Tazarotene-Induced Gene 1 (TIG1), a Novel Retinoic Acid Receptor-Responsive Gene in Skin

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Retinoids exert their effect through ligand-dependent transcription factors, retinoic acid receptors (RARα, β, and γ) and retinoid X receptor (RXRα, β, and γ), which belong to the superfamily of steroid/thyroid/vitamin D₃ nuclear receptors. Using a subtraction hybridization approach, we have identified a cDNA sequence, Tazarotene Induced Gene 1 (TIG1), which is highly upregulated in skin raft cultures by an RAR/γ-selective retinoid AGN 190168 (tazarotene/ethyl 6-[4-(4,4-dimethylthiophen-6-yl)-ethyl]nicotinate), which is effective in the treatment of psoriasis. The retinoid-mediated upregulation in the expression of TIG1 was confirmed by Southern and sequencing, TIG1 was found to be a novel cDNA which encodes a protein of 228 amino acids whose sequence suggests that it is a transmembrane protein with a small N-terminal intracellular region, a single membrane-spanning hydrophobic region, and a large C-terminal extracellular region containing a glycosylation signal. We demonstrate that TIG1 is also upregulated by AGN 190168 in skin raft cultures prepared from psoriatic fibroblasts and normal keratinocytes and in primary fibroblast and keratinocyte cultures. We also show that TIG1 is upregulated by retinoic acid receptor but not by retinoid X receptor-specific synthetic retinoids. Finally, we demonstrate that TIG1 is induced by AGN 190168 in psoriatic lesions during the course of clinical treatment of the disease. 


Retinoic acid (RA) and its synthetic analogs exert their biological effects through two families of nuclear receptors, retinoic acid receptors (RARα, β, and γ) and retinoid X receptor (RXRα, β, and γ), which belong to the superfamily of steroid/thyroid hormone nuclear receptors (Chambon, 1994; Mangelsdorf et al, 1994). RARs and RXRs upregulate gene expression by binding to the promoter regions of retinoid responsive genes as transcriptionally active RAR-RXR heterodimers (Naggal et al, 1993) or RXR homodimers (Zhang et al, 1992). Although retinoids are therapeutically effective in the treatment of various dermatological diseases (Peck et al, 1994; Boehm et al, 1995), their mode of action in skin is poorly understood. A number of retinoid-induced genes have been identified in various systems (Mangelsdorf et al, 1994), but cellular retinoid acid-binding protein II (CRABP II) is the only marker known to be induced by RA in normal skin (Elder et al, 1993). CRABP II is downregulated by RA in submerged keratinocyte cultures (Elder and Cromie, 1993), however, indicating that its promoter contains another enhancer function, which under certain conditions dominates the enhancer action of its retinoid acid response element. This notion is further strengthened by the observation that CRABP II is overexpressed in psoriasis (Didierjean et al, 1991), a disease that responds to retinoid treatment. Therefore, CRABP II does not appear to be an efficacy marker of retinoid action in diseased skin, and it may possibly contribute to the pathogenesis of psoriasis by sequestering RA in the cytoplasm. In order to understand the mechanism of retinoid action in diseased skin, we systematically looked for the genes induced by a novel RAR/γ-selective retinoid, AGN 190168 (tazarotene) (Naggal et al, 1995), which is topically effective for the treatment of psoriasis (Esgeleyes-Ribot et al, 1994). By subtractive hybridization in 3-dimensional cultures of skin (skin rafts), we have identified a novel RAR-responsive gene, tazarotene-induced gene 1 (TIG1), which is induced, in various 2- and 3-dimensional cultures of skin cells, in a retinoid-dependent manner. We also demonstrate that TIG1 is induced by the retinoid AGN 190168 in psoriatic lesions, where retinoids exert their therapeutic action. Thus, TIG1 is a true retinoid-responsive gene and appears to be a marker of retinoid therapeutic action in skin.

MATERIALS AND METHODS

Skin Raft Cultures Normal skin raft cultures (Model ZK 1300, Advanced Tissue Sciences, CA) are 3-dimensional human skin tissues that have dermal, epidermal, and corneal 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 an epidermis consisting of multilayered stratum corneum, basal, spinous, and granular layers.

