Retinoic Acid Receptor Gamma-Induced Misregulation of Chondrogenesis in the Murine Limb Bud In Vitro

Eugene Galdones and Barbara F. Hales

Department of Pharmacology and Therapeutics, McGill University, Montréal, Québec, Canada H3G 1Y6

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Vitamin A derivatives modulate gene expression through retinoic acid and retinoid receptor (RAR/RXR) heterodimers and are indispensable for limb development. Of particular interest, RARγ is highly expressed in cartilage, a target affected following retinoid-induced limb insult. The goal of this study was to examine how selective activation of RARγ affects limb development. Forelimbs from E12.5 CD-1 mice were cultured for 6 days in the presence of all-trans RA (pan-RAR agonist; 0.1 or 1.0 μM) or BMS-189961 (BMS961, RARγ-selective agonist; 0.01 or 0.1 μM) and limb morphology assessed. Untreated limbs developed normal cartilage elements whereas pan-RAR or RARγ agonist-treated limbs exhibited reductive effects on chondrogenesis. Retinoid activity was assessed using RAREβ2 (retinoic acid response element β2)-lacZ reporter limbs; after 3 h of treatment, both drugs increased retinoid activity proximally. To elucidate the expression profiles of a subset of genes important for development, limbs were cultured for 3 h and cRNA hybridized to osteogenesis-focused microarrays. Two genes, matrix GLA protein (Mgp; chondrogenesis inhibitor) and growth differentiation factor-10 (Gdf10/Bmp3b) were induced by RA and BMS-189961. Real-time PCR was done to validate our results and whole mount in situ hybridizations against Mgp and Gdf10 localized their upregulation to areas of cartilage and programmed cell death, respectively. Thus, our results illustrate the importance of RARγ in mediating the retinoid-induced upregulation of Mgp and Gdf10; determining their roles in chondrogenesis and cell death will help further unravel mechanisms underlying retinoid teratogenicity.

Key Words: matrix GLA protein; growth differentiation factor 10; retinoic acid receptor gamma; retinoic acid; limb development; chondrogenesis.

All-trans retinoic acid (RA), the most bioactive metabolite of vitamin A (retinol), is a potent morphogen that plays crucial roles during embryo development (Kochhar, 1967). Retinoid concentrations are tightly regulated during embryogenesis. Both deficiency (Hale, 1930) and excess can cause malforma-

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Single RAR isoform ablation studies have uncovered the importance of RARγ in controlling axial development and patterning, but receptor redundancy has been linked to the lack of effect seen in the appendicular skeletons of RARγ-deficient mice (Chambon, 1994; Lohnes et al., 1995). Notwithstanding, previous work in our lab has shown that limbs deficient in RARγ (on an z1-null background) were less susceptible to retinoid-induced limb dysmorphogenesis. Chondrogenesis and overall limb growth in these transgenic mice were vastly improved following retinoid insult, when compared with their retinoid-treated wild-type counterparts, indicating the importance of RARγ in mediating limb teratogenesis (Galdones et al., 2006).

Contrary to RARγ ablation, the objective of the present study was to determine the outcomes of aberrant RARγ activation on limb morphology. To do so, an in vitro limb bud culture system was employed (Kwasigroch and Skalko, 1983) and aberrant RARγ activation was induced pharmacologically using BMS-189961 (BMS961), a highly RARγ-selective agonist (Klaholz et al., 2000). Given the predominantly high expression of RARγ in cartilage during limb development, we hypothesized that retinoid-induced limb dysmorphogenesis is mediated by a RARγ-dependent misregulation of genes important for proper chondrogenesis.

From a pharmacological standpoint, RARγ is an extensively pursued drug target for the treatment of several medical conditions including polycystic acne, psoriasis, diabetes, and various cancers (Johnson and Chandraratna, 1999). Unfortunately, such treatments are contraindicated for women of childbearing age. Understanding the modes of RARγ-mediated toxicity during development will effectively aid in the development of safer, less teratogenic retinoid analogs.

