Identification of Biomarkers Modulated by the Rexinoid LGD1069 (Bexarotene) in Human Breast Cells Using Oligonucleotide Arrays

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Abstract

Retinoids have been found to be promising chemopreventive agents that play an important role in regulating cell growth, differentiation, and apoptosis. The action of retinoids is mediated by retinoid receptors (retinoic acid receptors and retinoid X receptors), which are nuclear transcription factors that, when bound to retinoids, regulate gene expression. LGD1069 is a highly selective RXR agonist that has reduced toxicity compared with retinoids. Our previous studies have shown that RXR-selective ligands (or “rexinoids”), including LGD1069, can inhibit the growth of normal and malignant breast cells and can suppress the development of breast cancer in transgenic mice. For the current study, we attempted to identify biomarkers of the chemopreventive effect of the RXR-selective retinoid LGD1069. In these experiments, we used Affymetrix microarrays to identify target genes that were modulated by LGD1069 in normal human breast cells. Afymetrix and dChip analysis identified more than 100 genes that were up-regulated or down-regulated by LGD1069 treatment. We then tested 16 of these genes in validation experiments using quantitative reverse transcription-PCR and Western blotting of independently prepared samples, and found that 15 of 16 genes were modulated in a similar manner in these validation experiments as in the microarray experiments. Genes found to be regulated include known retinoid-regulated genes, growth regulatory genes, transcription factors, and differentiation markers. We then showed that the expression of several of these retinoid-regulated biomarkers is modulated in vivo in mammary glands from mice treated with LGD1069. These critical growth-regulating proteins will be promising targets of future agents for the prevention and treatment of breast cancer. (Cancer Res 2006; 66(24): 12009-18)

Introduction

Despite significant improvements in early detection and treatment, breast cancer remains the second leading cause of cancer-related deaths in women (1). Thus, more effective strategies for the prevention and treatment of breast cancer are urgently needed. Antiestrogen selective estrogen receptor modulators, such as tamoxifen and raloxifene, and aromatase inhibitors have been shown to be promising agents for reducing the risk of breast cancer (2–5). However, whereas these anti-hormonal therapies are useful for the prevention of estrogen receptor–positive breast cancer, they have no effect on the development of estrogen receptor–negative breast cancer (6, 7). Thus, there remains a critical need to develop agents that will prevent estrogen receptor–negative breast cancer.

Several agents currently being investigated for the prevention of estrogen receptor–negative breast cancer include cyclooxygenase-2 (COX-2) inhibitors, tyrosine kinase inhibitors, and retinoids (8). Although retinoids are promising chemopreventive agents in animals and humans, they are not generally used for cancer prevention because of their toxicity (9, 10). The naturally occurring retinoids affect cellular processes by binding to nuclear receptors, including retinoic acid (RA) receptors (RAR) and retinoid X receptors (RXR), which interact with specific DNA response elements. The RXR can also heterodimerize with other nuclear hormone receptors, such as vitamin D receptor, thyroid hormone receptor, peroxisome proliferator–activated receptors, and others, to produce a variety of complexes that can regulate different sets of target genes (11, 12). Naturally occurring and synthetic retinoids activate different retinoid receptors. All-trans RA binds only RARs, whereas 9-cis RA is an agonist for both RARs and RXRs. The synthetic retinoid LGD1069 is a highly selective RXR agonist with low affinity for RARs (13).

To develop agents that will prevent cancer with reduced toxicity and increased efficacy, we investigated the cancer-preventive activity of RXR-selective rexinoids. We and others have previously shown that the rexinoid LGD1069 suppresses the development of estrogen receptor–positive and estrogen receptor–negative breast cancer in rats and mice (13–16). In addition, we have shown that LGD1069 exhibits similar preventive activity as the pan-agonist 9-cis RA, without the toxicity of this retinoid (17, 18). Our previous studies have revealed that RXR-selective rexinoids inhibit the proliferation of normal and malignant breast cells and suppress mammary tumor development in a transgenic mouse model (16, 18). Here, we describe our investigation of the genes and pathways modulated by LGD1069 to identify critical proteins that can be used as candidate surrogate end-point biomarkers for clinical chemoprevention trials.

