Tazarotene-induced Gene 2 (TIG2), a Novel Retinoid-Responsive Gene in Skin

Sunil Nagpal,* Sheetal Patel,† Heidi Jacobe,‡ Daniel DiSepio,* Corine Ghosn,* Monica Malhotra,* Min Teng,* Madeleine Duvic,† and Roshantha A.S. Chandraratna*

*Retinoid Research, Departments of Biology and Chemistry, Allergan, Inc., Irvine, California, U.S.A.; and †Departments of Dermatology and Internal Medicine, University of Texas Medical School at Houston, Texas, U.S.A.

Retinoids exert their biologic effects through two families of nuclear receptors, retinoic acid receptors (RARs) and retinoid X receptors (RXRs), which belong to the superfamily of steroid/thyroid hormone nuclear receptors. By using a subtraction hybridization approach, we have identified a cDNA sequence TIG2 (Tazarotene-induced gene 2), whose expression is up-regulated by the treatment of skin raft cultures with tazarotene. TIG2 encodes a putative protein product of 164 amino acids. TIG2 is neither expressed nor induced by tazarotene in primary keratinocyte and fibroblast cultures. Thus, TIG2 is expressed and induced by tazarotene only when keratinocytes and fibroblasts form a tissue-like 3-dimensional structure. We further demonstrate that RAR-specific retinoids increase TIG2 mRNA levels. In contrast, neither RXR-specific retinoids nor 1,25-dihydroxyvitamin D₃ increased TIG2 levels. Finally, we demonstrate that TIG2 is expressed at high levels in nonlesional psoriatic skin but at lower levels in the psoriatic lesion and that its expression is up-regulated in psoriatic lesions after topical application of tazarotene.

Key words: retinoic acid receptor, psoriasis, subtractive hybridization. J Invest Dermatol 109:91–95, 1997

Pharmacologic and physiologic retinoid signals in skin are transduced through two families of nuclear receptors, namely, retinoic acid receptors (RARs, β, and γ) and retinoid X receptors (RXRs, β, and γ) (Chambon, 1994; Mangelsdorf et al., 1994; Boehm et al., 1995; Nagpal and Chandraratna, 1996). RARs, which belong to the superfamily of steroid/thyroid/vitamin D₃ nuclear receptors, readily heterodimerize with RXRs in vitro (for references see, Nagpal and Chandraratna, 1996) and function as such in vivo (Nagpal et al., 1993). RARs are ligand-dependent transcription factors that activate the expression of retinoid-responsive genes by cooperative action of their activation functions, AF-1 (ligand-independent activation function), and AF-2 (ligand-dependent activation function) (Nagpal et al., 1992, 1993). Retinoids have been extensively used for the treatment of various nonmalignant (psoriasis and acne), and malignant (squamous cell carcinoma, actinic keratoses, basal cell carcinoma, and Kaposi’s sarcoma) skin diseases, and they hold significant potential as a therapeutic modality in various epithelial cancers (Peck and DiGiovanna, 1994; Boehm et al., 1995; Nagpal and Chandraratna, 1996). Despite their therapeutic efficacy in various dermatologic diseases, the molecular basis of retinoid action in skin is poorly understood. Various genes whose expression is induced in cell culture systems by retinoids have been described. Of these, cellular retinoic acid binding protein II (CRABPII) is the only marker whose expression is induced by retinoic acid (RA) in normal skin (Elder et al., 1993). In contrast, CRABPII expression is downregulated by RA in submerged keratinocyte cultures (Elder and Cromie, 1993), and it is also overexpressed in psoriasis (Didierjean et al., 1991), a hyperproliferative and inflammatory condition of skin (Krueger and Duvic, 1994) that can be treated by retinoids (Esleegyes-Ribot et al., 1994; Weinstein, 1996). The increased levels of CRABPII in psoriatic plaques indicates that it may not be a useful efficacy marker of retinoid action in psoriatic lesions. In addition, several lines of evidence suggest that CRABPII functions to modulate retinoid action by sequestering RA away from the nucleus and by enhancing RA metabolism (Boylan and Gudas, 1991; Fiorella and Napoli, 1991).