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Subtractive Hybridization
Skinraft cultures (Advanced Tissue Sciences) were kept overnight in serum-free maintenance medium (Advanced Tissue Sciences) and either mock-treated or treated with AGN 190168 (1 μM) on day 1 and 3, and harvested on day 4 by directly immersing them in guanidine thiocyanate solution for total RNA preparation. cDNA prepared from AGN 190168-treated skin raft cultures was directionally cloned into Sal I-Not I sites of pSPORT-2 (Life Technologies Inc.) and single-stranded DNA (ssDNA) was generated with the help of M13K07 (>1×10^6) input/ml, Life Technologies Inc.) (Li et al., 1994). Another directioncDNA library was constructed in Sal I-Not I sites of pSPORT-2 (Life Technologies Inc.) using cDNA from mock-treated skin raft cultures, linearized with Sal I and in vitro transcribed in the presence of biotin-14-CTP as described (Li et al., 1994). pSPORT-1 ssDNA was blocked with a 5′-oligo primer 5′-GGCGGCGGCGCCG-3′ by incubating at 37 °C with 100 μM dTTP and Taq DNA polymerase. Poly dA-blocked ssDNA (600 ng) was hybridized with biotinylated RNA (80 μg) in 2× hybridization buffer (80% formamide, 100 mM N-2-hydroxy-ethylpiperezine-N′-2-ethanesulfonic acid, pH 7.5, 2 mM ethylenediamine tetraacetic acid, and 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 with phenol/chloroform extraction (five times), unhybridized ssDNA was precipitated and electrophoresed into DH112S (Life Technologies Inc.) cells.

Retinoids
All-trans retinoic acid was purchased commercially (Sigma Chemical Co., St. Louis, MO). AGN 190168 (tazarotene), AGN 190299 (6′-(4′,4′-dimethyl-1′-cyclohexene-1′-yl)-3′-butan-1′-yl) benzoic acid), AGN 191183 (TTPNB; 4′-[2′-5′,6′,7,8-tetrahydro-5′,8′-tetramethyl-2′-naphthylamino]-2′-propenyl]benzoic acid], SR 11217 ([1′-4′-2′.6′-dimethyl-1′-cyclohexene-1′-yl]-3′-butan-1′-yl) benzoic acid], and SR 11237 (2′,4′-carboxyphenyl-2′-5′,6′,7,8-tetrahydro-5′,8′-tetramethyl-2′-naphthylamino-1′,3′-dioxolane) were synthesized in the Department of Chemistry, Retinoid Research, Allergan Inc. (Irvine, CA).

Southern Hybridization
For Southern hybridization, Sal I-Not I-digested cDNA clones were electrophoresed in duplicate, transferred to nitro, and probed with labeled total cDNA from either mock-treated or AGN 190168-treated skin rafts. Probes were hybridized overnight (42°C) and the membranes were washed (0.1× SSPE/1% sodium dodecyl sulfate) at 65°C for 45 min.

Preparation of Psoriatic Skin Raft Cultures
Human cultured psoriatic fibroblasts (200,000 cells) obtained from psoriatic lesion punch biopsies were mixed with type I collagen (3.5 mg/ml, Collaborative Biochemicals Products, Bedford, MA), neutralized with NaOH (1 N) and plated in 24-well plates. Human primary foreskin keratinocytes (500,000 cells/well) were layered after 24 h on top of the collagen layer. The collagen gels containing fibroblasts and keratinocytes were raised to the air-liquid interface after 24 h as described (Bell et al., 1979). Rafts were maintained in FAD medium supplemented with fetal bovine serum (10%), hydrocortisone (400 ng/ml), cholera toxin (10−10 M, Sigma), epidermal growth factor (10 ng/ml), fungicid (1%), adenosine (0.089 mM), and insulin (0.105 IU/ml), as described (Ducic et al., 1994), grown for 10 days and used for experiments on day 11. Psoriatic rafts were kept overnight (day 0) in serum-free medium, treated with retinoids on day 1 and day 3 (1 μM each) and harvested on day 4 by fixing in paraformaldehyde (4%).

PCR Amplification of TIG1 in Cultured Keratinocytes
For the isolation of keratinocytes, fresh human foreskins were washed in ethanol (70%) for 10 s followed by two washings in keratinocyte growth medium (Clonetics). Foreskins were cut into small pieces (4-mm diameter), incubated with trypsin (0.05%, Life Technologies, Inc.) for 24 h at 4°C, centrifuged, and harvested after 6 min, filtered through a nylon mesh (40 μm), and cultured in keratinocyte growth medium containing 10% fetal bovine serum. The media was replaced after 3 days with keratinocyte growth medium without fetal bovine serum, and keratinocytes were maintained in serum-free media and treated with AGN 190168 (1 μM) after three passages for 24 h. Total RNA was isolated from AGN 190168-treated and mock-treated cultures by guanidine thiocyanate (Promega) method, reverse transcribed using oligo(dT) and polymerase chain reaction (PCR)-amplified using TIG1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (internal control, Clontech) primers. TIG1 message was amplified using oligo pairs 5′-TCAGTGGTTTTTCTCTTTTACGTG-3′ and 5′-ACCTGGTTTACAGCAAAATG-3′. PCR products (10 μl) were removed after every three PCR cycles starting from 12 to 36 cycles as shown in Figs 3A and B. The PCR products were electrophoresed on agarose gel (2%) and viewed on a ultraviolet transilluminator after ethidium bromide staining.