In this study, we demonstrate the teratogenic action of a RARγ-selective agonist on the developing limb. Retinoid-responsive reporter mice (RARβ2-lacZ) were employed to assess the location and extent of retinoid activity following activation of RARγ. Gene expression analysis using chondrogenesis-focused micrarrays has identified two RARγ-responsive genes (Mgp [matrix GLA protein] and Gdf10 [growth differentiation factor-10]) that may have novel uncharacterized roles in transducing the teratogenic signal in the limb. Furthermore, in situ hybridization (ISH) has localized Mgp to areas of developing cartilage and Gdf10 to areas of programmed cell death in the limb, suggesting independent functions for each of these candidate genes.

MATERIALS AND METHODS

Limb bud culture and drug treatments. Pregnant gestational day (GD) 12 CD-1 mice were euthanized, embryos were explanted and forelimbs were excised just lateral to the somites and cultured in vitro in a chemically defined culture medium, as previously described (Ali-Khan and Hales, 2003). All-trans RA (Sigma, St Louis, MI) and BMS-189961, a RARγ-selective agonist (BMS961; a gift from Bristol-Myers Squabb, Wallingford, CT), were dissolved in 100% EtOH. Limbs were exposed to low and high concentrations of RA (0.1 and 1.0 μM) or BMS961 (0.01 and 0.1 μM) at the onset of culture. All animal studies complied with the guidelines established by the Canadian Council on Animal Care.

Limb morphology. Limbs were cultured for 6 days, with one change of the medium that was not supplemented with RA or BMS961, on day 3, as previously described (Galdones et al., 2006). Briefly, limbs were fixed and stained with 0.1% toluidine blue (Fisher Scientific, Montreal, Canada) and examined under a dissecting microscope (Wild Heerbrugg 99067, Wild Leitz, Ottawa, Canada) and photographs acquired with a JVC digital camera (JVC GCQX3HD, Tokyo, Japan). The extent of proper limb development was quantified using a limb morphogenetic scoring system (Neubert and Barrach, 1977). Five separate replicates were completed and examined for morphological changes or markers.

RARγβ2-hsp68lacZ reporter mice and staining. To examine the extent and localization of retinoid activity, RAREβ2-hsp68lacZ double-transgenic males were mated with wild-type CD-1 females to produce single copy transgene offspring. These embryos were explanted at E12.5 and limbs cultured for 2 and 12 h as described above, in the absence or presence of RA or BMS961. Tissues were fixed immediately with 0.25% glutaraldehyde then stained with X-gal for 2–4 h, as previously described (Rossant et al., 1991). Limbs were rinsed in phosphate-buffered saline (PBS) then visualized under a dissecting microscope. Results were acquired from three independent limb culture experiments.

RNA extraction. After 3 h in vitro, each group of six to eight forelimbs was removed from culture, rinsed with PBS and stored at −80°C in RNalater RNA Stabilization Reagent (Qiagen, Mississauga, Canada) until further processing. Total RNA was extracted using the RNeasy Micro Kit (Qiagen) and quality and purity/quantity assessed by agarose gel electrophoresis and spectrophotometric ultraviolet analysis in 10 mM Tris, pH 8.0 buffer, respectively. Five separate independent cultures of each treatment group were completed and used for microarray and quantitative RT-PCR analysis.

cRNA synthesis and hybridization. Starting with 2 μg of total mRNA, the TrueLabeling-AMP 2.0 Kit (Superarray Bioscience, Frederick, MD) was used to produce, amplify and biotinylate antisense cRNA for hybridization following the manufacturer’s guidelines. The cRNA product was purified with ArrayGrade cRNA Cleanup (SuperArray Bioscience) and then hybridized overnight to Mouse Oligo GEArray Osteogenesis Microarrays (OMM-026, SuperArray Bioscience), using 3 μg of cRNA per array and following the manufacturer’s suggested HybTube Standard Protocol. The following day, the membranes were stained with an alkaline-phosphatase-conjugated streptavidin antibody (1:8000, SuperArray Bioscience) and the chemiluminescence signal was developed using the Chemi-Drop Kit (SuperArray Bioscience). Five arrays, each from independent limb cultures, were completed for each treatment group.

