Cultured Human Uterine Smooth Muscle Cells Are Retinoid Responsive (44113)

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Abstract. **Primary cultures of human uterine smooth muscle cells have been widely used as a model system to evaluate agents that may play a role in the regulation of both normal and abnormal proliferative responses. We have used this *in vitro* system to determine if human uterine smooth muscle cells are responsive to treatment with a potent natural derivative of vitamin A, all-*trans* retinoic acid (ATRA). These studies were also designed to determine if there is a difference in retinoid responsiveness between normal smooth muscle and adjacent leiomyoma (a benign tumor of uterine smooth muscle). When cells were cultured in the presence of ATRA, a dose-dependent inhibition in proliferation was observed. This inhibition in proliferation was accompanied by an alteration in smooth muscle cell morphology. Both the inhibition in proliferation and the altered morphology were reversible when ATRA treatment was discontinued. Responsiveness to retinoids is determined, in part, by the expression of ligand-specific receptors belonging to the steroid/thyroid superfamily (RARs and RXRs); we have therefore identified the pattern of retinoid receptor transcript expression in human uterine smooth muscle cells. The data indicate that human uterine smooth muscle cells express retinoic acid receptors RAR α, β, and γ, and retinoid X receptors RXR α and β. No difference in retinoid responsiveness or in the pattern of retinoid receptor expression was observed between normal smooth muscle and adjacent leiomyoma. This is the first observation of an antiproliferative effect of ATRA in uterine smooth muscle cells and the first report of retinoid receptor expression patterns in this cell type. Since retinoids are common pharmacologic tools in the treatment of a wide variety of hyperproliferative disorders, these observations may have both therapeutic and toxicologic implications.** [P.S.E.B.M. 1997, Vol 215]

The importance of vitamin A and its naturally occurring derivatives (retinoids) in the maintenance of normal growth, differentiation, vision, and reproduction is well documented (1–3). Pharmacologically, both natural and synthetic retinoids have been used in the treatment of a number of hyperproliferative disorders, including psoriasis, acute promyelocytic leukemia, and cervical cancer (4–6). Retinoids have also been suggested as adjuvant therapy in the treatment of breast cancer (7). Although epithelial cells have historically been viewed as the major target of retinoid action, cells of mesenchymal origin also respond to retinoid treatment (8, 9). We have recently determined that administration of all-*trans* retinoic acid (ATRA) to immature rats inhibits estrogen induced uterine stromal and myometrial cell proliferation without an equivalent effect on epithelial cell proliferation (10). Since the retinoid was administered systemically in those studies, it was not possible to determine if the antiproliferative effect was due to a direct action on uterine cells or was secondary to retinoid effect at other sites. The current study was designed to determine if ATRA has a direct effect on uterine smooth muscle cells.

Primary cultures of human uterine smooth muscle cells have been widely used as a model system to evaluate agents that may play a role in the regulation of both

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normal and abnormal proliferative responses, and thus are an ideal system in which to assess the direct effects of ATRA on uterine smooth muscle cells. Previous studies have identified a number of growth factors (e.g., IGF, EGF, PDGF, etc.) that stimulate uterine smooth muscle cell proliferation *in vitro* (11, 12). However, few studies have identified agents that may inhibit proliferation. To date, little is known about the responsiveness of normal uterine smooth muscle cells to *in vitro* retinoid treatment. However, recent studies have indicated that micromolar concentrations of retinoic acid inhibit the proliferation of aortic smooth muscle cells (13). In addition, *in vitro* studies have demonstrated that high concentrations of retinoic acid inhibit the growth of a uterine leiomyosarcoma cell line (14). These and our *in vivo* data suggest that human uterine smooth muscle cells may be retinoid responsive. Identifying the human uterus as a potential target of retinoid action may have both therapeutic and toxicologic implications.

