All-*trans* Retinoic Acid– and N-(4-hydroxyphenil)-Retinamide–Induced Growth Arrest and Apoptosis in Orbital Fibroblasts in Graves' Disease

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In this study, we evaluated by reverse transcription-polymerase chain reaction (RT-PCR) the expression pattern of retinoic acid receptors (RAR) α, β, and γ and cellular retinoic acid binding protein-I (CRBP-I) genes in 12 primary cultures of fibroblasts (F) from orbital tissue of Graves' ophthalmopathy (GO) patients. We also studied the in vitro effects of all-*trans* retinoic acid (RA) and N-(4-hydroxyphenil)-retinamide (4HPR), a less toxic and better tolerated synthetic derivative of RA, on cell morphology, growth, apoptosis, and cyclic adenosine monophosphate (cAMP) accumulation. All primary cultures expressed RAR α, β, γ, and CRBP-I. FGO treated with RA and 4HPR (10^{-7} mol/L) presented morphologic changes and significantly inhibited cell growth after 72 hours. At 96 hours of drug exposure, apoptosis was detected in 15% and 50% of RA- and 4HPR (10^{-7} mol/L)-treated cells, and p53 protein increased in cell lysates. 4HPR induced a 70% decrease of Bcl-2 protein. After 30 minutes of RA and 4HPR (10^{-7} mol/L) exposure, a 20% decrease of basal cAMP accumulation was seen, and forskolin cAMP-induced increase was abolished. The expression of RAR α, β, γ, and CRBP-I in primary cultures of FGO indicates that they are targets for retinoids. Moreover, we show that RA and 4HPR are able to induce morphologic changes, inhibition of cell growth, and apoptosis in FGO exerting their effects through RAR-modulated pathways. The rapid inhibition of cAMP accumulation indicates that a novel nonclassic retinoid pathway may also be involved. Finally, the potent in vitro effects of 4HPR, a retinoid derivative with fewer adverse reactions in vivo, could justify further investigations on a clinical application of retinoids in GO.

GRAVES' OPHTHALMOPATHY (GO) is an autoimmune disorder characterized by increased orbital adipose volume and swelling of extraocular muscles and activation of fibroblasts (F) with glycosaminoglycan (GAG) deposition.[1,2] Despite recent progresses, the complex GO pathogenesis is still under debate. It seems that F are key effector cells in GO, responsible for the connective tissue remodeling, and are a rich source of inflammatory mediators.[3] An emerging concept is that F are not homogeneous, but rather consist of subsets, capable of producing regulatory mediators that control regional inflammatory responses.[2,4] Medical treatment of GO remains an important problem. In severe active forms of the disease, high-dose glucocorticoids are used to control symptoms and signs, but orbital radiotherapy or orbital decompression represents the mainstay of treatment.[5] All-*trans* retinoic acid (RA), the biologically active form of vitamin A, regulates the growth and differentiation of a wide variety of cell types and plays a crucial role in the physiology of vision and as morphogenic agents during embryonic development.[6-8] RA and its synthetic analogs exhibit potent antiproliferative action, normalization of differentiation and anti-inflammatory activities, which appear to account for their therapeutic effects in hyperproliferative disorders, such as promyelocytic leukemia, and inflammatory diseases.[9-16] These therapeutic effects are achieved by the ability of RA to regulate complex programs of gene expression in target cells by binding to nuclear receptors, the RA receptor (RAR) α, β, and γ, which are ligand-dependent transcription factors. Homeostasis of RA is maintained by developmentally regulated vitamin A metabolism enzyme systems and by retinoid binding proteins. They are involved in extracellular and intracellular transport and metabolism of retinoids, including the retinol binding protein (RBP), cellular retinol binding protein type I (CRBP-I), and type II (CRBP-II) and 2 cellular RA binding proteins.[17,18] A synthetic retinoid with minimal toxicity and favorable pharmacokinetics during long-term administration to patients in clinical trials, such as 4-hydroxyphenyl-retinamide (4HPR) is active in the prevention and treatment of a variety of tumors in animal models[19] and in clinical trials.[20-23] Different studies have demonstrated that RA has inhibitory effects on collagen and hyaluronate accumulation in cultured human skin F.[24,25] Smith[25] demonstrated that confluent F cultures treated with RA accumulated less [^{3}H]GAG than those without the compound. Another study demonstrated that RA leads to an accumulation of hyaluronate in the superficial layers of epidermis by stimulating its synthesis in keratinocytes.[24] Because the phenotype of orbital F appears to be distinct from that of other types of F,[2] the effect of retinoids in this subset of F is unknown. In this report, we demonstrated by reverse transcription-polymerase chain reaction (RT-PCR) that primary cultures of orbital F derived from GO patients expressed RARs and CRBP-I gene transcripts. Moreover, we showed that RA and 4HPR are able to inhibit growth and induce apoptosis in a p53-dependent manner in these F.

