Retinoic Acid Inhibits the Proliferative Response Induced by CD40 Activation and Interleukin-4 in Mantle Cell Lymphoma

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Abstract

Mantle cell lymphoma (MCL) is an aggressive B-cell non-Hodgkin's lymphoma with poor response to therapy and unfavorable prognosis. Here, we show that retinoic acid (RA) isomers significantly inhibit the proliferation of both primary MCL cultures (n = 7) and established cell lines (Granta 519 and SP-53) as shown by [^{3}H]thymidine uptake and carboxyfluorescein diacetate succinimidyl ester labeling coupled with cyclin D1 staining. RA induces cell accumulation in G_0-G_1 together with a marked up-regulation of $p27^{Kip1}$ by inhibiting ubiquitination and proteasome-dependent degradation of the protein. The $p21^{Cip1}$ inhibitor was also up-regulated by RA in Granta 519 cells, whereas the expression of cyclin D1 is unaffected. Most of RA-induced $p27^{Kip1}$ was bound to cyclin D1/cyclin-dependent kinase 4 complexes, probably contributing to the decreased cyclin-dependent kinase 4 kinase activity and pRb hypophosphorylation observed in RA-treated cells. Experiments with receptor-selective ligands indicate that RA receptor α cooperates with retinoid X receptors in mediating RA-dependent MCL cell growth inhibition. Notably, RA isomers, and particularly 9-*cis*-RA, also inhibited the growth-promoting effect induced in primary MCL cells by CD40 activation alone or in combination with interleukin-4. Immunohistochemical analysis showed that significant numbers of CD40L-expressing lymphoid cells are present in lymph node biopsies of MCL patients. These results therefore further strengthen the possibility that triggering of CD40 by infiltrating CD40L+ cells may continuously promote the growth of MCL cells *in vivo*. On these grounds, our findings that RA inhibits basal MCL proliferation as well as MCL growth-promoting effects exerted by microenvironmental factors make these compounds highly attractive in terms of potential clinical efficacy in this setting. (Cancer Res 2005; 65(2): 587-95)

Introduction

Mantle cell lymphoma (MCL) is a distinct B-cell non-Hodgkin's lymphoma, the normal counterpart of which is likely represented by the pre-germinal center, naive B cell of the mantle zone of lymph nodes (1). MCL is characterized by advanced stage at presentation, frequent extranodal localization, and aggressive clinical behavior, with poor response to conventional therapeutic regimens and a very unfavorable prognosis, even when the disease is treated with high-dose therapy and autologous bone marrow transplantation (1). More than 95% of MCLs show the t(11;14)(q13;q32) translocation that leads to overexpression of cyclin D1 and subsequent deregulation of the critical cyclin D/Rb protein pathway of the cell cycle (1, 2). Cyclin D1, however, is a weak oncogene and its overexpression does not induce a fully transformed phenotype and is not related to the proliferative activity of MCL (1, 3, 4). These findings suggest that other factors are required to further deregulate MCL growth and induce malignant progression of the disease. Besides additional genetic alterations, recent evidence indicates that microenvironmental stimuli may cooperate with inherent cyclin D1 deregulation leading to enhanced cell cycle progression of MCL cells. In particular, CD40/CD40L pathway is a major determinant in promoting migration of naive mantle B cell to germinal center and rescuing them from activation-induced apoptosis (5); furthermore, CD40 activation is sufficient to promote lymphoma cell proliferation in primary MCL cultures, thus enforcing the suggestion of a pathogenic role of CD40 stimulation *in vivo* (6, 7). Moreover, other stimuli involved in the proliferative burst of mantle naive B cells [i.e., interleukin (IL)-4 (8) and IL-10 (7)] were shown to enhance the proliferative responses induced in MCL cells by CD40 triggering. On these grounds, therapeutic approaches able to interfere with CD40 activation or inhibit CD40-induced signaling in MCL may prove effective in the management of this still incurable lymphoma.

Retinoids are a group of natural and synthetic vitamin A analogues that exert profound effects on a wide array of physiologic processes, including embryonal morphogenesis, visual response, reproduction, growth, cell differentiation, and immune function. These pleiotropic effects are mediated by the binding to and activation of two different families of specific nuclear receptors, the retinoic acid (RA) receptors (RAR) and retinoid X receptors (RXR; refs. 9–11). The RARs bind both all-*trans* RA (ATRA) and its 9-*cis* stereoisomer (9-*cis*-RA), whereas the RXRs bind only 9-*cis*-RA (9–11). Like other members of the nuclear receptor superfamily, RARs and RXRs become transcriptionally active on ligand binding and transactivate their target genes by interacting with *cis*-acting DNA response elements (9–11). Retinoids were shown to inhibit the growth of transformed cells of different histotypes through direct modulation of the levels of cell cycle regulatory proteins.

Note: M. Guidoboni and P. Zancai equally contributed to this work.

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Notably, these compounds may interfere with cell cycle regulation by enhancing cyclins D1 and E proteolysis (12, 13). In fact, treatment of human embryonal carcinoma cells with ATRA induced G_1 arrest through the accelerated ubiquitination of cyclin D1 (14). Retinoids were also shown to modulate the levels of cyclin-dependent kinase (Cdk) inhibitors, particularly of $p21^{Cip1}$ (15–17) and $p27^{Kip1}$ (18–24). Retinoids were proven effective chemopreventive and therapeutic agents in several experimental systems as well as in a variety of human neoplasms (25, 26). In particular, ATRA induces complete remission in most patients with acute promyelocytic leukemia (27). Moreover, retinoids, alone or in combination with other drugs, were shown to have some activity also in hematologic malignancies different from acute promyelocytic leukemia, including juvenile chronic myeloid leukemia, myelodysplastic syndrome, and cutaneous T-cell lymphoma (25, 26). Nevertheless, the possible efficacy of retinoids in the control of B-cell lymphoproliferations has been scarcely explored.

We have recently shown that RA may induce marked antiproliferative responses in immortalized and fully neoplastic B lymphocytes (18, 19, 28). Nevertheless, only little information is currently available on the effects exerted by RA in MCL. On these grounds, we investigated whether RA is able to induce significant antiproliferative responses also in MCL cells, in the light of the possible use of RA in the treatment of this lymphoma. The study was particularly focused on the effects induced by RA in primary MCL cell cultures exposed to factors, such as soluble CD40L alone or in combination with IL-4, which presumably promote the growth of MCL *in vivo*. Moreover, the biochemical mechanisms underlying RA-induced MCL cell growth inhibition were analyzed in MCL-derived cell lines. Finally, considering that RA may induce apoptosis in aggressive lymphomas (29), the effects of RA on MCL cell survival were also investigated.

Materials and Methods

Patient Samples. Seven patients with MCL (six with leukemic phase disease) were identified based on morphologic, immunophenotypical, and molecular criteria according to WHO lymphoma classification. Six patients (all with leukemic phase disease) showed typical morphology, whereas the nodal case had blastic/blastoid morphology (Table 1). All patients had been off treatment for >30 days at the time of the sample procurement. The study was done in accordance with protocols approved by the local ethical committee, and all patients gave their informed consent to participate. In all cases in the leukemic phase, the percentage of circulating CD5+/CD19+ MCL cells was always >80%. Mononuclear cells were isolated by density gradient centrifugation from peripheral blood samples or from unicellular suspension obtained from mechanically minced lymph nodes. After washing, cells were resuspended and cryopreserved in 10% DMSO until further study. Before use, the cells were thawed, washed, and resuspended in RPMI 1640 (Life Technologies, Grand Island, NY) containing 10% FCS and antibiotics. Non-B cells (T cells, natural killer cells, monocytes, granulocytes, platelets, and erythroid precursor cells) were removed using magnetic immunobeads (B-cell Isolation kit, Miltenyi Biotech, Calderara di Reno, Italy). In all cases, >95% of purified cells were cyclin D1 positive as shown by fluorescence-activated cell sorting analysis. Moreover, no change in the percentage of cyclin D1–positive cells was observed after mitogen stimulation or RA treatment.