In Situ Hybridization
In situ hybridization was performed on paraffin-embedded paraformaldehyde-fixed sections using Genius kit (Boeringer Mannheim, Indianapolis, IN). Proteinase K (2.5 μg/ml) digestion was carried on for 30 min and hybridization with digoxigenin labeled anti-sense probe (0.4 ng/ml) was performed at 44°C. Nonspecific binding was blocked with normal sheep serum (2%), and specific binding was detected by incubation (3 h) with alkaline phosphatase conjugated anti-digoxigenin antibody followed by 4-h incubation with nitroblue tetrazolium substrate at 37°C. Sense probes were used in parallel as negative controls and showed no background staining.

RESULTS

Identification of a Novel Retinoid-Responsive Gene, TIG1, by Subtraction Hybridization
AGN 190168, an RAR/β-selective retinoid, is effective in the treatment of psoriasis (Esleyges-Ribot et al., 1994), a hyperproliferative and inflammatory skin disease. Using a subtraction hybridization procedure (Li et al., 1994) for the isolation of AGN 190168-induced genes in 3-dimensional skin raft cultures, we have identified a cDNA sequence which was upregulated by retinoid treatment. Southern analysis of the subtracted cDNA clones showed the upregulation of #B27 and #B16 in nytran blots probed with labeled total cDNA from AGN 190168-treated cultures compared with the control where cDNA from mock-treated raft cultures was used (Fig 1A). The upregulation of #B27 and #B16 was further confirmed by Northern analysis of total RNA from mock-treated and AGN 190168-treated skin raft cultures and 12P-labeled #B27 and #B16 cDNAs (Fig 1C,D). The blot was probed with labeled GAPDH cDNA to ensure that equal quantity of control and retinoid-treated RNA was used in the Northern analysis (Fig 1B). Upon sequencing, #B27 and #B16 were found to be identical and hereafter will be referred to as TIG1. TIG1 was 862 bp in length, contained an open reading frame of 228 amino acids (Fig 2A). It had a poly A signal (5′-AATAAA-3′) at the 826 bp position (Fig 2A). Homology searches from DNA (GenBank and EMBL) and protein (Swiss-Prot) databases did not provide any identity to known DNA and protein sequences, thus indicating TIG1 to be a new cDNA sequence. The putative protein sequence of TIG1 contained a hydrophobic stretch of 19 amino acids (amino acids 24 to 45) and a glycosylation signal (NVT, amino acids 142 to 144) and a hyaluronic acid binding motif (KCSARVF, amino acids 125 to 133) (Fig 2A,B). Therefore, TIG1 appears to code for a transmembrane protein with a long extracellular domain of 194 amino acids, a membrane-spanning region of 19 amino acids and a small intracellular region of 15 amino acids. The hydropathic profile of TIG1 resembles that of CD38 (Jackson and Bell, 1990), another retinoid-responsive gene present in immune cells (Malavasi et al., 1994). CD38 also has a small intracellular domain (21 amino acids), a single transmembrane region and a long extracellular region (Jackson and Bell, 1990). Thus, TIG1 might be structurally related and analogous in function to CD38 since both are retinoid responsive and display homology in their hydropathic profiles. TIG1 was also upregulated by AGN 190168 in primary cultured keratinocytes (Fig 3A) and dermal fibroblasts (Fig 3C). GAPDH controls for RNAs are shown in Fig 3B and D for keratinocytes and fibroblasts, respectively. TIG1 was also induced by all-trans retinoic acid in dermal fibroblasts (Fig 3C) and skin raft cultures (data not shown), thus demonstrating it to be a classical retinoid-responsive gene.