MATERIALS AND METHODS

Origin of Tissues and Cell Cultures

Orbital connective tissue was obtained from 12 patients with GO during orbital decompression surgery. The diagnosis was based on endocrine and ophthalmologic criteria, including laboratory determi-

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nation of hormones and antibodies and imaging study (ultrasonography, computerized tomography, or magnetic resonance scan) of orbits. The patients (9 women, 3 men; aged 31 to 68 years), currently euthyroid, had never been treated with steroids or had been off therapy for over 6 months. Tissue explants were minced and placed directly on plastic culture dishes for incubation with the appropriate medium in a humidified incubator (37°C, 5% CO_2). F were allowed to proliferate in modified Eagle medium (MEM) supplemented with L-glutamine, 10% (vol/vol) fetal bovine serum (FBS), and antibiotics (GibcoBrl, Life-Technologies, Milan, Italy) as previously described.[26] F were used for experimental protocols at first-second culture passage, and at least 4 cell cultures from GO tissue specimens deriving from 4 different GO patients were used in experiments, which were repeated at least 3 times.

mRNA Isolation and RT-PCR

Total RNA was isolated from the cell cultures at first passage. Total RNA was recovered with TRIzol (Invitrogen, Milan, Italy). Residual DNA was removed by RNase free DNase I treatment (Promega, Florence, Italy). RT-PCR was performed as previously described.[26] RNAs were reversely transcribed using 5 μg total RNA after annealing with 0.2 nmol/L oligo (deoxythymidine) for priming of cDNA in the presence of reverse transcriptase (Superscript; Invitrogen, 200 U, Milan, Italy) at 37°C for 1.5 hours. The reaction was stopped by incubation at 95°C for 5 minutes. To obtain a negative control for the amplification reactions, we performed a RNA transcription without adding reverse transcriptase. Complementary DNA (400 ng cDNA) obtained by RT of RNAs was amplified in the total volume of 50 mL Tris HCl 10 mmol, 1.5 mmol $MgCl_2$, 50 mmol KCl pH 8.3, and 100 ng of 5′ to 3′ end primers. To evaluate variability in the expression of RARs and CRBP-I, a semiquantitative PCR was performed in which these genes were amplified with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as previously described.[27] Briefly, before performing semiquantitative PCR, the number of cycles was chosen in the middle of the exponential phase of the reaction separately for each gene type. To establish the number of cycles, GAPDH was amplified at 15, 22, 32, and 40 PCR cycles, RAR α, β, and γ were subjected to 25, 32, and 40 amplification cycles in separate experiments (data not shown). PCR conditions were as previously described, and the reaction consisted of 32 cycles of amplification for RAR α, β, and γ and 22 cycles for GAPDH. The specific primers for GAPDH were added to the PCR reaction after the first 10 cycles. The levels of mRNAs, quantified by densitometry scanning of the amplification products electrophoresed on agarose gels, are expressed as ratio between the density of each gene products and coamplified GAPDH. We used oligonucleotide sequences for RAR α, RAR β, RARγ, CRPB-I, and GAPDH as previously described.[27] PCR products were then separated on a 1.2% agarose gel containing ethidium bromide using 100-bp DNA ladder (Gibco BRL, Life Technologies, Milan, Italy) as size marker.