Cellular responsiveness to ATRA is determined, in part, by the expression of ligand-activated receptors belonging to the steroid/thyroid superfamily (15–20). Two families of retinoid receptors have been identified, the RARs and the RXRs (reviewed in Refs. 19 and 20). These receptors differ in their ligand specificities (RARs bind both all-*trans* and 9-*cis* retinoic acid, while RXRs bind only 9-*cis* retinoic acid with high affinity) and their ability to interact with other members of the steroid receptor superfamily (21, 22). Differential expression of RARs and RXRs, and differential production of retinoid receptor ligands help to determine the retinoid responsiveness of a given cell type and hence the balance between proliferation and differentiation. Both epithelial and stromal cells of the human uterus have been shown to express retinoid receptors (23, 24); the present study has determined the pattern of retinoid receptor expression in human uterine smooth muscle cells.

These studies were designed to determine if human uterine smooth muscle cells respond to retinoid treatment. The data indicate that all-*trans* retinoic acid inhibits the *in vitro* proliferation of human uterine smooth muscle cells in a dose-dependent, reversible manner. We have also assessed retinoid receptor mRNA expression patterns in these cells. The results of RNAse protection assays indicate that human uterine smooth muscle cells express both RARs and RXRs. These studies have also determined that cultures derived from normal smooth muscle and those derived from adjacent benign uterine smooth muscle tumors (leiomyomas) are retinoid responsive. These data provide the first evidence that primary cultures of human uterine smooth muscle cells are retinoid responsive.

Materials and Methods

Tissue Collection and Myometrial Smooth Muscle Cell Isolation. Portions of normal uterine smooth muscle and adjacent leiomyoma were obtained from nonpregnant premenopausal women undergoing hysterectomy for medically indicated reasons, excluding endometrial cancer. Tissues were collected under sterile conditions into ice-cold culture medium without $NaHCO_3$, containing antibiotics (penicillin/streptomycin, 100 U/ml). Use of tissues for these experiments was approved by the University of Texas institutional review board, with informed patient consent; no identifiers were attached to any specimens. Representative samples were taken from distinct nondegenerating subserosal or intramural leiomyomas and from a separate site for normal myometrium. Tissues were obtained from three individuals, in three different phases (proliferative, secretory, and ovulatory, respectively) as determined by menstrual calendar dating. Each patient served as her own control for the purpose of determining any difference in retinoid responsiveness between normal and leiomyomatous cells.

Samples of leiomyoma and myometrium from the same patient were dissected from endometrial cell layers, washed in phosphate-buffered saline (PBS) and minced into small pieces under sterile conditions. Tissues were transferred to collagenase solution (2.5 mg/ml in PBS containing Ca and Mg) and incubated at 37°C for 3–4 hr. The isolated smooth muscle cells were centrifuged at 500*g* for 3 min, washed twice with PBS to remove collagenase, and resuspended in 20 ml Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). Cells were maintained in DMEM with 10% FBS with 100 U/ml penicillin/streptomycin at 37°C in an atmosphere of 95% air and 5% CO_2. For all experiments, cells were used in early passage (passages 3–6) to retain as much of the normal phenotype as possible. Both α actin and desmin staining was used to verify the smooth muscle phenotype of all cultures.

Cell Proliferation Assay. Confluent cultures of each pair of early passage cells were trypsinized with 0.05% trypsin/EDTA (Gibco, Grand Island, NY) and seeded into 24-well tissue culture plates at a density of 1×10^4 cells/well; triplicate wells were used for each treatment in each time point. Cells were allowed to adhere overnight, then were treated under reduced lighting conditions with concentrations of all-*trans* retinoic acid (ATRA) ranging from 10^{-7} to 10^{-11} *M*. ATRA was diluted in 100% ethanol from a stock solution solubilized in DMSO at 20 mg/ml. Stock solutions of ATRA were made prior to each experiment, and stored in light protected containers at −20°C until use. Controls received an equivalent dose of DMSO diluted in 100% ethanol. The final concentration of ethanol in each well was <0.1%. Medium (and retinoid) was changed every 48 hr with termination of the experiments at 9 days of culture. At each time period media was aspirated from each well and cells washed with PBS. Cells were then

trypsinized and counted in a hematocytometer; each well was counted twice. Cell viability was determined using trypan blue exclusion.

