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Effects of Retinoids on Cancerous Phenotype and Apoptosis in Organotypic Cultures of Ovarian Carcinoma

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Background: **Retinoic acid analogues, called retinoids, have shown promise in clinical trials in preventing breast and ovarian cancers. Classic retinoids bind to retinoic acid receptors, which regulate cell growth. Some novel retinoids, such as fenretinide, i.e., *N*-(4-hydroxyphenyl)retinamide (4-HPR), induce apoptosis through retinoic acid receptor-independent mechanisms; however, they appear to do so only at concentrations above those achieved in clinical chemoprevention trials. At lower concentrations (≤1 μ*M*), 4-HPR acts like classic retinoids, by inducing differentiation through a receptor-dependent mechanism. Our goal was to compare the effects of novel receptor-independent (apoptotic) retinoids with those of classic growth-inhibitory retinoids at clinically achievable doses on growth, differentiation, and apoptosis in ovarian tissue.** *Methods:* **Four receptor-independent (apoptotic) and seven growth-inhibitory retinoids, including synthetic, low-toxicity compounds called heteroarotinoids, were administered at concentrations of 1 μ*M* to organotypic cultures of ovarian primary and cancer cell lines: OVCAR-3, Caov-3, and SK-OV-3. After fixation, embedding, and sectioning, the growth fraction was quantified by measuring expression of the proliferation marker Ki-67/myb, differentiation was assessed by expression of mucin, and apoptosis was evaluated by the TUNEL assay. Spearman correlation analysis was performed on the data, and all *P* values were two-sided.** *Results:* **All 11 retinoids reversed characteristics associated with the cancerous phenotype in all neoplastic cultures. Glandular structures were observed consistently in retinoid-treated, but not in untreated, OVCAR-3 and Caov-3 cultures. All retinoids decreased growth fractions, and some increased mucin expression. All receptor-independent retinoids and two receptor-dependent retinoids induced apoptosis, and the induction correlated significantly with increased expression of the mucin MUC1 (r = .83; P = .03). Retinoids with ester-linking groups did not induce apoptosis but decreased the growth fraction in correlation with MUC1 induction (r = –.93; P = .02).** *Conclusions:* **At clinically achievable concentrations, all retinoids tested decrease the growth fraction, induce differentiation and apoptosis. Induction of MUC1 expression is implicated in the mechanisms of action. [J Natl Cancer Inst 2001;93: 516–25]**

Ovarian cancer is the leading cause of gynecologic cancer-related mortality in the United States, with about 14 000 deaths per year *(1).* Despite advances in the surgical and medical management of these patients, the overall prognosis is still poor, with a 5-year survival of only 20%–30% in patients with advanced disease *(2).* Besides developing new strategies for treatment, it is important to divert efforts toward ovarian cancer prevention. These preventive efforts would be especially important in women with germ-line mutations in the BRCA1 or BRCA2 genes, which are known to increase risk of ovarian cancer *(3).* Current methods of risk reduction in these women include prophylactic oophorectomy and ultrasound screening, but the extent of risk reduction by these methods is unknown *(4).*

A promising candidate drug for ovarian cancer chemoprevention is fenretinide, i.e., *N*-(4-hydroxyphenyl)retinamide (4-HPR), which belongs to a family of synthetic retinoic acid (RA) analogues called retinoids. Its promising clinical activity was observed in a randomized clinical trial conducted at the Istituto Nazionale Tumori in Italy. In this trial, women who had surgery for breast cancer were randomly assigned to receive 200 mg of 4-HPR per day or a placebo for 5 years to determine whether 4-HPR reduced the incidence of second primary tumors *(5,6).* A secondary finding of this trial was that statistically significantly fewer ovarian cancers developed in women who received 4-HPR. This result, which suggests that 4-HPR may reduce the risk of ovarian cancer, has led to several clinical trials to evaluate its efficacy as a chemoprevention agent.

Retinoids are considered to be one of the most promising classes of cancer chemoprevention agents by the Chemoprevention Working Group to the American Association for Cancer Research *(7).* These drugs elicit biologic effects by regulating gene transcription via a series of nuclear receptors, which are ligand-inducible transcription factors that belong to the steroid receptor superfamily *(8,9).* There are two classes of retinoid receptors known as RA receptors (RARs) and retinoid X receptors (RXRs), each with three distinct subtypes (α, β, and γ). The RARs bind to DNA as homodimers with self or as heterodimers with other nuclear receptors. The retinoid receptors function

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only as heterodimers with RXRs, while RXRs function both as homodimers and as heterodimers with RARs *(8–10),* the thyroid hormone receptors *(8),* the vitamin D receptor *(8),* the peroxisome proliferator-activated receptor *(11),* and several orphan receptors *(12–14).* The retinoid receptor dimers regulate gene expression by binding to specific DNA sequence elements found in retinoid-responsive gene promoters termed RA-responsive elements (RAREs) *(8,9,15).*

The 4-HPR compound is similar to the classic retinoids in that it can induce transactivation of RAREs in intact cells *(16).* Since 4-HPR does not directly bind to retinoid receptors, this transactivation may be due to 4-HPR's ability to inhibit RA catabolism, thus causing increased concentrations of intracellular RA *(17).* Another similarity between 4-HPR and classic retinoids is that both can induce F9 mouse teratocarcinoma cell differentiation in a receptor-dependent manner at 1 μ*M (18).* At concentrations of 3–12.5 μ*M,* however, 4-HPR differs from classic retinoids in that it induces apoptosis in a variety of cell lines *(18–24).* The finding that 4-HPR can induce apoptosis in retinoid receptor null cells indicates that the molecular mechanism of apoptosis induction is independent of retinoid receptor activation *(18).* Other promising novel retinoids, such as CD437 *(25,26),* and some of the retinoid class, called heteroarotinoids, also induce apoptosis (Liu S, Kamelle S, Guruswamy S, Dhar A, Klucik J, Brown C, et al.: manuscript in preparation). The classic retinoids, on the other hand, are poor inducers of apoptosis as single agents, although they can enhance apoptosis when combined with DNA-damaging agents *(20,26,27).* Therefore, retinoids that can induce apoptosis through receptor-independent mechanisms may have very different biologic effects depending on the physiologic concentrations achieved in clinical trials [*(26);* Liu S, Kamelle S, Guruswamy S, Dhar A, Klucik J, Brown C, et al.: manuscript in preparation]. Since concentrations of retinoids greater than 1 μ*M* are difficult to achieve physiologically, it is most likely that apoptotic retinoids will act similarly to classic retinoids in clinical trials *(28).* In the trial conducted by Istituto Nazionale Tumori, the plasma concentration of 4-HPR achieved in treated women ranged from 340 to 868 nM *(29).* Therefore, the concentrations in the ovarian tissue of treated women were not likely to have reached the levels required for apoptosis, and we hypothesize that induction of differentiation in ovarian cancer cells contributed to the chemoprevention observed.