Morphologic Evaluation

The cells from primary monolayers were detached with trypsin-EDTA and seeded in 60-mm culture dishes to grow at 70% to 80% confluence. Cells were starved for 24 hours in MEM without fetal calf serum (FCS) and incubated in 1% FCS-supplemented medium in the presence of RA or 4HPR 10^{-7} and 10^{-8} mol/L or solvent (control cells) up to 96 hours. RA and 4HPR stock solutions were dissolved in ethanol, and the final ethanol concentration in the experimental medium per plate was 0.001%. The same amount of solvent was added to the control cells during the experimental protocol The solvent had no effect on cell morphology.Morphology was assessed using an inverted microscope (Nikon Duofot, Nikon, Japan).

Cell Proliferation Assay

Cell proliferation was evaluated with the tetrazolium salts (MTT) method (Roche, Milan, Italy). As previously described,[26,27] the cell proliferation kit is a colorimetric assay (MTT based) for the nonradioactive quantification of cell proliferation and viability. Only metabolically active cells cleave MTT to form a formazan dye detected by ultraviolet (UV) absorption (the absorbance spectrum is between 550 and 600 nm. Cells were seeded in microtiter plates in a final volume of 100 μL complete culture medium at a concentration of 2×10^3 cells/well and grown for 24 hours at 37°C, 5% CO_2. Cells, starved for 24 hours in MEM without FCS, were incubated in 1% FCS-supplemented medium in the presence of RA or 4HPR 10^{-8} mol/L, 10^{-7} mol/L, and 10^{-6} mol/L, or solvent (control cells) for up to 96 hours. The final ethanol concentration, used as solvent, in the experimental medium per plate was 0.001%. The same amount of solvent was added to the control cells during the experimental protocol. A total of 10 μL of the MTT solution was then added to each well, and plates were further incubated for 4 hours. Next, 10 μL of solubilization solution was added to each well, and plates were kept overnight in the incubator according to the manufacturer protocol. The absorbency was read at 550 nm using a microtiter plate reader.

Apoptosis Detection

The In Situ Cell Death Detection Kit (Roche) (TdT-mediated dUTP nick-end labeling [TUNEL]) was used to detect apoptosis and quantify DNA strand breaks in individual cells. The cell monolayers were grown directly on sterilized slides (Superfrost; Carlo Erba, Milan, Italy), starved for 24 hours in MEM without FCS, then incubated in 1% FCS-supplemented medium in the presence of RA or 4HPR 10^{-8}, 10^{-7} mol/L, or solvent (control cells) up to 96 hours. The slides were then fixed in buffered paraformaldehyde, permeabilized with triton-X and labeled with TUNEL reaction mixture according manufacturer instructions. Samples were analyzed using a Leitz Diaplan microscope (Eynst Leitz, Wetzlar, Germany) equipped with epifluorescence. A negative control (obtained by incubating a slide with labeled solution without terminal transferase) and a positive control (obtained by treating a slide with DNAase I solution) were included in each assay run.

Electrophoresis and Western Blot Analysis

P53 and Bcl-2 protein level was evaluated by Western blot analysis of protein extracts made from 3 different strains of F. For electrophoresis and Western blot analysis, the cells were harvested after a few minutes of incubation with phosphate-buffered saline (PBS) containing 0.2 mmol/L EDTA, centrifuged, and the pellets containing 10^{-6} cells were resuspended in 1:1 vol/vol of denaturing lysis buffer (2 times) containing 0.25 mol/L Tris-HCl pH 6.8, 5% sodium dodecyl sulfate (SDS), 8 mol/L urea 10 mmol/L EDTA, 0.1mol/L 1,4-dithiothreitol (DTT). The cell lysates were centrifuged for 10 minutes at maximum speed (7,000*g*) at room temperature to separate DNA, after which the supernatant was boiled for a few minutes before loading on gels. Protein concentrations were normalized and equal volumes of samples were loaded on the gels. Electrophoresis was performed on 12% polyacrylamide (1:40 mono-/bis acrylamide) containing SDS, according to standard SDS-polyacrylamide gel electrophoresis (PAGE) procedures. After separation on gel, proteins were electrophoretically transferred overnight to 0.45-μmol/L nitrocellulose sheets for Western blot analysis in transferring buffer containing 20% methanol, 10 g/L glycine, 4 g/L Tris, and 0.2 g/L SDS. Nitrocellulose reactive groups were then blocked with Western blot buffer (3 g/L Na_2HPO_4, 0.3 g/L NaH_2PO_4, 12 g/L NaCl, 0.05% Nonidet P-40, 0.05% Tween-20) containing 4% nonfat dried milk (Blocker; BIORAD, Rome, Italy) and 1% bovine serum albumin (BSA) (pH 8.0). After 1 hour of incubation with blocking solution at room temperature, the sheets were briefly washed

