Effect of retinoic acid on cell proliferation and differentiation as well as on lipid synthesis, lipoprotein secretion, and apolipoprotein biogenesis

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Grenier E, Maupas FS, Beaulieu J-F, Seidman E, Delvin E, Sane A, Tremblay E, Garofalo C, Levy E. Effect of retinoic acid on cell proliferation and differentiation processes, lipid esterification, apolipoprotein (apo) biogenesis and lipoprotein secretion along with nuclear factor gene transcription. Treatment of Caco-2 cells with RA at different concentrations and incubation periods revealed the reduction of thymidine incorporation in 60% preconfluent cells. Concomitantly, RA revealed the reduction of thymidine incorporation in 60% preconfluent cells. Concomitantly, RA, induced D-type cyclins by reducing the mitogen-sensitive cyclin D1 and upregulating cyclin D3 expressions and 2) caused a trend of increase in p38 MAPK, which triggers CDX2, a central protein in cell differentiation. RA remained without effect on lipoprotein output and apo synthesis, even for apo A-I that possesses RARE in its promoter. RA, in combination with 22-hydroxycholesterol, could induce apo A-I gene expression without any impact on apo A-I mass. Only the gene expression of peroxisome proliferator-activated receptor (PPAR)β, retinoic receptor (RAR)β, and RARγ was augmented and no alteration was noted in PPARα, PPARγ, liver X receptor (LXRα), LXRβ, and retinoid X receptors. Taken together, these data highlight RA-induced cell differentiation via specific signaling without a significant impact on apo A-I synthesis.

vitamin A can lead to an uncontrolled proliferation of epithelial stem cells that fail to differentiate into the normal phenotype in many lining epithelium (59). Although the small intestine is the first gateway for contact with dietary retinol as well as the unique organ for its absorption and metabolism, limited information is available as to the effects of RA on the mechanisms governing functional maturation.

Earlier studies established that mammalian apolipoprotein (apo) A-I is synthesized principally in the small intestine in addition to liver (15, 76). Apo A-I represents the major protein component of high-density lipoprotein (HDL) particles and has been attributed antatherogenic properties in view of its potential to initiate the removal of excess cholesterol from peripheral tissues by enhancing cholesterol efflux and delivering to the liver through the reverse cholesterol transport pathway (16, 33). Apo A-I exhibits supplementary functions, which include the protection against oxidative stress, inflammation, endothelium vasoconstriction, and thrombosis (3, 52, 55, 58). Therefore, multiple efforts have been exerted to find out nutrients and chemical agents that raise the levels of apo A-I, which may provide new therapeutic options for the prevention of atherosclerotic cardiovascular disease. There have been several tantalizing clues that revealed diverse influences on apo A-I expression in liver vs. intestine in response to developmental, dietary, hormonal, toxic, and pharmacological stimuli (23, 50, 54, 64, 67). Retinoid status seems to regulate hepatic apo A-I gene expression. The administration of vitamin A to rats lowered hepatic apo A-I mRNA (78), and treatment of rat hepatocytes with RA also resulted in a decrease in apo A-I gene expression (5). These observations indicate that RA suppressed the expression of apo A-I in rats. In contrast, a stimulatory effect on apo A-I synthesis was noted in hepatoma cell lines (6) and in primary cultures from cynomolgus monkey (30). The reason for the discrepancy among studies is not fully clear. Inconsistent findings were also reported in intestinal cells (22, 71), which prompted us to investigate the regulation of apo A-I by RA in Caco-2 cell line, an interesting model frequently used to study lipid transport and lipoprotein assembly (37). A considerable body of evidence supports the concept that intestinal cell differentiation is an obligatory prerequisite for apo biogenesis. Studies using pulse-labeling experiments in isolated rat enterocytes (11) and dot-blot hybridization analysis of rat mucosal scrapings (13) showed that apo synthesis was primarily localized in villus-associated enterocytes. Further-
more, rat intestinal cell line IEC-6, which retains the characteristics of normal rat crypt jejunal cells (57), was unable to synthesize apoproteins and lipoproteins (13, 45). We have, therefore, surmised that RA-mediated cell differentiation might promote apo synthesis. An additional aim of the present work was to determine the expression profile of various nuclear factors representing transacting proteins known to bind to the RARE that has been identified in the promoter region of the apo A-I gene promoter (60).