For reversibility experiments, cells were cultured in the presence of ATRA (10^{-7}–10^{-11} M, see above) for 6 days. On Day 6, ATRA treatment was stopped, and the cells were incubated in control media for an additional 6 days. As above, media was changed every 48 hr. The experiment was terminated at Day 12. At each time period, media was aspirated from each well and cells washed with PBS. Cells were then trypsinized and counted in a hematocytometer; each well was counted twice. Cell viability was determined using trypan blue exclusion.

RNAse Protection Assay. Paired normal and leiomyomatous cells were passaged into 60-mm culture dishes and grown to confluence. For each sample, RNA was obtained from three to four culture dishes per treatment group. To verify the integrity of RNA to be used for RNAse protection assays, samples of total RNA (10 μg) from each group was denatured for 30 min in 15 mM methylmercuric hydroxide (Johnson Matthey Electronics, Ward Hill, MA) and separated on a 1% agarose gel containing 20% (v/v) formaldehyde. After electrophoresis, gels were stained with ethidium bromide and the ribosomal bands viewed under ultraviolet light to verify the integrity of RNA.

The RNAse protection assay was performed as previously described (25). Ten micrograms of total RNA was used per probe in each assay. [^{32}P]UTP-labeled receptor subtype specific antisense riboprobes of human RAR α, RAR β, RAR γ, RXR α, RXR β, and γ actin were generated by inserting 100- to 150-bp–long fragments of their respective cDNAs into a pGEM4Z vector. Autoradiograms were scanned and digitalized, and the relative intensity of the protected bands was normalized to the intensity of the bands of the internal γ-actin control.

Chemicals. All-*trans* retinoic acid (Sigma Chemical Co., St. Louis, MO) was solubillized in 100% DMSO under reduced lighting conditions. Aliquots of this stock were frozen in light-protected containers and thawed immediately prior to use. All other chemicals and regents used were of the highest quality available.

Statistical Analysis. Statistical significance was determined at $\alpha < 0.02$. Comparison of means was achieved using the Student t test.

Results

The effect of ATRA treatment on the proliferative responses of normal and leiomyoma cells in culture was assessed in a standard proliferation assay. Autologous cultures were incubated in the presence or absence of varying concentrations of ATRA for 9 days. The effects of three dosages of ATRA (10^{-7}, 10^{-9}, and 10^{-11} M) was quantitated at all time periods. The effect of 10^{-9} M ATRA (or the corresponding vehicle control) on cell proliferation is described in Figure 1. The dose-response curves of ATRA treatment are presented in Figure 2. The data indicate that proliferation of both normal smooth muscle cells and of those derived from adjacent leiomyoma was inhibited by 10^{-7} and 10^{-9} M ATRA. No inhibition was seen at 10^{-11} M ATRA.

In addition to its antiproliferative effect on uterine smooth muscle, ATRA treatment dramatically altered uterine smooth muscle cell morphology. Normally, uterine smooth muscle cells in culture grow in parallel arrays of long fusiform cells (Fig. 3A). Cells grown in the presence of ATRA (at both 10^{-7} and 10^{-9} M), however,