Data acquisition and gene expression analysis. The array images were recorded using X-ray film, digitized using a desktop scanner, saved as 16-bit TIFF files and uploaded onto GEArray Expression Analysis Suite (GEArray, SuperArray Bioscience). Array alignment and raw intensity quantification were done using GEASuite, then the data were imported into Agilent GeneSpring 7.3 GX (Agilent Technologies, Palo Alto, CA) where background correction and all further analysis was completed. For each individual array, the gene detection threshold was set at a raw signal intensity of 2× the background intensity; to minimize array-to-array variation, expression values were normalized to the median of all measurements on that individual array. Upon identification of the uniquely and commonly expressed genes in each group, statistical comparisons between control and RA or BMS961 treated groups were done using Student’s t-tests (p ≤ 0.05). Genes were only included in the significantly regulated group of genes when significance was detected in at least three out of five replicates, and the changes were all in the same direction (i.e., either all up- or all downregulated).

Quantitative real-time PCR. RNA was diluted to a final working solution of 10 ng/μl and Quantitect One-Step SYBR Green RT-PCR (Qiagen) was done...
using the Roche LightCycler (Roche Diagnostics, Laval, Canada). The quantitative real-time PCR (qRT-PCR) cycling parameters were as follows: 95°C for 15 min (one cycle) and then 94°C for 15 s, 55°C for 30 s, and 72°C for 20 s (for 50 cycles) and each sample was measured in duplicate. Embryonic head tissue was also collected, and RNA extracted to provide 1, 10, and 50 ng/l RNA stocks used to generate standard curves for quantification. The primers were generated with Primer3 software (http://frodo.wi.mit.edu) and produced by alpha DNA (Montreal, Canada; see Table 1 for sequence information). To normalize the output, the expression of each gene of interest was divided by 18S gene expression, a commonly used housekeeping gene whose expression remains unchanged in murine limbs following retinoid exposure (Ali-Khan and Hales, 2006). Melting-curve analyses were done following each PCR to determine the output and detection quality (i.e., the formation of primer-dimers). Each treatment consisted of RNA from four separate culture experiments and each sample was measured in duplicate.

Whole mount ISH. Limbs cultured for 3, 6, or 12 h were rinsed with PBS containing 0.1% Tween 20 (PBT) and fixed overnight at 4°C in 4% paraformaldehyde. The following day, the limbs were dehydrated through a methanol/PBT gradient and stored at 0°C until further use. For whole mount ISH, a DIG RNA labeling kit (Roche Diagnostics) was used to synthesize Mgp and Gdf10 antisense probes from plasmids kindly provided by G. Karsenty (Columbia University, New York, NY) and S.-J. Lee (Johns Hopkins University, Baltimore, MD), respectively. Whole mount ISH was done as previously described (Decimo et al., 1995). After hybridization, limbs were rinsed in PBT and photographed under a dissecting stereomicroscope (Wild Leitz). Three separate whole mount ISH experiments were done for each gene of interest.

RESULTS

Aberrant Activation of RARγ is Teratogenic to the Developing Limb

Because retinoid insult in the limb is attenuated in the absence of RARγ (Galdones et al., 2006), our first goal was to determine if and how aberrant RARγ activation affects limb development. In vitro cultured murine limbs were treated with either RA (pan-RAR agonist) or BMS961 (RARγ-selective agonist) at E12.5. The treatment of limbs with either drug resulted in dose-dependent detrimental effects on gross morphology after culture for 6 days, with marked effects on chondrogenesis, as evidenced by decreases in toluidine blue stained cartilage (Fig. 1A). After exposure to low concentrations of RA or BMS961 (RA: 0.1μM, BMS961: 0.01μM), the zeugopod (long bones) appeared growth retarded, whereas the autopod (paw) remained intact. However, at high exposures (RA: 1.0μM, BMS961: 0.1μM) both limb regions were equally undifferentiated.

A limb scoring system was used to determine the quality and extent of morphogenetic differentiation of the cartilage elements that comprise the forming limb after exposure to the pan- or RARγ-selective agonist (Neubert and Barrach, 1977). In accordance with the gross morphology, limb scores were compromised following treatment with either the pan- or RARγ-selective agonist (Fig. 1B).