MATERIALS AND METHODS

Cell culture. Caco-2 cells (American Type Culture Collection, Rockville, MD) were grown at 37°C with 5% CO2 in minimal essential medium (MEM) (GIBCO-BRL, Grand Island, NY) containing 1% penicillin-streptomycin and 1% MEM nonessential amino acids (GIBCO-BRL) and supplemented with 10% decomplemented fetal bovine serum (FBS) (Flow, McLean, VA). Caco-2 cells (passages 30–40) were maintained in T-75-cm2 flasks (Corning Glass Works, Corning, NY). Cultures were split (1:6) when they reached density of 1 x 106 cells/well on 24.5-mm polycarbonate Transwells (Millipore, Bedford, MA) (44, 46). Cell viability was assessed by Trypan blue exclusion (46). Furthermore, DNA and protein content was evaluated as previously described (46). All these parameters were tested in the presence or absence of RA. Since no differences were noted between different RA isoforms, we have decided to use only 9-cis RA at a concentration of 10 μM that was found optimal for various biological effects (data not shown).

Cell proliferation analysis by [3H]thymidine incorporation. The effect of RA on Caco-2 cell proliferative activity was determined by omitting FBS for 18 h of incubation. Thereafter, Caco-2 cells were incubated with or without RA, in the presence of unlabeled oleic acid (as above) for 4 h. [3H]thymidine (10 μCi; sp act, 20 μCi/μmol; Du Pont, Markham, ON, Canada) was added to each well. Cells were incubated for 4 h, washed with MEM (serum free), and then harvested with a rubber policeman. Cells were sonicated and the incorporation of [3H]thymidine into DNA was measured in homogenates (counts·min−1·DNA−1). DNA was quantified by a microfluorometric method.

Measurement of lipid synthesis and secretion. Lipid synthesis and secretion were assayed as previously described (38, 46, 65). Briefly, radiolabeled [14C]oleic acid (sp act, 53 mCi/mmol; Amersham, Oakville, ON, Canada) was added to unlabeled oleic acid and then solubilized in fatty acid-free bovine serum albumin (BSA) (BSA/oleic acid, 1:5 [mol:mol]). The final oleic acid concentration was 0.7 molar (0.45 μCi)/well. Cells were first washed with phosphate-buffered saline (PBS) (GIBCO), and the [14C]oleic acid-containing medium was added to the upper compartment. RA was added to the upper chamber in serum-free MEM. At the end of a 24-h incubation period, cells were washed and then scraped with a rubber policeman in a PBS solution containing antiproteases (phenylmethylsulfonyl fluoride), pepstatin, EDTA, aminocaproic acid, chloramphenicol, leupeptin, glutathione, benzamidine, dithiothreitol, sodium azide, and Trasylol, all at a final concentration of 1 mM). An aliquot was taken for lipid extraction by standard methods (38, 46, 65) in the presence of unlabeled carrier [phospholipids (PL), monoglycerides, diglycerides, triglycerides (TG), free fatty acids, free cholesterol, and cholesteryl ester (CE)].

The various lipid classes synthesized from [14C]oleic acid were then separated by thin-layer chromatography (TLC) using the solvent mixture of hexane, ether, and acetic acid (80:20:3, vol/vol/vol), as previously described (38, 46, 65). The area corresponding to each lipid was scratched off the TLC plates, and the silica powder was placed in a scintillation vial with Ready Safe counting fluid (Beckman, Fullerton, CA). Radioactivity was then measured by scintillation counting (LS 5000 TD, Beckman). Cell protein was quantified by the Bradford method and results were expressed as disintegrations per minute per milligram of cell protein. Lipid secreted in the basolateral compartment was analyzed and quantified, as described above, after centrifugation (2,000 rpm for 30 min at 4°C) to remove cell debris.