To further understand the molecular mechanisms of retinoid therapeutic action in skin, we sought to identify genes whose expression is induced in a 3-dimensional skin culture system (skin rafts) by an anti-psoriatic synthetic retinoid, tazarotene (AGN 190168) (Weinstein, 1996). Since tazarotene is topically effective in the treatment of psoriasis, studying its mechanism of action would provide insight into how retinoids affect the disease. Recently, by using a subtractive hybridization approach in skin rafts, we identified a novel gene, tazarotene-induced gene 1 (TIG1), whose expression was induced in skin rafts and psoriatic lesions by tazarotene (Nagpal et al., 1996). With a similar strategy for identification of differentially expressed transcripts, we have now identified another novel gene, tazarotene-induced gene 2 (TIG2), whose expression is up-regulated by this anti-psoriatic retinoid in skin rafts. Interestingly, unlike TIG1, TIG2 is not expressed in...
2-dimensional cultures of keratinocytes and fibroblasts. The basal and retinoid-induced expression of TIG2 is observed only when keratinocytes and fibroblasts form a tissue-like structure as in skin rafts and psoriatic lesions. We also demonstrate that TIG2 is induced by RAR-specific but not significantly by RXR-specific retinoids and 1,25-dihydroxyvitamin D3. Finally, we demonstrate that TIG2 is expressed at high levels in nonlesional psoriatic skin but only at lower levels in the psoriatic lesion and that topical application of tazarotene to psoriatic plaques results in a rapid upregulation of TIG2 expression.

MATERIALS AND METHODS

Skin Rafts and Primary Cultures of Keratinocytes and Fibroblasts

Normal skin raft cultures (model ZK 1300, Advanced Tissue Sciences, San Diego, CA) are 3-dimensional models of human skin tissues that dermal, epidermal, and cornified layers. These skin rafts were made by the manufacturer by seeding neonatal fibroblasts (pooled from 10 donors) onto a nylon mesh, which gave rise to a dermal tissue. Keratinocytes seeded on top of the dermal tissue differentiated into multilayered epidermis consisting of basal, spinous, and granular layers and stratum corneum. Primary cultures of foreskin keratinocytes and fibroblasts were purchased (Cloneetics, San Diego, CA).

Subtractive Hybridization

Skin raft cultures were either mock-treated or treated with the retinoid tazarotene (1 μM) on days 1 and 3 and harvested on day 4 for total RNA preparation as described previously (Nagpal et al, 1996). eDNA prepared from tazarotene-treated skin raft cultures was directionally cloned into Sph-Not sites of pSPORT-1 (Life Technologies, Gaithersburg, MD) and single-stranded DNA (ssDNA) was prepared from 108 transformants as described previously (Li et al, 1994; Nagpal et al, 1996). Another directional cDNA library was constructed in Sph-Not sites of pSPORT-2 (Life Technologies, Gaithersburg, MD), by using cDNA from mock-treated skin rafts, linearized with Sph and in vitro-transcribed in the presence of biotin-14-CTP as described (Li et al, 1994). pSPORT-1 ssDNA was blocked with a Not oligo primer 5'-GCGGCCGCCCT-3' by incubation at 75°C for 20 min with 600 μM dTTP and Taq DNA polymerase. Poly(dA)-blocked ssDNA (600 ng) was hybridized with biotinylated RNA (80 μg) in 2X hybridization buffer (80% formamide, 100 mM N2-(hydroxyethyl)piperazine-N'- (2-ethanesulfonic acid), pH 7.5, 2 mM ethylenediamine tetracetic acid, 0.2% sodium dodecyl sulfate) for 24 h at 42°C with shaking (200 rpm). The control experiment contained all the components except biotinylated RNA. After hybridization, streptavidin (25 μg) was added and the mixture was incubated at room temperature (5 min). Common ssDNA/RNA sequences were removed by phenol/chloroform extraction (five times), and unhybridized ssDNA was precipitated and electroporated into DH12S (Life Technologies, Gaithersburg, MD) cells.

Retinoids

All-trans-retinoic acid (RA) and 1,25-dihydroxyvitamin D3 were purchased (Sigma, St. Louis, MO, and Calbiochem, La Jolla, CA). Tazarotene (ethyl 6-[2-(4,4-dimethylthiochroman-6-yl)-ethyl]nicotinate) was synthesized in the Department of Chemistry, Retinoid Research, Allergan, Inc. (MacDNASIS). eDNA clone B1, obtained from subtracted cDNA library, was confirmed to be tazarotene-responsive by northern blot hybridization. Total RNA (10 μg) from mock-treated or tazarotene-treated skin raft cultures was subjected to northern blot hybridization. GAPDH and B1 sequences were detected with labeled cDNA probes. The northern blot signals, quantitated in terms of total volume (an arbitrary unit) by Phosphorimager (Molecular Dynamics, Sunnyvale, CA), are also presented. The overnight exposure of the blot in the Phosphorimager screen is presented. The message level of B1 cDNA clone was induced 4-fold after tazarotene treatment of skin raft cultures.