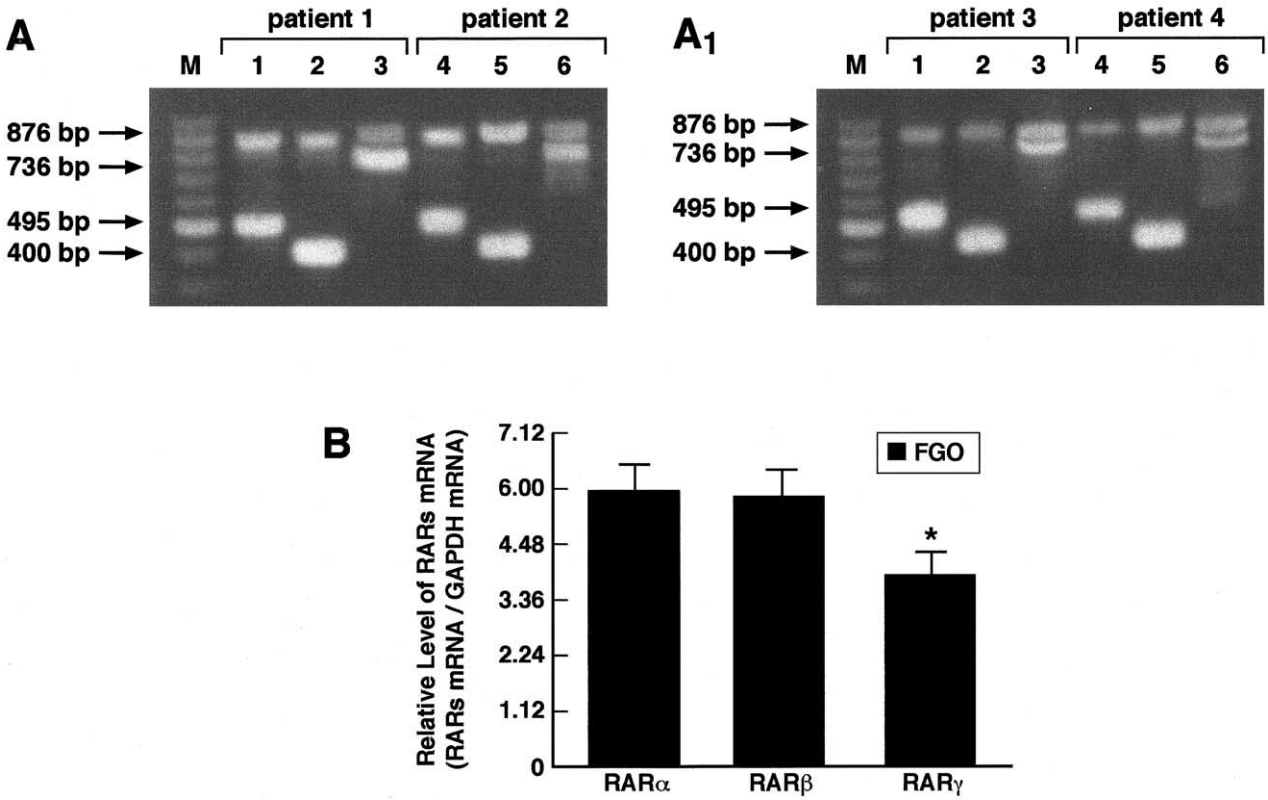

Fig 1. RAR α, β, and γ mRNA levels in 4 representative samples of retro-orbital F in primary culture from 4 patients affected by GO. (A) Patient 1 (lanes 1 through 3), patient 2 (lanes 4 through 6); (A_1) Patient 3 (lanes 1 through 3) , patient 4 (lanes 4 through6). M: 100-bp DNA size marker; lanes 1 and 4 (A, A_1): 495-bp products of RAR α; lanes 2 and 5 (A, A_1): 400-bp products of RAR β; lanes 3 and 6 (A, A_1): 736-bp products of RAR γ ; lanes 1 through 6 (A, A_1) 876-bp products of GAPDH. (B) Quantification of relative RAR α, β, and γ mRNAs in FGO. The RAR α, β, and γ bands were normalized to GAPDH mRNA control. Each column represents the mean ± SE of 3 separate experiments. *$P < .007$.

with Western blot buffer (pH 8.0), incubated overnight, and shaken at 4°C with primary antibodies diluted in Western blot buffer containing 1% nonfat dried milk and 0.25% BSA (pH 8.0). For p53 we used the p53 PAN 122 (Roche) at 2 μg/mL working concentration and for Bcl-2 immunoreaction the monoclonal antibody (Roche) at a working concentration of 1:800. At the end of incubation, blots were washed once for 15 minutes and 3 times for 5 minutes with Western blot buffer (pH 8.00). The antibody reaction was revealed by incubating it for 45 minutes at room temperature with horseradish peroxide-coupled antigoat or antimouse IgG serum (Amersham, Milan, Italy), 1:10,000 diluted in Western