The objective of this study was to compare the effects of clinically achievable concentrations of apoptotic retinoids and classic growth-inhibitory retinoids on growth, differentiation, and apoptosis in ovarian tissue. To evaluate therapeutic effects, we developed an organotypic culture model of ovarian tumors by growing established cell lines and primary cultures inside collagen gels. The retinoids used in this study include a series of compounds belonging to a class of synthetic retinoids called heteroarotinoids that consist of aromatic structures (arotinoids) containing heteroatoms in a ring. The presence of the heteroatom has been shown to decrease the toxicity of arotinoids *(30).* Heteroarotinoids with similar biologic activity profiles to 9-*cis*-RA, but with various receptor specificities, were compared with heteroarotinoids with similar profiles to 4-HPR and receptor-independent induction of apoptosis [Liu S, Kamelle S, Guruswamy S, Dhar A, Klucik J, Brown C, et al.: manuscript in preparation; *(30–34)*]. It was hypothesized that retinoids would increase the expression of mucins, which are markers of ovarian epithelial differentiation, and specifically increase the MUC1 mucin, which is a marker of glandular differentiation *(35,36).*

Materials and Methods

Cell lines and primary cultures. The OVCAR-3, Caov-3, and SK-OV-3 ovarian carcinoma cell lines were obtained from the American Type Culture Collection (Manassas, VA). The OVCAR-3 cultures were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and .01 mg/mL insulin. The Caov-3 cultures were maintained in modified Eagle medium (MEM) supplemented with 10% FBS, nonessential amino acids, sodium pyruvate, antibiotics, and antimycotics (complete MEM). The SK-OV-3 cultures were maintained in McCoy's 5a medium supplemented with 10% FBS, antibiotics, and antimycotics. A primary culture of a benign ovarian cyst (O3) and one of a serous papillary ovarian cancer (O2) were established from specimens obtained from two consenting patients undergoing surgery at the University of Oklahoma Health Sciences Center, Oklahoma City. The Institutional Review Board at the University of Oklahoma Health Sciences Center approved this study. The specimens were cut into small fragments, vortexed vigorously, and plated onto 100-mm tissue culture dishes containing complete MEM. Monolayer cultures that developed from single floating cells and outgrowths of the small pieces were passaged once before cryogenic preservation. Organotypic cultures were made from passage 2 in which the majority of cells were epithelial. Fibroblasts began to outgrow the epithelial cells after passage 4. Only lots of FBS with undetectable levels ($<10^{-8}$ *M*) of retinol and RA isomers as determined by high-performance liquid chromatography were used.

Retinoids. Structures of arotinoids and heteroarotinoids used in these experiments are given in Fig. 1; their biologic characteristics are described in Table 2. The arotinoid and heteroarotinoids in the laboratory of K. D. Berlin at Oklahoma State University, Stillwater, and were synthesized and their receptor specificity was determined as described previously [Nhet90 and NHet17 are compounds 2 and 3, respectively *(32),* OHet72, SHet50, OHet26, and Arot21 are compounds 6, 16, 8, and 11, respectively *(31),* and SHetA2, SHetA3, and SHetA4 are compounds 11, 12, and 13, respectively *(34)*]. The 4-HPR compound was a gift from R. W. Johnson Pharmaceutical Research Institute, Raritan, NJ, and 9-*cis*-RA was obtained from a commercial source (Bio-Mol, Plymouth Meeting, PA). All retinoids were dissolved in dimethyl sulfoxide (DMSO) and stored as aliquots of 1000× stocks at −70 °C. Since the retinoids are sensitive to light, all manipulations involving retinoids were performed under subdued lighting. Each experiment included control cultures that received the same volume of DMSO solvent that was administered in the retinoid treatment. The concentration of DMSO in each experiment was always less than or equal to 0.01%, which is not toxic and does not induce differentiation.

Cytotoxicity assays. Cultures were plated in 96-well microtiter plates in volumes of 150 μL at a concentration of 1000 cells/well. The next day, retinoids were added at 4× concentrations in 50 μL of medium, resulting in a 10-μ*M* final concentration of each retinoid. After 3 days of treatment, the cell density in each well was quantitated by use of a sulforhodamine B (SRB) assay. Briefly, the cells were fixed in trichloroacetic acid, the cytoplastic proteins were stained with SRB (which was solubilized with Tris–HCl), and the optical density (OD_{595}) of each culture was read with a MR600 microtiterplate reader. The OVCAR-3 cell line excreted substantial amounts of clear lipid droplets that interfered with the SRB assay. Therefore, the *in vitro* growth-inhibition assay for OVCAR-3 was performed by use of the CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay (Promega Corp., Madison, WI), which is composed of a novel tetrazolium compound that is metabolized by viable cells into a soluble formazan that can be quantitated by reading the OD. Each experiment was performed in triplicate, and the three values for each treatment were averaged. The average OD of the treated cultures was divided by that of the control cultures treated with solvent alone. For the determination of the percent cell decrease, this ratio was subtracted from 1 and multiplied by 100.

Organotypic cultures. Single-cell suspensions of the ovarian carcinoma cell lines and the primary ovarian cultures, at passage 2, were quantitated with a particle counter. Volumes containing 10^5 ovarian cells and 10^5 3T3 fibroblasts were mixed and centrifuged. The cell pellet was resuspended in a 4 °C solution containing complete MEM and rat tail collagen I (Collaborative Biomedical Products, Bedford, MA), a solution that is liquid at 4 °C. The suspension was poured into Falcon cell culture inserts with transparent membranes containing 0.4-μm pores (Becton Dickinson, Franklin Lakes, NJ) that were placed in tissue culture plates. After the collagen was allowed to solidify at 37 °C, a layer of

