Stony Brook University

Conversion of Vitamin A1 to Vitamin A2 and Its Involvement in the Response of Cultured Human Keratinocytes to UV Irradiation

A Dissertation Presented

by

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to

The Graduate School

in Partial Fulfillment of the

Requirements

for the Degree of

Doctor of Philosophy

in

Oral Biology and Pathology

Stony Brook University

August 2008

Stony Brook University

The Graduate School

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ABSTRACT OF THE DISSERTATION

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2008

This study focuses on the significance of Vitamin A1 to A2 conversion and its involvement in the response of human keratinocytes to UV irradiation. Previous work has shown that the Vitamin A system is a direct target of UV radiation and participates in the adaptive response of the cells. Human epidermal keratinocytes are unique in their ability to convert Vitamin A1 to Vitamin A2

retinoids. Even though retinol metabolism is studied extensively, little is known about this conversion. Even less is known about the biological role of this form of retinoid in epidermal cells.

For the first time in this work an *in vitro* conversion of Vitamin A1 to A2 was performed. This was achieved by using sonicates from normal human keratinocytes and SCC13. This converting activity showed enzymatic characteristics and the majority of it was recovered in the fraction containing mostly plasma membrane. Ketoconazole, a known inhibitor of p450 enzymes, inhibits the *in vivo* and *in vitro* conversion of Vitamin A1 to A2. Conversion of Vitamin A1 to A2 increases with progression of culture confluence.

The data presented shows that Vitamin A1 to A2 conversion is up-regulated after UV irradiation. Vitamin A2 esters in cultured keratinocytes are more stable than Vitamin A1 esters under UV radiation. Vitamin A2 esters are capable of better protection of epidermal keratinocytes after UV irradiation to undergo apoptosis than Vitamin A1 esters. This was shown by using cell death detection ELISA plus assay and normal human foreskin keratinocytes.

It was demonstrated that Vitamin A2 acid is able to promote transcription of the responsive genes. Screening of 84 genes related to the p53 pathway was performed by using PCR - Based Gene Expression Profiling experiments. Also differential expression of FASLG gene was observed in presence of Vitamin A1 or A2 acids.

In conclusion the Vitamin A2 form of retinoids provides better protective functions than Vitamin A1 after UV irradiation. Also Vitamin A2 acid probably has a different role in promoting transcription of responsive genes than Vitamin A1 acid.

With all my love, I dedicate this work to my family

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LIST OF ABBREVIATIONS

3T3	standard fibroblast cell line from Swiss mouse embryo tissue
5,6-epoxy-RP	5,6-epoxyretinyl palmitate
ABC complex	avidin—biotin—peroxidase enzyme complex
ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid
AP-1	activator protein 1
AR	anhydroretinol
ARAT	acyl CoA:retinol acyltransferase
BAI1	brain-specific angiogenesis inhibitor 1
Bax	Bcl2-associated X Protein
BCL-2	B-cell CLL/Lymphoma 2
BNL	Brookhaven National Laboratory
BPE	bovine pituitary extract
BSA	bovine serum albumin
CARM1	coactivator-associated arginine methyltransferase 1
CAT assay	chloramphenicol acetyltransferase assay
CBP/p300	creb-binding protein/histone acetyltransferase
CDK4	cyclin-dependent kinase 4
c-jun	mitogen-activated protein kinase 9
CRABP	cellular retinoic acid-binding proteins
CRBP	cellular retinol binding protein
CYP	cytochrome p450
DMEM	Dulbecco's modification of Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DR	direct repeats
E2F1	E2F transcription factor 1

E2F3	E2F transcription factor 3
EDTA	ethylene diamine tetra acetic acid
ELISA	enzyme-linked immunosorbent assay
ESR1	estrogen receptor 1
FABP	fatty acid binding protein
FADD	Fas (TNFRSF6)-associated via death domain
FAD(H)	flavin adenine dinucleotide (reduced)
FasL (FASLG)	TNF superfamily, member 6
FasR	Fas receptor
FBS	fetal bovine serum
Gadd45	growth arrest and DNA damage-inducible protein 45
HaCaT	spontaneously immortalized human keratinocyte cell line
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	high pressure liquid chromatography
IFNb	interferon, beta,
IL6	interleukin 6 (interferon, beta 2)
KBM	keratinocytes basal medium
KGM	keratinocytes growth medium
LRAT	lecithin:retinol acyltransferase
MAPK	mitogen-activated protein (MAP) kinase
MMP	matrix metalloproteinases
NAD(H)	nicotinamide adenine dinucleotide (reduced)
NADP(H)	nicotinamide adenine dinucleotide (reduced)
NER	nucleotide excision repair
NFAT	nuclear factor of activated T cells
NHEK	human epidermal keratinocytes
NHEK-F	normal human foreskin keratinocytes
p53	tumor protein p53
PBS	phosphate buffered saline
PC	positive factor
PCR	polymerase chain reaction

PM	plasma Membrane
PPAR	peroxisome proliferator-activated receptor
PRMT	protein arginine N-methyltransferase
PUVA	a combination of 8-methoxypsoralen and UVA radiation
RalDH	retinal dehydrogenases
RAR	retinoic acid receptor
RARE	retinoic acid response elements
RBP	retinol binding protein
RNA	ribonucleic acid
RoDH	retinol dehydrogenases
ROS	reactive oxygen species
RP	retinylpalmitate
RT	room temperature
RT-PCR	reverse transcriptase – polymerase chain reaction
RXR	retinoid X receptor
SA	specific activity
SCC	squamous cell carcinoma cell lines
SDR	short-chain dehydrogenase/reductase
SOCS	suppressors of cytokine signalling
SPF	sun protection factor
STAT	signal transducers and activator of transcription proteins
Stdev	standard deviation
TATA box	DNA sequence in the promoter region of most genes in eukaryotes and Archaea (Goldberg-Hogness box)
TP73	tumor protein p73
TRAIL	tumor necrosis factor ligand superfamily, member 10
UV	ultraviolet radiation
WAF1/CIP1/p21	cyclin-dependent kinase inhibitor-1A

ACKNOWLEDGEMENTS

I would like to thank my advisor Prof. Marcia Simon for her help and for her guidance in transforming me into an independent scientist, worker and thinker. From the beginning, Prof. Simon gave me a chance to be a part of the Living Skin Bank family. With her support I was able to study and develop my love and knowledge in the field of skin biology.

I would like to give my special recognition and thankfulness to my husband Dr. Stefan Tafrov for being there for me factually in every step with his infinite help, support and valuable advice.

Also I would like to thank Prof. Kathleen Parker and her graduate student Erik Stolarzewicz, Department of Chemistry, SUNY at Stony Brook for developing a procedure and performing the synthesis of Vitamin A2 acid.

I am thankful to Dr. Nelly Markova for her time and advice and for providing the pBLCAT2-DR5 plasmid.

I would like to thank Dr. Adriana Pinkas-Sarafova for her help and providing the standard curve for determining the cellular concentration of pyridine dinucleotides.

I am very grateful to Dr. Betsy Sutherland, Dr. John Sutherland and John Trunk for giving me the opportunities to perform my UV irradiations experiments in their laboratories in BNL. Also I especially appreciate their advice, support and understanding.

My thanks goes out to Dr. Jay Gao who was always there to provide me with constructive advice. Dr. Constantine Chipev's support and advice was always able to assist me in times of need.

From the moment I stepped in the world of tissue culture Gabriele Hatch was there to guide me and mother me through my journey. Alice Shih was always there to offer me help in the lab and guidance in real life situations. The spirit of Marie Barnouw made life in the lab enjoyable and pleasant. As graduate students, Nevena Karaman-Jurokovski and I shared memories and experiences.

I would like to thank Dr. Keith Randolph and Dr. Vladimir Jurokivski for introducing me to the world of retinoids and lipids.

INTRODUCTION

The Retinoids

The retinoids (Vitamin A analogs) are a large family of natural and synthetic compounds. Vitamin A1 alcohol (retinol) is the prototype of all other natural retinoids. It is composed of a β-inone ring, a polyprenoid side chain and a carbonyl end group (129). The association of Vitamin A with survival is well established. Postnatal Vitamin A deficiency is associated with immune deficiencies leading to morbid infection, with anemia, night blindness, and death. Retinoids also regulate embryonic development and thus in excess can be teratogenic.

Dietary sources of natural retinoids include carotenoids produced by plants and the long chain fatty acid esters of Vitamin A1 alcohol (retinyl esters) present in animal tissues. In the intestinal epithelium, carotenoids undergo oxidative cleavage to form Vitamin A1 aldehyde (retinal), which in turn is reduced to Vitamin A1 alcohol (25). The Vitamin A1 esters first undergo hydrolysis to produce Vitamin A1 alcohol and are then taken up by the intestinal epithelium. Vitamin A1 alcohol derived either from the carotenoids or from Vitamin A1 esters is esterified for transport via the lymphatics to the liver, which is the main Vitamin A1 storage site of the body. Transport into cells again requires ester hydrolysis. In the hepatocyte, Vitamin A1 alcohol is bound to retinol binding protein (RBP) and transported to the target tissues via the blood stream or transferred to Ito cells for storage as ester. Under Vitamin A1 sufficiency, RBP/retinol is bound to transthyretin and circulates at concentrations in the order of 1-2 μM (14, 109). Specific receptors for the RBP/Vitamin A1 alcohol complex on the plasma membrane of target tissue help regulate uptake. Vitamin A1 acid (retinoic acid) is also present in the circulation but only at 4-14 nM bound to serum albumin (29, 32).