<table>
<thead>
<tr>
<th>Name</th>
<th>Symbol</th>
<th>Genbank no.</th>
<th>Left primer</th>
<th>Right primer</th>
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<td>AAA CGG CTA CCA CAT CCA AG</td>
<td>CCT CCA ATG GAT CCT GGT TA</td>
</tr>
<tr>
<td>Matrix GLA protein</td>
<td>Mgp</td>
<td>NM_008597</td>
<td>GCG AAG AAA CAG TCA TTT GGT</td>
<td>TCA ACC GGC AGA AGG AAG</td>
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<tr>
<td>Growth differentiation factor 10</td>
<td>Gdf10</td>
<td>NM_145741</td>
<td>ATG CCC AGA ATT TCC ACA AG</td>
<td>AAG TCC AGC ACC TGA GAG GA</td>
</tr>
<tr>
<td>Cathepsin K</td>
<td>Ctsk</td>
<td>NM_007802</td>
<td>GAA CGA GAA AGC CCT GAA GA</td>
<td>CAC ACC TCT GCT GAA AAA CTG G</td>
</tr>
<tr>
<td>Decorin</td>
<td>Dcn</td>
<td>NM_007833</td>
<td>GTC TGG CCA ATG TTC CTC AT</td>
<td>AGG TCA TTT GTC GAA AAA ACT GC</td>
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</tbody>
</table>

FIG. 1. The effects of pan-RAR and RARγ-selective activation on limbs following 6 days in culture. Limbs were collected from CD-1 mice at embryonic day 12.5 and cultured in vitro for 6 days in the presence or absence of pan-RAR agonist, all-trans RA (0.1, 1.0μM) or RARγ-selective agonist, BMS961 (0.01, 0.1μM). (A) Limbs were stained with 0.1% toluidine blue (wt/vol 70% ethanol). Both RA and BMS961 treatment result in marked effects on chondrogenesis, namely long bone outgrowth and digit formation. (B) The limb morphogenetic scores measured the extent of differentiation and chondrogenesis following treatment.
RAR\(_\gamma\)-Selective Agonism Induces Retinoid Activity in RARE\(_{\beta2}\)-hsp68lacZ Reporter Limbs

To determine the amount and distribution of retinoid activity in limbs following RA or BMS961 exposure, we treated transgenic limbs from a retinoid reporter strain containing a transgene under the control of the retinoid sensitive RAR\(_{\beta2}\) response element (Rossant et al., 1991). In the absence of retinoid, the lacZ transgene was strongly expressed in the interdigital zones and observable along the axes of the forming long bones after 3 h in vitro (Fig. 2). With the addition of 0.1 or 1.0\(\mu\)M RA for 3 h, transgene expression was strongly upregulated in the proximal mesenchyme, necrotic zones, dorsal ectoderm, and interdigital webbing. At 3 h, BMS961 treatment (0.01 or 0.1\(\mu\)M) increased transgene expression in the proximal mesenchyme as well, but only in a modest fashion; transgene expression in the webbing was affected similarly in BMS961 and RA-treated limbs.

After 12 h in vitro, vehicle-treated limbs exhibited minimal transgene expression. In contrast, both pan-RAR and RAR\(_\gamma\)-selective activation induced restricted transgene expression in the zone of polarizing activity, the limb signaling center responsible for anterior-posterior patterning (Tickle, 2006). Additionally, although RA did not induce transgene expression in the interdigital zones at this later time point, BMS961 exposure resulted in a faint induction of staining in the webbing. Thus, because maximal effects on lacZ transgene expression were observed after 3 h in vitro, subsequent experiments were done at that early time point.

Changes in Gene Expression Induced by RA or BMS961 Treatment

It is clear that retinoids have detrimental effects on chondrogenesis (Jiang et al., 1995) yet the various mechanisms underlying this process are still unresolved. We adopted a gene array approach to assess how the regulation of chondrogenesis-related genes in the limb was affected following pan-RAR or RAR\(_\gamma\)-selective insult. Limbs were treated for 3 h with either RA or BMS961 and RNA was hybridized to Mouse Oligo Osteogenesis Microarrays from GEArray containing approximately 100 genes important in various aspects of cartilage and bone development.