Lipid carrier. Blood was drawn 2 h after the oral intake of a fat meal by human volunteers, and postprandial plasma was prepared to serve as a carrier for the lipoproteins synthesized by Caco-2 cells as described previously (38, 39).

Isolation of lipoproteins. For the determination of secreted lipoproteins, Caco-2 cells were incubated with the lipid substrate as described above, in the presence or absence of RA. The medium supplemented with antiproteases (as described above) was first mixed with a plasma lipid carrier (4:1, vol/vol) to efficiently isolate de novo lipoproteins synthesized. The lipoproteins were then isolated by sequential ultracentrifugation using a TL-100 ultracentrifuge (Beckman), as described previously (30). Briefly, chylomicrons (CM) were isolated after ultracentrifugation (20,000 rpm for 20 min). Very-low-density lipoprotein (VLDL) (1.006 g/ml) and low-density lipoprotein (LDL) (1.063 g/ml) were separated by spinning at 100,000 g for 2.26 h with a tabletop ultracentrifuge 100.4 rotor at 4°C. The HDL fraction was obtained by adjusting the LDL infranatant to density of 1.21 g/ml and centrifuging for 6.5 h at 100,000 g. Each lipoprotein fraction was exhaustively dialyzed against 0.15 M NaCl and 0.001 M EDTA, pH 7.0, at 4°C for 24 h.

De novo apo synthesis. The effect of RA on newly synthesized and secreted apoproteins (A-1, A-IV, B-48, B-100, and E) was determined as described previously (38, 46, 65). To first induce apo synthesis, cells were incubated apically with unlabeled oleic acid bound to albumin in serum-free medium, 24 h before [35S]methionine incubation. The concentration of the unlabeled lipid was equivalent to the labeled substrate described above. During this time, RA was again added to the apical chamber. After 24-h incubation, cells as well as the outer chambers were rinsed twice with PBS (GIBCO). The apical compartment was replaced with 1.5 ml of methionine-free medium containing the unlabeled substrate and 100 μCi/ml [35S]methionine (50 mCi/mmol, Amersham Life Sciences). After incubation for 4 h at 37°C with 5% CO2, the medium from the basolateral compartment was collected. Cells were scraped off the inserts in the cell lysis buffer, as described above. The medium and cell lysates were supplemented with an antiprotease cocktail. To assay a considerable amount of de novo apo synthesis, the material from two wells was pooled.

Immunoprecipitation of apo. The medium and cell lysates were first supplemented with unlabeled methionine to act as a carrier (final concentration, 0.1 mM). Immunoprecipitation was performed in the presence of excess polyclonal antibodies to human apoproteins (Boehringer Mannheim) at 4°C overnight (38, 46). Samples were then washed with Nonidet P-40 (0.05%). They were subsequently centrifuged and resuspended in sample buffer (1.2% SDS, 12% glycerol, 60 mM Tris, pH 7.3, 1.2% β-mercaptoethanol, and 0.003% bromophenol blue) and analyzed by a linear 4–15% polyacrylamide gradient preceded by a 3% stacking gel, as described previously. Radioactive molecular weight standards (Amersham Life Sciences) were run in the same conditions. Gels were sectioned into 2-mm slices and counted after an overnight incubation with 1 ml of Beckman tissue solubilizer (0.5 N

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quaternary ammonium hydroxide in toluene) and 10 ml of liquid scintillation fluid (Ready Organic, Beckman). Results for each apo studied were expressed as percent disintegrations per minute per milligram protein to assess the specific effect of RA on apo synthesis and secretion.

*RT-PCR.* PCR experiments for the various genes as well as β-actin (as a control gene) were performed by using the GeneAmp PCR System 9700 (Applied Biosystems) as described previously (61). Approximately 30–40 cycles of amplification were used at 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s. Amplicons were visualized on standard ethidium bromide-stained agarose gels. Importantly, we have established the experimental conditions relative to RT-PCR, which correspond to the linear portion of the exponential phase for every gene expression.