The goal of these studies is to identify the set of genes that are modulated by rexinoids in breast cells, the protein products of which regulate growth. In this study, we used expression microarrays to identify target genes that are modulated by LGD1069 in normal breast cells and validated that a subset of these genes are
modulated at the RNA and protein levels in human breast cells. We also showed that some of the genes that were modulated in vitro were also modulated in vivo in mammary glands from mouse mammary tumor virus (MMTV)-erbB2 transgenic mice treated with the retinoid. These markers will be tested as biomarkers of the chemopreventive effect of this retinoid in future cancer prevention clinical trials.

Materials and Methods

Cell Lines and Retinoid Treatment
Normal human mammary epithelial cells (HMEC) were obtained from Clonetics (San Diego, CA). These cells are primary cultures derived from healthy women who had undergone reduction mammoplasties. Cells between passages 6 and 12 were used. Cells were grown and maintained in mammary epithelial basal medium supplemented with 50 μg/ml bovine pituitary extract, 5 μg/ml insulin, 10 ng/ml human recombinant epidermal growth factor, 0.5 μg/ml hydrocortisone, 30 μg/ml gentamicin, and 15 ng/ml amphoterin-β (Clonetics). Cells were cultured in a humidified environment at 37°C with 5% CO₂ in the air.

The RXR-selective retinoid LGD1069 (Targretin; Bezaxotene) was obtained from Ligand Pharmaceuticals, Inc. (San Diego, CA). It was received as a powder and dissolved in DMSO. The cultured cells were treated with 1 μM LGD1069 for 1 and 24 hours, or with 0.1% DMSO alone for vehicle-treated control.

RNA Isolation
Total RNA was isolated using the RNeasy RNA isolation kit from Qiagen, Inc. (Valencia, CA) as described by the supplier. Triplicate RNA samples were independently prepared from each vehicle-treated or retinoid-treated HMECs at 1 or 24 hours. These samples were used for array analysis. For the validation experiments, another set of triplicate RNA samples was independently prepared from another batch of HMEC treated with vehicle or LGD1069 at 1 or 24 hours. Total RNA was also isolated from paraffin-embedded mammary gland tissues from MMTV-erbB2 mice treated with LGD1069 for 4 months using MasterPure complete RNA purification kit (Epicentre Biotechnologies, Madison, WI).

Affymetrix Microarray Experiments
cDNA synthesis and cRNA labeling. Total RNA (10 μg) was used in the first strand cDNA synthesis with 100 pmol of T7-(dT)24 primer [5'-GGCCCGGTAGATATTGATATACGACTCACTATAGGGAGGCGG-(dT)24-3']. Second-strand cDNA synthesis was carried out at 16°C by adding 10 units of Escherichia coli DNA ligase, 40 units of E. coli DNA polymerase I, 2 units of RNase H, and 10 units of T4 DNA polymerase to blunt the ends of newly synthesized cDNA. The double-strand cDNA was purified through phenol/chloroform and ethanol precipitation. To synthesize biotin-labeled antisense cRNA, an in vitro transcription reaction was done using a BioArray HighYield RNA transcript labeling kit (Enzo Diagnostics, Farmingdale, NY). The labeled cRNA was then purified with an RNeasy mini kit (Qiagen) according to the manufacturer’s protocol and by ethanol precipitation. The purified cRNA was fragmented in 1× fragmentation buffer (40 mmol/L Tris-acetate, 100 mmol/L KOAc, 30 mmol/L MgOAc) at 94°C for 35 minutes.

Hybridization and scanning. Fragmented cRNA probe (15 μg) was incubated with 50 μmol/L control oligonucleotide B2, 1× eukaryotic hybridization control (1.5 μmol/L BioB, 5 μmol/L BioC, 25 μmol/L BioD, and 100 μmol/L Cre), 0.1 mg/ml herring sperm DNA, 0.5 mg/ml acetylated bovine serum albumin, and 1× manufacturer-recommended hybridization buffer in a 45°C rotisserie oven for 16 hours to hybridize with the Affymetrix oligonucleotide human GeneChip U95Av2. The chips were washed in a fluidic station and the phycocerythrin-stained array was scanned as a digital image file as described in the Affymetrix GeneChip protocol (Affymetrix, Inc., Santa Clara, CA).