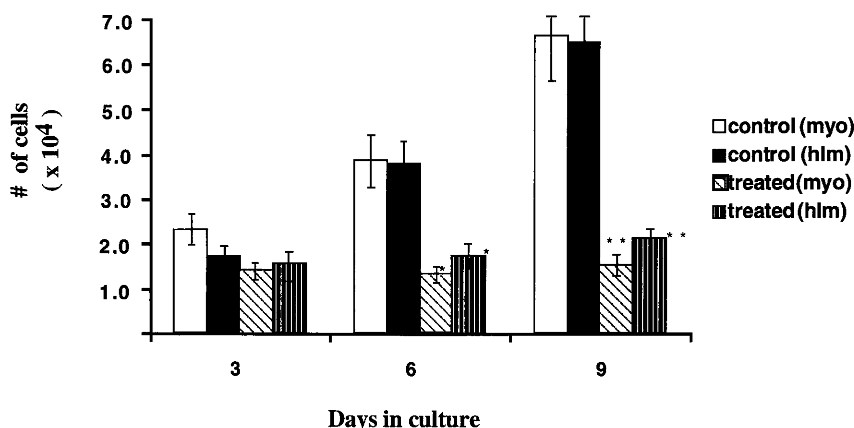

FIGURE 1. The effect of ATRA on human uterine smooth muscle cell proliferation. Proliferation assays were performed as detailed in Materials and Methods. Briefly, smooth muscle cells derived from normal myometrium (myo) or the adjacent leiomyoma (hlm) were plated into 24-well culture dishes at approximately 1×10^4 cells/well and grown in DMEM supplemented with 10% FBS in the presence of the vehicle control or different concentrations of all-*trans* retinoic acid. At various time intervals, cells were washed with PBS, trypsinized, and counted in a hematocytometer. For clarity, this figure represents the effects of treatment with 10^{-9} M ATRA versus the vehicle control. Data are the results of three separate triplicate experiments from three different patients and are expressed as the number of cells ± SEM. *$\alpha < 0.01$ versus vehicle-treated control; **$\alpha < 0.001$ versus vehicle-treated control.

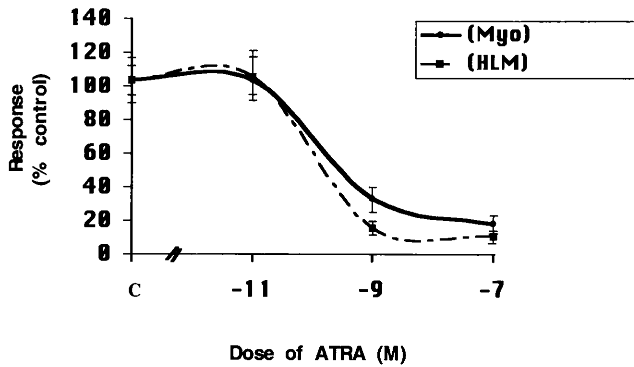

FIGURE 2. ATRA inhibits human uterine smooth muscle cell proliferation in a dose-dependent manner. Smooth muscle cells derived from normal myometrium (Myo) or the adjacent leiomyoma (HLM) were plated into 24-well culture dishes and grown for 9 days in the presence of varying concentrations of ATRA or in the presence of the vehicle control. At the end of the experiment, cells were washed with PBS, trypsinized, and counted in a hematocytometer. Data are the results of three separate triplicate experiments from three different patients and are expressed as a percentage of the Day 9 control value ± SEM.

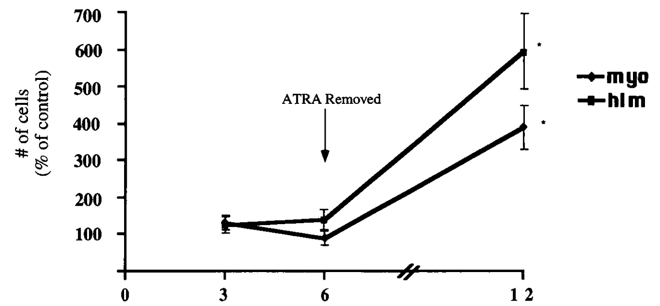

FIGURE 4. The effect of all-*trans* retinoic acid on uterine smooth muscle cell proliferation is reversible. Smooth muscle cells derived from normal myometrium (myo) and the adjacent uterine leiomyoma (hlm) were plated into 24-well culture dishes and grown for 12 days. For the first 6 days, cells were incubated in varying concentrations of all-*trans* retinoic acid (or the vehicle control, data not shown). On Day 6, all-*trans* treatment was stopped (arrow), and cells were incubated in the presence of DMEM supplemented with the vehicle control for an additional 6 days. At Days 3, 6, and 12, cells were trypsinized and counted in a hematocytometer. Data are expressed as a percentage of the Day 3 control; each point represents three replicates from a single patient ± SEM. The effects of ATRA were reversible in all cultures examined. *$\alpha < 0.01$ versus Day 6 value.