In comparing the vehicle-treated limbs to those treated with 0.1 or 1.0\(\mu\)M RA, 55 genes were commonly expressed in all groups (Supplementary Table 1). Four genes were expressed uniquely under control conditions, whereas one gene was expressed only after exposure to 1.0\(\mu\)M RA for 3 h. (C) Genes that were significantly up- or downregulated when comparing control-treated limbs to those treated with either low and high RA and/or BMS961 treatment.

Osteogenesis Microarrays from GEArray containing approximately 100 genes important in various aspects of cartilage and bone development.

In comparing the vehicle-treated limbs to those treated with 0.1 or 1.0\(\mu\)M RA, 55 genes were commonly expressed in all groups (Supplementary Table 1). Four genes were expressed uniquely under control conditions, whereas one gene was expressed only after exposure to 1.0\(\mu\)M RA (Fig. 3A). Only two genes were expressed in two out of three treatment groups (0 and 0.1\(\mu\)M; genes listed in Fig. 3A). Limbs treated with BMS961 exhibited a similar outcome, with the majority of
genes commonly expressed between all groups (58; Supplementary Table 2) although one gene was expressed solely after exposure to low concentrations; four genes were expressed in both control and 0.01 \( \mu M \) BMS961-treated limbs, but not in 0.1 \( \mu M \) exposed limbs (genes listed in Fig. 3B). All uniquely expressed genes were only expressed at minimal baseline levels, therefore our analysis was limited to the groups of commonly expressed genes.

**Activation of RAR\( \gamma \) is Implicated in the Misregulation of Mgp and Gdf10 Gene Expression**

To identify genes important in mediating retinoid-induced limb teratogenesis, the lists of commonly expressed genes in all RA or BMS961 treatment groups were analyzed for statistical significance \( (p < 0.05) \) using Agilent GeneSpring 7.3 GX software. Comparison between vehicle and the two RA-treated groups identified nine genes that were significantly up- or downregulated by treatment (Table 2). Our work identified a subset of genes that were significantly changed (either up- or downregulated) following RA exposure (Table 2). Several of the identified genes are related to bone morphogenetic protein (BMP)/transforming growth factor beta (TGF-\( \beta \)) signaling (Smad2, Bmp4, and Gdf10) and components of cartilage and extracellular matrix (Coll11a1, Spp1, and Dcn), whereas others are linked to matrix breakdown (Ctsk), oxidative stress (Nfkb1), and regulation of chondrogenesis (Mgp).

When comparing the control versus the two BMS961-treated groups, only two genes were identified as being upregulated by RAR\( \gamma \) activation (Table 2). The lists of significantly altered genes were compared; both of the genes altered by RAR\( \gamma \)-selective activation were also misregulated by RA exposure (Fig. 3C). These genes were Mgp, a potent inhibitor of chondrogenesis and osteogenesis (Price and Williamson, 1985).

### Table 2

List of Genes Significantly Up- or Downregulated following RA or BMS961 Treatment

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<tr>
<th>Gene</th>
<th>Symbol</th>
<th>Genbank</th>
<th>0( \mu M )</th>
<th>0.1( \mu M )</th>
<th>Direction</th>
<th>( p )</th>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Cathepsin K</td>
<td>Ctsk</td>
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and Gdf10, a bone morphogenetic protein of uncharacterized function in the developing limb (Cunningham et al., 1995).

Array Verification by qRT-PCR

We used qRT-PCR to assess the gene expression profiles of the two RARγ-responsive genes, Mgp and Gdf10. After culture for 3 h, the expression of both Mgp and Gdf10 was significantly upregulated by exposure to low or high concentrations of RA as well as the high concentration of BMS961; the expression of these genes was not responsive to the low concentration of BMS961 (Figs. 4A and 4B). Interestingly, after 12 h in culture, the expression of both genes did not differ from control in the RA or BMS961 exposed limbs, illustrating that the effect of the retinoids on the expression of these genes in the cultured limbs is transient; indeed, the expression of Gdf10 was significantly downregulated in 0.1 μM RA-treated limbs at 12 h.

