Retinoic Acid and its 4-Oxo Metabolites are Functionally Active in Human Skin Cells *In Vitro*

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Retinoic acid exerts a variety of effects on gene transcription that regulate growth, differentiation, and inflammation in normal and neoplastic skin cells. Because there is a lack of information regarding the influence of metabolic transformation of retinoids on their pharmacologic effects in skin, we have analyzed the functional activity of all-*trans*-, 9-*cis*-, and 13-*cis*-retinoic acid and their 4-oxo-metabolites in normal human epidermal keratinocytes (NHEKs) and dermal fibroblasts using gene and protein expression profiling techniques, including cDNA microarrays, two-dimensional gel electrophoresis, and MALDI-MS. It was previously thought that the 4-oxo-metabolites of RA are inert catabolic end-products but our results indicate instead that they display strong and isomer-specific transcriptional regulatory activity in both NHEKs and dermal fibroblasts. Microarray and proteomic analyses identified a number of novel genes/gene products that are influenced by RA treatment of NHEKs or fibroblasts, including genes for enzymes catalyzing biotransformation of retinoids, corticosteroids, and antioxidants and structural and transport proteins known to be essential for homeostasis. Our results expand current knowledge regarding retinoic acid action within skin cells and the target tissue/cell regulatory systems that are important for modulating the physiological and pharmacological effects of this important class of dermatological drugs.

Key words: dermal fibroblasts/epidermal keratinocytes/functional proteomics/gene expression profile
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Retinoids (vitamin A and its analogs) are crucial for maintaining the homeostasis of cells and tissues including proliferation and differentiation (Gudas *et al*, 1994). Aside from vision, the physiologic activity of retinoids is thought to be mediated primarily by the all-*trans*- and 9-*cis*-retinoic acid (RA) isomers that regulate transcription following binding to their cognate ligand-dependent transcription factors, the RA receptors (RAR), and retinoid X receptors (RXR) (Chambon, 1994, 1996; Mangelsdorf *et al*, 1994, 1995; Ross *et al*, 2000; Chawla *et al*, 2001; Clagett-Dame and DeLuca, 2002). Several hundred genes may be regulated by all-*trans*- and/or 9-*cis*-RA (Balmer and Blomhoff, 2002).

The skin is a stratified squamous epithelium and a major site of RA action (Peck and DiGiovanna, 1994; Roos *et al*, 1998; Blaner, 2001; Zouboulis, 2001). Vitamin A deficiency is known to result in squamous metaplasia of a variety of epithelia that is often associated with increased cell proliferation and hyperkeratosis in the skin (Peck *et al*, 1949). From studies of vitamin A-deficient animals and observation of vitamin A-deficient human infants, it was suggested that vitamin A deficiency gives rise to follicular keratoses resembling Darier's disease or pityriasis rubra pilaris (Peck and DiGiovanna, 1994). Initial attention focused on the naturally occurring all-*trans*- and 13-*cis*-RA isomers as potentially effective for treating skin disease (Peck and DiGiovanna, 1994; Roos *et al*, 1998; Shroot *et al*, 1999; Blaner, 2001; Zouboulis, 2001). More recently, a number of synthetic RA analogs with enhanced therapeutic efficacy have been developed (Peck and DiGiovanna, 1994; Roos *et al*, 1998; Shroot *et al*, 1999; Blaner, 2001; Zouboulis, 2001). It is important to emphasize that the skin has both a physiologic requirement for RA and responds to pharmacologic doses of natural or synthetic retinoids that are effective for treating patients with skin disease (Peck *et al*, 1949).

Since human skin cells express both RAR and RXR (Peck and DiGiovanna, 1994; Roos *et al*, 1998; Shroot *et al*, 1999; Zouboulis, 2001), it is reasonable to assume that retinoid effects in skin are mediated by retinoid-dependent transcription factors. Interestingly, the binding affinity of 13-*cis*-RA for the RAR and RXR and its activity in transactivation assays is substantially less than that for either all-*trans*- or 9-*cis*-RA (Mangelsdorf *et al*, 1992, 1994; Beard and Chandraratna, 1999). Yet, it is well established that 13-*cis*-

Abbreviations: CYP, cytochrome P450; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; IL, interleukin; LRAT, lecithin:retinol acyltransferase; MALDI-MS, matrix-assisted laser desorption ionization-mass spectrometry; MMP, matrix metalloproteinase; NHEK, normal human epidermal keratinocyte; PBS, phosphate-buffered saline; RA, retinoic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBS, tris-buffered saline

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RA exerts pharmacological effects that cannot be recapitulated by either all-*trans*- or 9-*cis*-RA, raising questions regarding the molecular basis underlying the remarkable efficacy of 13-*cis*-RA efficacy in acne vulgaris (Peck and DiGiovanna, 1994; Roos *et al*, 1998; Shroot *et al*, 1999; Blaner, 2001; Zouboulis, 2001). One possibility is that the superior clinical efficacy of 13-*cis*-RA in acne as compared with all-*trans*- or 9-*cis*-RA could relate to unique pharmacokinetic properties of this isomer (Peck and DiGiovanna, 1994; Blaner, 2001). In addition, the drug may act directly on proteins in skin cells by covalent and non-covalent binding. There is a substantial literature indicating that RA can covalently bind to proteins, thereby altering cell signaling pathways (Takahashi *et al*, 1991a, b; Takahashi and Breitman, 1992; Myhre *et al*, 1996; Hoyos *et al*, 2000; Radominska-Pandya *et al*, 2000; Imam *et al*, 2001; Korichneva *et al*, 2003). Another possibility is that the effects of 13-*cis*-RA in acne are indirect, acting for instance via competitive inhibition of enzymes involved in the bioactivation of androgenes (Karlsson *et al*, 2003). Alternatively, it is possible that one or more metabolites formed from 13-*cis*-RA could display transcriptional regulatory activity. To assess these possibilities, we have studied the metabolism and actions of 13-*cis*-RA in primary cultures of normal human skin cells. To conduct these studies, we used gene arrays and two-dimensional (2D) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) separations of proteins coupled with matrix-assisted laser desorption ionization-mass spectrometry (MALDI-MS) identification of the proteins to explore various modes of action of all-*trans*-, 13-*cis*-, 9-*cis*-RA, and their oxo-metabolites in these cells.