Table 1. MCL cases

Case	Sex/age (y)/stage	Type	Sample analyzed
MCL1	M/51/IV	Classic	Peripheral blood
MCL2	M/68/IV	Classic	Peripheral blood
MCL3	M/72/IV	Classic	Peripheral blood
MCL4	M/71/IIIS	Blastic/ blastoid	Lymph node biopsy
MCL5	M/66/IV	Classic	Peripheral blood
MCL6	M/57/IV	Classic	Peripheral blood
MCL7	F/62/IV	Classic	Peripheral blood

Cell Lines. The two MCL cell lines included in this study are Granta 519 (30) and SP-53 (31). Characterization of these cell lines has been reported recently (32). Granta 519 cells were cultured in DMEM supplemented with 10% heat-inactivated FCS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 20 mmol/L L-glutamine and maintained in a humidified 5% CO_2 incubator at 37°C. For SP-53, DMEM was substituted by RPMI 1640. In some experiments, the EBV-immortalized lymphoblastoid B-cell line HDE-14 was also used (18, 19).

Retinoids and Other Reagents. 13-*cis*-RA was purchased from Sigma (Milan, Italy). ATRA, 9-*cis*-RA, and Ro 40-6055 (RARα agonist) were kindly provided by Dr. W. Bollag (Hoffman-LaRoche, Basel, Switzerland). SR11237 (RXR agonist) was a kind gift of Dr. H. Gronemeyer (Institut de Génétique et de Biologie Moléculaire et Cellulaire, Strasbourg, France). The RXR agonist HX630 was kindly donated by Dr. H. Kagechika (University of Tokyo, Tokyo, Japan). The compounds were dissolved in DMSO at 10^{-1} mol/L and diluted in the culture medium so that the final concentration of the solvent was <0.01% (v/v). The stock solutions were stored at −20°C, protected from light and oxygen. As indicated, the cells were exposed to recombinant human soluble CD40L (rhsCD40L; Alexis, Rome, Italy) and IL-4 (R&D Systems, Minneapolis, MN). The proteasome inhibitors MG132 (Z-Leu-Leu-Leu-CHO, Biomol Research, Plymouth Meeting, CA) and LLnL (*N*-acetyl-Leu-Leu-norleucinal, Sigma) were prepared in DMSO and used at the final concentration of 40 and 150 μmol/L, respectively.

Proliferation Assays and Cell Cycle Analysis. Cell proliferation was evaluated by two different methods. For [^{3}H]thymidine uptake, cells were seeded in triplicate cultures in 96-well plates at an initial density of 10^6 cells per well in 200 μL of medium. At the time points indicated, cultures were pulsed with 1 μCi [^{3}H]methyl thymidine (specific activity 5 Ci/mmol/L; Amersham International, Bucks, United Kingdom) for 6 hours (Granta 519 and SP-53 cells) or 18 hours (primary MCL cultures) and subsequently harvested on Unifilter-96, GF/C filter plates (Packard, Meriden, CT). Radioactivity was measured with a liquid scintillation counter (Top Count NXT, Packard) and results were expressed as mean ± SD counts per minute of triplicate wells. For carboxyfluorescein diacetate succinimidyl ester (CFDA-SE; Molecular Probes, Inc., Eugene, OR) labeling (33), purified cells were stained with predetermined optimal concentration of the dye (0.5 μmol/L for 10 minutes at room temperature under continuous shaking), washed thrice with complete medium, and seeded at 10^6 cells/mL. After culture, cells were stained with either propidium iodide (PI; to monitor cell viability) or anti–cyclin D1 antibody (Upstate Biotechnology, Lake Placid, NY) after fixation with 4% paraformaldehyde and permeabilization with 0.5% saponin in PBS (for MCL cells gating). All flow cytometric analyses were done on a EPICS ALTRA flow cytometer (Beckman Coulter, Milan, Italy) and cell division was analyzed using the proliferation module of FlowJo 4.3 (Tree Star, Inc., San Carlos, CA) according to the reference manual. CFDA-SE is equally distributed after each cell division, resulting in a halved fluorescence intensity in daughter cells. All data were reported as "division index," defined as the average number of division that a cell present in the starting population has undergone. Results of CFDA-SE experiments are reported as mean ± SE and a paired, two-tailed Student's *t* test was used to compare assay results. In some experiments, proliferation was also evaluated by counting the number of viable cells (nine aliquots per time point) in a Bürker chamber in the presence of trypan blue. The influence of retinoids on cell cycle variables was investigated by flow cytometry after PI staining as described previously (19).

Extract Preparation, Immunoprecipitation, and Western Analysis. Whole cell lysates were prepared in lysis buffer [50 mmol/L Tris-HCl

(pH 7.5), 150 mmol/L NaCl, 2 mmol/L EDTA, 2 mmol/L EGTA, 2 mmol/L sodium orthovanadate, 25 mmol/L β-glycerophosphate, 25 mmol/L sodium fluoride, 1 mmol/L phenylmethylsulfonyl fluoride, 1 μmol/L okadaic acid, 5 μg/mL leupeptin, 5 μg/mL aprotinin, 0.2% Triton X-100, and 0.3% NP40] and lysed for 30 minutes on ice. Total protein extracts were obtained by centrifugation at 13,000 rpm for 30 minutes and protein concentration was determined by the Bio-Rad Protein Assay (Milan, Italy). Proteins were fractionated using SDS-PAGE and transferred onto nitrocellulose membranes. Immunoblotting was done using the enhanced chemiluminescence detection system (Amersham International). In immunoprecipitation studies, 500 to 1,000 μg of proteins were incubated with 1 μg of appropriate antibody and 50 μL of protein A-Sepharose CL4B (Amersham International) overnight, centrifuged, and washed thrice with lysis buffer. Proteins were eluted with Laemmli sample buffer and fractionated using SDS-PAGE, and Western blots were done. Antibodies were obtained from the following sources: $p27^{Kip1}$ and $p21^{Cip1}$ monoclonal antibodies from Transduction Laboratories (Lexington, KY); pRb monoclonal antibodies from Biosource International (Camarillo, CA); and $p27^{Kip1}$ (C-19), cyclin E (HE12), cyclin A (H-432), cyclin D1 (HD11), $p15^{Ink4b}$ (R-20), $p16^{Ink4a}$ (C-20), $p19^{Ink4d}$ (M-167), Cdk2 (M2), Cdk4 (H-22), ubiquitin (P4D1), and β-tubulin (H-235) from Santa Cruz Biotechnology (Santa Cruz, CA).

***In vitro* Degradation Assay.** Cells were gently lysed in ice-cold 10 mmol/L Tris-HCl (pH 8) by passing through a 22-gauge needle on ice. After centrifugation at 13,000 rpm at 4°C for 10 minutes, supernatants were collected, analyzed for protein concentration with Bradford's method, aliquoted, and stored at −80°C. Lysates (70 μg) were added to 50 μL of reaction buffer containing 50 mmol/L Tris-HCl (pH 8.3), 5 mmol/L $MgCl_2$, 2 mmol/L DTT, 5 μmol/L ubiquitin (Sigma), 3.5 mU/μL creatine kinase, 10 mmol/L creatine phosphate, and 2 mmol/L ATP. *In vitro* translated $p27^{Kip1}$ protein [50 ng; prepared from a $p27^{Kip1}$ cDNA cloned in pcDNA3 vector obtained from Dr. H. Zhang (Yale University, New Haven, CT) and translated using Promega (Promega Italia S.r.l., Milan, Italy) rabbit reticulocyte lysate TNT kit] was added to each sample. $p27^{Kip1}$ alone in 50 μL reaction buffer was used as positive control. As additional controls, lysate samples (70 μg) in 50 μL reaction buffer containing 50 ng $p27^{Kip1}$ were incubated with 40 μmol/L of the proteasome inhibitor MG132 or the vehicle alone (DMSO). After 18 hours of incubation at 37°C, aliquots of 3 μL were removed at desired time points, added to 30 μL of Laemmli buffer, loaded on 12% SDS-PAGE, and analyzed by immunoblotting with $p27^{Kip1}$ antibody.