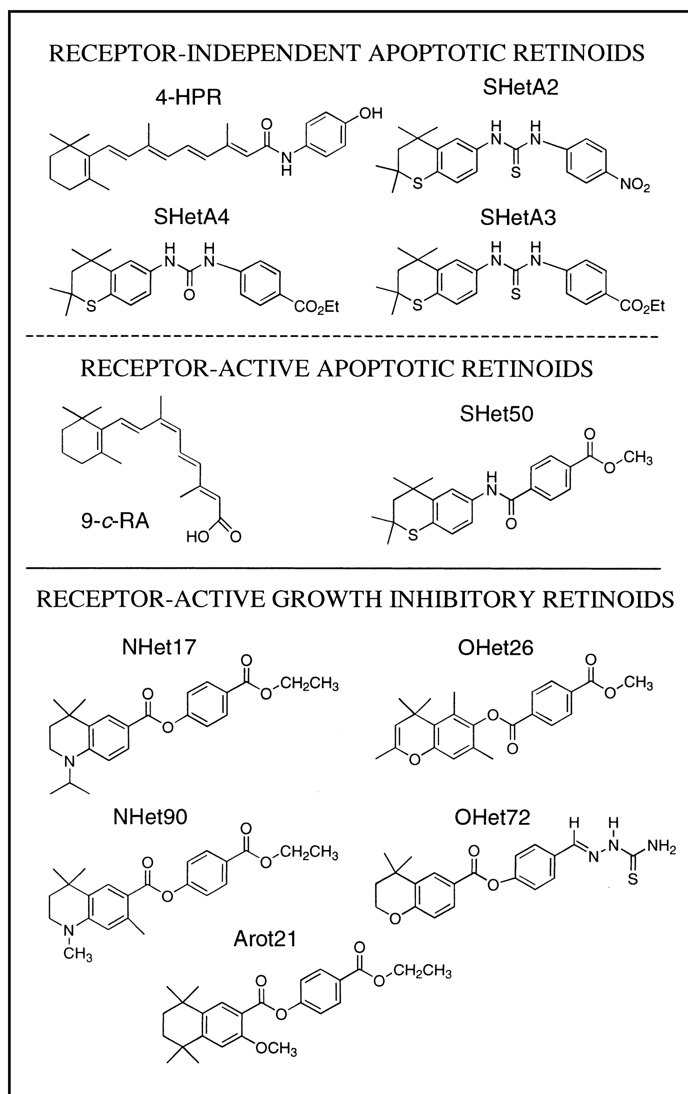

Fig. 1. Structures and classifications of retinoids. The **vertical dashed line** separates the apoptotic retinoids that induced cell death in both monolayer and organotypic cultures from the reclassified apoptotic retinoids that induced apoptosis in organotypic cultures but not in monolayer cultures. The growth-inhibitory retinoids did not induce apoptosis in monolayer or organotypic cultures. IUPAC names: SHetA2[N-4-[(2,3-dihydro-2,2,4,4-tetramethyl-2H-l-benzothiopyran-6-yl)-N′-4-nitrophenyl]thiourea]; SHetA3[(2,3-dihydro-2,2,4,4-tetramethyl-2H-l-benzothiopyran-6-yl)-N′-4-ethoxycarbonylphenyl]thiourea]; SHetA4[N-4-[2,3-dihydro-2,2,4,4-tetramethyl-2H-l-benzothiopyran-6-yl)-N′-4-ethoxycarbonylphenyl]urea]; SHet50 [methyl 4-[(2,3-dihydro-2,2,4,4-tetramethyl-2*H*-l-benzothiopyran-6-yl)carbamoyl]benzoate]; NHet17[ethyl 4-(4,4-dimethyl-*N*-isopropyl-1,2,3,4-tetrahydroquinolin-6-oyloxy)benzoate]; OHet26[methyl 4-(4,4,5,7-tetramethyl-6-coumaryl)benzoate]; NHet90[ethyl 4-(*N*,4,4,7-tetramethyl-1,2,3,4-tetrahydroquinolin-6-oyloxy)benzoate; OHet72 [4-[[4,4-dimethyl-3,4-dihydro-2*H*-benzo[*b*]pyran-6-yl)-carbonyl] oxy]benzaldehyde thiosemicarbazone]; Arot21[ethyl 4-[(3-methoxy-5,6,7,8-tetrahydro-5,5,8,8-tetramethylnaphthalen-2-yl)carbonyloxy]benzoate]. 4-HPR = *N*-(4-hydroxy-phenyl)retinamide; 9-*c*-RA = 9-*cis*-retinoic acid.

3×10^5 ovarian cells without fibroblasts was placed on top of the collagen gels. The liquid on top of the gel was allowed to evaporate, and the medium surrounding the insert was replenished every 2 days. Retinoid treatment was initiated after 1 week of growth and replenished with each medium change. Retinoids were administered by mixing liquid drug stocks into the medium surrounding the insert. Untreated control cultures were treated with the same volume of DMSO administered in retinoid treatments. After 2 weeks of retinoid treatment, the cultures were cut in half. One half was fixed in formalin and subsequently embedded in paraffin and sectioned, and the other half was snap-frozen in liquid nitrogen. The experiment was repeated two to five times for each cell line.

Histochemical and immunohistochemical analyses. Sections from the paraffin-embedded blocks were stained with hematoxylin–eosin (H & E) for histologic evaluation. Mucin expression was detected by staining with the periodic acid–Schiff (PAS) stain kit (American Master* Tech Scientific, Inc., Lodi, CA), and apoptosis was detected via the TUNEL assay (i.e., terminal deoxynucleodidyl transferase-mediated biotin-deoxyuridine triphosphate nick-end labeling) by staining with the Klenow-FragEL™ DNA Fragmentation Kit (Oncogene Research Products, Cambridge, MA) according to the manufacturer's instructions. A Ki-67 Kit (Zymed Laboratories Inc., South San Francisco, CA) was used to stain 5-μm sections of paraffin-embedded organotypic culture blocks according to the manufacturer's instructions. The slides were counterstained with Mayer's hematoxylin. Immunohistochemical staining for MUC1 was performed on 5-μm sections that were deparaffinized and subjected to antigen retrieval. The slides were incubated with diluted MUC1 antibody (1 : 75; Novocastra Laboratories Ltd., New Castle, U.K.) for 1 hour and subsequently washed with phosphate-buffered saline two times. The Histostain–Plus Kit (Zymed Laboratories, Inc.) was used for the secondary antibody and color development. A biotinylated second antibody was added and incubated for 20 minutes. This was followed by adding avidin–peroxidase complex for 30 minutes and the enzyme substrate aminoethyl carbozole for 7 minutes. Finally, the slides were counterstained with Mayer's hematoxylin for 1 minute, rinsed in 1% ammonia solution, and mounted in crystal mount.

Pathology review. All stained slides were labeled with a secret code and reviewed in a blinded manner by an experienced pathologist (S. Lightfoot), who did not know the meaning of the code. The following characteristic features in H & E-stained sections were used to identify apoptotic cells: nuclei that were shrunken and markedly hyperchromatic, multiple small pieces of nuclear material in the cell, cell cytoplasm that contained some fragment of a nucleus, and cell cytoplasm that resided as a small dense segment without nuclear material. In a separate assay, the TUNEL-stained slides were screened at 10× magnification, and then the individual cells or cell pieces were examined at 40× magnification. Cells that had granular brown pigment in the nucleoplasm were scored as positive. Cells were scored as negative if no staining was present or if the brown pigment was smooth and nongranular.