blot buffer (pH 8.0) containing 1% nonfat dried milk and 0.25% BSA, followed by a washing cycle (as above) and using chemiluminescent substrate (ECL, Amersham) according to the manufacturer's instructions. Visualization was obtained by autoradiography.

Cyclic Adenosine Monophosphate Accumulation

Confluent F monolayers in 6-well plates (Costar, Milan, Italy) were starved for 24 hours in medium without FCS and then shifted to medium supplemented with 1% FCS and phosphodiesterase inhibitor. The cells were treated with forskolin 10^{-5} mol/L, RA and 4HPR 10^{-7} and 10^{-8} mol/L or forskolin plus RA and 4HPR at the indicated doses. After 30 minutes, cultures were interrupted by adding cold 70% ethanol. After overnight incubation in ethanol, the supernatant was collected, centrifuged, and dried. The extracts were reconstituted with the appropriate buffer and processed for cyclic adenosine monophosphate (cAMP) assay using a RIA kit (Amersham).

Statistical Analysis

The data are reported as mean ± SE from at least 3 separate experiments performed in triplicate. The means were compared using analysis of variance.

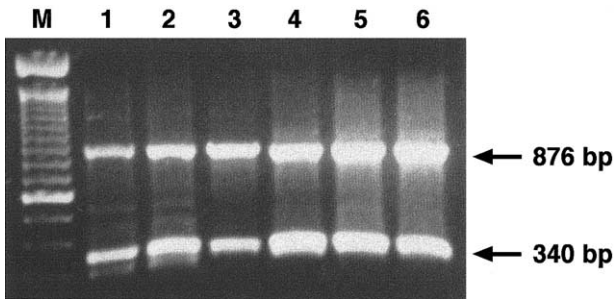

Fig 2. CRBP-I mRNA levels in retro-orbital F in primary culture from patients affected by GO. CRBP-I and GAPDH mRNAs in 6 FGO from 6 representative patients. M: 100-bp DNA size marker; lanes 1 through 6: 340-bp products of CRBP-I, 876-bp products of GAPDH.