TIG1 Is an RAR but Not an RXR Agonist-Responsive Gene
AGN 190168, 190121, and 191183 (TTPNB) activated gene expression only through RARs and not through RXRs (Nagpal et al., 1995). In contrast, SR 11217 and 11237 activated gene expression through RXRs (Lehmann et al., 1992) and not through RARs.
(our unpublished results). Further, AGN 190168 and AGN 190121 were RARβ/γ selective and exhibited only weak transactivation through RARα (Nagpal et al, 1995). To determine the receptor specificities of TIG1 expression, primary cultured keratinocytes were treated with RAR-specific (AGN 190168, 190121, and 191183) or RXR-specific (SR 11217 and 11237) synthetic retinoids (Fig 3G). Further, TIG1 was not induced by RXR-specific synthetic retinoids in skin raft cultures (data not shown). The RAR-specific retinoids induced TIG1 expression, whereas the two RXR-specific retinoids failed to significantly induce TIG1, thus demonstrating that the induction of TIG1 is mediated through RAR but not by RXR agonist–dependent signal transduction pathways.

Figure 1. Screening and identification of AGN 190168 induced cDNA clones. Subtracted cDNA clones were screened for their AGN 190168 upregulation by Southern analysis (A) and they were finally confirmed by Northern hybridization (B–D). A) Two clones (B27 and B16) which showed upregulation are indicated by arrows. B–D) Total RNA (10 μg) form mock-treated (CONTROL) and AGN 190168–treated skin raft cultures was subjected to Northern hybridization. GAPDH (B), #27(C), and #16 (D) sequences were detected with labeled cDNA probes.

TIG1 is Upregulated in Psoriatic Rafts in a Retinoid-Dependent Manner. Psoriatic fibroblasts have been shown to induce hyperproliferation of normal keratinocytes (Saigag et al, 1985). Psoriatic skin raft cultures prepared from psoriatic lesional fibroblasts and normal keratinocytes were either mock–treated or treated with AGN 190168 or its acid from AGN 190299 (6-/2-[4,4-dimethylthiochroman-6-yl] nicotinic acid). Northern analysis of control and treated RNAs revealed the induction of TIG1 by both AGN 190168 and AGN 190299 (Fig 3E) in comparison with GAPDH (Fig 3F). We further demonstrated the retinoid mediated upregulation of TIG1 in psoriatic rafts by in situ hybridization using digoxigenin–labeled TIG1 anti-sense RNA. In mock-treated psoriatic rafts, TIG1 was expressed only in the suprabasal layers of the epidermis (Fig 4B). Treatment of rafts with AGN 190168 (Fig 4C) or AGN 190299 (Fig 4D) induced the expression of TIG1 in all the...
layers of epidermis, thus further confirming the results obtained with Northern hybridization (Fig 3E). Treatment of raft sections with labeled sense TIG1 RNA did not show any staining (Fig 4A).

**Topical Tazarotene (AGN 190168) Induces TIG1 Expression in Psoriatic Lesional Biopsies.** In order to demonstrate the induction of TIG1 in psoriatic patients after AGN 190168 treatment, patients (n = 20) with long-standing bilateral plaque psoriasis were treated twice daily with AGN 190168 (0.1% gel) in a clinical study for up to 8 weeks. Skin punch biopsies were taken on day 0 (before the start of treatment), day 3 (after 3 d of application of AGN 190168), and day 14 (after 2 wk of treatment with AGN 190168). In situ hybridization using digoxigenin-labeled TIG1 antisense RNA showed little staining for TIG1 mRNA in nonlesional psoriatic skin (Fig 4F) as well as in day 3 mock-treated lesion skin (Fig 4G). In contrast, TIG1 was readily observed either at day 3 or 14 in AGN 190168-treated psoriatic lesions. A typical in situ demonstrating induction of TIG1 in a day 3 biopsy is shown (Fig 4H). Treatment of psoriatic lesion section with sense digoxigenin-labeled TIG1 RNA probe did not show any staining (Fig 4E). TIG1 upregulation in psoriatic lesions was also confirmed by reverse transcript PCR in pooled biopsies from responders (n = 16) after 2 wk of treatment with AGN 190168 (data not shown). Thus, the upregulation of TIG1 by AGN 190168 is observed not only in 2- and 3-dimensional cultures of keratinocytes and fibroblasts but also in the epidermis of psoriatic patients treated with AGN 190168. Although TIG1 is induced by AGN 190168 in cultured primary fibroblasts (Fig 3C,D), it is expressed in psoriatic lesional skin after AGN 190168 treatment mainly in epidermal keratinocytes and not significantly in dermal fibroblasts (Fig 4G,H). Alternatively, the lack of induction of TIG1 in dermal fibroblasts in vivo could be due to the inefficient penetration of the topically applied retinoid.