Statistical analysis. All values were expressed as means ± SE. Data were analyzed by two-tailed Student’s t-test.

RESULTS

**Effect of RA on Caco-2 cell proliferation and integrity.** Cell proliferation was assessed by [3H]thymidine incorporation at various periods of confluence. RA caused a decrease in Caco-2 cell proliferation as measured by [3H]thymidine incorporation (Fig. 1, A and B). On the other hand, RA remained without impact when the differentiation process was engaged, i.e., 10 and 21 days postconfluence (Fig. 1, C and Fig. 1D).

Following treatment with RA, Caco-2 cell integrity was assessed by the determination of cell monolayer transepithelial resistance and cell viability by Trypan blue exclusion. No cytotoxic effect of RA was observed (results not shown).

**Effect of RA on Caco-2 cell differentiation.** Since D-type cyclins are believed to be cell cycle-promoting agents, we first studied their protein expression in proliferative and differentiated states. The treatment of Caco-2 cells with RA lowered cyclin D1 and raised cyclin D3 (data not shown). Consequently, the cyclin D3/cyclin D1 ratio increased at 0, 10, and 21 days postconfluence (Fig. 2).

Then, we examined the protein expression of phosphatidylinositol 3-kinase (PI3K), an enzyme required for morphological and differentiation of enterocytes. The addition of RA resulted in little effect on PI3K protein expression at 60% as well as at 0 and 10 days postconfluence. However, RA was able to reduce PI3K protein expression at 21 days postconfluence (results not shown).

p38 Mitogen-activated protein kinase (p38 MAPK) has recently emerged as a key modulator of various vertebrate cell differentiation processes (26, 77). We therefore investigated whether it is modulated by RA. A significant raise was noted in its protein expression at 60% confluence, whereas only a trend was noted at confluence or at 10 and 21 days postconfluence (results not shown). Additionally, we tested the influence of RA on activated p38 MAPK by exposure of Caco-2 cells to environmental stress and found a fall at 10 days postconfluence (results not shown).

Finally, we sought to address the effect of RA on caudal-related homeobox 2 (CDX2), a protein capable of suppressing proliferation and promoting differentiation. Interestingly, RA significantly increased CDX2 protein expression at 60% (Fig. 3A) confluence and lowered it at 10 and 21 days postconfluence (Fig. 3, C and D).

**Effect of RA on lipid esterification and apo synthesis in Caco-2 cells.** We explored the modulation of lipid esterification and de novo apo synthesis by RA in the presence and absence of 22-hydroxycholesterol (22-OH), a natural ligand agonist for liver X receptor (LXR), solubilized in ethanol and used at a concentration of 4 μg/ml during 6- and 24-h incubation culture. No changes were observed in the esterification of TG, PL, and CE (results not shown). For the short incubation period, RA was unable to influence the biogenesis of apo A-I and apo A-IV (Fig. 4) as well as that of apo B-48, apo B-100, and apo E (Fig. 5). In contrast, 22-OH could increase the synthesis of most of the apos. However, the addition of RA to 22-OH suppressed the 22-OH-induced biogenesis.

During the long incubation period, RA only enhanced the apo A-IV synthesis whereas 22-OH was effective in activating the production of apos (A-I, A-IV, B-100, and E). The combination of RA and 22-OH did not induce any change in the level of synthesis of all the apos.

**Effect of RA on lipoprotein secretion by Caco-2 cells.** No substantial variations were observed in lipoprotein output following the addition of RA either at 4 or 24 h of incubation (results not shown).