Retinoids in the epidermis

Epidermis is one of the many target tissues of Vitamin A1. Over 90% of the epidermis is composed of keratinocytes which form the stratified squamous epithelium necessary to protect underlying tissues. The active metabolite of Vitamin A1 alcohol, Vitamin A1 acid (retinoic acid), is a transcription factor ligand, which when bound to its nuclear receptors can positively or negatively regulate gene transcription. It plays critical roles in epithelial homeostasis and in keratinocyte differentiation which require a narrow nM concentration range of Vitamin A1 acid.

Keratinocytes take up Vitamin A1 alcohol and Vitamin A1 acid from the circulation. Intracellularly, Vitamin A1 alcohol binds to cellular retinol binding protein (CRBP) which protects the alcohol from UV radiation and limits its metabolism to those enzymes that accept Vitamin A1 alcohol bound to CRBP as substrate (79). The majority of alcohol is sequestered as long chain fatty acid esters. The uptake and accumulation of Vitamin A1 acid is limited by its rapid metabolism in keratinocytes, intracellular binding proteins, and its depletion by dermal fibroblasts in tissue fluids (88).

Each of the three regions of Vitamin A1 alcohol are subject to modification: the polar end group which can be oxidized or esterified, the isoprene side chain which can be hydroxylated or isomerized and the β-ionone ring which can be desaturated or hydroxylated (Fig Introduction – 1) (80, 81). In cultured keratinocytes, more than 90% of the total internal retinoids exist as esters (87) whose formation is catalyzed by the microsomal enzymes lecithin: retinol acyltransferase (LRAT) (52) and acyl CoA:retinol acyltransferase (ARAT) (125). ARAT accepts only free Vitamin A alcohol as substrate and at high CRBPI levels does not contribute significantly to the ester pool (43). Access of the Vitamin A alcohol requires the action of retinyl ester hydrolases. The oxidation of the polar end group occurs in two steps. First the alcohol is oxidized to Vitamin A1 aldehyde by microsomal retinol dehydrogenases (RoDH) and then the aldehyde is oxidized to Vitamin A1 acid (retinoic acid) by cytosolic retinal dehydrogenases (RalDH) (77, 51). The aldehyde is readily reduced to the alcohol. In this way keratinocytes can increase the intracellular concentration of Vitamin A1 acid. In humans, ring desaturation at C3-C4 generates Vitamin A2 (3,4-didehydroretinol) and has only been demonstrated in keratinocytes (87; Fig Introduction – 1, black arrow). This retinoid was first identified as a component of the livers of certain species of freshwater fish (104) and only later was discovered in humans (128). Because Vitamin A2 is not found in the circulation, but can be synthesized in epidermis and in cultured epidermal keratinocytes, it is believed that, in humans at least the only source of epidermal Vitamin A2 is its biosynthesis from Vitamin A1 (87, 122, 96). Keratinoyctes cannot reverse this reaction. Vitamin A2 is present in epidermis in both esterified and unesterified form. These two forms, together with a small amount of Vitamin A2 acid (3, 4 - didehydroretinoic acid) can be observed in cultured human keratinocytes (87, 96). Although the enzymes that modify the terminal carbon and catalyze hydroxylation of the ring have been identified, little is known about the enzymes that catalyze ring desaturation. One of the goals of this work will be to characterize the enzymatic activity that is responsible for the conversion of Vitamin A1 to A2 in cultured human keratinocytes.

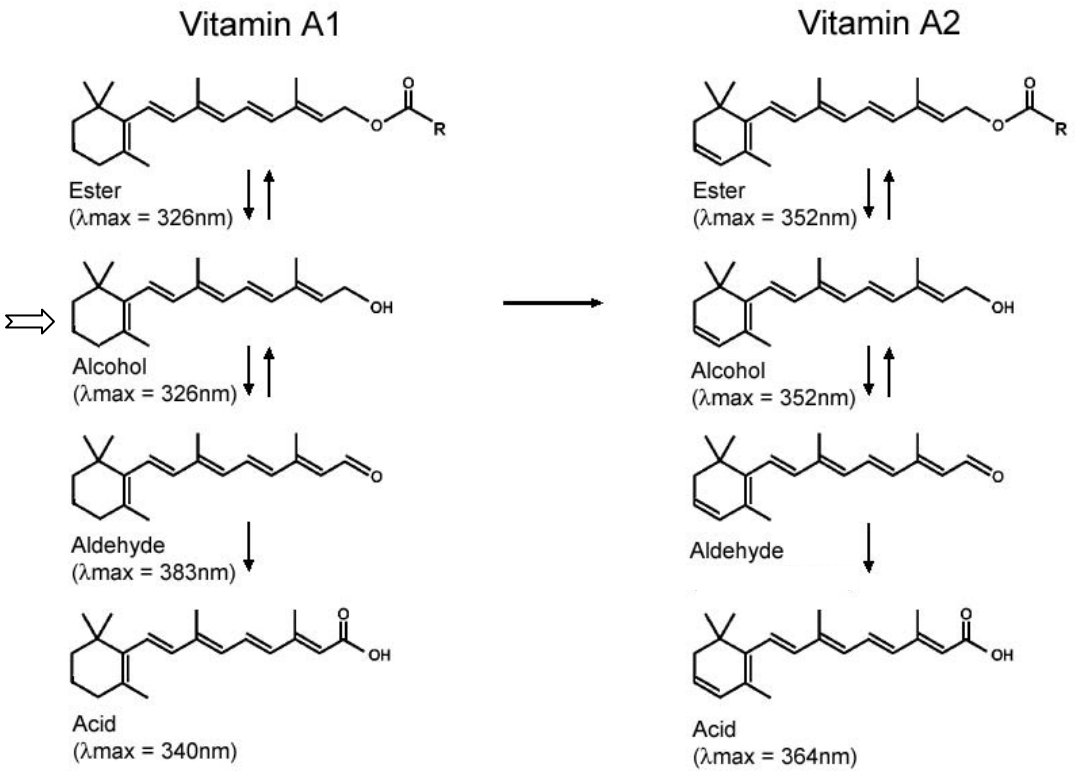


Fig I - 1: Molecular structure of the two Vitamin A families of endogenous retinoids (112)

Ultraviolet radiation (UV) radiation and its effects on the skin

Emissions from the sun include infrared, visible and UV radiation, proton particles, and heat. Solar UV radiation consists of three components – UVA (320 – 400 nM), UVB (280 – 320 nM), and UVC (200 – 280 nM) (67). Only UVC is prevented from reaching the Earth's surface by the ozone layer and the atmosphere that surrounds the Earth. UVA and UVB are responsible for the immense biological effects on the human body. UVB (6 %) represents only small portion of the total radiation while UVA (94 %) is the predominant fraction. UV radiation levels are influenced by sun elevation, latitude altitude, cloud cover and ground reflection (9).

Small amounts of UV radiation are beneficial and essential for the production of Vitamin D. UV radiation has also been used to treat hyperproliferative diseases (140). However, prolonged exposure to solar radiation may cause acute and chronic health effects. Acute exposure causes sunburn and tanning (60). Chronic exposure contributes to skin damage (photoaging and cancer), cataracts and immune suppression (67). UVB can penetrate the epidermis and to a lesser extent, the upper part of the dermis, while UVA radiation penetrates more deeply into dermis of human skin (114). UVB can be absorbed by proteins, nucleic acids and other macromolecules and cause direct damage to cells. UVA also causes indirect, cytotoxic and genotoxic effects by formation of oxidized radicals named sensitizers.

UV effects on Vitamin A1 alcohol and esters

In addition to their biological effects retinoids absorb UV radiation with a maximum from 326 to 383 nm (37, Fig Introduction – 1). UV exposure has been shown to decrease both Vitamin A1 alcohol and esters in skin (111, 127), and in so doing can induce local Vitamin A deficiency. Retinyl palmitate (RP) has been shown to be as efficient as commercial sunscreens with SPF 20 in preventing photobleaching and photooxidation of molecules encapsulated within liposomes (6) suggesting its potential usefulness as a sunscreen. Many cosmetics in fact contain retinoids either to protect the skin or to stimulate responses that will correct UV damage. Retinyl palmitate, which is more thermally stable than retinol, is found in over 600 cosmetic products (21). However, UV-induced photodecomposition of retinyl palmitate generates reactive oxygen species and lipid peroxides (21, 138, 47, 139). Lipid peroxidation in humans is associated with cancer, atherosclerosis, ischemia inflammation, liver injury, aging etc. (138). In one study it was found that UVA treatment of Jurkat T-cells in the presence of retinyl palmitate, or its decomposition products, anhydroretinol (AR) and 5,6-epoxyretinyl palmitate (5,6-epoxy-RP) increases DNA fragmentation (141). Other studies with mouse lymphoma cells demonstrated that RP (69) and its decomposition products (68) in combination with UVA exposure are photomutagenic. However, photodecomposition products are non-genotoxic in bacterial assays (21).