***In vitro* Kinase Assays.** Immune complexes were washed twice with lysis buffer and once with kinase buffer containing 20 mmol/L Tris-HCl (pH 7.4), 10 mmol/L $MgCl_2$, and 1 mmol/L DTT. Washed complexes were resuspended in 50 μL of kinase reaction buffer containing 5 μCi [γ-^{32}P]ATP, 30 μmol/L ATP, 1 μg glutathione *S*-transferase-Rb (Santa Cruz Biotechnology), or 1 μg histone H1 (Roche Molecular Biochemicals, Indianapolis, IN). Reaction mixtures were incubated for 30 minutes at 37°C and applied to SDS gels. Phosphoproteins were visualized by autoradiography.

Apoptosis Detection. Apoptosis was evaluated by fluorescence analysis after PI staining, nick translation labeling of DNA strand breaks [terminal nucleotidyl transferase–mediated dUTP nick end labeling (TUNEL)], and Annexin V labeling as reported previously (18, 28). Fas-dependent apoptosis was analyzed using soluble recombinant FasL (5 ng/mL) in the presence of cross-linking enhancer according to manufacturer's instructions (Alexis). One lymphoblastoid B-cell line (LCL) sensitive to Fas-dependent apoptosis was also included in these experiments as a positive control.

Reverse Transcription-PCR Analysis. Total cellular RNA was isolated by the guanidinium isothiocyanate method described by Chomczynski and Sacchi (34). Total RNA (1 μg) and oligo(dT) primers were used for reverse transcription, applying the avian myeloblastosis virus reverse transcriptase system according to the manufacturer's instruction (Promega). Sequences of primer pairs (described as 5′ and 3′ end primers, respectively) were as follows: RARα 5′-ACCCCCTCTACCCCGCATCTACAAG-3′ and 5′-CATGCCCACTTCAAAGCACTTCTGC-3′, RARβ 5′-GAAGCAAGAATGCACAGAGAGC-3′ and 5′-GATGGTCAAGCCAGTGAAACC-3′, RARγ 5′-TTCGAGATGCTGAGCCCTAGCTTCC-3′ and 5′-CATGCCCACTTCGAAGCACTTCTGT-3′, RXRα 5′-CATGAGTTAGTCGCAGACATGGA-3′ and 5′-AGCTGATGACCGAGAAAGGC-3′, RXRβ 5′-ATGCCACCCCCGCCACTGGGC-3′ and 5′-GGCTCCAGGATCCTGTCCACAGGC-3′, RXRγ 5′-CCCCTGGTCACACTGGCTCGACG-3′ and 5′-CACCAGAGACCCAGGGCTGGTGG-3′, $p27^{Kip1}$ 5′-ATGTCAAACGTGCGAGTGTCTAACG-3′ and 5′-TTACGTTTGACGTCTGAGGCCA-3′, $p21^{Cip1}$ 5′-CAAGCTCTACCTTCCCACGG-3′ and 5′-GCCAGGGTATGTACATGAGG-3′, and β-actin 5′-GTGGGGCGCCCCAGGCACCA-3′ and 5′-CTCCTTAATGTCACGCACGATTTC-3′.

Amplification conditions were as follows: for RARα, 35 cycles with denaturation at 94°C for 30 seconds, annealing at 65°C for 1 second, and extension at 72°C for 1 minute; for RARβ, 35 cycles with denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 1 minute; for RARγ, 37 cycles with denaturation at 94°C for 30 seconds, annealing at 65°C for 30 seconds, and extension at 72°C for 1 minute; for RXRα, 35 cycles with denaturation at 95°C for 1 minute, annealing at 60°C for 1 minute, and extension at 72°C for 2 minutes; and for RXR β and γ, 40 cycles with denaturation at 94°C for 30 seconds, annealing at 60°C for 90 seconds, and extension at 72°C for 90 seconds with an increment of 5 seconds at each cycle. LCLs known to express the various RARs and RXRs were used as positive controls. Amplification conditions were as follows: for β-actin, 35 cycles with denaturation at 94°C for 1 minute and annealing at 65°C for 1 minute; for $p27^{Kip1}$ and $p21^{Cip1}$, cDNA denaturation at 95°C for 1 minute, primer annealing at 60°C for 1 minute, and DNA extension at 72°C for 1 minute. Semiquantitative data of PCR products were obtained using the β-actin cDNA as internal control and comparing the intensity of the amplified fragment of interest (obtained in linearity conditions) with that of the internal control by densitometric scanning (Bio-Rad GS-670).

Immunohistochemistry. Analysis of CD40L expression was carried out by immunohistochemistry on tissue sections obtained from pathologic lymph nodes from 12 MCL cases retrieved from the archive of from the files of the Division of Pathology, Centro di Riferimento Oncologico, IRCCS-National Cancer Institute (Aviano, Italy). Nonspecific reactive lymphadenopathies (15 cases) were also examined. Tissues were fixed in Bouin solution or neutral buffered formalin. In all cases, a portion of unfixed tissue was snap frozen in liquid nitrogen and stored at −80°C. Anti-CD40L monoclonal antibody (M90, IgG1, kindly provided by R.J. Armitage, Immunex Research and Development Corp., Seattle, WA) was applied to frozen sections from all cases included in the study. Immunohistochemistry was done by the APAAP technique. Negative control experiments were done by incubating sections with irrelevant isotype-matched mouse immunoglobulin and by omitting the primary antibody. In each case, the percentage of CD40L+ lymphocytes was derived from the number of CD40L+ lymphocytes counted on a total of 100 nonneoplastic lymphocytes evaluated.

Results

RA Inhibits the Spontaneous Proliferation of Primary MCL Cells. In a first set of experiments, the antiproliferative activity of 9-*cis*-RA, 13-*cis*-RA, and ATRA (1 μmol/L) was investigated by [^{3}H]thymidine uptake in four B-cell–enriched primary MCL cultures (MCL1-4). Although basal proliferation was usually low, a significant antiproliferative effect was induced in all four cultures by the three RA isomers, with 9-*cis*-RA showing the most pronounced effects (Fig. 1*A*). RA treatment had no major effect on cell viability as shown by trypan blue exclusion. To specifically evaluate the proliferative response of MCL cells, avoiding possible interference by residual normal B cells, the effects of RA were also investigated using the CFDA-SE assay combined with intracellular cyclin D1 staining. Consistently with the results of [^{3}H]thymidine uptake, 9-*cis*-RA (1 μmol/L) significantly decreased the division index of cyclin D1–positive cells of all the seven primary MCL cultures analyzed at both 3 and 5 days of treatment (mean ± SE,

1.07 ± 0.13 versus 0.84 ± 0.004; $P = 0.029$ and 1.39 ± 0.05 versus 1.01 ± 1.01; $P = 0.001$, respectively; Fig. 1*B*). Besides confirming that RA inhibits spontaneous MCL cell growth, these findings also indicated that the CFDA-SE labeling coupled with cyclin D1 staining is a reliable method for the specific evaluation of MCL cell proliferation in primary cultures.

9-*cis*-RA Inhibits the Growth-Promoting Activity Exerted by CD40 Activation and IL-4 Costimulation. Consistently with previous findings, exposure to rhsCD40L alone (1 μg/mL for 3 days) significantly increased the division index of primary MCL cells in all cases (mean ± SE, 1.52 ± 0.13 versus 1.07 ± 0.12; $P = 0.00014$). A further slight increase in the division index was observed in most cases when IL-4 (1,000 units/mL) was added to rhsCD40L (mean ± SE, 1.68 ± 0.08 versus 1.52 ± 0.13; $P = 0.06$), although the statistical significance was not reached. Proliferative responses of primary MCL cells under these conditions were also evaluated after 5 days of treatment, obtaining comparable results. To assess whether RA is able to antagonize the growth-promoting activity of CD40 stimulation and IL-4, the effects of 9-*cis*-RA (1 μmol/L for 3 and 5 days) were investigated in seven primary MCL cultures treated with rhsCD40L alone or in combination with IL-4. Globally, 9-*cis*-RA significantly inhibited the growth of MCL cells stimulated with either rhsCD40L alone (mean ± SE, 1.51 ± 0.13 versus 1.01 ± 0.08; $P = 0.004$) or rhsCD40L plus IL-4 (mean ± SE, 1.68 ± 0.08 versus 1.06 ± 0.08; $P = 0.0005$) as shown by CFDA-SE labeling (Fig. 1*C-E*).