Quantification of PAS and MUC1 staining. A scoring system was used that assigned each stained section a numeric value on the basis of the staining intensity and the percent of positive cells. The numeric values assigned on the basis of staining intensity were as follows: 1) slight staining outlining the cell wall, 2) moderate staining with some staining of the cell proper, and 3) moderate to heavy staining with marked cell staining. The numeric values assigned on the basis of the percent of positive cells were as follows: 1) 0%–10%, 2) 10%–50%, 3) 51%–80%, and 4) greater than 80%. The score was then derived by multiplying the assigned category number of the staining intensity by the assigned category number of the percentage of positive cells.

Statistical analysis. All data were entered into Microsoft Excel 5.0. GraphPad Software was used to perform two-tailed *t* tests and correlation analysis for TUNEL and Ki-67 parametric results. For analysis involving the nonparametric PAS stain and MUC1 scores, the two-tailed Mann–Whitney *U* test and Spearman correlation analyses were performed. All *P* values of less than .05 were considered to be statistically significant.

Results

Classification of Retinoids

To evaluate the mechanism of retinoid action on ovarian cancer cells, a series of 11 retinoids were chosen on the basis of their *in vitro* growth-inhibition activity against OVCAR-3, Caov-3, and SK-OV-3 ovarian carcinoma cell lines. To classify the heteroarotinoids, a standard cytotoxicity assay was performed to differentiate between drugs that cause cell loss similar to 4-HPR and those that inhibit growth similar to 9-*cis*-RA. Since the doubling times of these cultures were approximately 48 hours and the cultures were treated for 72 hours (1.5 doubling times), a greater than 67% decrease in the number of cells would indicate that cell loss occurred. The three retinoids that exhibited a greater than 74% cell decrease, similar to 4-HPR, belong to a category of heteroarotinoids that are known to induce apoptosis

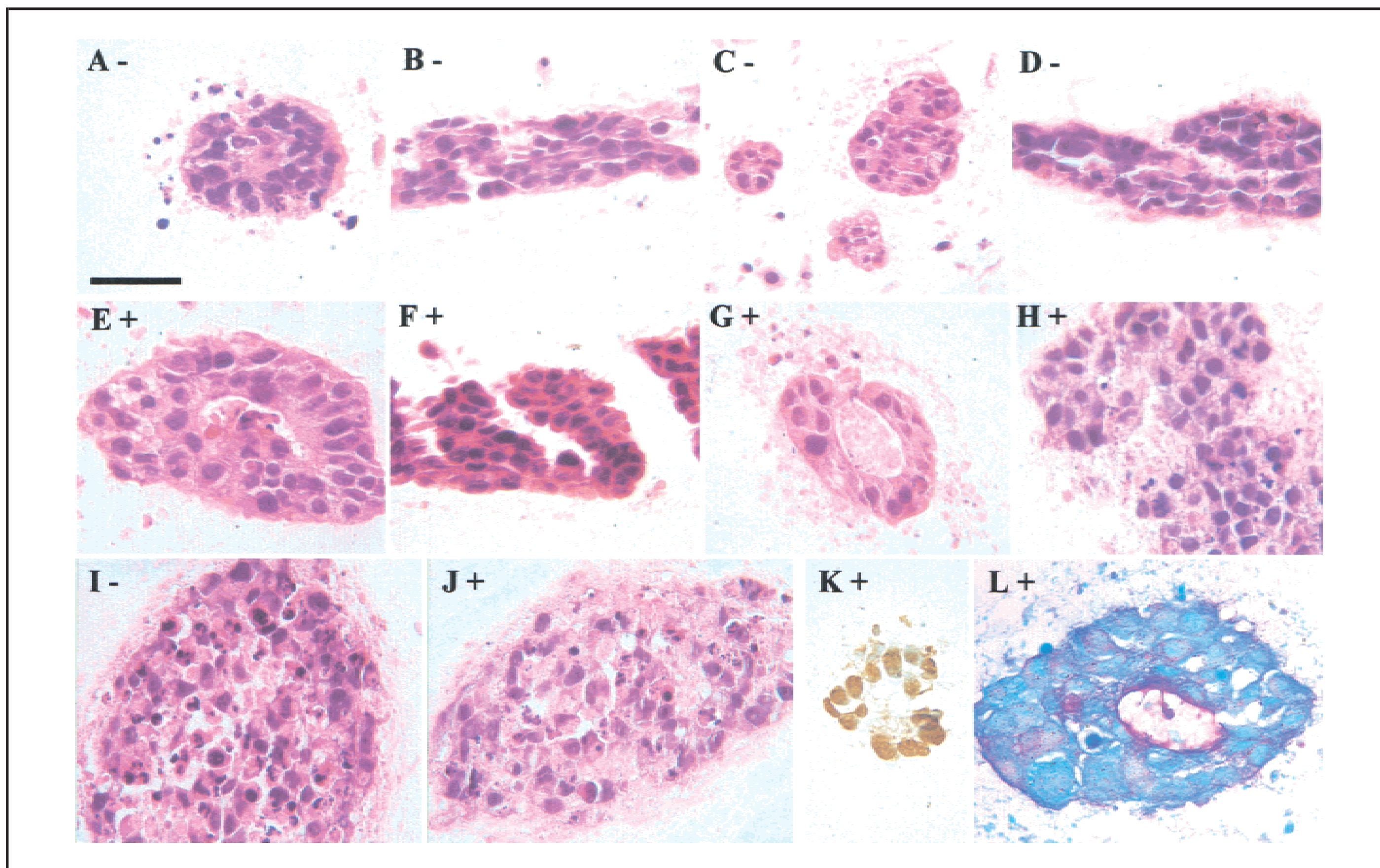

Fig. 2. Retinoid effects on ovarian carcinoma organotypic cultures. OVCAR-3 (**A, B, E, F, K** and **L**), Caov-3 (**C** and **G**), SK-OV-3 (**D** and **H**), and O2 (**I** and **J**) organotypic cultures were grown in the absence (–, **A** to **D** and **I**) or the presence (+, **E** to **H** and **J** to **L**) of retinoid SHetA4 at a concentration of 1 μ*M* in the medium. All sections were stained with hematoxylin–eosin except for **I,** which was stained with periodic acid–Schiff stain, and **J,** which was stained with the TUNEL assay (i.e., terminal deoxynucleodidyl transferase-mediated biotin-deoxyuridine triphosphate nick-end labeling). Original magnifications are ×20, and the **scale bar** in **A** represents 0.05 mm for all pictures.

and act through receptor-independent mechanisms (Fig. 1; Liu S, Kamelle S. Guruswamy S, Dhar A, Klucik J, Brown C, et al.: manuscript in preparation). The six retinoids that caused a less than 50% cell decrease similar to 9-*cis*-RA inhibit tumor cell growth and exhibited a wide range of RAR specificities [Fig. 1; receptor-active retinoids *(32,33)*]. Two of the receptor-active retinoids were classified as apoptotic on the basis of their activities in further experimentation as described in the next sections.