**DISCUSSION**

In this article, we describe the identification of a new retinoid-responsive gene, TIG1, by a subtraction hybridization approach to determine genes regulated by an anti-psoriatic retinoid AGN 190168. TIG1 is induced in an RAR-specific manner in a variety of human skin-related systems analyzed in this report (Figs 1, 3, 4). We also demonstrate that TIG1 is not significantly detected in nonlesional or lesional psoriatic skin (Fig 4F,G), it is significantly induced during the clinical treatment of psoriasis by AGN 190168 (Fig 4H), suggesting that it might play a role in the anti-psoriatic mechanism of action of retinoids. Further, TIG1 is not induced by RXR-specific retinoids, whereas it is induced by all the RAR-specific retinoids tested (Fig 3G), although human keratinocytes contain both RARs and RXRs (Fisher et al., 1994). This observation is in concordance with other observations that RXR-specific compounds are silent as suggested for a DRS element (Kurokawa et al., 1994). Also, the RXR homodimer pathway (Zhang et al., 1992) is either absent in keratinocyte systems or does not lead to TIG1 induction. Since retinoid-dependent activation events involve RAR–RXR heterodimers (Chambon, 1994; Mangelsdorf et al., 1994), TIG1 must get activated by RAR–RXR heterodimers in response to RAR agonist with RXR acting as a partner to facilitate the DNA binding of RARs in an RXR agonist-independent manner. Thus, TIG1 appears to be a specific RAR-responsive gene.
Figure 4. Retinoid-dependent induction of TIG1 in psoriatic rafts and lesional psoriatic skin biopsy. *In situ* hybridization using digoxigenin labeled anti-sense TIG1 RNA probes was performed in psoriatic skin rafts after mock-treatment (B) or treatment with AGN 190168 (C) or AGN 190299 (D). AGN 190168-treated psoriatic raft probed with sense digoxigenin labeled probe is also shown (A). *In situ* hybridization using digoxigenin-labeled antisense TIG1 probe was also performed in nonlesional psoriatic skin (F), lesional psoriatic skin mock-treated for 3 d (G), and lesional psoriatic skin after treatment with AGN 190168 for 3 d (H). *In situ* control with retinoid-treated psoriatic skin probe with digoxigenin-labeled sense TIG1 probe was also performed (E). Scale bars: A,B,D–H) 100 μm; C) 50 μm.
which may be a molecular marker of retinoid therapeutic efficacy and hence useful in the systematic development of retinoids for skin diseases such as psoriasis. In contrast, although CRABP II has been suggested as an inducible marker of retinoid action in normal skin (Elder et al, 1993), it is expressed at high levels in psoriatic skin (Didierjean et al, 1991) and it is downregulated by RA in submersed keratinocyte cultures (Elder and Cromie, 1993).

It is interesting to consider the potential functions of TIG1 and its role in RAR-mediated biology. Since it appears to be a transmembrane protein and contains a putative hyaluronic acid binding motif, it might function as a cell adhesion molecule whose expression on the cell surface might lead to better cell-cell contact and reduced proliferation. Another important phenotype of psoriasis is the presence of inflammatory cell infiltrate in the epidermis and dermis. How TIG1 might inhibit the inflammatory component associated with psoriasis, is presently unknown and the exact answer must await more understanding of the biology associated with TIG1. It is particularly interesting to consider the similarity that exists in the hydrodynamic profiles of TIG1 and CD 38 in spite of the lack of homology at the nucleotide and amino acid levels. CD 38, which is acutely upregulated by retinoids in immune cells, has been shown to have cyclic ADP-ribosyl (cADPR) cyclase, cADPR hydrolase, and NAD+ glycohydrolase activities. Further, CD 38 binds in vitro to hyaluronic acid and like TIG1 contains a hyaluronic acid binding motif in its large extracellular domain (Nishina et al, 1994) and has been implicated in cell-cell adhesion functions (Malavasi et al, 1994). Thus, molecules such as TIG1 and CD 38 might play some general but not as yet well-understood roles in RAR biology in a cell-specific manner. The importance of TIG1 in RAR-mediated physiological functions and in the therapeutic efficacy of retinoids in responsive diseases such as psoriasis would be clear only after elucidation of the exact function of TIG1.

Finally, it should be noted that in spite of the general understanding that has prevailed since the discovery of the retinoid nuclear receptors that retinoids are gene regulatory molecules, TIG1 is the first novel gene to be identified specifically because of its retinoid responsiveness.

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REFERENCES


Nagpal S, Friant S, Nakshhatry CH, Chambon P: RARs and RXRs: evidence for two autonomous transactivation functions (AF-1 and AF-2) and heterodimerization in vitro. *EMBO J* 12:2349–2360, 1993