Thus, even though Vitamin A and its derivative are used therapeutically against skin cancer, the role of dietary uptake of Vitamin A in UV induced carcinogenesis has been brought up. Exposure to UV radiation leads to photodecomposition of excess Vitamin A in the diet of hairless mice that has been shown to generate short-lived intermediates. These radicals could act as photosensitizers during cutaneous carcinogenesis (73).

Vitamin A1 acid (all-*trans* and 13-*cis*) in ethanol and dermatological cream preparations exposed to solar simulated light, UVA and visible light undergo isomerization and photolysis where the all-*trans* form of Vitamin A1 acid is more sensitive (117). Vitamin A1 alcohol and Vitamin A1 esters in different media undergo photodecomposition and generate reactive oxygen species (18) and have been found to decrease in concentration in the epidermis of hairless mice exposed to UVB and UVA (110, 111). These molecules cause lipid peroxidation leading to premature aging (photoaging) with induction of matrix metalloproteinases and inflammation as well as forming DNA adducts that can lead to photocarcinogenesis (43).

Vitamin A2 retinoids (3,4-didehydroretinoids) are more stable under UV exposure

Vitamin A2 is found in the eye lens of the diurnal gecko *Lygodactylus picturatus* is bound to ι-cristallin, a protein closely related to another retinol binding protein CRBP I (95, 135). The protein complex Vitamin A2/ ι-cristallin absorbs short wave length radiation leading to the proposal that not only does improve the optical quality but it also protects the gecko's retina from the harmful UV radiation (102). Additional studies have shown that Vitamin A2 alcohol is more stable and is depleted more slowly than Vitamin A1 after UV radiation of cultured human keratinocytes and melanocytes (3). Phototherapy of patients with chronic renal failure and healthy control treated with UVA (7.9 J/cm^2) and UVB (1.3 J/cm^2) showed reduction in the epidermal Vitamin A1 and no change of Vitamin A2 alcohol (13). Vitamin A2 depletion under UV radiation in rabbit skin *in vivo* and in human skin *in vitro* is less compared to the Vitamin A1 depletion (12). Together, the data support the conclusion that Vitamin A2 is protective as a photostable retinoid. It is also possible that these retinoids exert their effect by acting as antioxidants due to their physiological properties and their capabilities to quench oxygen free radicals and singlet oxygen (102).

Another goal of this work will be to test and compare the ability of Vitamin A1 and A2 to protect epidermal keratinocytes from undergoing apoptosis after UV irradiation.

Biological effect of UV radiation

Many of the biological effects of UV radiation on sun exposed tissues are initiated by the triggering of cell signal pathways that lead to alteration in gene expression (44). Solar UV radiation triggers several different cell signal transduction pathways and involves a number of proteins and cell structures. The

UV response includes activation of different protein kinases (42, 56), transcription factors (27, 30) and cell membrane receptors to induce a variety of biological effects. After UV exposure the skin releases a number of growth factors, cytokines and receptor ligands that can act in paracrine or autocrine fashion to activate receptors on target cells. As a result the intracellular signaling pathways are activated, some of which initiate the program for apoptotic cell death. UV induced cell death by apoptosis is considered to be a natural protective mechanism that removes damaged keratinocytes and lessens the risk of accumulating damage that results in malignant transformation (7).

Cell death after UV radiation could be the consequence of reactive oxygen species (ROS), severe DNA damage and/or death receptor activation (57). UV induced apoptosis is a highly complex process where the precise molecular events involved in the triggering of apoptotic death of keratinocytes are still unclear. Some studies point out that low dose UV radiation sensitizes the cells to TRAIL induced apoptosis (86). Other studies distinguish between three different apoptotic events. Immediate apoptosis that is triggered by singlet oxygen (O_2) or superoxide anions (O_2^-) and results in mitochondrial proapoptotic signal activation. Intermediate apoptosis initiated at the plasma membrane by activation of the Fas receptor. And delayed apoptosis which is induced by DNA damage and leads to either, AP-1 induced FasL upregulation and initiation of the intermediate apoptotic mechanism, or p53 induced Bax upregulation, which triggers the creation of channels in the mitochondrial membrane and cytochrome *c* release into the cytoplasm (40).

Central role of p53 in UV radiation response

The p53 pathway responds to a variety of intrinsic or extrinsic stress signals. UV radiation is one of the many detrimental events that trigger cellular responses orchestrated by the p53 protein. The wild type p53 gene is also called “guardian of the genome”, for its role in preventing the accumulation of genomic alterations (28). Keratinocytes regulate its activity by changing the total amount of the p53 protein in the cells and by introducing posttranslational modifications such as acetylation or phosphorylation at specific sites in the p53 molecule. These posttranslational modifications are necessary for the protein to become stabilized and active, and thus to fulfill its biochemical and cellular functions (20). One important function of p53 is transcriptional activation or repression of downstream target genes. For this purpose, a tetramer of activated p53 proteins binds promoters of p53 responsive genes and activates or represses their activities.

The skin has specialized mechanisms to protect it from the damaging effects of UV radiation including cell cycle arrest to allow DNA repair and apoptosis to eliminate cells whose damage is too severe to repair. The p53 tumor suppressor gene codes for a DNA-binding protein that regulates the expression of numerous genes involved in each of these processes. For example, an important mediator of the G1 arrest is WAF1/CIP1/p21, which is a target gene of p53. p21 prevents the cyclin kinase-mediated phosphorylation of the

retinoblastoma protein, which is an essential step for the G1/S transition to proceed (7). p53 also plays a role in nucleotide excision repair (NER) that is one of the main pathways for repair of the UV-induced DNA damage. Components of the p53 pathway, in particular Gadd45, contribute appreciably to DNA repair. Recent studies showed that Gadd45 binds to UV-damaged chromatin, perhaps facilitating the access to the regions of DNA damage. The p53-associated nucleotide excision repair response may therefore be mediated at the level of chromatin accessibility to sites of DNA damage (108). Finally, p53 regulates UV induced apoptosis of keratinocytes by inducing transcription of genes involved in different stages of the apoptotic signal transduction. In addition, p53 mediates mitochondria dependent apoptosis through direct interaction with the mitochondrion itself and/or with members of the BCL-2 family of apoptosis regulating proteins (133).

There is high frequency of p53 gene mutations in many human cancers (45). Studies have shown an extreme sensitivity of p53-/- and +/- mice to tumor induction by UV radiation. This is another indication that p53 plays a critical role in protecting the cells against UV induced carcinogenesis. It supports the concept that detection of p53 mutations can serve as an early marker for or risk factor of skin cancer development (50).

Vitamin A1 acid plays a role in the signaling response after UV radiation

Retinoids are frequently used for treatment of photoaged skin. Vitamin A1 acid helps to protect human skin against such damage (23). On the other hand it was demonstrated that Vitamin A1 acid does not function as a sunscreen but most probably as a transcriptional regulator (27). This conclusion was based on clinical studies in which human volunteers were exposed to UV and redness compared after 1 minimal erythema dose on pretreated and untreated sites; the effect of Vitamin A1 acid is not by absorbing the UV radiation (70). It has been shown that UV radiation up regulates AP-1 and *in vivo* induces AP-1 – regulated matrix - degrading MMPs genes in human skin. Application of Vitamin A1 acid on the skin inhibits activation of AP-1 and thus reduces the induction of MMPs by UV radiation (34). It may antagonize UV activation of AP-1 by inhibiting induction of the c-jun protein that is executed by posttranscriptional modifications (35).

Vitamin A1 acid exerts its transcriptional effects as a ligand for heterodimeric nuclear retinoid receptors. The diverse activity of retinoids is primarily mediated by two families of nuclear retinoic acid receptors, the RARs and RXRs. Each family is composed of three members, α, β, and γ. Ligand – receptor complexes are formed upon binding of the ligand to these receptors. These complexes can bind to the retinoic acid response elements (RAREs) in the promoter region of the target genes which then recruit other proteins over the promoter. This triggers upregulation of the transcription from the target gene. On the other hand after binding with the ligand, both RARs and RXRs can inhibit expression of certain genes by antagonizing transcriptional activity of other receptors such as the activator protein-1 complex (19). The predominant retinoid receptors in human skin such as RARα, RARγ and RXRα are rapidly down

regulated in cultured human keratinocytes after UVB irradiation, which can cause alteration in the retinoid signaling (4, 16).

It has been shown that cells pretreated with 1μM Vitamin A1 acid before UVB irradiation undergo massive apoptosis compared to control cells that have not been pretreated with Vitamin A1 acid (74). In these cells the mRNA and the protein level of the p53 and several caspases that participate in the apoptotic response were strongly upregulated. Over expression of p53 by itself does not induce apoptosis. Importantly experimental evidence indicates that the proapoptotic activity of retinoids depends on the presence of wild-type p53 (74). Currently there is no developed model for the interaction between p53 and the retinoids although the experimental data suggests that there is active cross talk between these two major signaling pathways.