In addition, to further validate the results from the array experiments, we selected two genes that were pan-RAR, but not RARγ-responsive. Dcn (decorin), a proteoglycan associated with collagen (Scott and Orford, 1981) and ctsk (cathepsin K), a cysteine protease expressed in bone (Garnero et al., 1998) were both downregulated by exposure to 1.0 μM RA for 3 h (Figs. 4C and 4D). As anticipated from the array data, exposure to either 0.1 μM RA or BMS961 had no effect on the expression of these genes in limbs for 3 h in vitro.

Distribution of Mgp and Gdf-10 Gene Transcripts Following pan-RAR and RARγ-Selective Activation

Whole mount ISHs were done to determine the distribution of Mgp and Gdf10, our gene transcripts misregulated by RARγ activation. Vehicle-treated limbs exhibited very little Mgp staining at 3 h; Mgp expression was induced by RA in a concentration-dependent manner. The induction of Mgp expression was restricted to the forming long bones at low RA concentrations and was extended distally into the rays at high RA levels. Mgp expression was upregulated strongly by BMS961 in the forming long bones, as well as the proximal mesenchyme, but was not induced in the digits by exposure to either concentration of BMS961 (Fig. 5A). At 6 h, Mgp transcripts were detected in the long bones in vehicle exposed limbs; exposure to either RA concentration further upregulated Mgp expression at this time point, whereas BMS961 did not. Interestingly, by 12 h in vitro, little Mgp expression was found in any treatment group, echoing the results seen by qRT-PCR where a transient decrease in expression was observed (Fig. 4A).

Localization of the expression of Gdf10 was distinct from that of Mgp. Gdf10 expression was faintly observable in the interdigital webbing under control conditions at 3 h (Fig. 5B). Ectopic expression was induced by exposure to either the low or high concentration of RA and shown to be highly localized to the interdigital webbing as well as the anterior and posterior necrotic

FIG. 4. qRT-PCR validation of the changes in expression of genes responsive to pan-RAR and RARγ-selective activation. Limbs were cultured in vitro for 3 (open bars) or 12 (closed bars) h in the absence or presence of RA (0.1, 1.0 μM) or BMS961 (0.01, 0.1 μM). (A) Mgp gene expression is upregulated by RA and BMS961 treatment after 3 h in culture but returns to baseline expression levels by 12 h. (B) Gdf10 expression is upregulated in response to RA and BMS961 treatment following 3 h, but not 12 h in vitro. (C) Dcn and (D) Ctsk are both downregulated by RA but not BMS961 after 3 h in culture. Each bar represents the mean gene expression of four separately conducted limb cultures normalized against 18S mRNA expression. Statistical analysis was done using two-way ANOVA and the Holm-Sidak post hoc multiple-comparison test. *Significant increase, p < 0.05; #significant decrease, p < 0.05.
zones, all areas that are predestined to apoptose and help sculpt the developing limb. In addition, high concentrations of RA induced \textit{Gdf10} transcripts in the proximal regions of the forming digits. BMS961 treatment resulted in similar expression patterns, albeit not as intense as their RA-treated counterparts. At 6 h, when control limbs expressed very little \textit{Gdf10}, RA and BMS961 induced ectopic expression in the regions of programmed cell death and proximal rays. By 12 h \textit{in vitro}, in RA-exposed limbs only faint staining was observable in the digits; however, BMS961-treated limbs still expressed low levels of \textit{Gdf10} in the webbing, necrotic zones and proximal rays (Fig. 5B).

DISCUSSION

Although the effects of excess retinoids on development have been described, the mechanisms by which normal limb chondrogenesis and patterning are disrupted, and specifically the roles of distinct RARs, are not completely understood. Given that RAR\(\gamma\) is highly expressed in the forming cartilage (Dolle \textit{et al.}, 1989), we hypothesized that RAR\(\gamma\) mediates retinoid-induced limb dysmorphogenesis. Specifically, the goal of this study was to examine how pharmacological activation of RAR\(\gamma\) affects limb chondrogenesis.