Ovarian Organotypic Cultures

The objective of this study was to determine the effects of clinically achievable concentrations of these retinoids on ovarian tissue. Monolayer cultures of cells are not accurate representations of *in vivo* tissue because they lack the types of interactions that cells have with other cells and with the extracellular matrix *in vivo.* These interactions are known to influence gene expression and, thus, the constitution and behavior of cells. We have developed an ovarian organotypic model system that allows ovarian tissue growth inside as well as on top of a collagen gel (Fig. 2, A and B). The growth of tumor cells inside the collagen allows a representation of tumors that have invaded stromal tissue. In the presence of fibroblasts, established cell lines and primary cultures of ovarian cancer formed colonies inside the collagen, while normal ovarian epithelial cells remained as single cells inside the collagen. The primary ovarian carcinoma cultures did not consistently form colonies in the absence of added fibroblasts. Fibroblasts were, therefore, included in all experiments to standardize the procedure.

In this study, the three cell lines and the two primary cultures derived from serous papillary (O2) and benign (O3) ovarian tissue specimens were cultured inside and on top of a collagen gel seeded with 3T3 fibroblasts for 1 week. After a further 2 weeks of incubation in the presence or absence of 1 μ*M* retinoid (medium concentration), the cultures were fixed and sectioned for histochemical and immunohistochemical analyses (Fig. 2). The neoplastic cultures consisted of scattered single cells and colonies inside the collagen. The OVCAR-3 and Caov-3 cell lines consistently formed colonies inside the collagen, while the SK-OV-3 cell line formed colonies in only two of five identical experiments. The primary O2 culture formed much larger colonies than the cell lines, and the O3 benign culture remained as single cells. Only the OVCAR-3 cell line consistently formed multilayered sheets of surface epithelium. The histologic and morphologic features of each culture are described in Table 1. In general, the arrangement of cells inside the colonies was chaotic with crowding and no discernible pattern. Central areas of necrosis were evident in some of the colonies, as shown in the example in Fig. 2, A.

Retinoid treatment of the ovarian cancer cell line cultures reversed characteristics associated with the cancerous phenotype (Fig. 2 control cultures in the top row versus treated cultures in

the middle row, summarized in Table 1). In general, this was observed as follows: increased cytoplasm, decreased nuclear-to-cytoplasmic ratio, decreased nuclear pleomorphism, decreased size and number of nucleoli, and a more orderly organization of the cells. In retinoid-treated OVCAR-3 and Caov-3 cultures, the majority of colonies exhibited central areas containing debris and some apoptotic cells. Rare, well-formed glands were observed as cells arranged around a central space, with the individual cells having the cytoplasm pointed toward the space and their nuclei located at the outer periphery of the cells (Fig. 2, E and G). To determine if the observation of glandular structures in the presence of retinoid treatment and not in the controls was due to sampling error, the experiment was repeated with larger numbers of cultures. Six parallel organotypic cultures of the OVCAR-3 cell line were grown and divided into two groups of three cultures each. One group was treated with the solvent control and the other with one of the most potent differentiating-inducing retinoids, SHetA4. Pathologic review noted 27 glandular structures in 458 colonies (6%) in sections of the SHetA4-treated cultures and in none in the untreated cultures. Three repeats of this experiment found a statistically significantly higher number of glands in the treated cultures, with an average of 0.33 ± 0.58 (95% confidence interval [CI] = 1.10 to 1.77) glands per section in the untreated cultures and 6.67 ± 1.53 (95% CI = 2.87 to 10.46) glands per section in the treated cultures (P = .01; two-tailed t test). This indicates that the observance of glandular structures in the treated cultures is not a random event.

Retinoid treatment also reversed the cancerous phenotype of the O2 primary serous papillary cultures but did not induce glandular formation (Fig. 2, I and J).

Apoptosis

Microscopic evaluation of H & E-stained sections revealed that, in the absence of retinoids, apoptosis occurred predominantly in cells that were located at the periphery of the colonies. In the presence of retinoids, apoptosis was observed at both the center and at the periphery of the colonies. To quantitate retinoid effects on apoptosis, the TUNEL assay was performed and quantitated by blinded pathologic review (Fig. 2, K; Table 2). The OVCAR-3 and Caov-3 control cultures exhibited 10% and 5% cells undergoing apoptosis, respectively. All of the retinoids that were categorized as apoptotic based on the monolayer cytotoxicity assays induced high percentages of cells undergoing apoptosis in the OVCAR-3 cultures. While five of the retinoids classified as growth inhibitory induced low to no increases in apoptosis, two of this class, 9-*cis*-RA and SHet50, induced levels equal to those of the apoptotic class of retinoids. Therefore,