Vitamin A2 acid - ligand for retinoic acid receptors

In vitro and *in vivo* studies have shown that Vitamin A2 acid has biological potency similar to that of Vitamin A1 acid (2, 91, 119). It is biologically active and is found in the chicken limb (118) and in certain mammalian tissue (122, 123). In order to understand better the mechanism of action of Vitamin A2 acid its interaction with cellular retinoic acid-binding proteins (CRABP), RARs and RXRs were studied in several model systems. The results showed that Vitamin A2 acid binds with comparable affinity as Vitamin A1 acid to all of the aforementioned proteins; Vitamin A2 acid binds RXRα with somewhat higher affinity than the Vitamin A1 acid. Evaluation of the transcriptional activation potential of Vitamin A2 acid showed that this retinoid induced RARα-mediated transcription to the same magnitude as the Vitamin A1 acid. In comparison to Vitamin A1 acid, Vitamin A2 acid produced a 2- to 3-fold higher activation of the transcription mediated by RXRα homodimers, as well as RARβ-RXRα heterodimers (100). These results suggest that the biological activity of retinoids in the skin is complex and may be achieved by the activity of both Vitamin A1 and A2 acids.

Based on the data obtained to date, it is reasonable to think that Vitamin A1 and A2 acids could build and shape the UV response of the skin cells jointly. It is also reasonable to expect that in some cases they could generate similar phenotypes and in other cases they could generate different features or traits. One of the goals of my thesis is to compare the impact of both Vitamin A1 acid and Vitamin A2 acid on the expression of genes that participate in the cellular response to UV radiation. As p53 protein plays a central role in this response, to test the hypothesis that Vitamin A2 retinoids differentially regulate keratinocyte response to irradiation, gene expression profiling was carried out on p53 related genes.

AIM OF THE STUDY

The long term goal of this work was to better understand the biological functions of the Vitamin A2 retinoids. In humans, these retinoids are selectively synthesized and accumulate in epidermal keratinocytes. Because the epidermis is subject to UV induced damage and because these retinoids are more stable to photodecomposition than the Vitamin A1 retinoids, we postulate that they offer better protection to the skin from the harmful effects of UV radiation than do the Vitamin A1 retinoids. We also postulate that Vitamin A2 acid functions as a transcription factor ligand *in vitro* and together with Vitamin A1 acid regulates the response of the epidermal cells to UV irradiation.

To determine the validity of the hypothesis

1. An assay for *in vitro* conversion of Vitamin A1 to A2 was developed and used to assess the interaction of UV with retinoid metabolism.

2. The ability of Vitamin A1 and A2 retinoids to protect keratinocytes against UV-induced apoptosis was compared.

3. Differences between the ability of Vitamin A1 and A2 acid to regulate genes responsive to UV were measured by gene expression profiling of p53 responsive genes as proof of concept.

MATERIALS AND METHODS

Cell culture

Normal human epidermal keratinocytes (strain NHEK-O or D033), normal human foreskin keratinocytes (NHEK-F), human epidermal squamous cell carcinoma cell lines (SCC13 and SCC12b) and HaCaT cell line were used for all experiments. For some of the experiments, keratinocytes were grown in submerged culture utilizing γ-irradiated mouse 3T3 feeder cells as described by Rheinwald and Green,1975 (92). The cells were grown in a modified basal medium consisting of a 3:l (v/v) mixture of Dulbecco's minimum essential medium and Ham's F-12 medium containing adenine (1.8×10^{-4} M), penicillin (1.7×10^{-4} M), streptomycin (6.9×10^{-5} M), hydrocortisone (1.1×10^{-6} M), insulin (8.6×10^{-7} M), epidermal growth factor (1.6×10^{-9} M), and cholera toxin (1×10^{-7} M). This medium also contains 5% fetal bovine serum (FBS) (HyClone, Logan, UT). This medium will be referred to as 3 – 1 medium in this work. Primary keratinocytes of passage less than five were plated at a density of $2 \times 10^3/cm^2$ into dishes or flasks containing lethally irradiated 3T3 cells at a density of $11 \times 10^3/cm^2$. The cells were incubated at 37^oC in a humidified 7 % CO_2 incubator. The cells were supplemented with fresh medium every other day until 3-4 days post-confluence for UV radiation experiments and up to 7 days for *in vitro* assay development. For maintenance of stock cultures and for experiments, keratinocytes were passaged at approximately 70% confluence. They were detached from the flasks by treatment with trypsin, 0.1 % and 5×10^{-4} M EDTA (ethylene diamine tetra acetic acid). For some experiments the cells were grown in KGM which was prepared by supplementing KBM medium (Clonetics, Walkersville, MD) with penicillin, and streptomycin (100 units and 100 μg/ml), hydrocortisone (0.4 μg/ml), insulin (5 μg/ml), epidermal growth factor (10 ng/ml), cholera toxin (1.2×10^{-10} M), and bovine pituitary extract (BPE) (Clonetics, Walkersville, MD) with final concentration of 2 ml/l. For maintenance of stock cultures and for experiments, keratinocytes were passaged at approximately 70 % confluence. They were detached from the flasks by treatment with trypsin, 0.1 % and 5×10^{-4} M EDTA. For neutralization of the trypsin was used soybean trypsin inhibitor (Sigma, St Louis, MO) in final concentration of 1 mg/ml.

Delivering of retinoid and ^{3}H-retinoid substrates to the cells

Vitamin A1 alcohol ([11,12-^{3}H(N)]-all-trans-retinol), 44.4 Ci/mmol, and Vitamin A1 acid ([11,12-^{3}H(N)]-all-trans-retinoic acid), 44.4 Ci/mmol, were purchased from PerkinElmer Life and Analytical Sciences, Boston, MA. Vitamin

A1 alcohol (all-trans-retinol) and Vitamin A1 acid (all-trans-retinoic acid) was purchased from Sigma, St Louis, MO.

All retinoids were stored in dark under argon at - 80^{o} C for long-term storage or - 20^{o} C for shorter less than several months of storage. The retinoids were handled under amber fluorescent lighting (GE F-40 Gold) (Bulbtronics Inc., Farmingdale, NY). The necessary amount of retinoid or ^{3}H - retinoid substrates were freshly purified by reverse phase HPLC and dried under a stream of argon. At the delivery time the retinoid were dissolved into 50 μl dimethyl sulfoxide (DMSO) and added to a sterile 10 mg/ml solution of fatty acid free bovine serum albumin (BSA), which was used after 10 time dilution in the desired media. This way of delivering of retinoids was used for both – 3 – 1 and KGM medium. The cells were supplemented with freshly prepared medium every other day for the duration of the experiments.

Preparation of whole cell sonicates for retinoid analysis and subcellular fractionation

Cells at the desired growth stage were washed twice with calcium and magnesium-free phosphate buffered saline, pH7.4 (PBS). After the last wash the cells were scraped off the plastic surface for retinoid analysis or trypsinized for *in vitro* assay analysis, and transferred to 15 ml sterile plastic centrifuge tubes. After centrifugation at 3000 rpm (Beckman GPKR centrifuge) at 4 ^{o}C the cells were resuspended in 1.2 ml PBS for retinoid analysis or 400 μl homogenate buffer (0.01 M potassium phosphate buffer, pH 7.25, 280 mM sucrose, and 10 % glycerol) for *in vitro* assays. The cells were sonically disrupted using 20 bursts for retinoid analysis and two times 12 bursts each for *in vitro* assay analysis at 50 watts of output from a Branson Sonifer. Protein concentrations were determined by using Bio-Rad Protein Assay kit (Bio-Rad Life Sciences, Hercules, CA) with fatty acid-free bovine serum albumin (Sigma, St Louis, MO) as a standard.

Procedures for extraction of retinoids

For extraction of retinoids after the termination of the *in vitro* reactions I used the Bligh and Dyer extraction procedure (15). In brief the reaction was stopped by adding 2 ml of 100 % methanol, extracted with 2 ml of 100 % chloroform and the final separation of the organic and aqueous layers were achieved by addition of 1ml water. After 20 minutes centrifugation at 3000 rpm (Beckman GPKR centrifuge) the organic phase was placed into a new tube and dried under argon. The retinoids were dissolved in ethanol and evaluated by HPLC.

For the extraction of retinoids from whole cell homogenates I used the Barua extraction protocol (10). The cells were prepared as described above. After the sonic disruption, 1ml from sonicates was used for the extraction with ethanol, ethyl acetate, hexanes and water as described (10). After the separation of the organic and the aqueous phases by centrifugation at 3000 rpm (Beckman GPKR centrifuge) for 20 minutes the organic phase was transferred to a new

tube and dried under argon. After that the retinoids were dissolved in ethanol and evaluated by HPLC.