Our results show that treatment of limbs with either RA (pan-RAR agonist) or BMS961 (RAR\(\gamma\)-selective agonist) \textit{in vitro} is detrimental to limb chondrogenesis and growth. Microarray-based expression analysis of chondrogenesis-related genes identified a group of RA-responsive genes and two RAR\(\gamma\)-responsive genes (\textit{Mgp} and \textit{Gdf10}). The localization of upregulated \textit{Mgp} transcripts to chondrocytes, and those of \textit{Gdf10} to interdigital webbing/necrotic zones, suggests that they play novel roles during retinoid-induced limb dysmorphogenesis; namely the misregulation of chondrogenesis and programmed cell death, respectively.

RAR\(\gamma\)-Induced Limb Defects

Both pan-RAR and RAR\(\gamma\)-selective activation are detrimental to limb morphogenesis, significantly affecting the proper chondrogenesis of long bones, carpalia, and digits (Fig. 1). Specifically, although RA-treated limbs exhibited marked reductive effects on all cartilage elements at 0.1 and 1.0\textmu M RA concentrations, BMS961 elicited phenotypically similar effects at concentrations that were 10-fold less (0.01 and 0.1\textmu M). Although there is evidence that BMS961 can exhibit weak affinity for RAR\(\beta\), the effective concentration (EC\textsubscript{50}) at which BMS961 binds RAR\(\beta\) is threefold higher than our highest treatment (Klaholz \textit{et al.}, 2000), suggesting that BMS961 is indeed acting as a RAR\(\gamma\)-selective agonist in our limb bud cultures.

Retinoid Activity

The pattern of retinoid-induced lacZ upregulation in areas of endogenous RAR\(\beta\) expression has been observed previously (Rossant \textit{et al.}, 1991). Given that transgene expression is driven by three copies of the highly retinoid-responsive RARE of RAR\(\beta\) (RARE\(\beta\)), the specific sequence of RAR\(\beta\) may not have a strong binding affinity for RAR\(\gamma\). Indeed, RAR\(\gamma\)-associated retinoid activity is most likely higher than observed, because RAR\(\gamma\)-selective activation induced gene expression changes in areas (i.e., chondrocytes) where no lacZ transgene expression was detected (Fig. 5A).

Pan-RAR Responsive Genes

Several groups, including our own, have studied global gene expression changes in the limb following retinoid excess (Ali-Khan and Hales, 2006; Qin \textit{et al.}, 2002). To refine our search, and because chondrogenesis is severely affected by exogenous retinoid exposure, we assessed the expression changes of genes known to participate in aspects of cartilage and bone development.
Interestingly, the direction in which the genes were regulated was mixed; this is contrary to previous work illustrating the positive unidirectionally of the gene changes following exogenous retinol (vitamin A) exposure (Ali-Khan and Hales, 2006), a discrepancy that may be explained by the kinetics of the drug used. The immediate bioactivity of RA treatment leads to direct effects on their respective targets whereas retinol must be activated into RA, thus it would be of particular interest to assess retinol-induced gene changes following time points later than 3 h in vitro.

Classically, in the absence of ligand, RARs remain associated with RAREs and transcriptionally repress target genes; however, once a ligand is bound, the RARs change conformation, release their associated corepressors and in turn recruit coactivators and the transcriptional machinery that drive gene expression (Weston et al., 2003). In our study, although several genes were upregulated by RA treatment (Mgp, Gdf10, Spp1, and Bmp4), others (Ctsk, Coll11a1, Nkx1.5, Smad2, and Dcn) were significantly downregulated. How this down-regulation occurs is not well understood, yet at least two explanations exist: (1) RA may upregulate an intermediate gene upstream, which in turn inhibits the transcription of the observed target gene, or (2) the liganded RAR/RXR heterodimers may bind negative response elements (NRE) that repress rather than activate transcription. These NREs have been identified upstream of several retinoid-responsive genes, including Mgp (Kirfel et al., 1997). However, because we have observed an upregulation of Mgp in our system, the activity of the NRE must be cell and tissue dependent.

We propose that these RA-responsive genes may play important novel roles in mediating retinoid-induced limb dysmorphogenesis. Because the scope of this study was focused on the RARγ-mediated regulation of gene expression, future studies will be required to determine how the misregulation of these newly identified RA-responsive genes affects limb morphogenesis.