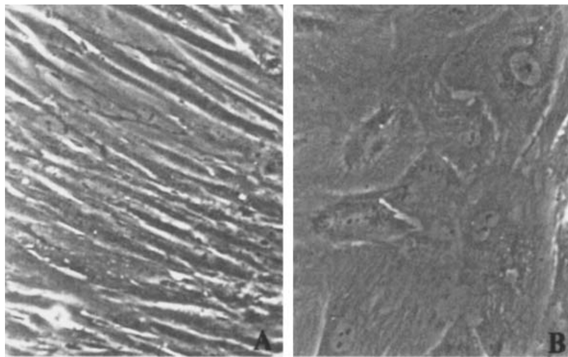

FIGURE 3. ATRA treatment alters uterine smooth muscle cell morphology. Representative phase contrast micrograph of the morphology of normal uterine smooth muscle cells incubated for 6 days in the presence of the vehicle (A) or 10^{-7} *M* ATRA (B). Morphological changes observed with cells derived from adjacent leiomyoma (at both 10^{-7} and 10^{-9} *M* ATRA) were identical to those seen in normal smooth muscle (data not shown).

exhibit an increased cytoplasmic-to-nuclear ratio (Fig. 3B). This altered morphology was observed within 96 hr of exposure to ATRA. These cells retain the smooth muscle cell characteristic α-actin staining and are viable as determined by trypan blue exclusion (data not shown).

To determine if the effect of ATRA on cell proliferation and on cell morphology was reversible, retinoid treatment was discontinued on Day 6, and the cultures given 6 days to recover. A significant increase in cell number is seen 6 days after removal of either 10^{-7} or 10^{-9} *M* ATRA from the culture medium (Fig. 4), with a concomittant reversion of cells to their normal morphology (Fig. 5, compare cellular morphology of Panel B with that of Panel D). Again, no difference was observed beween cells derived from normal uterine smooth muscle and those derived from adjacent leiomyoma.

Retinoid responsiveness is, in part, determined by the expression of ligand-activated receptors belonging to the steroid/thyroid superfamily. Two major classes of retinoid receptors have been identified, the RARs and the RXRs. Each class of receptor includes at least three discrete genes (RAR α, β, and γ, RXR α, β, and γ) encoding distinct proteins. RNAse protection assays were used to determine the pattern of retinoid receptor expression in cells derived from normal uterine smooth muscle and adjacent leiomyoma (RXR γ was not measured). Figure 6 shows the pattern of protected fragments for each of the radiolabeled receptor probes (identified along the margin) following incubation of total RNA from cultured normal or leiomyoma cells. Note that RAR β and γ were electrophoresed in the same lane. Multiple protected bands differing by a single nucleotide are generated for each receptor probe as a result of partial degradation of the ends of the protected fragments ("breathing" of hybrids) (25). Human γ actin was used as an internal standard for each sample (see Materials and Methods).

Discussion

Our previous data indicated that all-*trans* retinoic acid inhibited estrogen-stimulated uterine smooth muscle cell proliferation in an *in vivo* (immature rat) model system. This potent, naturally occurring derivative of vitamin A has been used for the treatment of a number of hyperproliferative disorders—most notably, acute promyelocytic leukemia (APL) (5). Other derivatives of vitamin A have been used as antiproliferative agents to treat psoriasis, cervical cancer, and breast cancer (4,