Table 1. Histologic and morphologic features of organotypic cultures*

Culture	Colonies and surface epithelium	Cell morphology
Control OVCAR-3 (Fig. 1, A and B)	The cells were present as single cells and as colonies, in which the cells were arranged chaotically with crowding and marked cohesiveness. The surface epithelium was 4 to 7 cells in thickness. The cells were crowded, jumbled, and appeared tightly cohesive with no discernible pattern.	The nuclear to cytoplasmic ratio was high. The nuclei ranged from oval to spindle to angular and were uniformly hyperchromatic. The minimal cytoplasm that was present was granular.
Retinoid-treated OVCAR-3 (Fig. 1, E and F)	In comparison to the untreated culture, the colonies exhibited less disarray. Rare well-formed glands were present, in which the cells were arranged around a central space with their cytoplasm pointing toward the space and their nuclei located at the outer periphery of the cells. In the surface epithelium, acinar formation and some two-layered papillations were present.	In comparison to the untreated culture, the nuclear to cytoplasmic ratio was decreased, primarily because of increased amounts of cytoplasm. The nuclei were less pleomorphic, and the majority was oval. Nuclear hyperchromatism remained marked.
Control Caov-3 (Fig. 1, C)	The cells were present as single cells and as colonies in which the cells were arranged chaotically with crowding and marked cohesiveness.	The single cells were characterized by eccentric nuclei that were markedly irregular, angular, and hyperchromatic with prominent nuclei. The cytoplasm was modest in quantity and granular to foamy.
Retinoid-treated Caov-3 (Fig. 1, G)	In contrast to the control, there were fewer single cells, and the majority of colonies exhibited central areas of debris and some apoptotic cells. Rare well-formed glands were present with similar arrangements to OVCAR-3 glands described above.	The single cells have modestly pleomorphic hyperchromatic nuclei, which tend to be eccentrically located. The cytoplasm is granular and moderate in quantity.
Control SK-OV-3 (Fig. 1, D)	The malignant cells formed disorderly colonies in two of five experiments. In the other three experiments, the cells remained single. Modest numbers of mitoses were seen.	The nuclear to cytoplasmic ratio was high. The nuclei ranged from oval to anuglar, and numerous nicks could be seen. The nuclei were markedly hyperchromatic and located primarily in the centrum of the cell. The cytoplasm was foamy to granular.
Retinoid-treated SK-OV-3 (Fig. 1, H)	The colonies were smaller than in the control cultures. No mitoses were seen, and modest to marked apoptosis was present.	The nuclear to cytoplasmic ratio was decreased because of the increased amounts of cytoplasm that was eccentrically located. The nuclei were predominantly round to oval but still hyperchromatic.
Control O2 (Fig. 1, K)	The cells were present mostly in large colonies, with very few single malignant cells. The cells within the colonies were crowded and cohesive. Some peripheral cells undergoing apoptosis were noted.	The nuclear to cytoplasmic ratio was very large. Most nuclei were centrally located, irregular, pleomorphic, and hyperchromatic. The cells had minimal cytoplasm.
Retinoid-treated O2 (Fig. 1, L)	In comparison to the control, the cells inside the clumps were more loosely connected but tended to be more orderly and less crowded. No mitoses were seen. Cells undergoing apoptosis were noted primarily in the center of the colonies.	In contrast to the control, the cells had decreased nuclear to cytoplasmic ratios, and the nuclei tended to be oval.

*Organotypic cultures were treated with retinoids for 2 weeks and fixed and embedded in paraffin. Sections were cut and stained with hematoxylin–eosin. This pathologic review was performed by light microscopic examination.

Table 2. Receptor specificity and biologic activities of apoptotic and growth-inhibitory retinoids in OVCAR-3 and Caov-3 organotypic cultures*

		OVCAR-3				Caov-3		
Drug	Specific retinoic acid receptor activation†	TUNEL % positive	Ki-67 % positive	PAS score	MUC1 score	PAS score	TUNEL % positive	Ki-67 % positive
None	Not applicable	10	48	6	1	1	5	82
		Apoptotic retinoids						
SHetA2	No activation	95	40	12	9	1	0	48
4-HPR	No activation	95	18	6	9	2	0	45
SHetA4	No activation	80	44	12	3	2	15	68
9-*c*-RA	All 6 receptors	80	40	2	9	1	0	12
SHetA3	No activation	75	31	4	6	4	0	24
SHet50	All 6 receptors	75	22	4	3	2	0	48
Average		83 ± 9‡	33 ± 11	7 ± 4	7 ± 3	2 ± 1	4 ± 8	46 ± 18
		Growth inhibitory retinoids						
NHet17	All except RARγ	35	32	2	9	2	1	38
OHet26	Inverse agonist	20	18	6	12	2	10	15
NHet90	All 6 receptors	15	36	4	9	9	5	62
OHet72	RXR receptors only	10	34	9	9	2	1	46
Arot21	Not determined	10	22	4	12	1	13	26
Average		18 ± 10‡	28 ± 11	5 ± 2	10 ± 4	3 ± 3	4 ± 5	35 ± 18

*The specific retinoic acid receptors activated by the retinoids are listed in the second column. Ki-67 and TUNEL (i.e., terminal deoxynucleodidyl transferase-mediated biotin-deoxyuridine triphosphate nick-end labeling) values are presented as the percentage of cells that stained positively, and periodic acid–Schiff (PAS) and MUC1 values are presented as a score determined by the number and intensity of positively stained cells as described in the "Materials and Methods" section. Het represents heteroarotinoid and Arot represents arotinoid.

†Activation of individual retinoic acid receptors by heteroarotinoids was published previously as cited in the "Materials and Methods" section.

‡Denotes that the percent TUNEL-positive cells of the OVCAR-3 cultures treated with apoptotic retinoids were statistically significantly greater than those treated with growth-inhibitory retinoids ($P<.0001$; two-tailed t test).

these two compounds were reclassified into the apoptotic group. The group of retinoids classified as apoptotic on the basis of the organotypic culture assay induced significantly higher levels of apoptotic cells than the growth-inhibitory retinoids ($P<.0001$; two-tailed t test). It was predicted that apoptosis would be observed inside the lumina of the glandular structures induced by retinoids, but this was not observed on microscopic evaluation of sections stained by the TUNEL assay. In general, it appeared that the colonies were either differentiating or undergoing apoptosis and that these two activities were not being induced simultaneously in the same colonies.

Ki-67/myb Expression

To determine if both classes of retinoids were capable of decreasing the growth fraction, the sections were immunohistochemically stained for Ki-67, which is considered to be a marker of proliferating cells *(37).* All of the retinoids decreased the percentage of Ki-67-positive cells (Table 2). There was no significant difference between the inhibition of Ki-67 induced by the apoptotic class of retinoids and the growth-inhibitory class of retinoids ($P = .49$; two-tailed t test). No statistically significant correlations were observed between the effects of the retinoids on the Ki-67 and the TUNEL assays (linear regression: $r^2 = .24$ and $P = .24$ for apoptotic retinoids, $r^2 = .04$ and $P = .70$ for growth-inhibitory retinoids, and $r^2 = .00$ and $P = .99$ for all retinoids combined).

Mucin Expression

To determine whether mucin was being produced in these glands, sections of the organotypic cultures were stained with PAS. As can be seen in Fig. 2, L, positive PAS staining was observed in some cells and inside the glandular lumina. In the absence of retinoids, OVCAR-3 expressed high levels of mucin, with a score of 6 of 12; Caov-3 expressed a low level, with a score of 1 of 12; and neither SK-OV-3 nor the primary serous ovarian cancer culture O2 produced mucin. Both classes of retinoids increased PAS staining of mucin. The intensity and percentage of cells staining PAS positive were estimated semiquantitatively by pathology review and were used to generate a score as described in the "Materials and Methods" section (Table 2). The levels of PAS staining in the cultures treated with the different classes of retinoids were not statistically significantly different (two-tailed Mann–Whitney U tests; $P = .66$ for both OVCAR-3 and Caov-3) and did not correlate with the results of the TUNEL or Ki-67 assays as separate groups or as a single group (Spearman correlations: PAS and TUNEL—$r = .31$ and $P = .50$ for the apoptotic retinoids, $r = -.56$ and $P = .24$ for the growth-inhibitory retinoids, and $r = .15$ and $P = .65$ for all retinoids combined; PAS and Ki67—$r = .26$ and $P = .60$ for apoptotic retinoids, $r = .15$ and $P = .80$ for growth-inhibitory retinoids, and $r = .23$ and $P = .47$ for all retinoids combined).