Fig. 1. Effects of retinoic acid (RA) on Caco-2 cell line proliferation. The incorporation of [3H]thymidine was studied in Caco-2 cells at various conditions: 60% confluence (A), 0-day confluence (B), 10 days postconfluence (C), 21 days postconfluence (D). Experiments were performed in duplicate and values are expressed as means ± SE for n = 4 in each group of experiments. *P < 0.05 vs. controls.
Effect of RA on nuclear factors in Caco-2 cells. To approach the mechanisms triggered by RA, we assessed the gene expression of peroxisome proliferator-activated receptors (PPARs), LXR, RXR, and RAR in Caco-2 cells at 4 and 24 h of incubation. RA and 22-OH did not exhibit an influence on PPARs at the short incubation period (Fig. 6A). A significant lowering effect was recorded with 22-OH and 22-OH + RA (Fig. 6B). RA elicited an enhancement in the gene expression of PPARγ at 4 and 24 h of incubation (Fig. 6, C and D). The combination of RA and 22-OH significantly lowered PPARβ gene expression (Fig. 6D). RA and 22-OH alone or in combination reduced the PPARγ gene expression (Fig. 6, E and F).

When we tested the influence on RA and 22-OH on gene expression of LXRα and β, we could notice only a trend of increase (results not shown).

We also explored the impact of RA treatment on RXRs in Caco-2 cells. We mostly noted an increase in RXRα (Fig. 7B) and RXRβ (Fig. 7D) following the administration of RA to Caco-2 cells.
Caco-2 cells at 24 h of incubation. 22-OH alone or in combination with RA seemed to cause a decline in their gene expression (Fig. 7, B and D) without altering RXRγ (data not shown).

We finally tackled the RAR regulation by RA and 22-OH (Fig. 8). In general, a raise of RARα, RARβ, and RARγ resulted from the addition of RA. A trend of decrease was apparent in the gene expression of these nuclear factors fol-

Fig. 4. Effects of RA and 22-hydroxycholesterol (22-OH) on the synthesis of apolipoproteins (apo) A-I and A-IV in Caco-2 cells. These epithelial cells at 21 days postconfluence were incubated with [35S]methionine in the presence of unlabeled oleic acid for 4 or 24 h to stimulate the biogenesis of apos. At the end of the labeling period, cells were washed, homogenized, and centrifuged. Supernatants from the cell homogenates were then reacted with excess antibodies for 18 h at 4°C to precipitate specific apos. Immune complexes were washed and analyzed by linear 4–20% SDS-PAGE. After electrophoresis, gels were sliced and counted for radioactivity. Data represent means ± SE of 4 experiments in each group: apo A-I (4-h incubation) (A), apo A-I (24-h incubation) (B), apo A-IV (4-h incubation) (C), apo A-IV (24-h incubation) (D). Values represent means ± SE for n = 4 in each group of experiments and are reported as percent difference relative to control values representing 100%. *P < 0.05 vs. controls.

Fig. 5. Effects of RA and 22-OH on the synthesis of apos (B-48, B-100, and E) in Caco-2 cells. These epithelial cells at 21 days postconfluence were incubated with [35S]methionine in the presence of unlabeled oleic acid for 4 or 24 h to stimulate the biogenesis of apos. At the end of the labeling period, cells were washed, homogenized, and centrifuged. Supernatants from the cell homogenates were then reacted with excess antibodies for 18 h at 4°C to precipitate specific apos. Immune complexes were washed and analyzed by linear 4–20% SDS-PAGE. After electrophoresis, gels were sliced and counted for radioactivity. Data represent means ± SE of 4 experiments in each group: apo B-48 (4-h incubation) (A), apo B-48 (24-h incubation) (B), apo B-100 (4-h incubation) (C), apo B-100 (24-h incubation) (D), apo E (4-h incubation) (E), apo E (24-h incubation) (F). Values represent means ± SE for n = 4 in each group of experiments and are reported as percent difference relative to control values representing 100%. *P < 0.05 vs. controls.
following the addition of 22-OH, and an enhancement was recorded in RARβ when RA and 22-OH were combined (Fig. 8C).