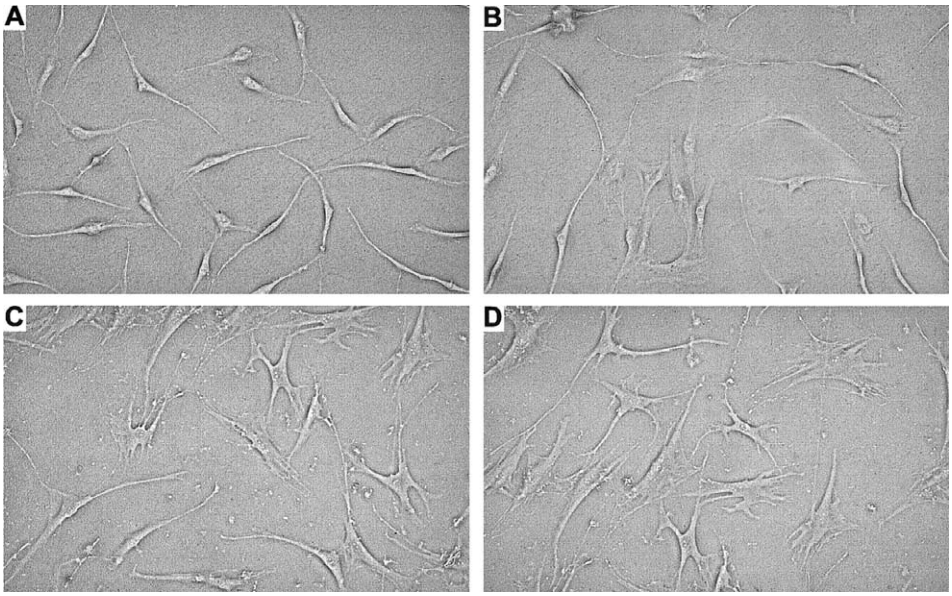

Fig 3. Change in the morphology of F from a patient affected by GO in primary culture after 48 hours of 10^{-7} mol/L RA or 4HPR treatment and after 72 hours of RA 10^{-7} mol/L. (A) Untreated FGO. (B) FGO treated with RA 10^{-7} mol/L for 48 hours. (C) FGO treated with 4HPR 10^{-7} mol/L for 48 hours. (D) FGO treated with RA 10^{-7} mol/L for 72 hours (phase contrast, 320 ×).

RESULTS

RAR α, β, γ, and CRBP-I Are Expressed in F From Orbital Tissues From Patients Affected by GO

All primary cultures of F obtained from orbital tissues (F) from patients affected by GO studied expressed RAR α, β, and γ (Fig 1) and CRBP-I (Fig 2). The level of the transcripts analyzed by semiquantitative RT-PCR of RAR α and β was clearly higher than RARγ mRNA ($P < .007$) (Fig 1B), while no significant differences were found in CRBP-I expression in different samples.

FGO Treated With RA and 4HPR Present Morphologic Changes, Growth Arrest, and Different Degrees of Apoptosis

FGO were incubated for up to 96 hours with RA and 4HPR at 10^{-8}, 10^{-7}, and 10^{-6} mol/L. Morphologic changes were observed after 48 hours of 4HPR 10^{-7} mol/L treatment and after 72 hours of RA 10^{-7} mol/L exposure in FGO (Fig 3). Cells became thinner and more oblong, with the presence of cytoplasm extensions projecting from their surface; the cytoplasm contained numerous vacuoles (Fig 3). The effect of 4HPR (10^{-7} mol/L) on cell morphology was achieved faster than RA (10^{-7} mol/L) treatment (48 hours *v* 72 hours) (Fig 3). The dramatic changes on the morphology observed after 72 hours of RA and 4HPR 10^{-7} mol/L treatment were reversed 48 hours after drug washout (data not shown), and the cells returned to a morphology similar to the control cells. FGO treated with RA or 4HPR 10^{-8}, 10^{-7}, and 10^{-6} mol/L for 48, 72, and 96 hours showed a significant decrease of cell growth after 72 hours when treated with RA and 4HPR 10^{-7}, and 10^{-6} mol/L ($P < .01$ and $P < .005$ *v* untreated controls) (Fig 4). The arrest of cell growth was present for up to 96 hour of drug exposure (Fig 4). RA and 4HPR at lower doses (10^{-8} mol/L) did not