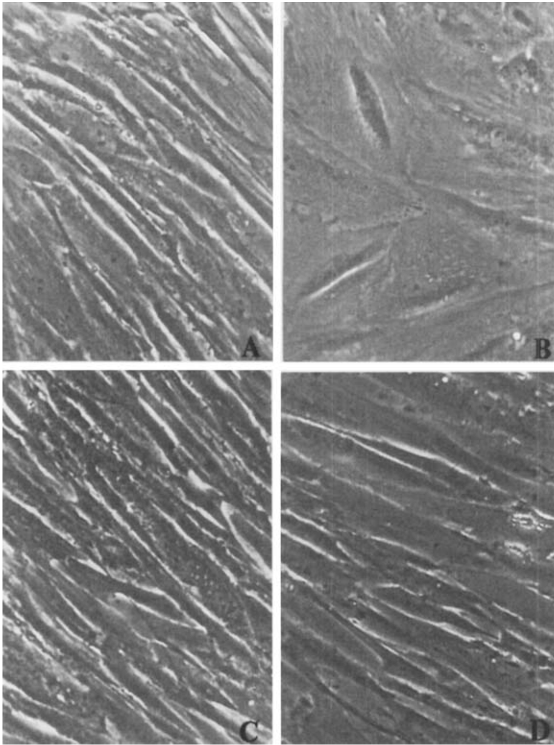

FIGURE 5. The effect of ATRA on uterine smooth muscle cell morphology is reversible. Phase contrast micrographs compare the morphology of cells derived from normal uterine smooth muscle incubated for 6 days in the presence of the vehicle (A) or 10^{-7} *M* all-*trans* retinoic acid (B) with cells incubated for 12 days in the presence of the vehicle (C) or those cells whose all-*trans* treatment was stopped at 6 days and grown for an additional 6 days in the presence of the vehicle (D). Normal uterine smooth muscle cell morphology is observed in Panels A, C, and D. The altered morphology observed in Panel B is completely reversed by the removal of ATRA from the media (cf., Panels B and D). Although not shown, the morphology of cells derived from the adjacent leiomyoma in the context of both the vehicle and the retinoid treatment (and subsequent reversibility) was identical to that of the normal myometrium.

6, 26). Our *in vivo* data, and the utility of all-*trans* retinoic acid as a differentiation-promoting, antiproliferative agent, prompted the current studies to determine if human uterine smooth muscle cells are responsive to ATRA in an *in vitro* setting.

To determine the effect of ATRA treatment on uterine smooth muscle cell proliferation, cells were cultured in the presence of 10% fetal bovine serum. In this manner, determination of retinoid effects in the presence of the wide variety of mitogens (e.g., growth factors, hormones) contained in serum could be assessed. Our data indicate that *in vitro* treatment of human uterine smooth muscle cells with ATRA results in complete suppression of proliferation. This effect is dose dependent and evident at physiological levels (10^{-9} *M*) of ATRA (27). In addition, this effect is accompanied by an alteration in cell morphology. Inhibition of proliferation and alterations in cell morphology with retinoid treatment have been observed in many cell types (28–30), but this is not a universal response of cultured cells to retinoid treatment. Indeed, some fibroblast cell lines are insensitive to ATRA, while proliferation of Swiss 3T3 cells is enhanced by retinoid treatment (reviewed in Ref. 31). Although the physiological significance of this observation has yet to be determined, this is the first study that has identified human uterine smooth muscle cells as potential targets of retinoid action.

Since inhibition in cell proliferation occurred at physiological levels of ATRA (10^{-9} *M*), this effect is unlikely to be due to cytoxicity. The reversibility of both growth inhibition and changes in cell morphology upon discontinuation of retinoid treatment (Fig. 4) supports this observation, although we cannot rule out the possibility that this reversibility may be a "recovery period" from ATRA's sublethal effects. Although we have yet to determine the full time course of reversibility, it is clear that, 6 days after the removal of the retinoid, cells have begun to resume growth and have also resumed the fusiform smooth muscle cell morphology.