Array data analysis. To assess the quality of the cRNA labeling and hybridization, the scanned image was analyzed using Microarray Suite 5.0 (Affymetrix). The following control factors were observed: low noise (RawQ < 15), low background (<600), low 3’ to 5’ ratios for both actin and glyceraldehyde-3-phosphate dehydrogenase (ratio < 1.5), and the presence of control genes Cre, BioD, and BioC. Arrays that met these quality control criteria were used in low-level analysis using invariant set normalization (baseline chip was 1-hour DMSO-B) and estimation of expression using the model-based expression index (MBEI) algorithm (PM only) as implemented in the dChip Analysis software package (19). To identify retinoid-regulated genes in 1- or 24-hour treatment groups, the probes on the array were filtered for a present call in all three array sets. An initial relatively nonstringent analysis was done to generate a long list of potentially changed genes and to generate a visual display by hierarchical clustering. By comparative analysis between vehicle- and retinoid-treated samples, the genes with lower-bound 90% confidence intervals of >1.2-fold change and with a two-sample t test P < 0.05 were analyzed. Retinoid up-regulated or down-regulated genes were then selected by querying for genes that were up-regulated (fold change ≥1.4; P < 0.05) or down-regulated (fold change ≤ 1.4; P < 0.05) by LGD1069 treatment. Gene lists queried for the Gene Ontologies containing cell cycle, cell growth, apoptosis, stress response, transcription factors, signal transduction and cell communication, ligand binding, and nucleic acid binding were generated using dChip.

Validation of Array Results
Quantitative reverse transcription-PCR. The amount of specific RNA transcripts was assayed by quantitative real-time PCR using gene-specific double fluorescence-labeled probes and an ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA). The PCR reaction mixture consisted of 300 nmol/L of each primer, 100 nmol/L of probe, 0.025 units/μL of Taq polymerase, 125 μmol/L of each deoxynucleotide triphosphate, 3 mmol/L MgCl₂, and 1× Taq polymerase buffer. Cycling conditions were 94°C for 1 minute, followed by 40 cycles at 94°C for 12 seconds and 60°C for 1 minute. All primers and probes were designed with Primer Express 1.0 software (Applied Biosystems). 6-Carboxy-fluorescein (FAM) was used as the 5’ fluorescent reporter whereas tetramethylrhodamine (TAMRA) was added to the 3’ end as the quencher. Standard curves for the quantification of each transcript and β-actin were generated using a serially diluted solution of synthetic templates. Genome equivalent copies were calculated from the standard curve. For each sample, TaqMan PCR reactions were done in triplicate for each gene of interest and for a reference gene (β-actin) to normalize for input cDNA. The ratio between the values obtained provided relative gene expression levels. Statistical significance was determined on triplicate samples using a Student’s t test.

Western blotting analysis. HMEC lysates were prepared by treating with lysis buffer [50 mmol/L Tris-HCl (pH 8.0), 2% SDS, and a protein kinase inhibitor cocktail]. The protein lysates for mouse mammary gland epithelial cells were also prepared from MMTV-erbB2 mice treated with LGD1069 for 4 months. To collect mouse mammary epithelial cells, mammary gland tissues were removed and minced into very small pieces and were thoroughly digested in digestion medium (DMEM/F12, 20 mg/ml collagenase, 100 unit/ml hyaluronidase). The mixtures were then washed five times with the PBS containing 5% adult bovine serum. Total protein lysates were sheared on ice using a 22-gauge needle and centrifuged at 10,000 × g for 30 minutes. The protein concentration of the supernatant was measured by bichinonic acid protein assay (20). Protein (30 μg) was resolved on a 10% SDS-polyacrylamide gel. The proteins were transferred to a nitrocel-lulose membrane, which was then blocked in 5% nonfat dry milk TBST [10 mmol/L Tris (pH 8.0), 150 mmol/L NaCl, 0.05% Tween 20] at room temperature for 1 hour. Membranes were probed with the following primary antibodies in 1% nonfat dry milk/TBST: I-1 (Santa Cruz Biotechnology, Santa Cruz, CA), COX-2 (Cayman, Ann Arbor, MI), insulin-like growth factor binding protein 6 (JGFRP6; Auzel Biologicals, San Ramon, CA), actin (Sigma, St. Louis, MO), keratin (Chemicon, Temecula, CA), and cyclin D1 (NeoMarkers, Fremont, CA). Membranes were then probed with the corresponding horseradish peroxidase–conjugated secondary antibodies in 1% nonfat dry milk/TBST. The blots were visualized using the enhanced chemiluminescence Western blot detection system (Amersham Life Sciences, Piscataway, NJ).
Results

We and others have previously shown that LGD1069 suppresses estrogen receptor–positive (13) and estrogen receptor–negative (16, 18) breast cancer development in preclinical models. To identify potential RXR-modulated biomarkers in human breast cells, we did global gene expression profiling of human breast epithelial cells treated with the RXR-selective retinoid LGD1069 using Affymetrix oligonucleotide microarrays. Normal HMECs were treated with LGD1069 for 1 hour to identify immediate-early gene responses, and for 24 hours to identify late gene responses. Real-time quantitative reverse transcription-PCR (RT-PCR) assays and Western blot analyses were also done to verify critical changes identified by the microarrays using a set of three independently prepared samples.