**RARγ Responsive Genes**

In contrast to limbs treated with RA, BMS961-treated limbs exhibited a significant upregulation of only two genes: Mgp and Gdf10 (Fig. 3C). Whole mount ISHs were performed to determine the extent and localization of gene upregulation; Mgp was expressed and significantly upregulated in chondrocytes (Fig. 5A). Of interest, Gdf10 upregulation was limited to the interdigital webbing and the necrotic zones (Fig. 5B). These differential locations of expression suggest that each of these genes performs unique functions following retinoid-induced misregulation.

**Inhibition of Chondrogenesis by Mgp**

MGP, a γ-carboxyglutamic acid-rich, vitamin K–dependent protein (Price and Williamson, 1985), has been characterized as a potent inhibitor of chondrocyte maturation and mineralization (Yagami et al., 1999). Mice lacking Mgp are viable, but exhibit spontaneous calcification of arteries and cartilage (Luo et al., 1997). Our array experiments and follow-up qRT-PCR analysis show that Mgp expression is significantly upregulated by RA and BMS961. These results are consistent with those of Cancela and Price (1992) who identified a putative RARE upstream of Mgp bearing a striking resemblance to the RAREβ (de The et al., 1990) and showed the RA-dependent upregulation of Mgp in various human tissues, including chondrocytes (Cancela and Price, 1992). However, although the RARγ-selective agonist did significantly upregulate Mgp, it did not do so to the same extent as RA (Fig. 3A), suggesting that other RARs (α and β) may participate in its complete transcriptional regulation.

Although Mgp transcripts were not detected by whole mount ISH in untreated limbs, RA, and BMS961-treated limbs aberrantly expressed Mgp in areas of chondrocyte development. Because chondrogenesis is a major target in retinoid-induced limb dysmorphogenesis, the distribution of Mgp transcripts may correlate with the reductive effects associated with retinoid insult. This is the first report implicating Mgp in a teratological context. MGP has been identified as playing a role as a functional inhibitor of BMP-2 and BMP-4 function (Yao et al., 2007). Given that regulated BMPs are required for proper limb development, further work is needed to determine whether Mgp is indispensable for mediating specific retinoid-induced aberrations during development.

**Gdf10 and Programmed Cell Death**

GDF10 is a member of the TGFβ superfamily and is highly related to BMP-3 (Cunningham et al., 1995), hence Gdf10 is also commonly referred to as BMP-3b. Expression of Gdf10 during organogenesis is highly localized in uterus, adipose and brain and less so in bone; ablation of Gdf10 resulted in no apparent effects on developmental outcome (Zhao et al., 1999). Many TGF-β superfamily members have been linked to various aspects of limb development: BMP-2, -4, and -7, the most highly characterized, play crucial roles in precardilaginous and chondrocyte differentiation, apical ectodermal ridge-dependent limb outgrowth, anterior-posterior patterning and interdigital cell death (Kawakami et al., 1996), whereas TGF-β and GDF5 have been implicated in joint development (Storm and Kingsley, 1999). Gdf10 function in the limb has not been previously characterized (Zhao et al., 1999). Experiments targeting osteoblast function in vitro have recently revealed that GDF10 can inhibit BMP-2–dependent alkaline phosphatase activity, hence illustrating the negative regulation of Gdf10 on osteogenesis (Hino et al., 1999). We are the first to show that Gdf10 can be transcriptionally regulated by retinoids and that its upregulation is localized specifically to domains of programmed cell death in the limb, namely the webbing and necrotic zones (Fig. 5B). We postulate that Gdf10 plays an integral role in regulating the apoptosis associated with retinoid teratogenicity (Ali-Khan and Hales, 2003).
SUMMARY

These results show the importance of RARγ agonists in mediating retinoid-induced limb dysmorphogenesis. Our chondrogenesis-focused gene expression analysis has identified a handful of retinoid-regulated candidate genes that may play novel roles in limb dysmorphogenesis. Notably, our work is the first indication that Mgp and Gdf10 are significantly upregulated following pan-RAR or RARγ-selective activation. The identification of these candidate genes and their association with RARγ will help to unravel new mechanisms underlying retinoid-induced insult during development.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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