**Figure 1. Identification of a tazarotene-induced eDNA clone.** cDNA clone B1, obtained from subtracted cDNA library, was confirmed to be tazarotene-responsive by northern blot hybridization. Total RNA (10 μg) from mock-treated or tazarotene-treated skin raft cultures was subjected to northern blot hybridization. GAPDH and B1 sequences were detected with labeled cDNA probes. The northern blot signals, quantitated in terms of total volume (an arbitrary unit) by Phosphorimager (Molecular Dynamics, Sunnyvale, CA), are also presented. The overnight exposure of the blot in the Phosphorimager screen is presented. The message level of B1 cDNA clone was induced 4-fold after tazarotene treatment of skin raft cultures.

**RESULTS**

Identification of a Novel Retinoid-Responsive Gene, TIG2, by Subtraction Hybridization

Tazarotene (AGN 190168), an RARβ/γ-selective retinoid, is topically effective in the treatment of psoriasis (Esugleyes-Ribot et al, 1994; Weinstein, 1996), a skin disease characterized by keratinocyte hyper-proliferation and infiltration of immune cells into epidermis and dermis. To identify tazarotene-responsive genes in skin, we used a subtraction hybridization procedure (Li et al, 1994; Nagpal et al, 1996) to isolate differentially regulated genes from skin raft cultures. Differentially expressed genes in tazarotene-treated skin rafts, isolated after subtraction of common cDNA sequences between vehicle-treated and tazarotene-treated skin rafts, were analyzed by northern blot hybridization. The expression of one of the subtracted cDNA clones, B1, was induced after tazarotene treatment of skin rafts (Fig 1). The blot was also probed with labeled Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (a housekeeping) cDNA to ensure that equal quantities of control and retinoid-treated RNA were used in the northern blot analysis (Fig 1). Quantitation of the northern message showed a 4-fold increase in B1 expression after tazarotene treatment. Upon sequencing, B1 was found to be 830 bp in length, contained an open reading frame from 157 to 648 bp, and coded for a putative protein product of 164 amino acids (Fig 2).

The ATG (157-159 bp) protein initiation codon was confirmed by the presence of an in-frame stop codon (TGA) 12 bp upstream of the ATG codon (Fig 2A). It had a canonical poly(A) signal (5'-AATAAA-3') at bp 750 and a long poly(A) tail starting at bp 769 (Fig 2). Homology searches from DNA (GenBank and EMBL) databases showed significant homology to a 176-bp mRNA sequence (GenBank accession number, L07329) isolated from retinal pigment epithelial cells (Fig 3A). Further homology searches from protein (Swiss-Prot) database did not show identity to known protein sequences. We therefore conclude that B1 is a novel cDNA sequence. Subsequently, another cDNA clone, B13, was identified from the same library as a tazarotene-induced gene and upon sequencing was found to be identical to B1. Because we have previously reported a tazarotene-induced gene as TIG1, B1 will hereafter be referred to as tazarotene-induced gene 2, or TIG2. A hydrophobic stretch of 20 amino acids in the N-terminal region of the protein (Fig 3B) indicated that the TIG2 protein might be a membrane-associated protein. TIG2 also contained putative signals for casein kinase II phosphorylation (amino acids 50-53 and 54-57), myristylation (amino acids 16-21 and 29-34), and protein kinase C phosphorylation (amino acids 76-78). These putative motifs were identified by MacDNASIS PROSITE program. The consensus sequences used for searching phosphorylation sites were (S/T)-X-X-(D/E) and (S/T)-X-(R/K), respectively, for casein kinase II and protein kinase C phosphorylation motifs (MacDNASIS).

Interestingly, TIG2 was not up-regulated by tazarotene or RA in 2-dimensional cultures of keratinocytes (Fig 4A) or fibroblasts (Fig...
GAPDH controls for RNAs are also shown in Fig 4A and B for keratinocytes and fibroblasts, respectively. Also, TIG2 was expressed exclusively in the epidermis of nonlesional and lesional psoriatic skin and was up-regulated in psoriatic lesions by tazarotene treatment (vide supra, Fig 5). These findings suggested two plausible reasons why TIG2 was not retinoid-inducible in keratinocyte and fibroblast cultures: (i) the retinoid might induce a fibroblast-specific factor that then up-regulates TIG2 expression in keratinocytes or (ii) TIG2 might be expressed and induced in a retinoid-dependent manner only when keratinocytes and fibroblasts form a "tissue" structure as in skin raffs and skin. To test the first possibility, keratinocytes were treated with tazarotene in the presence of fibroblast-conditioned medium. These keratinocytes also exhibited neither basal nor tazarotene-induced expression of TIG2 (Fig 4C), therefore ruling out the possibility of a retinoid-responsive fibroblast-specific factor in the induction of TIG2. The GAPDH control for the keratinocytes grown in fibroblast-conditioned medium is also shown (Fig 4C).