DISCUSSION

RA is known to exert pleiotropic effects given its ability to modulate gene expression of various genes involved in growth, adaptation, and function. We have, therefore, undertaken systematic studies to examine the impact of RA treatment on intestinal epithelial cells to examine its regulatory role in cellular proliferation and differentiation, as well as on lipid transport processes and transcriptional programs elicited by nuclear factors. Our data indicate that RA acted as a differentiation factor since, at 60% preconfluence and at 0-day confluence, it lowered labeled thymidine incorporation; 2) modulated D-type cyclins (cell cycle regulatory proteins) by downregulating the mitogen-sensitive cyclin D1 expression (thereby blocking DNA synthesis and cell proliferation) and upregulat-

Fig. 6. Effects of RA and 22-OH on peroxisome proliferator-activated receptor (PPAR) gene expression in Caco-2 cells. PCR analysis was performed on Caco-2 cells at 21 days postconfluence to analyze mRNA of PPARα (4-h incubation) (A), PPARα (24-h incubation) (B), PPARβ (4-h incubation) (C), PPARβ (24-h incubation) (D), PPARγ (4-h incubation) (E), PPARγ (24-h incubation) (F). Values represent means ± SE for n = 4 in each group of experiments and are reported as percent difference relative to control values representing 100%. *P < 0.05 vs. control; **P < 0.01 vs. control.
ing cyclin D3 expression (thereby inducing cell differentiation); and 3) showed a trend of increase in p38 MAPK, which triggers CDX2, a central protein in cell differentiation. Despite its capacity to diminish cell proliferation, RA was unable to increase intestinal apo A-I gene expression, apo A-I synthesis, and HDL production. Furthermore, it remains without effect on the esterification of TG, CE, and PL; the production of CM, VLDL, and LDL; and the biogenesis of apos (A-IV, B-48, B-100, and E). Moreover, RA augmented the gene expression of various nuclear receptors, such as PPARγ, RARα, and RARγ. Finally, when combined with 22-OH, RA could induce apo A-I gene expression without any impact on apo A-I mass.

Caco-2 cells, an intestinal cell line derived from a human colorectal carcinoma that spontaneously differentiates under standard culture conditions, lends itself to the in vitro study of human gut in view of its efficient intestinal transport processes (37). Their distinct cellular polarity is characterized by apical microvilli with associated brush border hydrolases and basolaterally positioned nuclei, features comparable to those of normal, mature intestinal absorptive cells. Caco-2 cells have the ability to form a high transepithelial electrical resistance, indicating a functional tight junction barrier. They are among the few intestinal cell lines capable of synthesizing sucrase-isomaltase, a well-characterized marker for functional differentiation of enterocytes. Among their multiple biological functions are those related to the absorption, transport, and metabolism of lipids and lipoproteins (37). The present work has described the regulation of cell differentiation, apo and lipoprotein synthesis, and nuclear factor expression by RA. However, these findings obtained in Caco-2 cells should be interpreted with caution since there are still different shortcomings intrinsic to the model, which have to be taken into consideration when using the Caco-2 model as a screening tool, including the absence of mucus, the lack of cytochrome P450 enzymes, and the inability to study regional intestinal differences in oral absorption. Further studies are necessary before extrapolating the results to human intestine in vivo.

In our studies with Caco-2 cells, RA diminished cell proliferation and raised cell differentiation in conditions of preconfluence and postconfluence. Similarly, a number of in vivo and in vitro studies using various experimental models have revealed that RA plays a key role in embryonic development, the prevention and treatment of tumors, as well as cell proliferation and differentiation (10, 12). In particular, our data are in line with many reports suggesting that RA is more active in inhibiting proliferation and inducing differentiation in rela-
tively undifferentiated systems, i.e., murine F9 teratocarcinoma cells, promiocyctic HL-60 cells, L-6 melanoma cells, and neuroblastoma and embryonal stem cells P19 (2, 20, 49). In contrast, in other cell lines, such as keratinocytes (66), embryonal lung cells (63), and chick embryo hepatocytes (70), proliferation is stimulated. Differentiation is also blocked in chondrocytes, osteocytes, and adipocytes (49). The reported contrasting effects of RA can be explained by the existence of many types of RA-nuclear receptors, regulated both temporally and in a tissue-specific fashion. Moreover, the divergent effects of RA in various tissues may be explained by the different levels of cellular retinol binding protein (RBP) necessary for retinol binding and transfer across the plasma membrane (72).