HPLC analysis of retinoids

Retinoids were extracted as described above and resolved by reverse-phase HPLC which utilizes gradient elution and detection by a photodiode array as previously described (87), but using a Waters 2996 photodiode array detector equipped with automated injection. A NovaPackHRC18 column (Waters, Milford, MA) was used for retinoid separation with a flow rate of 1ml/minute. For purification of Vitamin A2 acid a semi-preparative NovaPackC18 column with flow rate 4ml/minute and SunFireC18 column with flow rate of 1ml/minute were used. For all three columns the mobile phase gradient system described by Barua (11) was utilized. This gradient system is able to resolve retinoid acids, alcohols and esters present in keratinocytes. The gradient consists of 15 minutes linear gradient from 10 mM ammonium acetate in methanol: water (68:32) to methanol: dichloromethane (4:1) followed by a 15 minutes hold. Column elutes was monitored at 326 nm for mass determination. Radiolabeled retinol and its metabolites were measured by in-line scintillation counting. The identity of the retinoid peaks was determined by comparing the retention times and spectra of each peak to authentic retinoid standards.

Permeabilization of plasma membrane (PM) with Digitonin

SCC13 was grown in submerged culture as described above till 7 days post confluence. At the day of the experiment the cells were washed twice with PBS. After that two different ways of treatment with Digitonin (Sigma, St Louis, MO) were tested. In the first type of experiments the cells were first incubated for 10 minutes at 4^{o}C with different concentrations of Digitonin in 75 mM potassium acetate, 2.5 mM magnesium acetate, 1.8 mM calcium chloride, and 25 mM HEPES, pH 7.2 After that they were washed twice with PBS and supplemented with ^{3}H Vitamin A1 alcohol (25 nM; 10,000 dpm/pmol) in 3 – 1 medium and incubated for 1 hour at 37^{o}C. In the second type of experiment, the cells were pulsed first with ^{3}H Vitamin A1 alcohol (25 nM; 10,000 dpm/pmol) in 3 – 1 medium and incubated for 1hour at 37^{o}C. After the labeling the cells were washed twice with PBS and permeabilized by incubation in PBS buffer containing different concentrations of Digitonin for 10 minutes at 4^{o}C. In both type of experiments after the treatment the cells were washed twice with PBS. The cells were detached from the solid support by incubation in PBS/EDTA solution for 15 minutes, harvested by scraping and analyzed by HPLC.

Extraction and measurement of pyridine dinucleotides in SCC13

The pyridine dinucleotides were extracted and analyzed according to the procedure described previously by Klaidman et al. (55) and Pinkas-Sarafova et al. (85). SCC13 cell pellets were homogenized in chilled buffer containing 60 mM

KOH, 200 mM KCN, and 1mM bathophenanthroline at 100 μl buffer per 10 mg wet cells. The homogenate was centrifuged in a microcentrifuge at 14,000 rpm for 15 minutes at 4°C to remove the mitochondria. The supernatant was rapidly extracted with chloroform/isoamylalcohol (24:1) several times until minimal precipitate was visible at the interface of buffer and chloroform. The aqueous phase was passed through a 0.45 μm Ultrafree-MC filtration device (Millipore, Billerica, MA) by centrifugation at 5000 rpm (Beckman GPKR centrifuge) for 10 minutes at 4°C to remove any residual DNA and protein contaminations. Then the aqueous phase was diluted 1:1 or 1:3 with mobile phase to reduce the pH before loading onto the HPLC column.

To measure the content of pyridine dinucleotides I used an YMC-PACK ODS-AQ, 5 μm, 250 x 4.6 mm, reverse-phase C-18 column (Waters, Milford, MA). The fluorescent detector from Waters was set up on excitation wavelength 330 nm and emission was detected at 460 nm. The mobile phase consisted of 0.2 M ammonium acetate, pH 6.9, and 4% methanol.

To prepare a standard, the four cofactors (six different amounts from 6.5 to 500 ng each) were mixed in the presence of cyanide buffer, incubated for 5 minutes at room temperature, and extracted 4 times with chloroform/isoamylalcohol (24:1), as described for the sample. The standard curve was made based on 20 different injections for each standard point and the deviation of the single points was 13-18% (courtesy of Dr. Pinkas-Sarafova). Prior to each set of experiments the detection sensitivity of the instrument was verified for three points of the standards that were injected and analyzed on the HPLC.

Conditions for *in vitro* conversion of Vitamin A1 to A2

Keratinocytes were grown in 3 – 1 medium until the desired time post-confluence. The cells were washed two times with PBS, harvested by trypsinization and sonically disrupted in homogenation buffer (potassium phosphate pH 7.25 containing 280 mM sucrose and 10% glycerol). 100-200 μg protein/ 200 μl reaction was used. The buffer for assay was 100 mM Tris-HCl pH 7.4 containing 2 mM $CaCl_2$, 1.5 mM $MgCl_2$, and 2 mM NADPH. ^{3}H-Vitamin A1 alcohol was used as a substrate for the reaction with specific activity 10,000 dpm/pmol. The necessary amount of the substrate was dried under stream of argon, dissolved in DMSO (2.5%) and bovine serum albumin (BSA) (1mg/ml) and added to the reaction mix. The reaction was carried out in 37°C for 1hour. After the incubation period the retinoids were extracted using the Bligh and Dyer extraction procedure and analyzed by HPLC.

Isolation of plasma membrane (PM) ghosts by sucrose gradient centrifugation

Plasma membrane ghosts were isolated according the procedure described in Atkinson, P.H., and Summers, D.F., (8). In brief, ten days post confluent SCC13 cultures (1.0×10^8) harvested by trypsinization and followed

centrifugation at 3000 rpm (Beckman GPKR centrifuge) were resuspended in 20 times cells volume of 10 mM potassium phosphate buffer, pH 7.25. The salt concentration in the hypotonic buffer kept below 50mM to enhance cell disruption. The cells were allowed to swell 10 minutes on ice and then were ruptured with three to five strokes of a glass tissue homogenizer. The level of cell disruption was evaluated by phase microscope observation and the homogenization was continued for five more strokes if needed. Excessive homogenization was avoided because it could cause fragmentation of the plasma membrane ghosts or rupture of the nuclei. Immediately after the cells were disrupted, 0.1 volumes of 30 mM $MgCl_2$, 100 mM NaCl were added to the cell homogenate to stabilize the nuclei. The nuclei and the remaining whole cells were removed by centrifugation at 1500 rpm (Beckman GPKR centrifuge) for 30 secunds. The pellet was resuspended in four times its volume in 10 mM potassium phosphate buffer, pH 7.25, 3 mM $MgCl_2$, 10 mM NaCl and centrifuged again at 1500 rpm (Beckman GPKR centrifuge) for 10 seconds. The two combined supernatants, a volume of approximately 10 ml, contained most of the whole ghosts, and the pellet contained the nuclei and unbroken cells. The supernatant was divided into four aliquots of 2.5 ml each and layered onto discontinuous sucrose gradients composed of 15 ml of 30 % w/w sucrose over 5 ml of 45 g w/w sucrose, both in 10 mM potassium phosphate buffer. The gradients were centrifuged at 7000 x g for 20 minutes in a Sorvall (HB4 rotor) centrifuge. The plasma membrane ghosts were observed as a white opaque layer at the 30% to 45% sucrose interface, and were carefully removed with a syringe and 14 gauge cannula. To verify the purity of the isolated membrane ghosts, a small aliquot of the preparation was stained with Trypan blue and observed on phase contrast microscope. The preparation contained exclusively whole membrane ghosts or large fragments of plasma membrane.

Inhibition of A1-A2 conversion by ketoconazole

NHEK-O and SCC13 were grown in 3 - 1 medium as described above till 2 days post confluence. For the *in vivo* experiments the cells were pretreated with the inhibitor for 1hour followed by pulse labeling of the cell with ^{3}H- Vitamin A1 alcohol (25 nM; 10,000 dpm/pmol). After a 24 hours treatment with the inhibitor and the ^{3}H- Vitamin A1 alcohol the cells were harvested by scraping and retinoids were extracted using Barua extraction method and analyzed by HPLC as described above. For the *in vitro* experiments the cells were harvested by trypsinisation and sonically disrupted. The *in vitro* conversion of Vitamin A1 to A2 was performed as described above in presence of different concentrations of ketoconazole. The retinoid metabolites were extracted using the Bligh and Dyer extraction procedure and analyzed by HPLC.

UVA and UVB irradiation experiments

For UVA irradiation, a black light blue fluorescent lamp (40W T12 BLB, General Electric Co., Fairfield, CT) with the emission spectrum of 300 nm - 400

nm and a maximum at 366 nm was used. Radiation below 320 nm was eliminated by a UV32 filter (HOYA Corporation Optics Division, Fremont CA) positioned in front of the samples. For UVB irradiation, a Full Spectrum - 20 lamp (F20T12/FS, Westinghouse Lighting Corp., Philadelphia, PA) with the emission spectrum of 275 nm – 350 nm and a maximum at 310 nm was used. The emission below 300 nm was eliminated by UV30 filter (HOYA Corporation Optics Division, Fremont CA) positioned in front of the samples. UVA and UVB radiation produced by the two sources was monitored with a Jagger meter (49) calibrated versus a Molectron PR200 pyroelectric radiometer (Molectron Corporation, Sunnyvale, CA). The irradiance (intensities) measured in μA was calculated into $J/m^2/sec$ (UVA – 18.61 μA = 1 $J/m^2/sec$, UVB – 100 μA = 1 $J/m^2/sec$) and used to determine the irradiation time for different doses.