Effects of RA on Primary MCL Cell Survival. Possible proapoptotic effects of RA were investigated in B-cell–enriched cultures from seven MCL cases by CFDA-SE/PI labeling without cell permeabilization. Identification of apoptotic cells by double gating on low-scatter/PI-positive events showed that, in all cases, 9-*cis*-RA (1 μmol/L for 3 and 5 days) did not significantly enhance basal MCL cell apoptosis (mean ± SE, 28.5 ± 6.2% versus 31.3 ± 5.7%; P = NS). These findings were confirmed by TUNEL assay (Fig. 2*A*) and Annexin V/PI staining (data not shown) in primary MCL cells from two cases with >95% leukemic blasts (MCL1 and MCL2).

Stimulation with rhsCD40L and IL-4 decreased basal apoptosis in primary cultures from all seven MCL cases, a phenomenon that was however unaffected by 9-*cis*-RA (1 μmol/L for 3 and 5 days) as assessed by CFDA-SE/PI vital staining (data not shown). TUNEL assay and Annexin V/PI staining carried out in MCL1 and MCL2 cases confirmed these results (Fig. 2*A*). Treatment with recombinant FasL (5 ng/mL) for 24 hours did not increase the rates of apoptosis of primary cultures from MCL1 (Fig. 2*A*), MCL2, and MCL3 cases (data not shown) either basally or after stimulation with rhsCD40L and IL-4, consistently with the known resistance of MCL cells to FasL-induced apoptosis (35). Furthermore, 9-*cis*-RA failed to sensitize MCL cells to FasL-induced apoptosis.

Effects of RA on the Proliferation and Survival of Established MCL Cell Lines. 13-*cis*-RA and 9-*cis*-RA inhibited the proliferation of both Granta 519 and SP-53 cells in a dose-dependent fashion, being active at concentrations as low as 0.01 μmol/L (Fig. 2*B*). Similar findings were induced by ATRA (data not shown). Proliferation of SP-53 cells but not of Granta 519 cells was enhanced by exogenous administration of rshCD40L, IL-4, or their combination (Fig. 2*C*), indicating that SP-53 cells are similar to primary MCL cultures with regard to the responsiveness to microenvironmental stimuli. Notably, 9-*cis*-RA significantly inhibited the growth-promoting activity induced in SP-53 cells by rshCD40L, IL-4, or

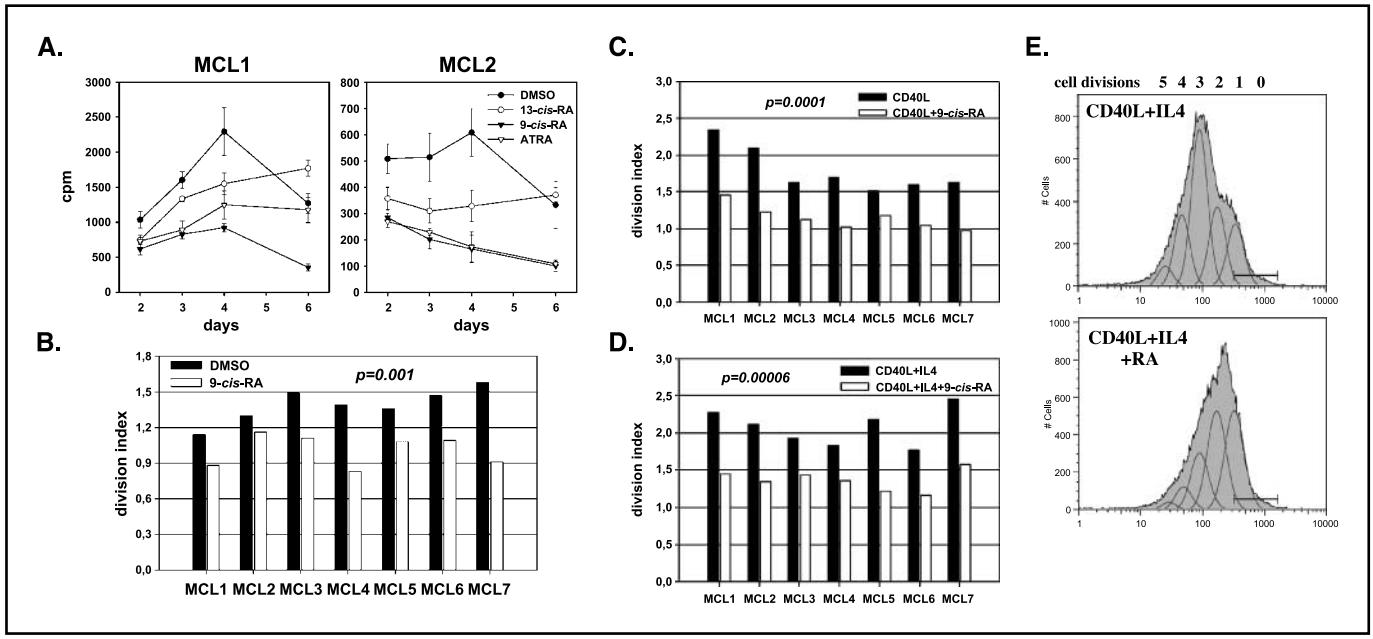

Figure 1. RA inhibits the spontaneous proliferation of primary MCL cells. *A*, antiproliferative activity of 9-*cis*-RA, 13-*cis*-RA, and ATRA in B-cell–enriched primary cultures of the MCL1 and MCL2 cases investigated by [^{3}H]thymidine uptake. Cell proliferation was evaluated after 2, 4, and 6 days of continuous treatment with 1 μmol/L of each RA isomer. *Points*, mean from triplicate wells; *bars*, SD. *B*, effects of 9-*cis*-RA on the division index of seven primary MCL cultures evaluated by CFDA-SE labeling coupled with cyclin D1 staining. Results are relative to a 5-day culture period. Globally, cultures treated with 1 μmol/L 9-*cis*-RA showed a significantly lower division index compared with controls (mean ± SE, 1.39 ± 0.05 versus 1.01 ± 1.01; $P = 0.001$). Effects of 1 μmol/L 9-*cis*-RA were also investigated using the same approach in seven primary MCL cultures stimulated by rhsCD40L alone (*C*) or in combination with IL-4 (*D*). Results are relative to a 3-day culture period. Globally, 9-*cis*-RA significantly decreased the division index of primary MCL cells stimulated with either rhsCD40L alone (*C*) or rhsCD40L + IL-4 (*D*). *E*, CFDA-SE labeling of cyclin D1–positive cells of the MCL2 case after 5 days of stimulation with rhsCD40L + IL-4 in the presence and absence of 1 μmol/L 9-*cis*-RA. CFDA-SE is equally distributed after each cell division, resulting in a halved fluorescence intensity in daughter cells. Exposure to 9-*cis*-RA markedly decreases the number of cell divisions (*numbers on top*).