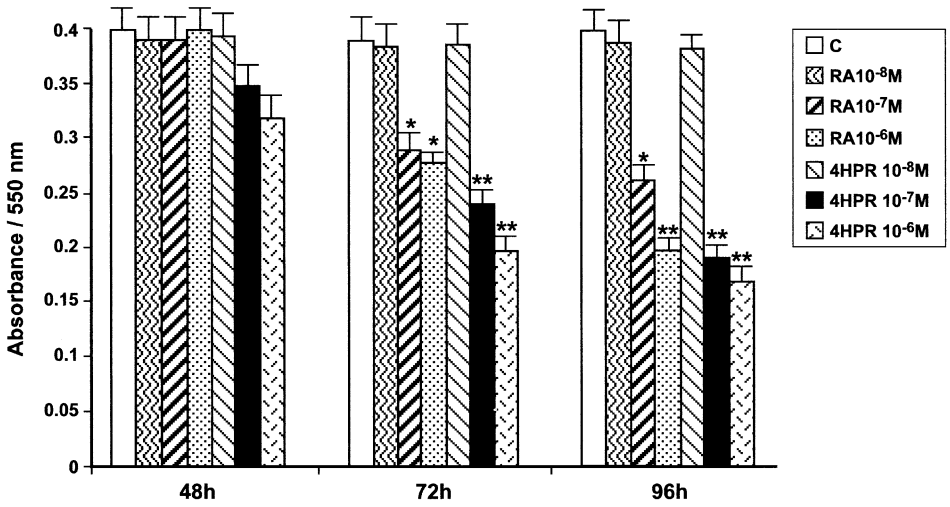

Fig 4. RA- and 4HPR-induced growth inhibition of F from GO patients in primary culture. The cells were seeded at 2,000 cells per well and treated with RA or 4HPR at 10^{-8}, 10^{-7}, and 10^{-6} mol/L for 48, 72, and 96 hours. The results were expressed as the A_{550} of MTT-derived formazan developed by untreated control (C) and treated cells. Each column represents the mean ± SE of 3 separate experiments, *$P < .01$; **$P < .005$.

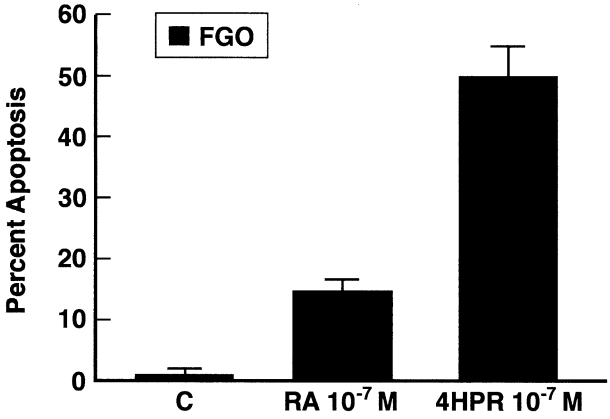

Fig 5. Effect of RA and 4HPR on apoptosis induction in F from GO patients in primary culture. FGO were treated with 10^{-7} mol/L RA and 4HPR for 96 hours. After treatment, apoptosis was detected by the TUNEL method. Values represent the mean of 3 independent experiments performed in duplicate (± SE).

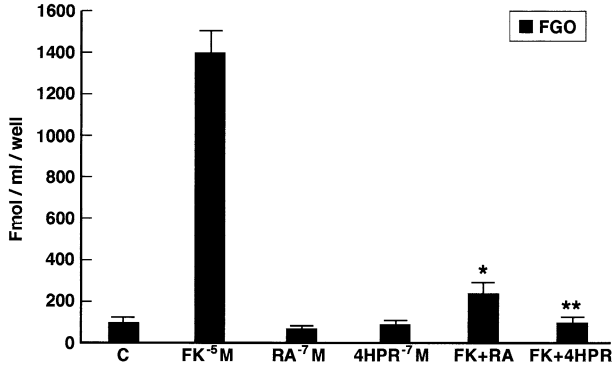

Fig 7. RA- and 4HPR-induced inhibition of forskolin (FK)-stimulated cAMP accumulation in F from patients affected by GO. The cells were treated with FK 10^{-5} mol/L with or without RA and 4HPR 10^{-7} mol/L for 30 minutes. Each column represents the mean ± SE of 3 separate experiments, *$P < .01$; **$P < .005$ *v* FK-stimulated cAMP accumulation.