In proliferating eukaryotic cells, the progression from G1 phase to S phase of the cell cycle is controlled by the expression of a subset of the cell cycle control genes. These include the D-type cyclin genes, which consist of three family members, cyclins D1, D2, and D3. The regulatory function of the D-type cyclins is mediated by their interactions with cyclin-dependent kinases (CDK)2, CDK4, and CDK6 (43). Abnormal

Fig. 8. Effects of RA and 22-OH on RAR gene expression in Caco-2 cells. PCR analysis was performed on Caco-2 cells at 21 days postconfluence to analyze mRNA of RARα (4-h incubation) (A), RARα (24-h incubation) (B), RARβ (4-h incubation) (C), RARβ (24-h incubation) (D), RARγ (4-h incubation) (E), and RARγ (24-h incubation) (F). Values represent means ± SE for n = 4 in each group of experiments and are reported as percent difference relative to control values representing 100%. *P < 0.05 vs. control; **P < 0.01 vs. control.
CDX2 is selectively localized in the nuclei of fetal and adult mucosal epithelial cells in the small and large intestines in both humans and mice (48). In our study, CDX2 protein expression was increased by RA at 60% confluence and decreased by RA at 10 and 21 days postconfluence. Although the reasons for the variable CDX2 modulation by RA during the processes of cell proliferation and differentiation remain unclear, it is possible to suggest a few explanations to enlighten this situation. The loss of CDX2 expression in the differentiation state may result from 1) defects in trans-acting pathways known to regulate CDX2 transcription in the 5'-flanking gene region (24); 2) CDX2 gene silencing by hypermethylation of the CpG island associated with the promoter region (75); and 3) the inhibition p38 MAPK pathways leading to the downregulation of CDX2 (25, 34), as exemplified by our findings showing the parallel trend of p38 MAPK and CDX2.

P38K belongs to a family of signal transducer heterodimeric enzymes composed of a 85-kDa regulatory subunit (p85α or β) containing SH2 and SH3 domains and a 110-kDa catalytic subunit (p110α or β) (9, 35) that phosphorylates the D3 hydroxyl in the inositol ring of phosphatidylinositol. P38K is involved in the regulation of normal cellular processes such as cellular growth, vesicular trafficking, insulin-regulated glucose uptake, and apoptosis. Recently, P38K has been shown to be an important regulator of differentiation in certain cell types, including myogenic, intestinal, and adipocyte differentiation (53). However, P38K may behave as a negative regulator of cellular differentiation (8, 56). In our studies, P38K did not change following RA treatment at 60% preconfluence or at 0 confluence. On the other hand, p38 MAPK was augmented, which is congruent with its important role in various mammalian cell-differentiating processes (53). Consistent with the notion that CDX2 has a critical function in intestinal cell differentiation within a regulatory network that establishes the differentiated phenotype of intestinal epithelial cells (25), we found a RA-induced enhancement in Caco-2 cells at 60% confluence.

