *RARβ2* signaling pathways by concomitant exposure to HDAC inhibitor and pharmacologic doses of RA may be a predictor of response in patients with epithelial tumors, particularly renal cell cancer.

In summary, this report shows that retinoid sensitivity can be restored in retinoid-resistant RCCs via targeted therapy with *RARβ2* agonists and chromatin remodeling drugs that produce epigenetic changes at *RARβ2*. A methylated *RARβ2* promoter and an inducible *RARβ2* may represent a rational predictor for tumor response in patients undergoing "differentiation" therapy with the combination of a HDAC inhibitor and a retinoid. Based also on these preclinical results, a Cancer Therapy Evaluation Program-National Cancer Institute–sponsored phase I clinical study of MS-275 in combination with CRA in metastatic progressive cancer is currently accruing patients at our institution.

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