MUC1 Expression

To evaluate the role of the MUC1 mucin protein in the mechanism of retinoid action, MUC1 expression was evaluated in treated and untreated OVCAR-3 cultures by immunohistochemistry. Clumps of cells in the untreated cultures exhibited low to undetectable levels of MUC1 protein, with a score of 1 of 12 (Fig. 3, A). In contrast, MUC1 was highly expressed on the luminal surface of cells in the glandular structures that developed in retinoid-treated cultures (Fig. 3, B and C). Scoring of the intensity and percent positive cells revealed that all of the retinoids increased MUC1 expression. The greater induction of MUC1 by the growth-inhibitory retinoids in comparison to the apoptotic retinoids approached statistical significance (Table 2; two-tailed Mann–Whitney U test; $P = .05$). The effects of ret-

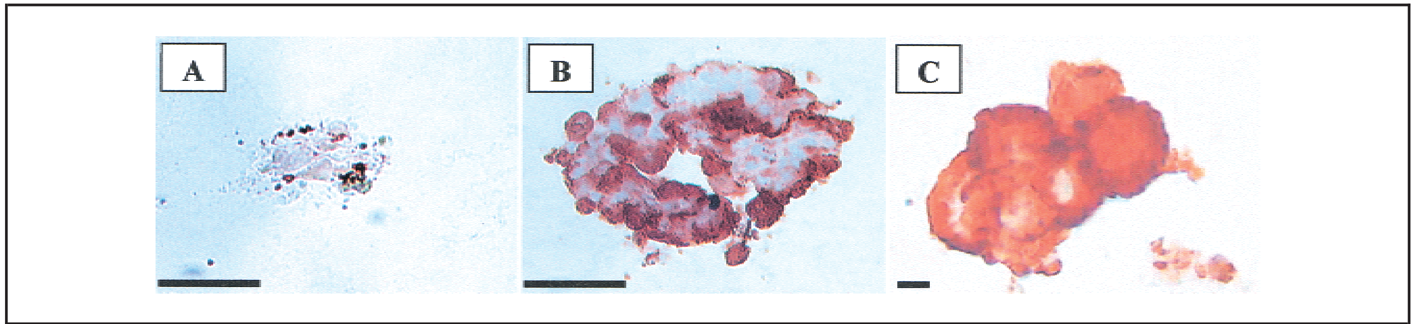

Fig. 3. Retinoid effects on MUC1 expression in OVCAR-3 organotypic cultures. Cultures grown in the absence (**A**) or presence (**B** and **C**) of SHetA4 were photographed at 25× (**A** and **B**) to demonstrate induction in a single glandular structure (**B**) and 4× to demonstrate multiple glands (**C**). The **scale bar** in the lower left-hand corner of each figure represents 0.05 mm.

inoids on the PAS scores and the MUC1 scores did not correlate, suggesting that additional MUCs are involved in the mechanism of retinoid induction of PAS staining (Spearman correlation: $P = .84$ for apoptotic retinoids, $P = .80$ for growth-inhibitory retinoids, and $P = .64$ for all retinoids combined). Grouping all of the retinoids together did not demonstrate a correlation between MUC1 staining and TUNEL, PAS, or Ki-67 (two-tailed Spearman correlation: $r = -.14$ and $P = .67$ for TUNEL, $r = -.15$ and $P = .64$ for PAS, and $r = -.53$ and $P = .08$ for Ki-67). Of interest, when the two classes of retinoids were evaluated separately, the effects of the growth-inhibitory retinoids exhibited a statistically significant correlation between increased MUC1 expression and decreased Ki-67 but not between MUC1 induction and the TUNEL assay (two-tailed Spearman correlation: $r = -.93$ and $P = .02$ for MUC1 and Ki-67 and $r = .20$ and $P = .71$ for MUC1 and TUNEL). In contrast, the apoptotic retinoids exhibited a statistically significant correlation between an increase in MUC1 and the TUNEL assay but not between MUC1 and Ki-67 (two-tailed Spearman correlation: $r = .83$ and $P = .03$ for MUC1 and TUNEL and $r = -.49$ and $P = .27$ for MUC1 and Ki-67).

Discussion

This study demonstrated that clinically achievable concentrations of retinoids decreased the growth fraction of and induced glandular differentiation and apoptosis in ovarian cancer cells and tissue. The induction of glandular differentiation was accompanied by an increase in mucin expression. This is, to our knowledge, the first report of induction of glandular differentiation and mucin expression by retinoids in ovarian tissue and has substantial clinical implications. Efforts to treat acute promyelocytic leukemia by inducing differentiation with RA have been successful *(38).* Although glandular differentiation is not a normal feature of the single layer of surface epithelium from which epithelial ovarian tumors arise, the tumors are often glandular in nature. Pathologists use the type of glandular formation observed in H & E-stained sections of ovarian tumors to classify the tumors as serous, mucinous, or endometrioid and to determine the degree of differentiation. Well-differentiated tumors are considered to be less aggressive and are associated with better patient prognosis than are poorly differentiated tumors *(39).* The induction of glandular differentiation and a glandular MUC1 expression pattern by retinoids in ovarian cancer tissue demonstrates reversal of the tumorigenic phenotype. The overall effect of retinoids on the ovarian cancer cell morphology (decreased nuclear-to-cytoplasmic ratios, decreased nuclear pleomorphism, and decreased size and number of nucleoli) also demonstrates a reversal of the tumorigenic process.