Normal human keratinocytes and squamous cell carcinoma keratinocytes from the SCC13 cell line were used for all the experiments. The cells were grown in p-60 cell culture dishes submerged in 3 – 1 culture medium as described above until they reached 3-4 days post-confluence. For a typical experiment, the cells were transported to Brookhaven National Laboratory (BNL) for irradiation. All cells were transported in containers provided by BNL in accordance with the safety standards for work with human cells. During transportation, the cells were kept warm in a 7 % CO_2 atmosphere. Before the experiments the cells were washed three times with 3 ml PBS, covered with 2 ml PBS and exposed to a single dose of UVA or UVB radiation. The UV radiation absorption and transmission of the PBS solution was measured by a Hewlett Packard Model 8452A Diode Array Spectrophotometer with 2 nm resolution between 190 to 820 nm range. The measurements did not show any significant UV absorption in the range that was used for the irradiations. The transmission for the same range was 100%. After the irradiation with the desired dose, the PBS was replaced with medium and the cells were transported back to SUNY at Stony Brook. The cells were grown in 3:1 medium for an additional 24 hours in a humidified 7 % CO_2 incubator, harvested by trypsinization and subjected to *in vitro* analyses. For the *in vivo* studies, after the cells were transported back to SUNY at Stony Brook, cells were pulsed labeled with radioactive ^{3}H-vitamin A1 alcohol (25 nM; 10,000 dpm/pmol), incubated for 24 hours in a humidified 7% CO_2 incubator at 37^{o}C and then harvested and evaluated by HPLC.

For the cell death assays normal human keratinocytes from foreskin were grown for several passages in serum-free KGM medium to deplete completely the internal content of retinoids. The cells with depleted retinoid content were plated in 6 wells plates in densities of 1.66×10^5 per well. The cells were supplemented with a fresh medium every other day until four days post confluence. 24 hours before the irradiations, the cells were supplemented with a medium containing either 0.1 μM Vitamin A1 alcohol or 0.1 μM vitamin A2 alcohol or no retinoids in case of the control. The medium contained also 1.5 mM Ca^{++} and 0.5 μM ketoconazole. Before the irradiations the cells were washed three times with PBS, and 1 ml PBS was placed over the cells. They were then irradiated with a single dose of UVB (0.5 J/cm^2 or 0.6 J/cm^2) or UVA (35 J/cm^2 or 40 J/cm^2) radiation. After the irradiations the PBS was replaced with medium.

The cells were transported the same way as described above to SUNY at Stony Brook and incubated for 16 hours in a humidified 7% CO_2 incubator at 37^{o}C. The cells were harvested by trypsinization and were assayed for cell death using cell death detection ELISA plus assay.

Evaluation of apoptosis after UVA and UVB irradiations using cell death detection ELISA plus assay

Apoptosis after UVA and UVB irradiations was detected using the Cell Death Detection ELISA^{plus} kit (Roche-Applied Science, Indianapolis, IN), according to the manufacturer's procedure. In brief, after harvesting, the cells were counted and 5000 cells were used for each assay. They were resuspended into 200 μl Lysis Buffer and incubated for 45 minutes at room temperature. After that, the lysates were centrifuged at 200 x g for 10 minutes. 20μl from the supernatant was transferred into streptavidin coated micro plates for analysis. After adding 80 μl of the immunoreagent, the plates were incubated with gentle shaking (300 rpm) for 2 hours at 24^{o}C and then were washed 3 times with incubation buffer and developed with ABTS solution for approximately 20 minutes. The reaction was stopped with ABTS stop solution. All the solutions and reagents were provided in the kit. The blank was measured at 405 nm against ABTS solution plus ABTS stop solution and the reference wavelength was at 490 nm. The protection function of the retinoids is expressed as extend of reduction of the enrichment of mono- and oligonucleosomes released into the cytoplasm (extent of reduction of apoptosis) and was calculated using the following formula:

$$\text{Reduction of apoptosis} = \frac{\text{Absorbance of the UV irradiated sample (supplemented with retinoids)}}{\text{Absorbance of the UV irradiated control (no retinoids)}}$$

Cloning of DR1, DR2 sequences into reporter plasmid pBLCAT2

The retinoic acid response elements (RARE) DR1 and DR2 sequences were introduced (the motif is repeated three times) in to the plasmid vector pBLCAT2 by cloning between the HindIII and XbaI restriction enzymes sites. Double strand DNA for the DR1 and DR2 elements were obtained by synthesizing and annealing of oligonucleotide sequences that harbor the following elements: the response element sequence; the needed restriction enzyme sequences, flanking the RARE sequence; each oligonucleotide sequence starts and ends with 4 unrelated nucleotides to facilitate the restriction enzyme digestion of the final double stranded (ds) DNA product. The oligonucleotides were synthesized by Sigma – Genosys. Dr. N. Markova from our laboratory kindly provided a construct harboring the RARE DR5 sequence in the plasmid pBLCAT2 cloned similarly between the HindIII and XbaI restriction sites. The sequences of the oligonucleotides used in this study are shown in below.

DR1	GGAGAAGCTTGGGGTCAAAGGTCAAAGGTCATCTAGACTGC
DR1 reverse	GCAGTCTAGATGACCTTTGACCTTTGACCCCAAGCTTCTCC
DR2	GGAGAAGCTTGGGGTCATGGGGTCATGGGGTCATCTAGACTGC
DR2 reverse	GCAGTCTAGATGACCCCATGACCCCATGACCCCAAGCTTCTCC
DR5	GGAGAAGCTTGGTTCACCGAAAGTTCACTCGGGAGGGTTCACCGAAAGTTCACTCAGACTGC
DR5 reverse	GCAGTCTGAGTGAACTTTCGGTGAACCCTCCCGAGTGAACTTTCGGTGAACCAAGCTTCTCC

The corresponding forward and reverse oligonucleotides were dissolved in water and equimolar amounts were mixed, denaturated by heating and allowed to anneal by slowly cooling the mix. The resulting dsDNA was desalted through Chroma spin columns (Clontech, Mountain View, CA). The dsDNA for each RARE and the vector were cut with HindIII and XbaI restriction enzymes (New England BioLabs, Inc., Ipswich, MA). The short dsDNA for each RARE element were purified using QIAquick Nucleotide Removal Kit (QIAGEN, Valencia, CA). The vector DNA was purified by electrophoresis through 0.8 % agarose gel and extracted from the gel by MinElute Gel Extraction Kit (QIAGEN, Valencia, CA). It is possible that vector molecules cut only by one of the restriction enzymes during ligation to self-anneal and produce undesirable background. To decrease this possibility the vector was subject to dephosphorylation by Calf Intestinal Phosphatase (New England BioLabs, Inc., Ipswich, MA) and subsequent purification. The digested dsDNA were ligated to the digested and dephosphorylated vector using Rapid DNA Ligation Kit (Roche-Applied Science, Indianapolis, IN). The resulting ligation products were transformed into chemically competent *E. coli* cells (One Shot TOP 10 Competent Cells, Invitrogen Life Technologies, Carlsbad, CA). From each ligation mix five independent single colonies were chosen for further analysis. Each clone was independently amplified and plasmid DNA was isolated using QIAprep Spin Miniprep Kit (QIAGEN, Valencia, CA). The correctness of the anticipated RARE element was verified by DNA sequencing. Purified plasmid DNA from each clone was prepared and the introduced DNA sequenced by the DNA Sequencing facility, Stony Brook University. The sequence of the inserted DNA was compared to the RARE sequence and correct clones were identified. From each correct clone plasmid DNA was isolated using the HiSpeed Plasmid Purification Midi Kit (QIAGEN, Valencia, CA). These plasmid DNAs were introduced into keratinocyte cells by transfection.

Transfection of HaCaT cells

HaCaT cells were grown in serum free KGM medium for several passages in order to deplete completely their internal content of Vitamin A1 acid and Vitamin A2 acid. These cells were seeded into 6 wells plates at density of 1 x 10^6 cells per well and fed with KGM. Four hours after seeding they were at 90 % confluence and at that time they were transfected with the constructed plasmids (pBLCAT2 vector containing DR1, DR2 or DR5 sequences respectively) using Lipofectamine Reagent and PLUS Reagent both from Invitrogen Corporation,

Carlsbad, CA. For each transfection, 1 μg of plasmid DNA was diluted into 100 μl KBM and pre-complex with 8 μl PLUS reagent. The mixture was incubated for 15 minutes at room temperature. After the 15 minutes incubation for each transfection 4 μl Lipofectamine Reagent was diluted into 100 μl KBM and added to the pre-complex DNA. The solution was incubated at room temperature for an additional 15 minutes. While complexes were forming the cells were washed 2 times with PBS and 800 μl fresh KBM medium was added. After the DNA-PLUS-Lipofectamine Reagent complexes were formed they were added to the cells. The cells were incubated with the DNA complexes for 4 hours at 37°C in a humidified 7 % CO_2 incubator. After the last incubation the medium was removed and the cells were shocked for 2 minutes with 1 ml 15 % glycerol in PBS. The glycerol was replaced with KGM supplemented with 10 nM or 100 nM Vitamin A1 acid; 10 nM or 100 nM Vitamin A2 acid or 1 mg/ml BSA. The cultures were incubated an additional 40 hours at 37°C in a humidified 7 % CO_2 incubator and then analyzed by CAT reporter assay.