Results

cDNA microarray analysis of the effects of RA and its oxo-metabolites on normal human epidermal keratinocytes (NHEK) and dermal fibroblast gene expression To explore the mechanism whereby all-*trans*-, 13-*cis*-, and 9-*cis*-RA isomers and their 4-oxo-metabolites might differentially affect gene expression when added to cultured NHEK and fibroblasts, each of the RA isomers and 4-oxo-RA species was added to primary cultures of NHEK or fibroblasts for 24 h, and analysis of differential gene expression was performed using DNA-Chip technology (Fig 1). We analyzed the expression of sequence-validated cDNA arrayed on PIQOR Skin Microarray using ImaGene software version 4.1 (Table I and Tables S1 and S2). There were substantial differential effects of the RA isomers on gene expression. Incubation of NHEK with 10^{-6} M of each RA isomer for 24 h led to upregulation of keratins 7, 15, and 19 as well as interleukin-1 (IL-1)β, tissue-type plasminogen activator, and others (Table I and Table S1). We detected a significant downregulation of keratins 1, 10, 14, and 17 as well as matrix metalloproteinase (MMP)-10, vascular endothelial growth factor (VEGF)-C, thrombospondin-1, plasminogen activator inhibitor-2, cyclooxygenase-2, psoriasis-associated fatty acid-binding protein (PA-FABP), and filaggrin and other genes by all-*trans*-, 13-*cis*-, and 9-*cis*-RA (Table I and Table S1). Expression of S100 calcium-binding protein A8 was upregulated by 13-*cis*- but downregulated by and all-*trans*- and 9-*cis*-RA.

Interestingly, the 4-oxo-metabolites had very similar effects on expression profiles as those observed for their parent compounds (Table I and Table S1). The 4-oxo-metabolites of RA, especially 4-oxo-13-*cis*-RA, are generally thought to be inert catabolic end-products that are destined for elimination. As evidenced in Table I and Table S1, however, these metabolites, which cannot be converted back to RA, have substantial transcriptional activity. Incubation of NHEK with these substances for 3 h revealed minor changes in gene expression profiles (Table S2).

Incubation of *dermal fibroblasts* with the RA isomers for 24 h revealed more substantial changes than those observed in NHEK, including downregulation of p-450-dependent enzymes involved in xenobiotic/endogenous biotransformation, including cytochrome P450 (CYP)2D, CYP3A7, CYP4A1, corticosteroid 11β-dehydrogenase, prostaglandin-endoperoxide synthase 1, dual specificity phosphatase 11, glutathione S transferase M1, and transport proteins like solute carrier family 16 (SLC16A1) (Table S3). Similar effects were seen for most of the RA isomers and their 4-oxo-RA isomers. The RA and their 4-oxo metabolites downregulated fibroblast Fas-activated serine/threonine kinase, growth factor receptor-bound protein 7, epidermal growth factor receptor, collagen types VI and XVII, as well as protease inhibitor 8 and 9 (4-oxo-) 13-*cis*-, (4-oxo-) all-*trans*-, and (4-oxo-)-9-*cis*-RA. Expression of annexin A1, annexin A5, thymosin β4, ribosomal protein S13, apolipoprotein D, and insulin-like growth factor-binding protein 3 was upregulated in skin fibroblasts by 4-oxo-13-*cis*-, all-*trans*-, and 4-oxo-all-*trans*-RA but not by 9-*cis*-, 4-oxo-9-*cis*-, and 13-*cis*-RA.

Real-time PCR (RT-PCR) confirmation of gene array data RT-PCR analysis in NHEK incubated with 10^{-6} M retinoids using specific primers for the detection of cytokeratins 1, 10, 13, and 19 confirmed strong upregulation of cytokeratins 13 and 19 by 13-*cis*-RA but a weaker upregulation by all-*trans*-, 9-cis-, 4-oxo-13-*cis*-, 4-oxo-all-*trans*-, and 4-oxo-9-*cis*-RA (Fig 2*b* and *d*). In accordance with the cDNA microarray data, we also observed downregulation of cytokeratins 1 and 10 and MMP-3 by all-*trans*-, 9-*cis*-, and 13-*cis*-RA and their 4-oxo-metabolites (Fig 2*a*, *c*, and *e*). Since there were no available cDNA probes specific for the detection of lamin B1 on the microarrays used in this project, our 2D-gel data were also directly confirmed by real-time PCR (Fig 3*f*). Only a weak upregulation of lamin B1 mRNA expression in NHEK occurred after incubation with each of the six different retinoids, especially after incubation with 13-*cis*- or 4-oxo-13-*cis*-RA. Real-time PCR analyses of NHEK incubated with 10^{-7} M concentration of the retinoids revealed similar effects (Table S4). When cells were incubated with these substances at a lower concentration (10^{-8} M), upregulation of keratins 13 and 19 and downregulation of MMP-3 were still detectable, whereas expression of keratins 1, 10, and lamin B1 was not significantly regulated (Table S4).

Immunofluorescence analysis of the effects of 13-*cis*- and 4-oxo-13-*cis*-RA on NHEK expression of cytokera-