Expression of the APO A-I gene is regulated at both the transcriptional and posttranscriptional level. The APO A-I gene promoter contains a TATA-like motif close to the transcriptional start site and several cis-elements that regulate expression of the gene in either a positive or negative manner in response to various hormonal or metabolic signals. Various transcription factors assemble into multiprotein complexes on key regulatory regions of the gene (40). General transcription factors (TFs) associated with polymerase II (Pol II), such as TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIFH, are recognized by core promoter sequences and are responsible for the basal expression of the gene. The multistep assembly of these factors is involved in APO A-I gene modulation. Data of our study clearly demonstrated that RA does not regulate the expression of apo A-I in the Caco-2 cells, although Hargrove et al. (23) have reported that retinoids induce APO A-I promoter activity and gene expression through proximal promoter elements located between nucleotides -235 and -144 (relative to the transcriptional start site, +1). It seems therefore possible that RA might act by simultaneously blocking a liver-specific enhancer element such as that described by Sastro et al. (62). In fact, several transcriptional repressors have been shown to suppress APO A-I gene transcription (23), although the physiological relevance of each of these transcription elements is not always clear. For example, binding of a thyroid hormone receptor monomer to a negative thyroid hormone response element located 3' of the APO A-I gene TATA-box may suppress APO A-I gene transcription only when removed from the full-length promoter (69). Nevertheless, additional studies are needed to clarify whether RA modifies the 3 apos (A-I, C-III, and A-IV), which are grouped together in a cluster on 17 kb of human chromosome 11 (31). Apparently, apo A-I transcription rates are strongly influenced by an APO C-III gene enhancer element, part of the APOs (A-I, C-III, A-IV) gene cluster (29). Thus one cannot exclude the possibility that RA may affect the distal regulatory region of the APO C-III promoter that acts as a common enhancer for the three closely linked genes (32). For example, as a scenario, prevention of the binding of SP1 to the enhancer by RA could diminish the promoter/enhancer activity (32).

The retinoid signal is transduced by two families of nuclear receptors, the RARs and RXRs, which work as RXR/RAR heterodimers. Each family consists of three isotypes (α, β, and γ) encoded by separate genes (41). RARs are activated by all-trans RA and its 9-cis isomer, whereas RXRs are only activated by 9-cis RA. Our data showed that RA was able to induce RXRα and RXRβ only at the 24-h incubation, whereas RXRγ gene expression was not altered by the presence of RA. On the other hand, all the RAR isoforms responded to RA either at 4-h incubation or 24-h incubation except RXRα at 4-h incubation. Our results imply that a long period of incubation is necessary to enhance the mRNA levels of RXRα and RXRβ in Caco-2 cells. Moreover, RXRγ seems resistant to regulation by RA, suggesting a low dissociation constant for the bound retinoids or limited role in Caco-2 cells.

Importantly, posttranslational modification of transcription factors by phosphorylation provides a rapid cellular response to environmental changes. Phosphorylation processes are critical for the transcriptional activity of RAR and RXR because an abnormal phosphorylation might result in the dysregulation of RA target genes (7). Moreover, a link between the phosphorylation status of the receptor and its ubiquitination/degradation has recently been proposed, possibly implicating this mechanism in the proliferative and carcinogenic processes (1, 42). The precise mechanisms underlying the effects of retinoids in our study are still to be elucidated since RAR and RXR were assessed only at the molecular level and the specific role of the nuclear receptors phosphorylation and degradation through the ubiquitin-proteasome pathway should be addressed in future investigation. Evidently, confirmation of the positive modulation of the RAR and RXR factors at the protein levels by...
RA may uncover changes in their properties, including alterations in DNA binding capacity, ligand binding, dimerization, coactivator recruitment, and transcriptional activation.

PPARs forms a heterodimer with RXR, which enhances its binding to DNA sequence elements termed peroxisome proliferator response elements (19, 21, 36). In our studies, a parallel decline in the gene expression of PPARs and RXRs was noticed with the administration of RA + 22-OH compared with RA alone. At present, the mechanism by which RA and RA + 22-OH differently impact on the gene expression of PPARs is not clear. In view of the repression of PPARs and RXRs by LXRα reported previously (27), one may only suggest that the RA + 22-OH-mediated trend of increase of LXRα might behind the reduction of PPARα.

Although much is known about the modulation of apo A-I by diet, hormones, and development, there remains a dearth of information regarding the intestinal regulation of apo A-I synthesis and HDL production by natural and synthetic retinoid members of the vitamin A family. Our studies have revealed the potential of RA to induce cell differentiation without modulating apo A-I synthesis. A better understanding of the regulatory mechanisms in the future may offer us useful information to manipulate apo A-I expression and to benefit patients with coronary events.

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GRANTS

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