CAT reporter assay

The cells were washed two times with PBS and lysed with 5 x Reporter Lysis Buffer (Promega, Madison, WI) according to the manufacturer's procedure. The lysates were transferred into microcentrifuge tubes and incubated for 10 - 15 minutes at 65°C. The extracts were centrifuged at 13000 rpm (Maraton Micro A microfuge, Fisher Scientific) for 10 minutes and the supernatant was transferred into new tubes and assayed. The protein content into the samples was measured using Bio-Rad Protein Assay (Bio-Rad Life Sciences, Hercules, CA) with fatty acid-free bovine serum albumin (Sigma, St Louis, MO) as a standard. The CAT assay was performed as follows: In a 5 ml scintillation vial I mixed 100 μl H_2O, 50 μl from the cell extract and 100 μl CAT detection solution (0.3 M Tris, HCL, pH 8, 3 mM chloramphenicol, 25 μM Acetyl CoA and 1 μl Acetyl Coenzyme A, [Acetyl-^{3}H]-, CAT Assay Grade with a specific activity 200 mCi/mmol (PerkinElmer Life and Analytical Sciences, Boston, MA). The mixture was carefully overlaid with 2 ml scintillation fluid Econofluoro-2 (PerkinElmer Life and Analytical Sciences, Boston, MA.). The samples were incubated at 37°C for 4 hours and then counted using liquid scintillation counter (LKB Wallac, 1214 Rackbeta, Gaithersburg, MD). For measuring the background as a control I used extracts prepared from untransfected cells.

Vitamin A1 and A2 acid uptake and metabolism experiments

For these experiments normal human foreskin keratinocytes (NHEK-F) were grown at 37° C in a humidified 7% CO_2 incubator for several passages in serum-free KGM medium to exhaust completely the internal content of Vitamin A1 and A2 retinoids. The retinoid depleted cells were plated in p-100 plates in densities of 5 x 10^5 cells per well and were incubated in KGM and were fed every other day until one day postconfluence. At that point the cells were supplemented with medium containing 1.5 mM Ca^{++}. 24 hours post addition of Ca^{++} the cells

were pulsed with 1 μM of radiolabel ^{3}H-Vitamin A1 or 1 μM of radiolabel ^{3}H-Vitamin A2 acid or 0.25 μM of radiolabel ^{3}H-Vitamin A1 acid with specific activity of 30 dpm/pmole. The retinoids were delivered to the cells using BSA as a carrier to final albumin concentration of 1mg/ml. The cells were allowed to uptake and metabolize the labeled retinoids for 2, 6, and 24 hours at 37^o C in a humidified 7% CO_2 incubator. After the incubation period the cells were washed two times with PBS and harvested by trypsinization. The cell suspension was centrifuged at 3000 rpm (Beckman GPKR centrifuge) for 5 minutes at 4^o C. The cell pellet was resuspended into 1 ml PBS and sonically disrupted using 20 bursts of a Branson Sonifer. Protein concentrations were determined using the Bio-Rad Protein Assay kit with fatty acid-free bovine serum albumin as a standard. The radiolabel ^{3}H-Vitamin A1 and A2 acids were extracted using Bligh and Dyer extraction procedure and were evaluated by HPLC.

Involucrin immunocytochemistry staining

For these experiments NHEK-F were grown at 37^o C in a humidified 7% CO_2 incubator for several passages in KGM to deplete the internal content of Vitamin A1 and A2 retinoids. These cells were then plated onto 6 well glass slides (Lab-Tek Chamber Slide System,Nalge Nunc Int., Rochester, NY) at densities of 1.5 x 10^5 cells per well. The cells were grown in KGM and fed every other day until one day postconfluence. At that point replicate wells were incubated either with low or high calcium (1.5 mM Ca^{++}) KGM. In parallel NHEK-F were grown in 3 – 1 medium until two days post-confluence as a positive control. The cells were washed with PBS two times. For immunohistochemistry, cultures were fixed in methanol for 10 minutes at -20^o C, washed twice with PBS, and then incubated with 0.3% H_2O_2 in PBS for 30 minutes at room temperature (RT). The cells were then rinsed twice with PBS and blocked with a 10% goat serum solution in PBS for 30 minutes at RT. After decanting the blocking solution, rabbit anti-human involucrin antibody (prepared by Dr. Marcia Simon) at a dilution of 1:5000 in 10% goat serum solution in PBS was added, and the slides were incubated in a moist chamber overnight at 4^o C.
The next day the cells were washed with PBS three times and incubated with peroxidase rabbit IgG (Vectastain ABC kit, Burlingame, CA) in 1% BSA for 1hour at RT, washed with PBS three times and incubated with ABC complex provided in the Vectastain ABC kit for 30 minutes at RT. The cells were rinsed with tap water and briefly counterstained with hematoxylin. They were mounted with BioGenex aqueous mounting medium (BioGenex Laboratories, San Ramon, CA).

PCR - Based Gene Expression Profiling experiments

For these experiments normal human foreskin keratinocytes (NHEK-F) were grown at 37^o C in a humidified 7% CO_2 incubator for several passages in serum-free KGM medium to deplete completely the internal content of Vitamin A1 and A2 retinoids. The retinoid depleted cells were plated in p-100 plates in densities of 5 x 10^5 per well and were incubated in KGM and fed every other day

until one day post-confluence. At that point the cells were supplemented with medium containing 1.5 mM Ca^{++}. For assessment of retinoid uptake, cultures replicate cultures were supplemented with 0.25 µM ^{3}H-Vitamin A1 acid, 1.0 µM ^{3}H-Vitamin A1 acid or 1.0 µM ^{3}H-Vitamin A2 acid for 2, 6, or 24 hours. Retinoids were delivered bound to BSA with a final BSA concentration of 1 mg/ml. For gene expression profiling, cultures were incubated at 37^{o}C for 4 hours in high calcium KGM supplemented with non-labeled 0.25 nM Vitamin A1 acid, 1.0 nM Vitamin A1 acid, 1.0 nM Vitamin A2 acid, or with retinoid-free BSA. After the incubation period the cells were harvested by trypsinization, counted and 3 x 10^{6} cells were used for total RNA extraction. For this purpose was used RNeasy Mini Kit (QIAGEN Inc., Valencia, CA) according to the manufacturer's recommendations. Quantification of the RNA was determined by absorbance at 260 and 280 nm using a UV spectrophotometer. The quality and integrity of the samples were verified by electrophoresis of 1µg of the total RNA on 2 % agarose gel. RNA was visualized by ethidium bromide (0.5 µg/ml) staining. Samples (2 µg) were reverse transcribed using RT First Strand Kit (SuperArray Bioscience Corp., Frederick, MD) and cDNA templates were used to determine the gene expression profile of 84 pathway focused genes using the RT Profiler PCR Array System (SuperArray Bioscience Corp., Frederick, MD) according to the manufacturers' recommendations. Real - time PCR and detection was performed using the Applied Biosystems 7300 Real - Time PCR System (Applied Biosystems, Foster City, CA). Data was obtained by performing a two-step cycling program. First step consists of one cycle for 10 minutes at 95^{o}C. The second step consists of 40 cycles: 15 seconds at 95^{o}C followed by 1 minute at 60^{o}C. A melting curve program was performed immediately after the above cycling program in order to generate a first derivative dissociation curve for each well in the entire plate using the instrument's software. The analysis of the data was performed using the free software provided by the manufacturer (PCR Array Data Analysis Web Portal: http://www.superarray.com/pcrarraydataanalysis.php, SuperArray Bioscience Corp., Frederick, MD).

Real - time two step RT - PCR

The total RNA that was used for PCR - Based Gene Expression Profiling experiments was used to perform real-time two steps RT – PCR experiments. From the total RNA I synthesized cDNA by reverse transcription using QuantiTect Reverse Transcription Kit (QIAGEN Inc., Valencia, CA) according to the manufacturer's recommendations. The synthesized cDNA templates were used to further verify the gene expression profile of 12 genes that showed change in the expression levels in the experiments described above. The primers for the chosen genes were purchased from QIAGEN (QuantiTect Primer Assay, QIAGEN Inc., Valencia, CA). The real-time PCR reactions were assembled and the amplification and the detection of the PCR products were performed using the QuantiTect SYBR Green PCR Kit (QIAGEN Inc., Valencia, CA) and the DNA Engine Opticon System (Bio-Rad, Hercules, CA) according to the manufacturer's recommendations. The cycling program consists of two steps.

First step – 1 cycle for 15 minutes at 95^{o}C and the second step – 40 to 48 cycles: 30 seconds at 94^{o}C, 30 seconds at 55^{o}C, 1 minute at 72^{o}C. A melting curve program was performed immediately after the above cycling program in order to generate a first derivative dissociation curve for each sample and dilution series by using the instrument's software. The data was analyzed by using the relative quantification method described by Michael W. Pfaffl (84).