TIG2 Responds to RAR but Not to RXR or Vitamin D₃
Receptor (VDR) Agonists

Tazarotene (AGN 190168) activates gene expression only through RARs and not through RXRs (Nagpal et al., 1995, 1996). Further, AGN 190168 is RAR/γ-selective and exhibits only weak transactivation through RARα (Nagpal et al., 1995). In contrast, SR 11217 and SR 11237 are RAR-specific and do not activate gene expression through RXRs (Lehmann et al., 1992). 1,25-Dihydroxyvitamin D₃ is a ligand for VDR and induces the expression of VDR-responsive genes through its cognate nuclear receptor. To determine the receptor specificities of TIG2 expression, skin raffs were treated with RAR-specific (Tazarotene), RXR-specific (SR11217 and SR11237), or VDR-specific (1,25-dihydroxyvitamin D₃) ligands. Only the RAR-specific ligand significantly induced TIG2 expression (Fig 4D), thus demonstrating that the induction of TIG2 is mediated through RAR but not by RXR or VDR agonist-dependent signal transduction pathways.

**Topical Application of Tazarotene**

The high expression of TIG2 message in uninvolved relative to lesional skin suggested that it may play a role in maintaining the normal physiology of skin and that of other tissues. Thus, various normal adult tissues were analyzed for TIG2 expression. Apart from skin, TIG2 was also expressed in pancreas, liver, spleen, prostate, ovary, small intestine, and colon but not in heart, brain, placenta, lung, skeletal muscle, kidney, thymus, testis, or peripheral blood.

**Figure 3. Nucleotide homology and hydrophobic profile of TIG2.**

The nucleotide and putative protein sequences of TIG2 were analyzed by MacDNASIS (Hitachi, San Bruno, CA) program for nucleotide and protein homologies from GenBank, EMBL, and Swiss-Prot databases. (A) The homology (approximately 60%) between TIG2 and the partial cDNA clone L07329, of 176 bp from retinal pigment epithelial cells, is shown. The upper strand represents the TIG2 cDNA forward sequence and the lower strand corresponds to the L07329 cDNA sequence. (B) Kyte-Doolittle hydrophobicity plot of TIG2 showing hydrophobicity (y axis) against amino acids (x axis) is presented. Regions of the protein greater than +0.50 are predicted to be membrane-embedded (MacDNASIS).

**Figure 2. Nucleotide and putative protein sequence of TIG2 (B1).**

B1 cDNA clone was sequenced and analyzed by MacDNASIS (Hitachi, San Bruno, CA) program for open reading frames. Nucleotide and putative protein sequence of TIG2 as obtained from our subtracted cDNA library after sequencing two independent cDNA clones (B1 and B13) in both directions is shown. GenBank accession number, U77594.

**Figure 4. TIG2 Expression in Psoriatic Lesions.**

To evaluate induction of TIG2 in vivo in psoriatic lesions, patients with long-standing plaque-type psoriasis were treated once daily with topical tazarotene gel (0.1%) for up to 8 wk. Skin-punch biopsies were taken before the treatment and after 3 and 14 d of tazarotene treatment. A biopsy was also taken from the nonlesional skin of the psoriatic patients before start of treatment. A typical in situ hybridization using digoxigenin-labeled TIG2 anti-sense RNA showed intense staining for TIG2 mRNA expression in the epidermis of the nonlesional skin biopsy of the psoriatic patient (Fig 5A), demonstrating that TIG2 is highly expressed in uninvolved psoriatic skin. In nonlesional skin, TIG2 was expressed in basal and suprabasal layers of the epidermis, hair follicles, and endothelial cells. A reduction in TIG2 expression was observed in the psoriatic lesion compared to nonlesional skin (Fig 5B). TIG2 was strongly induced in psoriatic lesions after 3 or 14 d of tazarotene treatment (Fig 5C, D). Hybridization of psoriatic lesional section with sense digoxigenin-labeled TIG2 RNA probe did not show staining (Fig 5E), thus demonstrating the specificity of TIG2 signal obtained with the anti-sense probe.