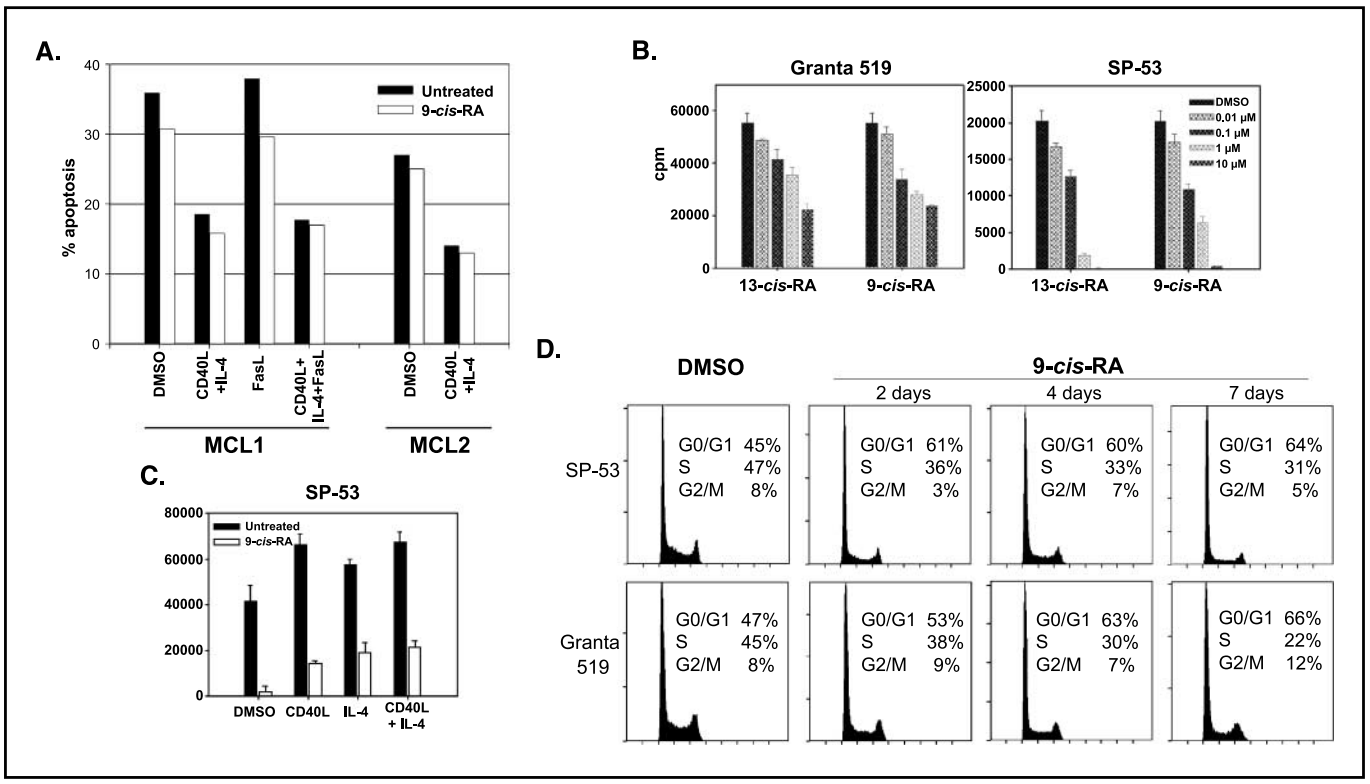

Figure 2. *A,* effects of RA on MCL cell survival. Treatment with 1 μmol/L 9-*cis*-RA did not enhance the apoptosis of primary MCL cells from the MCL1 and MCL2 cases cultured for 48 hours with the solvent alone (DMSO). In both cases, exposure to rhsCD40L + IL-4 decreased the spontaneous apoptosis that was however unaffected by 9-*cis*-RA. Treatment with recombinant FasL did not increase the apoptosis of primary cells of the MCL1 case, either basally or after stimulation with rhsCD40L + IL-4, and 9-*cis*-RA did not induce any relevant modification of these effects. Apoptosis was evaluated by TUNEL assay and flow cytometry as described in MATERIALS AND METHODS. Histograms represent percentage of TUNEL-positive cells. Effects of RA on the proliferation of Granta 519 and SP-53 MCL cell lines. *B,* 9-*cis*-RA and 13-*cis*-RA induce a dose-dependent inhibition of [^{3}H]thymidine uptake in Granta 519 and SP-53 cells. Cell proliferation was evaluated after 4 days of continuous treatment with RA concentrations ranging from 0.01 to 10 μmol/L. Representative of one of three experiments. *C,* rhsCD40L, IL-4, or their combination enhances basal proliferation of SP-53 cells evaluated by [^{3}H]thymidine incorporation after 3 days of culture. 9-*cis*-RA (1 μmol/L for 3 days) significantly inhibited the growth-promoting activity of rhsCD40L, IL-4, or their combination. Histograms represent mean ± SD of values obtained from triplicate wells. *D,* treatment with 9-*cis*-RA increased the number of Granta 519 and SP-53 cells in the G_0-G_1 phases of the cell cycle. Cells were treated with 9-*cis*-RA (1 μmol/L) for the indicated times and cell cycle variables were investigated by flow cytometry after PI staining. Representative of one of three experiments.

their combination (Fig. 2*C*). The resistance of Granta 519 to these factors may be related to the expression of EBV-encoded proteins, such as the latent membrane protein-1 (data not shown), which is known to constitutively activate part of the CD40-dependent signaling (36).

The effect of RA on cell cycle phase distribution was investigated in Granta 519 and SP-53 cells exposed to DMSO or (1 μmol/L) 9-*cis*-RA for 2, 4, and 7 days. In both cell lines, the analysis showed an increase in G_0-G_1 cells at the expense of those in S phase starting from the first 2 days of treatment (Fig. 2*D*). Nevertheless, the treatment did not induce a complete proliferative arrest as shown by the persistence of a small fraction of cells in the S phase and of a residual [^{3}H]thymidine uptake even after 2 weeks of continuous treatment (data not shown).

Similarly to what observed in primary MCL cells, RA did not enhance basal apoptosis of Granta 519 cells. The low level of basal apoptosis was not further decreased by stimulation with rhsCD40L and IL-4. Moreover, RA was unable to sensitize Granta 519 cells to Fas-induced apoptosis. These findings were also observed in SP-53 cells and using a different approach (Annexin V/PI labeling; data not shown).

Effects of RA on Cell Cycle Regulatory Proteins. In both Granta 519 and SP-53 cell lines, RA treatment (1 μmol/L 9-*cis*-RA for 72 hours) induced a decrease in the amount of hyperphosphorylated pRb protein, whereas the levels of cyclins D1, E, and A were not affected (Fig. 3*A*). Both MCL cell lines were negative for the expression of $p15^{Ink4b}$, $p16^{Ink4a}$, and $p19^{Ink4d}$ and none of these inhibitors was up-regulated by RA (data not shown). Conversely, a marked up-regulation of both $p27^{Kip1}$ and $p21^{Cip1}$ proteins was observed in Granta 519 exposed to RA, whereas only $p27^{Kip1}$ was up-regulated by RA in SP-53 cells (Fig. 3*A*). In Granta 519 cells, a significant increase in the level of $p27^{Kip1}$ and $p21^{Cip1}$ proteins was detectable since the first 24 hours of treatment (Fig. 3*B*), earlier than RA-induced cell cycle perturbations that were observed only after 48 hours of treatment (Fig. 2*D*). Semiquantitative reverse transcription-PCR showed no evidence of RA-induced transcriptional modulation of either $p27^{Kip1}$ or $p21^{Cip1}$ genes during the first 72 hours of treatment (data not shown). Exposure of Granta 519 cells to the proteasome inhibitor LLnL (150 μmol/L for 8 hours) markedly increased the expression levels of both $p27^{Kip1}$ or $p21^{Cip1}$ proteins (Fig. 3*C*), indicating that proteasome-mediated degradation is the main mechanism regulating the levels of these CDK inhibitors in Granta 519 cells.