Analysis of known retinoid-regulated genes. RNA prepared from LGD1069 (1 μmol/L)- or vehicle-treated cells for Affymetrix GeneChip analysis was first used to verify that known retinoid-regulated genes were modulated by this rexinoid using real-time RT-PCR. Known retinoid-regulated genes chosen for investigation were RARβ, retinoic acid hydroxylase (RA-OH), tissue transglutaminase 2 (TGM2), and collagenase (MMP-1; Fig. 1).

The expression of RARβ was not changed after 1 hour of treatment, but was induced ~3-fold in rexinoid-treated HMECs after 24 hours. RA-OH was significantly induced by 24 hours, and TGM2 was up-regulated by LGD1069 treatment at both 1 and 24 hours. The expression of collagenase, a gene previously reported to be down-regulated by LGD1069 (21), was down-regulated (Fig. 1). These RNA samples were then used for Affymetrix oligonucleotide array analysis.

Analysis of gene expression in LGD1069 treated breast cells using oligonucleotide arrays. The total RNA prepared from HMECs was next used to synthesize biotin-labeled cRNAs by in vitro transcription, followed by hybridization to Affymetrix human microarray chips as described in Materials and Methods. The experiments were conducted independently thrice on separate array chips to ensure reproducibility of the microarray results (as shown schematically in Supplementary Fig. S1).

The Affymetrix U95Av2 array set allows >12,000 genes to be screened in a single sample. Approximately 40% of the 12,000 genes on the array were expressed at a detectable level and were called “present” by the analysis program. To identify the maximum number of rexinoid-regulated genes in this first screen, we selected those genes that were found to be called present and that showed a fold change ≥1.2, or ≤−1.2 with P < 0.05. This gene set was then analyzed using hierarchical clustering. We selected those genes that showed a fold change ≥1.4 or ≤−1.4 for further analysis. Comparative data analysis using DNA Chip Analyser (dChip) identified 353 genes (2.9% of total genes) of which the expression was either induced or repressed in response to LGD1069.

Analysis of this microarray data using dChip analysis program showed 213 genes that were significantly (P < 0.05) up-regulated at the early or late times, and 140 genes that were significantly (P < 0.05) down-regulated after treatment with the rexinoid (Fig. 2). To interpret and organize the data, differentially expressed genes were grouped according to their temporal expression pattern using hierarchical clustering (19, 22). As shown in Fig. 2, the replicates of vehicle, DMSO-treated samples and LGD1069-treated samples clustered together (complete annotated results available in Supplementary data).

Genes modulated after 1-hour treatment. We next focused on those genes that were modulated after 1-hour treatment with LGD1069. Relatively few genes were modulated after 1 hour of treatment. Analysis of the 1-hour microarray data revealed 35 genes that were significantly (P < 0.05) up-regulated and 47 genes that were significantly (P < 0.05) down-regulated by the LGD1069 treatment (Supplementary Fig. S2A). The GenBank accession numbers and gene names of the 66 genes with the greatest fold change in expression are listed in Supplementary Table S1.

Several transcription factors, including delta sleep–inducing peptide immunoreactive peptide (DIP), zinc finger proteins (HTF1,

Figure 1. Expression of known retinoid-regulated genes by quantitative real-time RT-PCR. HMECs treated with vehicle (DMSO) and LGD1069 1 μmol/L for 1 and 24 hours. Columns, mean mRNA expression levels after normalization with h-actin; bars, SE. *, P < 0.05, LGD1069 significantly up-regulates RARβ, RA-OH, and TGM2 mRNA and down-regulates collagenase mRNA.
HTF4, and HTF6), v-maf, high-mobility group protein (up-regulated), activating transcription factor 3 (ATF3), and early growth response 3 (EGR3; down-regulated), were modulated after 1-hour treatment with LGD1069. LGD1069 also induced genes encoding cell cycle-related proteins, WEE1 kinase and SMC4 structural maintenance of chromosomes 4-like 1 (SMC4L1). MAD2 mitotic arrest deficient-like 1 (MAD2L1), a regulator of apoptosis, was up-regulated in HMECs in response to LGD1069 treatment.