decrease significantly cell growth. At 96 hours of drug exposure, nuclear fragmentation indicating programmed cell death by TUNEL was detected in 15% and 50% of RA and 4HPR (10^{-7} mol/L)-treated cells (Fig 5).

p53 and Bcl-2 Protein Levels During RA and 4HPR Treatment

To test if the p53 pathway was functionally activated during RA and/or 4HPR treatment, we determined whether p53 was present in FGO and whether its level increased after RA (10^{-7} mol/L) and 4HPR (10^{-7} mol/L) administration. In untreated FGO, a faint band was recognized by the monoclonal p53 antibody. After 72 hours of RA and 4HPR (10^{-7} mol/L) treatment, there was a selective increase in the labeling of the band representing the wild-type p53. Moreover, 4HPR induced a 70% decrease of Bcl-2 protein (Fig 6).

RA and 4HPR Effects on cAMP Accumulation

RA and 4HPR treatment for 30 minutes induced a 20% decrease of basal cAMP accumulation and abolished forskolin cAMP-induced increase (Fig 7).

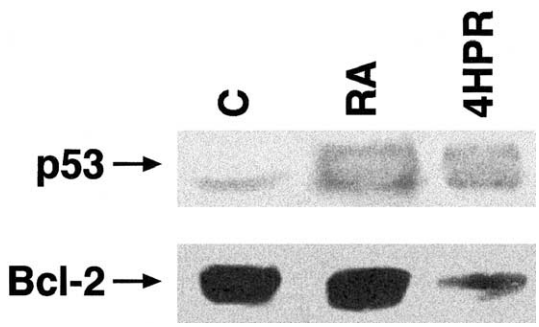

Fig 6. Western blot analysis of p53 and Bcl-2 protein expression in F from patients affected by GO cell lysates before (C) and after 72 hours of 10^{-7} mol/L RA and 4HPR treatment. Protein concentrations were normalized and equal volumes of samples were loaded on the gels.

DISCUSSION

RAR α, β, and γ transcripts were found in cultured orbital F from GO-affected subjects, showing that they are potential target for retinoids. Moreover, the presence of CRBP-I transcript in FGO demonstrated that these cells are able to metabolize retinoids. Retinoids modulate the growth and differentiation of several normal and malignant epithelial cell types in vitro and in vivo.[15] We found for the first time that retinoids inhibit cell growth and induce programmed cell death in orbital F from GO acting on p53 and Bcl-2 protein expression level, suggesting that RAR are functional. 4HPR, a less toxic synthetic derivative of RA, has been used in the prevention and treatment of a variety of tumors both in vivo and in vitro.[20-23] 4HPR induced cell growth inhibition and apoptosis in all RA-sensitive, as well as RA-resistant cells, demonstrating a wider spectrum of potency over RA. We demonstrated that 4HPR is able to inhibit FGO growth and induce programmed cell death in a more effective way than RA in a p53-dependent manner. 4HPR induced a downregulation of the antiapoptotic Bcl-2 protein expression, resulting in an increased sensitivity of these cells to apoptotic stimuli. Because 4HPR binds poorly to the RAR, the issue of whether 4HPR exerts its biologic effects via classical retinoic receptor pathways remains unclear. Recent data suggest that 4HPR acts, at least in part, via the RARβ[28] that we detected in orbital F from GO. RA and 4HPR treatment for 30 minutes decreased cAMP accumulation induced by forskolin in FGO, suggesting an alternative mechanism of action of retinoids in these cells. Specific nongenomic effects occur within a few minutes and are mediated by steroid-selective membrane receptors.[29,30] At this time, it is difficult to establish that the inhibition of cAMP accumulation seen in our cell system induced by RA and 4HPR could be linked to the effect on cell growth. Many events may occur in the length of time between these effects, but it is intriguing to think about the possible link and intracellular cooperation from

different signaling pathways probably induced by the same ligand.

In conclusion, these results shows that retinoids may modulate GO orbital F growth and differentiation acting on the cell morphology and apoptosis, and on cAMP production. These antiproliferative effects of RA and 4HPR in FGO may suggest a possible therapeutic use of retinoids in GO even if more studies in vitro and in vivo are necessary.

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