RA Inhibits Ubiquitination and Proteasome-Dependent Degradation of $p27^{Kip1}$ in MCL Cells. After RA treatment, up-regulation of $p27^{Kip1}$ but not $p21^{Cip1}$ was also observed in one additional MCL cell line (JVM-2) and in primary MCL cells from one patient (MCL1; data not shown), indicating that

$p27^{Kip1}$ is probably the main mediator of the antiproliferative activity exerted by RA in this system. These findings prompted us to investigate in more detail the mechanisms by which RA enhances $p27^{Kip1}$ expression. To this end, we evaluated the ability of extracts from Granta 519 cells cultured with or without RA to degrade recombinant $p27^{Kip1}$ *in vitro*. As shown in Fig. 4*A*, RA markedly increased the stability of the $p27^{Kip1}$ protein. Notably, preincubation of extracts of growing cells with the proteasome inhibitor MG132 prevented $p27^{Kip1}$ removal (Fig. 4*A*), confirming that the degradation of the protein requires MG132-sensitive proteolytic systems. To assess whether RA also affects $p27^{Kip1}$ ubiquitination, $p27^{Kip1}$ was immunoprecipitated from both 9-*cis*-RA-treated and control (DMSO) Granta 519 cells, both cultured with LLnL (150 μmol/L) to increase the half-life of ubiquitinated intermediates, and analyzed with an anti-ubiquitin antibody by immunoblotting. The analysis showed that proteasome inhibition caused a considerable increase in slowly migrating $p27^{Kip1}$ derivatives, whereas 9-*cis*-RA-treated cells showed undetectable levels of ubiquitinated forms (Fig. 4*B*). Immunoblotting analysis of whole cell lysates showed no detectable difference in total protein ubiquitination between control and 9-*cis*-RA-treated cells, confirming that the more slowly migrating intermediates detected in the $p27^{Kip1}$ immunoprecipitates coincided with polyubiquitinated forms of $p27^{Kip1}$ (Fig. 4*B*). These findings indicate that RA inhibits ubiquitination and proteasome-dependent degradation of $p27^{Kip1}$ in MCL.

$p27^{Kip1}$ and $p21^{Cip1}$ Proteins Are Sequestered by Cyclin D1/Cdk4 Complexes in RA-Treated MCL Cells. Physical interactions between RA-induced $p27^{Kip1}$ and $p21^{Cip1}$ proteins and cyclin D1, Cdk4, and Cdk2 were investigated in Granta 519 cells. Treatment of these cells with 9-*cis*-RA (72 hours) induced a marked increase in the amounts of both $p27^{Kip1}$ and $p21^{Cip1}$ proteins associated with cyclin D1 as shown by immunoprecipitation experiments (Fig. 5*A*). $p27^{Kip1}$ and $p21^{Cip1}$ also coimmunoprecipitated with Cdk4, with an evident increase in complexes formation in the presence of RA. In contrast, $p27^{Kip1}$ was not found to associate with Cdk2, whereas only poor levels of $p21^{Cip1}$ were recovered after Cdk2 immunoprecipitation. These data indicate that the majority of RA-induced $p27^{Kip1}$ and $p21^{Cip1}$ proteins are sequestered by cyclin D1/Cdk4 complexes. To assess the functional consequences of this association, the effects of RA on cyclin D1/Cdk4 and Cdk2 kinase activity were investigated. As shown in Fig. 5*B*, RA strongly reduced cyclin D1– and Cdk4-associated kinase activity against recombinant pRb protein, whereas the Cdk2 kinase activity toward the histone H1 remained unaltered.

Both RARs and RXRs Mediate the Antiproliferative Effects Exerted by RA in MCL Cells. The antiproliferative effects of RAR- and RXR-selective retinoids were investigated in B-cell–enriched primary cultures obtained from two cases for which a sufficient number of cells was available: one MCL involving a lymph node (MCL4) and one MCL in the leukemic phase (MCL1). The RARα agonist Ro 40-6055 was markedly active in both cases at concentrations ranging from 0.01 to 1 μmol/L (Fig. 6*A* and *B*). The RXR pan-agonists SR11237 and HX630 significantly inhibited the growth of MCL4 cells, whereas only a slight antiproliferative effect was induced by SR11237 in MCL1 cells (Fig. 6*A* and *B*). The same analysis carried out in Granta 519 cells confirmed that both Ro 40-6055 and SR11237 were active, although the entity of the antiproliferative effect was less pronounced compared with that of ATRA and 9-*cis*-RA (Fig. 6*C*). Of note, the combination of SR11237 with Ro 40-6055 resulted in a stronger inhibition of growth compared with that induced by these compounds when added alone (Fig. 6*C*), indicating that RARα cooperates with RXRs to mediate the antiproliferative effect exerted by RA in Granta 519 cells. These findings are also consistent with the pattern of RAR and RXR isoforms expressed by these cells. In fact, reverse transcription-PCR analysis showed that, with the exception of RXRγ, all RARs and RXRs were basally expressed by Granta 519 cells. Moreover, a marked up-regulation of RARβ mRNA expression was also observed after treatment with 1 μmol/L ATRA or 9-*cis*-RA (data not shown).

Detection of CD40L+ Cells within MCL Microenvironment. The finding that RA markedly inhibits the proliferative response induced in MCL cells by CD40 stimulation prompted us to verify in more detail whether CD40 triggering may indeed occur *in vivo* in MCL patients. On these grounds, a retrospective series of 12 MCL biopsies and 15 nonspecific lymphadenopathies were characterized for the presence of CD40L+ cells. In reactive human lymph nodes, the expression of CD40L was restricted to small lymphocytes localized in the mantle zone and germinal center light zone of secondary follicles, thus paralleling the

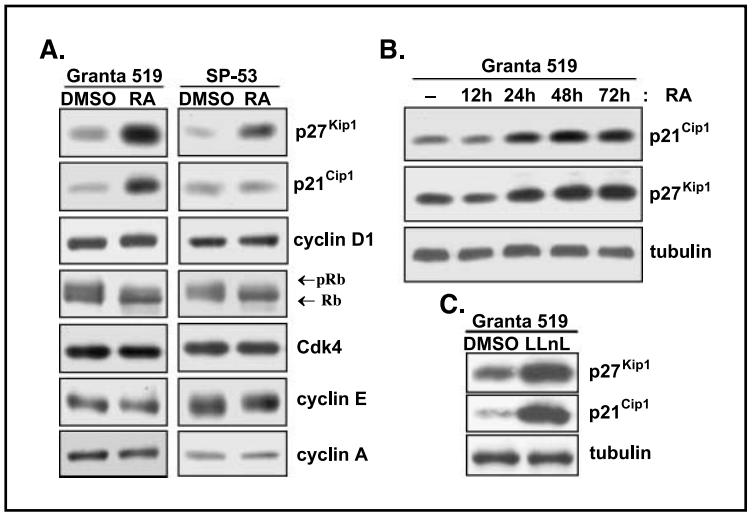

Figure 3. Effects of RA on cell cycle regulatory proteins. *A,* Granta 519 and SP-53 cells were treated with 1 μmol/L 9-*cis*-RA or the solvent alone for 72 hours. Total cell extracts (50 μg) were resolved by SDS-PAGE and subjected to immunoblot analyses for $p27^{Kip1}$, $p21^{Cip1}$, pRb, Cdk4, and cyclins D1, E, and A. In both cell lines, RA induces a decrease in the amount of hyperphosphorylated pRb protein. Moreover, RA markedly up-regulates $p27^{Kip1}$ and $p21^{Cip1}$ in Granta 519 cells, whereas only $p27^{Kip1}$ is up-regulated in RA-treated SP-53 cells. *B,* RA-induced $p27^{Kip1}$ and $p21^{Cip1}$ up-regulation is an early event, being detectable since the first 24 hours of treatment. Granta 519 cells were treated for 0, 12, 24, 48, and 72 hours with 1 μmol/L 9-*cis*-RA and analyzed by immunoblotting using the indicated antibodies. *C,* $p27^{Kip1}$ and $p21^{Cip1}$ protein expression is regulated by proteasome-dependent degradation in MCL cells. Granta 519 cells were treated with 150 μmol/L LLnL for 8 hours and cell extracts were analyzed for $p27^{Kip1}$ and $p21^{Cip1}$ proteins. Levels of β-tubulin were used as loading controls.