Figure 2. Hierarchical clustering of gene expression data. Analysis of this microarray data using DNA Chip Analyzer (dChip) showed that 213 genes were significantly up-regulated ($P < 0.05$) and 140 genes were significantly down-regulated ($P < 0.05$) at 1 or 24 hours after treatment with the rexinoid LGD1069 compared with vehicle (DMSO)-treated samples. The expression values for each gene are normalized to have a mean of 0 and a SD of 1, and the colored bars represent relative to the mean [green (-2.0) to red (+2.0)].
Additional cell cycle– and apoptosis-regulated genes were modulated on two of the three arrays per treatment group studied (p27, Bcl2, Id-1, and cyclin D1). The modulation of these growth-regulating genes may explain the biological effects of this rexinoid (cell cycle, apoptosis, and differentiation), and may in part account for the cancer-preventive effect of the rexinoid.

**Genes modulated after treatment with LGD1069 for 24 hours.** Many more genes were shown by the microarray to change after 24-hour treatment with LGD1069. One hundred eighty-seven genes were significantly up-regulated (P < 0.05) and 109 genes were significantly down-regulated (P < 0.05; Supplementary Fig. S2B). The GenBank accession numbers and gene names of the 66 up-regulated or down-regulated genes with the greatest fold change in expression are listed in Table 1, and more filtered gene names are listed in Supplementary Table S2.A and B.

After 24 hours of treatment, keratin 15 was shown to be highly up-regulated and keratin 17 was shown to be slightly down-regulated by LGD1069. Other up-regulated genes included DEPP (decidual protein induced by progesterone), RTP801 (hypoxia responsive transcription factor), lysyl oxidase (extracellular matrix remodeling enzyme), inositol 1,4,5-triphosphate receptor (calcium release channel), IGFBP-6 (signal transduction), short-chain dehydrogenase/reductase 1 (retinol metabolism), v-maf (transcriptional activator), SPARC (secreted protein associated with cell migration, matrix mineralization, cell cycle regulation, and angiogenesis), and stearoyl-CoA desaturase (regulatory enzyme involved in the synthesis of the monounsaturated fatty acids palmitoleate and oleate). Down-regulated genes included COX-2 (catalyzing enzyme for prostaglandin synthesis), EGR-3 (transcription factor), X-box binding protein, ODC (ornithine decarboxylase), uPAR (plasminogen activator, urokinase receptor), and v-maf-F (transcriptional inactivator). Several of these genes (IGFBP-6, SCD-1, COX-2, and ODC) are known retinoid-regulated genes (23–26).

Thus, treatment of HMECs with LGD1069 for 24 hours significantly alters the expression of genes encoding cell proliferation and cell cycle signaling proteins, cell death and apoptosis signaling proteins, stress response proteins, transcription factors, and proteins involved in retinoid signaling. Supplementary Table S3

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*Fold changes represent the ratio of gene expression in LGD1069 versus DMSO-treated HMECs.
shows a list of the genes subcategorized by their biological function.

Comparison of the genes modulated at both 1 and 24 hours shows that 22 genes were modulated at both time points. These rexinoid-regulated genes include 7 up-regulated genes and 15 down-regulated genes as shown in Supplementary Fig. S2C and Supplementary Table S4. Several of these genes were selected for further study in our validation experiments.

**Validation of array data.** To confirm the changes observed by our microarray analysis, we measured RNA or protein expression levels of selected LGD1069-regulated genes by semiquantitative RT-PCR using 32p-labeled primers, real-time quantitative RT-PCR, and Western blotting. Sixteen genes of which the products are involved in growth regulation and which had a fold change >2-fold were selected for further study. Three independent sets of total RNA and protein were prepared and analyzed in these validation studies.