These results stimulate questions regarding the role of differentiation and mucin expression in ovarian tumorigenesis and the potential of the retinoid differentiation-inducing activities to effectively prevent ovarian tumorigenesis and metastases. Epithelial mucins are a family of glycoproteins characterized by pleomorphic tandem repeat peptide sequences and *O*-linked carbohydrate side chains. Increased production of aberrantly glycosylated mucins has been observed in both malignant and low-malignant potential ovarian tissues as well as in many adenocarcinomas, including those in the breast, pancreas, lung, and colon *(40–43).* Although there have been no studies of the regulation of mucin expression by retinoids in the ovary, there have been reports of such regulation in other organ sites. Vitamin A and its natural metabolite, RA, are required for the normal growth and differentiation of epithelium. During vitamin A deprivation, the normal mucosecretory differentiation of trachea undergoes metaplasia to a squamous type of differentiation, and the normal vaginal and cervical squamous differentiation undergoes metaplasia to a columnar type of differentiation *(44).* Decreased expression of mucin genes has been observed during vitamin A-induced squamous metaplasia of tracheobronchial epithelium *(45).* Synthetic retinoids and RA can restore normal differentiation in these tissues in association with restoration of the normal expression pattern of mucin genes *(45,46).*

There is substantial evidence implicating MUC1 in the metastatic process. MUC1 is the best characterized mucin protein and is expressed on the apical surface of most normal glandular cells. The pattern of MUC1 expression and glycosylation in tumor tissue has been found to differ substantially from that found in normal tissue. Since the architecture of tumor tissue is usually disrupted, MUC1 is often expressed in an unorganized fashion throughout the cell surfaces in tumors, in contrast to the polarized expression limited to the apical surface of normal glands *(47).* In addition, the MUC1 proteins found on tumor cells are underglycosylated, with fewer and less complex carbohydrate side chains *(48).* The increased expression of aberrantly glycosylated MUC1 has been implicated in increasing metastatic potential *(49–51).*

In this study, the induction of MUC1 expression suggests caution with regard to using retinoids in the treatment of advanced disease with metastatic potential. The induction of MUC1 expression in this study was associated with the strongest

staining at the surface of glandular lumina, which is the pattern of expression in normal tissue. In contrast, increased MUC1 expression observed in tumors occurs in an unorganized pattern with expression on all of the cell surfaces *(47)*. Therefore, retinoid induction of glandular patterns of MUC1 expression may not increase metastasis, as has been suggested for the deregulated patterns of MUC1 expression observed in tumors. Retinoids offer substantial promise as chemopreventative agents, with the potential to act earlier than the metastatic phase of tumorigenic progression. The role of MUC1 in growth inhibition is supported by the statistically significant correlation between MUC1 induction and decreased Ki-67 expression after treatment with the growth-inhibitory retinoids; this role is clouded, however, by the lack of correlation of these two parameters by the apoptotic retinoids. Perhaps the final measurement of MUC1 over time in apoptotic retinoid-treated cultures is reduced relative to growth-inhibitory retinoid-treated cultures by loss of the most responsive cells because of apoptosis, which is suggested by the greater induction of MUC1 by the growth-inhibitory retinoids in comparison to the apoptotic retinoids.

The role of MUC1 in both growth inhibition and apoptosis suggests that these two activities share a common upstream pathway that branches off after MUC1 induction. The similar levels of Ki-67 repression by both classes of retinoids support this suggestion and are consistent with the observation that apoptotic retinoids can act similarly to classic retinoids at low concentrations. The simultaneous induction of MUC1 and inhibition of Ki-67 by retinoids in this study are consistent with the inverse relationship between growth and differentiation. While both classes of retinoids share these activities, the apoptotic retinoids appear to have additional functions that result in apoptosis. The correlation of MUC1 and apoptosis induction by the apoptotic retinoids may be a consequence of the potencies of individual retinoids and may not be due to a direct role of MUC1 in the mechanism of retinoid-induced apoptosis. Future studies are planned to test if MUC1 and other mucin genes are mediators or consequences of ovarian tumorigenesis and retinoid action.

The role of RXRs in the mechanism of retinoid action was tested with compound OHet72, a rexinoid that is specific for RXRs *(31)*. Substantial inhibition of Ki-67 staining and induction of PAS staining in OHet72-treated cultures indicate that RXR activation is sufficient for the decrease in growth fraction and induction of differentiation. This result does not rule out a role for RARs in these activities, however, because the complex interplay of these receptors as heterodimers suggests that some of the biologic activities of these receptors may be redundant. The potent induction of differentiation by the apoptotic group of retinoids could be due to indirect activation of RARs as is known to occur with 4-HPR *(16)*. The lack of induction of apoptosis by the rexinoid OHet72 suggests that RXR activation is not sufficient to induce this activity. However, although receptor-specific compounds can be used to rule out the requirement of nonactivated receptors in indirect biologic activities, their structural limitations may prevent sufficient potency on the activated receptors to induce the indirect biologic activity. Therefore, although the structure of OHet72 confers specificity for RXRs, it may not induce sufficient potency for induction of apoptosis.

Comparison of the heteroarotinoid structures with their biologic activities revealed that the compounds containing ester linkages were unable to induce apoptosis. The ester linkage is more freely rotating than the corresponding linkages of the apoptotic heteroarotinoids *(30)*. The apoptotic retinoids appear to hold greater promise as chemoprevention agents, since they induce similar levels of growth inhibition and differentiation induction as the growth-inhibitory retinoids, while inducing statistically significant greater levels of apoptosis. The observation that clumps of OVCAR-3 cells inside the collagen were undergoing either differentiation or apoptosis is consistent with the heterogeneous population of cells in this cell line. The clumps arise from single cells that have a variety of abnormalities. Our results suggest that retinoids will induce differentiation in cells that have retained differentiating potential and yet do not differentiate unless an appropriate stimulus is received. On the other hand, if the cell has lost differentiation potential, retinoids will induce the cell into the pathway of a natural form of cell death, apoptosis.

The SK-OV-3 cell line consists of a heterogeneous population of cells that exhibit resistance to retinoid treatment in monolayer culture *(52,53)*. In this study, increased amounts of cytoplasm and more rounded nuclei were induced in SK-OV-3 organotypic cultures by retinoids. Despite these improvements in cellular morphology, no glandular structures were observed, suggesting that this cell line may be too dedifferentiated to respond to retinoids at a histologic level. It also exhibited undetectable levels of basal or induced apoptosis, which may be a result of the lack of p53 protein expression in this cell line. These results demonstrate that organotypic cultures can be used to evaluate retinoid activities, such as histologic changes and observation of glandular structures, that are not detectable in monolayer cultures. In addition, two of the retinoids that did not induce apoptosis in monolayer cultures, 9-*cis*-RA and SHet50, were capable of inducing apoptosis in organotypic cultures.

In conclusion, clinically achievable concentrations of retinoids reverse the cancerous phenotype, decrease the growth fraction, and induce MUC1 expression in ovarian organotypic cultures. A subset of retinoids exhibits the additional activity of apoptosis induction. MUC1 induction is implicated in the mechanisms of apoptosis and growth inhibition. These activities demonstrate substantial promise for the effectiveness of these compounds as ovarian cancer chemoprevention agents.

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