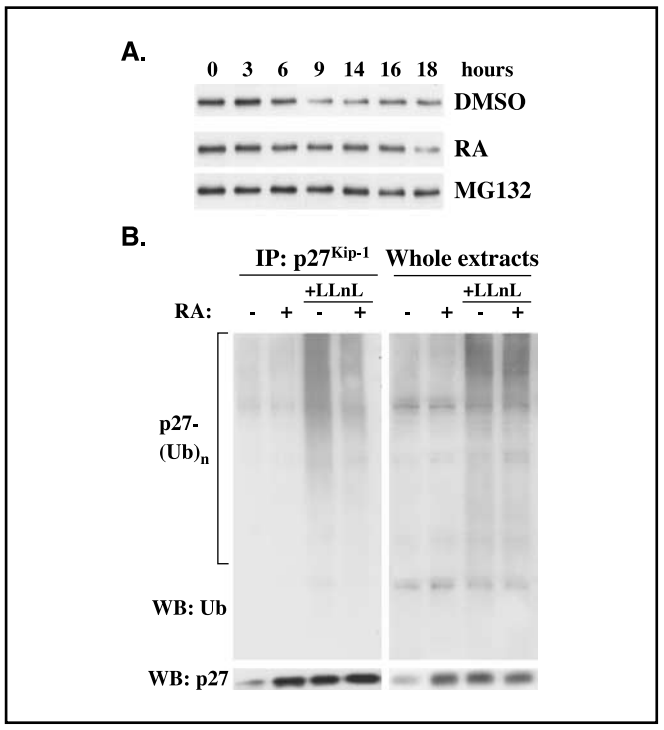

Figure 4. RA inhibits ubiquitination and proteasome-dependent degradation of $p27^{Kip1}$. *A, in vitro* degradation assay. Cell extracts were prepared from Granta 519 cells treated for 72 hours with 9-*cis*-RA or 0.01% DMSO as described in MATERIALS AND METHODS. *In vitro* translated $p27^{Kip1}$ protein was added to 70 μg of cell lysates in the presence of ATP regenerating system and incubated at 37°C and aliquots were taken at the indicated times. Sample added with the proteasome inhibitor MG132 was used as control. Levels of the $p27^{Kip1}$ protein were analyzed by immunoblotting with $p27^{Kip1}$ C-19 antibody. Experiments were repeated thrice; representative of one experiment. *B,* effects of RA on $p27^{Kip1}$ ubiquitination *in vivo*. Granta 519 cells were incubated with 9-*cis*-RA for 72 hours in the presence or absence of the proteasome inhibitor LLnL. Ubiquitinated forms of $p27^{Kip1}$ were analyzed by immunoprecipitation of $p27^{Kip1}$ from 500 μg of total cell lysates with the C-19 antibody and immunoblotting with anti-ubiquitin (*Ub*) antibody. RA-treated cells show impaired $p27^{Kip1}$ ubiquitination. Relative whole cell extracts were also examined by immunoblotting to verify the accumulation of ubiquitinated proteins in the presence of LLnL (*top*). Steady-state levels of $p27^{Kip1}$ protein in the samples were monitored by immunoblotting (*bottom*).

distribution of $CD4^+$ T lymphocytes in the follicles as well as the follicular dendritic reticulum cell network stained by the DRC-1 monoclonal antibody. In several of the follicles, positive cells accumulated along the boundaries between the germinal center and the mantle zone. CD40L-expressing cells were usually immunodetected within the interfollicular and paracortical areas. The same analysis carried out in MCL biopsies showed that lymphoma cells of all cases did not express immunodetectable CD40L. Conversely, in all MCL cases investigated, CD40L+ nonneoplastic lymphoid cells were detected in areas involved by lymphomas, although in low numbers (1-4%; data not shown). However, neoplastic MCL cells were often seen in close proximity to isolated or clustered CD40L+ lymphoid cells, suggesting the possible occurrence of functionally relevant interactions.

Discussion

In the present study, we provide evidence indicating that the RA isomers 9-*cis*-RA, 13-*cis*-RA, and ATRA induce significant antiproliferative responses in both primary cultures and established cell lines derived from MCL patients. This conclusion is based on the results of $[^3H]$thymidine uptake, particularly CFDA-SE labeling coupled with cyclin D1 intracellular staining. All three RA isomers were shown to inhibit the basal proliferation of primary MCL cultures as well as that of Granta 519 and SP-53 cells, although 9-*cis*-RA and ATRA generally induced slightly more pronounced effects. Considering that 9-*cis*-RA binds all RARs and RXRs and ATRA can be metabolized to 9-*cis*-RA, RAR-mediated activity cannot be distinguished from RXR-dependent effects in cells exposed to 9-*cis*-RA or ATRA (37). Therefore, receptor-selective ligands were used to assess which receptors were responsible for RA-mediated MCL cell growth inhibition. Treatment with a RARα-selective agonist induced marked antiproliferative responses in all cases, although to a lesser extent compared with 9-*cis*-RA or ATRA, consistently with an involvement of RARα. RXR-selective ligands also induced MCL cell growth inhibition, although the effect was more evident in primary cells from the MCL*4* case than in MCL1 and Granta 519 cells. Moreover, combined treatment of Granta 519 cells with selective agonists for RARα and RXR resulted in a growth inhibition comparable with that induced by 9-*cis*-RA or ATRA, suggesting that RARs (RARα in particular) cooperatively act with RXRs in mediating RA-dependent MCL cell growth inhibition. Consistently, reverse transcription-PCR analysis showed that the mRNA of all RARs as well as that of RXRα and RXRβ was basally expressed by Granta 519 cells. Moreover, the lack of RXRγ expression or induction after RA treatment indicates that RXRγ does not contribute to this effect. Intriguingly, the observation

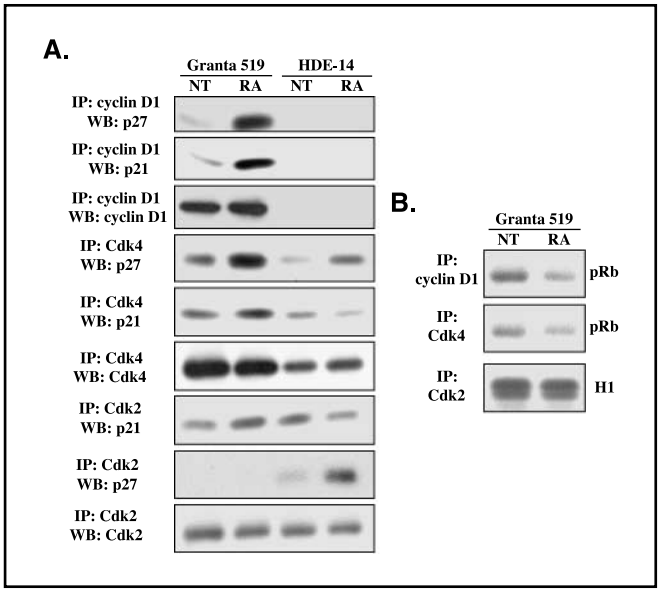

Figure 5. $p27^{Kip1}$ and $p21^{Cip1}$ proteins are sequestered by cyclin D1/Cdk4 complexes in RA-treated Granta 519 cells. *A,* Granta 519 and HDE-14 cell lines were treated with 1 μmol/L 9-*cis*-RA or DMSO for 72 hours and 500 μg of total cell extracts were immunoprecipitated with antibodies specific for cyclin D1, Cdk4, and Cdk2. Immunoprecipitated were subjected to immunoblot analyses with the indicated antibodies. The lymphoblastoid B-cell line HDE-14 was used as control for specific immunoprecipitation. *B,* RA inhibits cyclin D1/Cdk4 but not Cdk2 kinase activity in Granta 519 cells. Kinase activity toward glutathione *S*-transferase-Rb (for cyclin D1 and Cdk4) and histone H1 (for Cdk2) was measured in complexes immunoprecipitated from proliferating (DMSO) or RA-treated (1 μmol/L 9-*cis*-RA for 72 hours) Granta 519 cells.