We initially selected six of the genes that were shown to be highly regulated by LGD1069, including IGFBP-6, Id-1, SCD-1, COX-2, ODC, and keratin 17, for validation using this set of independent RNA samples. IGFBP-6 and Id-1 were confirmed to be up-regulated; COX-2 and ODC were confirmed to be down-regulated by 32p-labeled semiquantitative RT-PCR assay (data not shown). To more accurately quantitate differences in amounts of RNA expression, we developed real-time quantitative RT-PCR analysis assays for each of these genes. Figure 3A shows the results of these experiments and confirms that LGD1069 up-regulated the expression of IGFBP-6, SCD-1, and Id-1, and down-regulated COX-2. In addition, we have chosen another RXR agonist, LGD100268, which is even more selective for RXR than LGD1069, to determine whether this more selective rexinoid modulates the expression of these genes (results shown in Fig. 3B). LGD100268, the more RXR-selective reagent, induced the expression of Id-1, IGFBP-6, and SCD-1. However, the expression of COX-2 was...
not changed by LGD100268. These results suggest that certain LGD1069-regulated genes are induced through an RXR-dependent pathway (such as Id-1, IGFBP-6, and SCD-1), whereas other genes (such as COX-2) may be modulated by LGD1069 through its ability to also bind RAR.

We then investigated the expression of 10 other genes shown to be regulated by LGD1069 in our array analysis (IVL, RAI3, SDR1, cyclin D1, p27, DEPP, IGFBP-3, keratin 15, EGR3, and cMaf) using quantitative RT-PCR analysis, and found that 9 of these 10 genes were similarly up-regulated or down-regulated in this independent set of RNA samples (data not shown). The only gene found to not change in this set in a similar manner as seen in the array experiment was EGR3. This gene is a known early response gene that may be very sensitive to experimental conditions. Thus, for 15 of the 16 genes tested, the validation experiments confirmed the results of the microarray experiments. Therefore, for these highly selected genes, the validation experiments showed a 6.25% “discordant rate.”

We also measured the protein expression of several of the genes found modulated by LGD1069. As shown in Fig. 4, the expression of Id-1 and IGFBP-6 proteins was confirmed to be up-regulated and the expression of COX-2 and keratin 17 was down-regulated.

**LGD1069 modulates biomarkers in the mammary glands of mice.** We next investigated whether LGD1069 treatment modulated the expression of our newly identified biomarkers in vivo in mouse mammary glands. We have previously shown that LGD1069 suppresses mammary tumor formation in MMTV-erbB2 mice (16). Therefore, we determined whether LGD1069 would modulate the expression of our selected biomarkers in the mammary glands from MMTV-erbB2 mice. For these experiments, RNA and protein were isolated from mammary glands from MMTV-erbB2 mice treated with LGD1069 for 4 months as described in Materials and Methods. At this age, no palpable tumors had yet developed. We selected this time point to be analogous to breast tissue obtained from high-risk women before they develop cancer. Thus, changes in gene expression occurred in the normal or premalignant mammary epithelial cells in these mice before invasive cancer developed. As shown in Fig. 5A, the RNA expression of four biomarkers (SCD-1, ODC1, COX-2, and cyclin D1) was significantly modulated after 4 months of LGD1069 treatment in MMTV-erbB2 mouse mammary gland tissues. RNA expression of SCD-1 was up-regulated (by 3-fold; \( P < 0.05 \)) and ODC1, COX-2, and cyclin D1 were down-regulated (20%, 38%, and 39% respectively; \( P < 0.05 \)). As seen in Fig. 5B, cyclin D1 protein was also reduced (by 30%; \( P < 0.05 \)) by LGD1069 treatment in mammary epithelial cells isolated from MMTV-erbB2 mice treated with LGD1069 for 4 months. We have not been able to measure the expression of the other three markers because of a lack of available antibodies that can be used for Western blotting or immunohistochemistry in these mouse tissues. Thus, the results from the in vivo study are consistent with the gene regulation effect of rexinoids from the in vitro study, and suggest that these rexinoid-regulated biomarkers will be useful biomarkers to assess the response to rexinoids in human breast cancer prevention clinical trials.

**Discussion**

The above studies show that oligonucleotide arrays can be used to identify rexinoid-responsive biomarkers in normal HMECs. Our
studies identified 353 genes regulated after treatment with the rexinoid LGD1069 in HMECs (213 genes were up-regulated and 140 genes were down-regulated). We validated that 15 of 16 genes tested were modulated after rexinoid treatment at the RNA and/or protein level (for a discordant rate of 6.25%). Rexinoid-regulated genes included important growth regulatory genes such as cyclin D1, COX-2, IGFBP-6, and RARα. These results suggest that rexinoids act as “multifunctional chemopreventive agents” to affect many important growth regulatory pathways in normal and premalignant breast cells. This inhibition of the growth of normal cells seems to be associated with inhibition of progression to premalignant and subsequent malignant lesions. Indeed, it seems that the predominant effect of the rexinoids is to stop normal cell proliferation, thus preventing the acquisition of DNA mutations that promote malignant transformation.