The high expression of TIG2 message in uninvolved relative to lesional skin suggested that it may play a role in maintaining the normal physiology of skin and that of other tissues. Thus, various normal adult tissues were analyzed for TIG2 expression. Apart from skin, TIG2 was also expressed in pancreas, liver, spleen, prostate, ovary, small intestine, and colon but not in heart, brain, placenta, lung, skeletal muscle, kidney, thymus, testis, or peripheral blood.
Figure 4. TIG2 is retinoid-inducible in a 3-dimensional culture system but not in 2-dimensional cultures of keratinocytes and fibroblasts. Total RNA (10 µg) from mock-treated (Control) or tazarotene-treated (A), fibroblasts (B), and keratinocytes (C) cultured in fibroblast-conditioned medium were subjected to northern blot hybridization. TIG2 and GAPDH sequences were detected with 32P-labeled cDNA probes prepared by random priming. The overnight PhosphorImager exposures of the blots are presented. (D) TIG2 expression is induced in an RAR-ligand-dependent manner. Total RNA (10 µg) from mock-treated (Control), tazarotene-, SR11217-, SR11237-, or 1,25-dihydroxyvitamin D₃-treated skin raft cultures was subjected to northern blot hybridization. TIG2 and GAPDH sequences were detected with labeled cDNA probes.

leukocytes (Fig 6), demonstrating the tissue-specific expression of TIG2 in vivo. GAPDH control of the multiple tissue northern blot is also shown.

Chromosomal Localization of TIG2  BLAST search of TIG2 cDNA sequence against human sequence tag site genomic clones database identified a clone (HSKSTS 367; GenBank accession number, Y07966) that displayed 100% nucleic acid homology over a stretch of 68 bp. The location of this STS clone fragment in the human genome maps the TIG2 gene to p13.3 region of chromosome 17. Therefore, TIG2 is located at 17p13.3 position. Interestingly, this region is associated with pancreatic tumorigenesis (for references, see GenBank accession number Y07966).

DISCUSSION

In this manuscript, we describe the identification of TIG2, a new retinoid-responsive gene. TIG2 was isolated by a subtraction hybridization approach aimed at identifying genes regulated by tazarotene, an RAR/β/γ-selective anti-psoriatic synthetic retinoid. TIG2 is induced in a retinoid-dependent manner in skin rafts and in the epidermis of psoriatic lesions (Fig 1, 5). In contrast, it was neither expressed nor induced by retinoids in 2-dimensional cultures of keratinocytes and fibroblasts (Fig 4). Thus, TIG2 is a unique retinoid-responsive gene in that it is retinoid-inducible only when keratinocytes and fibroblasts form a 3-dimensional tissue structure as observed in skin rafts (Fig 1) and psoriatic lesions (Fig 5). We also demonstrate that TIG2 expression is reduced in psoriatic lesions in comparison to nonlesional skin (Fig 5A,B), suggesting that its expression may play a role in maintaining the normal phenotype of the skin. TIG2 expression was significantly induced in vivo in psoriatic lesions after 3 d or 2 wk of topical application of tazarotene (Fig 5C,D). The early induction of TIG2 expression in the epidermis of the psoriatic lesion after tazarotene treatment, along with the observation that TIG2 was induced in skin rafts in a retinoid-dependent manner, suggest that the TIG2 induction is a result of direct effect of tazarotene on keratinocyte gene expression rather than an overall tazarotene effect on the lesional therapeutic status. Whether TIG2 is a marker of retinoid efficacy in psoriasis can be determined only by elucidation of its biologic functions.

TIG2 was induced only by RAR and not by RXR or VDR-specific agonists (Fig 6), although human keratinocytes contain...
lesions are important first steps in understanding the mechanisms of retinoid action in psoriasis.

We thank S. Thacker and S. Frient for generously providing primary foreskin keratinocytes and fibroblasts. We also thank Drs. L. Wheeler and M. Rosenblath for critical review of the manuscript. M.D. is supported by Grant AR 39915 (National Institute of Arthritis and Musculoskeletal and Skin Diseases) from the National Institutes of Health.

REFERENCES


Nagpal S, Friant S, Nakashahi H, Champon P: RARs and RXRs: evidence for two autonomous transcription activities (AF-1 and AF-2) and heterodimerization in vivo. EMBO J 12:2349–2360, 1993