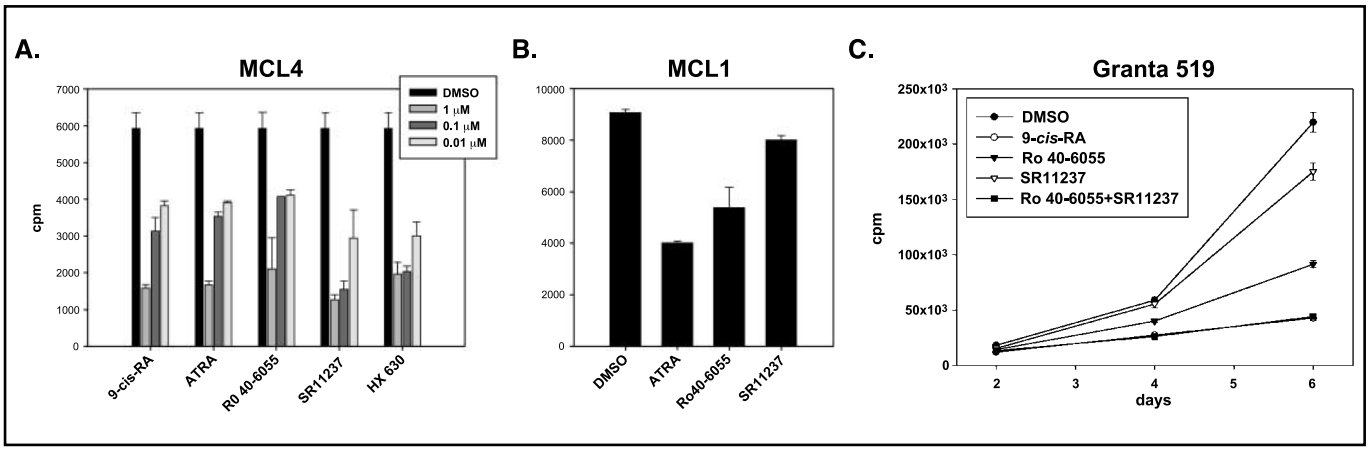

Figure 6. Both RARs and RXRs mediate the antiproliferative activity induced by RA in MCL cells. *A,* effect of the RARα-selective agonist Ro 40-6055 and the RXR pan-agonists SR11237 and HX630 on the [^{3}H]thymidine uptake of primary cultures derived from a lymph node biopsy of the MCL4 case. Data relative to the activity of 9-*cis*-RA and ATRA are also reported for comparison. Cell proliferation was evaluated after 4 days of continuous treatment with drug concentrations ranging from 0.01 to 1 μmol/L. *B,* antiproliferative activity of ATRA, Ro 40-6055, and SR11237 (1 μmol/L for 4 days) in primary MCL cultures derived from the peripheral blood of the MCL1 case, which was in the leukemic phase. *C,* the RARα-selective agonist Ro 40-6055 and the RXR pan-agonist SR11237 show additive effects in inhibiting the [^{3}H]thymidine uptake of Granta 519 cells. Antiproliferative activity induced by the combination of these selective agonists is similar to that exerted by the RAR and RXR pan-agonist 9-*cis*-RA. Cell proliferation was evaluated after 2, 4, and 6 days of continuous treatment with 1 μmol/L of each compound. *Points,* mean from triplicate wells; *bars,* SD. Representative of one of three experiments.

that two different RXR agonists (SR11237 and HX630) were as active as 9-*cis*-RA or ATRA in inhibiting the proliferation of primary cells the MCL4 case, a nodal blastoid variant, stimulates further studies aimed at verifying whether RXR-selective ligands may have effects of potential clinical significance in this aggressive MCL subtype. This appears of particular relevance considering that treatment with selective agonists may induce less pronounced toxic side effects compared with retinoids with pan-agonist activity.

Analysis of the effects of RA on basal MCL cell proliferation carried out in Granta 519 and SP-53 cells showed an increase in the number of cells in the G_0-G_1 phases at the expenses of those in the S phase. Unlike what observed in other cellular systems (12–14), these effects were not due to a down-regulation of cyclin D1. Our findings rather indicate that RA-induced MCL cell growth inhibition is the result of a marked up-regulation of $p27^{Kip1}$ and, in one cell line, also of $p21^{Cip1}$. In fact, virtually all RA-induced $p27^{Kip1}$ protein was bound to cyclin D1/Cdk4 complexes, similarly to what occurs to basal levels of $p27^{Kip1}$ in MCLs expressing this inhibitor (38). Nevertheless, whereas in untreated cells the levels of $p27^{Kip1}$ are insufficient to affect cyclin D1–dependent kinase activity, the marked up-regulation of the protein induced by RA results in a significant inhibition of cyclin D1/Cdk4 kinase activity and a decreased phosphorylation of endogenous pRb. In cells showing up-regulation $p21^{Cip1}$ on RA treatment, most of this inhibitor is associated with cyclin D1/Cdk4 complexes, whereas a limited amount of the protein also binds to Cdk2, thus cooperating with $p27^{Kip1}$ to inhibit pRb phosphorylation. The lack of evident Cdk2 inhibition by RA-induced $p27^{Kip1}$ and $p21^{Cip1}$ is probably one of the factors that allow MCL cells to retain a residual proliferative activity when treated with RA.

Similarly to what was observed in LCLs (19), RA-induced $p27^{Kip1}$ up-regulation in MCL cells does not involve transcriptional mechanisms but is the result of an enhanced stability of the protein. Notably, our results indicate that, in MCL cells, RA inhibits ubiquitination and proteasome-dependent degradation of the $p27^{Kip1}$ protein. This is of particular importance considering the enhanced proteasome-dependent degradation of this inhibitor that characterizes a significant proportion of typical MCL cases (39).

Even more relevant in terms of potential clinical application of RA in this setting is the demonstration that RA isomers also inhibit the growth-promoting effect induced by CD40 activation and IL-4 in primary MCL cultures as well as in SP-53 cells. These findings, together with the observation that RA increases the number of MCL cells in G_0-G_1, suggest that RA probably prevents the S-phase entry promoted by CD40 triggering and IL-4 stimulation (8). Previous *in vitro* studies have consistently provided evidence supporting the likely relevant role of factors present within MCL microenvironment in complementing the inherent cyclin D1 deregulation to enhance cell cycle progression and malignant progression of MCL cells (6–8). Besides confirming the strong growth-promoting activity exerted *in vitro* by CD40 activation and IL-4, the results of the present study also show that CD40L-expressing lymphoid cells are indeed present in lymph node biopsies of MCL patients. These results therefore add further support to the possibility that CD40 stimulation by infiltrating CD40L+ cells may trigger and/or sustain the proliferation of MCL cells *in vivo*. On these grounds, our finding that RA inhibits the growth-promoting effects of factors that are indeed present *in vivo* in MCL patients makes these compounds highly attractive in terms of potential clinical usefulness.

Finally, RA-mediated MCL cell growth inhibition was not associated with increased apoptosis as disclosed by CFDA-SE/PI or Annexin V/PI labeling and TUNEL assay. Moreover, considering that CD40-dependent signaling promotes B-cell survival, we also asked whether RA could interfere with the antiapoptotic effects of CD40 triggering in primary MCL cells. Although CD40 activation alone or in combination with IL-4 decreased basal apoptosis in all primary MCL cultures investigated, treatment with RA isomers was unable to antagonize this effect. We also showed that RA has no effect on the inherent resistance of MCL cells to Fas-dependent apoptosis (35).

In conclusion, the results of the present study show that RA isomers exert significant antiproliferative effects in MCL cells

without affecting their survival. In particular, RA was shown to markedly inhibit the growth-promoting activity of CD40 activation, a phenomenon that is probably of pathogenic relevance *in vivo*, as indicated by the demonstration of CD40L+ cells infiltrating MCL microenvironment. Considering that the most active RA isomer 9-*cis*-RA has been used in several clinical trials for different tumor histotypes (40–43), our findings provide the rational background to verify the possible effectiveness of schedules, including 9-*cis*-RA, in the treatment of MCL patients.

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