The regulation of growth and differentiation of normal, premalignant, and malignant cells by retinoids is thought to result from direct and indirect effects on gene expression. These effects are mediated by the RAR and RXR nuclear receptors, ligand-activated transcription factors that are members of the steroid hormone receptor superfamily. Retinoid receptors activate transcription in a ligand-dependent manner by binding as RAR/RXR heterodimers to retinoic acid response elements (RARE) or RXR response elements (RXRE) located in the promoter regions of target genes. RXRs can also form “nonpermissive” heterodimers (heterodimers that are not activated by rexinoids) with other nuclear receptors, such as vitamin D hormone receptors and thyroid hormone receptors, or “permissive” heterodimers (heterodimers that are activated by rexinoids) with peroxisome proliferator–activated receptor, liver X receptor, farnesoid X receptor, or nerve growth factor inducible-B (NGFI-B/Nur77; refs. 27–33). Examples of nonpermissive heterodimers include RXR/thyroid hormone receptor, RXR/vitamin D receptor, and RXR/RAR heterodimers. In nonpermissive heterodimer complexes, the binding of the nonpermissive partner to the RXR receptor suppresses the RXR ligand–induced transcriptional activity of the RXR receptor; thus, RXR can be described as a “silent partner.” However, if ligands for both receptors bind either permissive or nonpermissive heterodimer complexes, these complexes are synergistically activated (33).

Figure 5. Measurement of LGD1069-modulated biomarkers in mouse mammary tissues. RNA and protein were isolated from mammary gland tissue sections or mammary epithelial cell pellets from MMTV-erbB2 transgenic mice treated with LGD1069 or vehicle for 4 months (from 3 to 7 months of age). A, the mRNA level of each gene was measured using quantitative RT-PCR. Columns, average level of RNA from 10 mammary gland samples after normalization with β-actin; bars, SE. *, P < 0.05. LGD1069 significantly up-regulates SCD-1 RNA and down-regulates COX-2, ODC1, and cyclin D1 RNA in these mammary gland tissues. B, protein expression of cyclin D1 was measured by Western blot analysis. Each band shows the average level of protein expression in mammary gland epithelial cells pooled from four mice. Columns, mean value for control and LGD1069 treatment group after normalization with β-actin; bars, SE. *, P < 0.05.

Unpublished observations.
Our results show that genes known to be regulated by RAR activation, such as RARβ or TGM2, were up-regulated by the rexinoid LGD1069. We also found that Id-1, IGFBP-6, and SCD-1 were modulated by this rexinoid. Interestingly, Ma et al. (23) recently reported that Id-1 and IGFBP-6 were modulated by RAR activation using all-trans RA in human bronchial epithelial cells. We further investigated whether these genes are RAR-dependent or RXR-dependent biomarkers using the more RXR-selective rexinoid LGD100268. Our results showed that both LGD1069 and LGD100268 modulated Id-1, IGFBP-6, and SCD-1, suggesting that these genes are indeed RXR-modulated biomarkers. These results suggest that for these genes, either ligands that bind RXR or ligands that bind RAR can induce their expression. Activation of the promoters of these genes could occur either by activation of permissive RAR/RXR heterodimers or, alternatively, through the binding of distinct RARE and RXRE response elements and activation of gene expression through multiple response elements. We are now investigating the specific mechanism by which these genes are being activated by both RAR-selective or RXR-selective retinoids.

Previous work in our laboratory showed that the naturally occurring retinoid 9-cis RA is a promising agent for treatment and prevention of breast cancer (17). However, in human clinical trials, 9-cis RA has been found to have significant toxicity (34). Therefore, receptor-selective retinoids that have reduced toxicity are now being developed as potential chemopreventive agents. We have previously shown that RXR-selective retinoids suppress tumorigenesis with minimal toxicity compared with RAR-selective retinoids (18). In addition, retinoids can significantly prevent estrogen receptor–negative mammary tumor development with minimal toxicity in MMTV-erbB2 transgenic mice (16). Suh et al. (35) and Rendi et al. (36) previously reported that another rexinoid, LG100268, was active for prevention and treatment of chemically induced estrogen receptor–positive breast carcinomas in rats by promoting apoptosis. Furthermore, the ability of this rexinoid to promote apoptosis was increased by combining it with the selective estrogen receptor modulator arzoxifene (36). This report suggested that LG100268, in combination with arzoxifene, induced transforming growth factor β, inhibited nuclear factor-κB signaling, and induced phosphatase and tensin homologue (PTEN) expression. All of these changes would contribute to the cancer-preventive activity of this combination.