Since retinoid responsiveness is partly dependent upon the expression of nuclear retinoid receptors, we examined the retinoid receptor expression patterns in

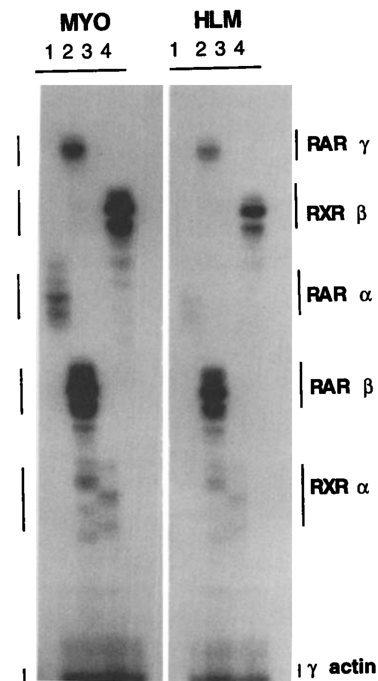

FIGURE 6. RNAse protection assay to identify retinoid receptor expression patterns in human uterine smooth muscle cells. Ten micrograms of total RNA was obtained from autologous primary cultures of cells derived from normal human uterine smooth muscle and adjacent leiomyoma. RNA was hybridized to [^{32}P]UTP labeled retinoid receptor–specific antisense riboprobes. Riboprobes for human RAR α, RAR β, RAR γ, RXR α, RXR β, and γ actin were used for each protection assay. The autoradiogram reflects the mobility of protected fragments of each of the specific retinoid receptors expressed by uterine smooth muscle cells. MYO: Lane 1, RAR α; Lane 2, RAR β and RAR γ; Lane 3, RXR α; Lane 4, RXR β. HLM: Lane 1, RAR α; Lane 2, RAR β and RAR γ; Lane 3, RXR α; Lane 4, RXR β. γ actin is included in each protection assay as an internal loading control.

uterine smooth muscle cells. The expression of all RARs and RXR α and β indicates that uterine smooth muscle cells have the potential to respond to retinoic acid at the transcriptional level. Redfern *et al.* have identified retinoid receptors in both epithelial and stromal cells of the human uterus (23, 24). Thus, all major uterine cell types express retinoid receptors. Taken together, data from our lab and others suggest that vitamin A and its naturally occurring derivatives may play an important, although as yet undetermined, role in uterine biology.

Paired autologous cultures of normal uterine smooth muscle and smooth muscle cells derived from adjacent leiomyoma were used to assess differences, if any, in retinoid responsiveness between normal and hyperproliferative smooth muscle cells. In this manner, each patient served as her own control. Retinoid responsiveness was observed in both normal and fibroid tissue in all samples assayed. Thus, cultured cells derived from leiomyomas have not lost their ability to respond to retinoids. As mentioned in the introduction, prophylactic use of retinoids in combination with tamoxifen have been proposed for breast cancer clinical trials (7). It would be interesting to determine if patients on this regimen have an alteration in the incidence of hyperproliferative disorders of the uterine smooth muscle.

These studies have identified uterine smooth muscle as a direct target of retinoid action. The effect of ATRA treatment on the proliferation of cultured human uterine smooth muscle cells is quite striking, although the mechanism of this effect has yet to be elucidated. Since the inhibition in proliferation is accompanied by a change in cellular morphology, alterations in cell surface characteristics and increased adhesiveness may play a role in this effect. Retinoids have been shown to alter surface properties in a number of cell types and can have profound effects on extracellular matrix production (32, 33). In addition, ATRA treatment has been shown to alter gap junctional communication. In murine cell lines C3H10T1/2 and BALB/c 3T3, dose-response curves for enhancement of gap junctional communication paralleled those for growth suppression (34). In dermal fibroblasts, expression of connexin 43, a member of the multigene family that is involved in gap junction assembly, is increased by ATRA (35). Connexin 43 is also expressed in uterine myometrial cells, but the influence of retinoic acid on connexin 43 expression has yet to be determined. It is clear that further studies are required to assess these and other possible mechanisms for the effect of ATRA on cultured uterine myometrial cells.

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