The mechanism of the antitumor effects of retinoids is not fully understood. A number of mechanisms have been proposed by us and others. In breast cancer cells, retinoids inhibit cell proliferation by decreasing expression or activity of growth stimulators (37, 38) or by increasing expression or activity of growth inhibitors (39, 40). Breast cell growth can also be suppressed by retinoid-induced inhibition of mitogenic signaling pathways that activate activator protein-1 (AP-1) transcription factor complexes (21, 41). Retinoid-induced inhibition of proliferation is complex and may be mediated in part by the induction of RARβ (42) and reduced AP-1 activity (21). Our previous studies of the biological effect of retinoids also suggest that inhibition of proliferation is in part caused by a reduction in cyclin D1 expression, which then induces a G1 cell cycle block (43). In addition, many retinoid-regulated genes identified in this study are growth regulatory genes. These include COX-2, IGFBPs, SPARC, p27, PTEN, SCD-1, WEE1 kinase, SMC4L1, MAD2L1, and cyclin D1. Thus, retinoids seem to be multifunctional chemo-preventive agents able to prevent cancer through modulation of many growth regulatory proteins.

Of the genes that have been identified as rexinoid-regulated, COX-2 is of particular interest. COX-2 is an immediate early response gene that is induced by a variety of mitogenic and inflammatory stimuli (44) and is commonly overexpressed in transformed cells and in human malignancies (45, 46). Over-expression of COX-2 in the mammary glands of multiparous MMTV-COX-2 transgenic mice is sufficient to induce mammary cancer (47). Interestingly, in human breast cancer, high expression of COX-2 is significantly associated with an estrogen receptor–negative phenotype, high expression of erbB2, and poor prognosis (45). In addition, COX-2 inhibitors have been shown to prevent breast cancer in animal models (48, 49). Based on these preclinical results, COX-2 inhibitors are now being tested in human clinical trials for the prevention of breast cancer (50). The present results showing that rexinoid LGD1069 down-regulated the expression of COX-2, along with previous studies showing that LGD1069 suppresses the development of estrogen receptor–negative tumors in animals (16), suggest that the combination of LGD1069 plus COX-2 inhibitors may be particularly effective for prevention of human breast cancer.

This study has identified rexinoid-modulated genes in HMECs using oligonucleotide microarrays. It is possible that additional genes are modulated by retinoids in vivo because gene expression changes induced by retinoid treatment of breast cells in culture may not completely reflect those modulated in vivo. For example, some genes may be modulated through epithelial-stromal cell interaction. Such rexinoid-regulated genes would not have been identified in our in vitro screen. However, the candidate biomarkers (SCD-1, ODC1, COX-2, and cyclin D1) identified from our in vitro studies were also modulated in vivo in the MMTV-erbB2 mice. Thus, the rexinoid-regulated biomarkers identified in these studies represent potentially useful biomarkers for testing retinoids in future clinical studies. The protein products of these genes could be (a) biomarkers of exposure to retinoids (keratins, DEPP, and SCD-1), (b) biomarkers involved in suppressing breast cell proliferation (RARβ, IGFBP-6, p27, and PTEN), or (c) important growth regulatory molecules that could serve as targets of future therapy (COX-2, collagenase). An early-phase breast cancer prevention clinical trial testing the rexinoid LGD1069 (Bexarotene) in women at high risk of breast cancer is being conducted at Baylor College of Medicine. The biomarkers identified in this report are now being used to assess the response to the rexinoid in these high-risk women. In addition, we are conducting studies to investigate the role of the identified gene products on the growth and transformation of breast cells. Such studies will allow us to develop even more effective chemopreventive agents for the prevention of breast cancer.

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References

Identification of Biomarkers Modulated by the Rexinoid LGD1069 (Bexarotene) in Human Breast Cells Using Oligonucleotide Arrays

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