Statistical analysis

Statistical significance ($p < 0.05$) was determined using a student's t-test.

CHAPTER 1

ASSAY DEVELOPMENT AND CHARACTERIZATION OF THE VITAMIN A1 TO VITAMIN A2 CONVERTING ACTIVITY OF CULTURED HUMAN EPIDERMAL KERATINOCYTES

A2/A1 ratio is greatest in SCC13 compared to SCC12b and NHEK

Because there can be strain to strain differences in the ability of cultured human epidermal keratinocytes to convert Vitamin A1 alcohol to Vitamin A2, prior to assay development a strain with high conversion ability was sought. For this purpose two NHEK (NHEK-O and D033) and two SCC cell lines (SCC12b and SCC13) were screened for their capacity to convert Vitamin A1 to A2. Cultures were grown in 3 – 1 medium and at one week post-confluence were labeled for 24-hours with ^{3}H-Vitamin A1 alcohol. Retinoids were extracted, resolved by reverse-phase HPLC and identified by retention time with in-line scintillation counting. Shown in Figure I – 1 are the results presented as a pmol/mg cell protein.

Both the NHEKs and SCC cell lines esterified Vitamin A1 and A2 alcohols with long chain fatty acids. Vitamin A oleate and palmitate (18:1, 16:0) were the predominant Vitamin A1 and A2 esters (3,4-didehydroretinyl esters) in all cell lines. Consistent with previous studies and with differences in Lecithin Retinol Acyl Transferase (LRAT) expression (52, 87), the NHEKs converted more than 97% of the Vitamin A1 taken up to retinylester. They also produced 2 to 5 times more A1 esters than the SCCs, and both NHEK strains accumulated 2-3 times more Vitamin A1 esters than A2 esters. The SCC cultures esterified only 67-82% of the Vitamin A1 taken up and converted a higher percentage of Vitamin A1 to Vitamin A2 (alcohol and ester combined), making the SCC lines preferred candidates for assay development.

A number of differences were found between the SCC cell lines, although both had nearly equivalent levels of Vitamin A1 and A2 alcohols. First, SCC12b had nearly equivalent Vitamin A1 and A2 esters, while SCC13 had 2.5 fold more Vitamin A2 ester than A1 ester. Second, the total ester level in SCC13 was 5-fold greater than SCC12b. Therefore, SCC13 was used to develop the *in vitro* conversion assay.

NADPH enhances Vitamin A1 to A2 conversion

Desaturation typically requires a complex of membrane bound enzymes that employ molecular oxygen and NAD(P)H as a cofactor (113). As formation of Vitamin A2 requires ring desaturation, it is possible that a similar mechanism is employed and that the same cofactors are required. Therefore, in order to determine the feasibility of converting Vitamin A1 to A2 *in vitro*, co-factor requirements were first assessed. In these experiments conversion was measured in SCC13 permeabilized with digitonin, which is a non–ionic detergent commonly used to solubilize membrane bound proteins. It replaces cholesterol in membranes, and disrupts their integrity. It selectively permeabilizes plasma membranes leaving nuclei intact due to differences in their cholesterol content (83).

The concentration of digitonin required to permeabilize the plasma membrane without causing loss of internal retinoids was first determined. SCC13 was grown to one week post confluence, washed with PBS and incubated for 10 minutes at 4^{o}C with two different concentrations of digitonin (100 μg/ml and 300 μg/ml). ^{3}H-Vitamin A1 alcohol in 3:1 medium was then added and the cultures were incubated for 1h at 37^{o}C after which cells were harvested, and retinoids were extracted and analyzed. To measure loss of cellular retinoids due to digitonin treatment, retinoid analyses were carried out on cultures that were first incubated with ^{3}H-Vitamin A1 alcohol for 1h at 37^{o}C, and then washed two times with PBS and incubated with digitonin (100 and 300 μg/ml) for 10 minutes at 4^{o}C.

As shown in Figure I – 2 A, B, when cells were permeabilized with increasing concentrations of digitonin and then incubated with ^{3}H-Vitamin A1 alcohol they progressively lost the ability to convert Vitamin A1 to A2. In cultures incubated with 0, 100, and 300 μg/mL digitonin, the percent conversion was 57, 29, and 8, respectively. The loss of Vitamin A2 formation was not due to reduced substrate Vitamin A1 alcohol, which was found at higher concentrations with both digitonin treatments; cultures treated with 300 μg/mL digitonin had twice as much Vitamin A1 as non-treated cultures. The amount of Vitamin A1 esters was not changed by the treatments. However, Vitamin A1 aldehyde levels increased in the digitonin treated cells and showed concentration dependency (Fig I – 2 B). As NAD(P) is required for the conversion of Vitamin A1 alcohol to aldehyde and NAD(P)H is required for the conversion of Vitamin A1 aldehyde to alcohol, this data suggests that the increase in plasma membrane permeability caused an increase in the ratio of NAD(P):NAD(P)H.

When cultures were incubated with ^{3}H-Vitamin A1 alcohol first and then subjected to 100 μg/mL digitonin, no significant difference in retinoid status was detected (Figure I – 2 C, D). These cultures did not accumulate Vitamin A1 aldehyde and generated Vitamin A2 retinoids. However, in cultures incubated with 300 μg/mL digitonin, a small, but significant loss of Vitamin A2 retinoids was observed and Vitamin A1 aldehyde levels increased (Figure I – 2 D). Therefore, to prevent loss of cellular retinoids into the media, the concentration of digitonin must be kept under 300 μg/ml.

As disruption of the plasma membrane caused a decrease in conversion of Vitamin A1 to A2, in the next series of experiments addition of FAD/FADH, NAD/NADH, and NADP/NADPH were tested for the ability to restore activity. SCC13 was cultured to five days post-confluence and treated with digitonin (200 µg/ml) in presence or absence of cofactors (2 mM). As before, after the digitonin treated cultures were supplemented with 3 – 1 medium containing ^{3}H Vitamin A1 alcohol in presence or absence of these cofactors. After 1hour at 37^{o}C retinoids were extracted and analyzed. Cultures not exposed to digitonin served as control.

In this set of experiments (Fig I – 3), the cultures accumulated more Vitamin A1 and A2 alcohol and more Vitamin A1 aldehyde (52% versus 74%) than those presented in Figure I – 2. However, the same trends were seen. Namely, digitonin treatment caused a reduction in conversion of ^{3}H-Vitamin A1 to ^{3}H-Vitamin A2; changes in Vitamin A1 aldehyde did not reach statistical significance. Addition of NAD, FAD, or FADH was without effect, and incubations with NADH did not significantly change the retinoid pattern. However, NADPH partially restored the ability of permeabilized cells to convert ^{3}H-Vitamin A1 to A2 (Fig I - 3). The amount of ^{3}H-Vitamin A2 in digitonin-treated cells was 74% lower than control whereas the amount of ^{3}H-Vitamin A2 in digitonin-treated cells supplemented with NADPH was 45% lower.

The cellular concentration of pyridine dinucleotides was next measured in order to determine whether addition of NADPH actually restored normal levels of this co-factor. As shown in Table I - 1, in non-permeabilized cells the levels of NAD, NADH, NADP, and NADPH were 0.692, 0.035, 0.046, and 0.132/10mg wet cells, respectively. Digitonin treatment caused a drop in all pyridine dinucleotides except for NADP. The levels of NAD, NADH and NADPH decreased to 0.299, 0.012, and 0.017/10mg wet cells, respectively. In incubations with 2 mM NADPH, the cellular content of NADPH increased but not to normal levels. The concentration of NAD, NADP and NADPH increased to 0.348, 0.187, and 0.050/10mg wet cells, respectively. The elevated levels of NADP and NAD suggest that oxidation and dephosphorylation of NADPH occurs under the experimental conditions and limits recovery of NADPH. This may explain in part why the converting activity of vitamin A1 to A2 could not be fully restored. However, other required factors may also have been lost from the permeabilized cells and the enzyme(s) responsible for the conversion may have been altered or damaged by the procedure.

An Assay for *in vitro* conversion of Vitamin A1 to A2

The next step was to partially purify the activity by fractionating disrupted cells and assaying fractions for their ability to convert vitamin A1 to A2 in presence of the 2 mM NADPH. SCC13 grown until one week post-confluence in 3 – 1 medium was harvested, sonically disrupted and fractionated by step centrifugation into four fractions (1). The first fraction was the pellet collected by centrifugation at 3000 rpm (Beckman GPKR centrifuge) which should contain nuclei, cytoskeleton, large pieces of plasma membrane and whole undisrupted

cells. The second fraction was the pellet collected by centrifugation at 17500 rpm (Sorvall centrifuge – SS-34 rotor) which should contain mitochondria, lysosomes, peroxisomes and smaller pieces of the plasma membrane. The third fraction was the pellet collected by centrifugation at 40,000 rpm (ultracentrifuge, 100,000 x g) and contains microsomes and small vesicles, and the fourth was the supernatant which comprises the cytosol with ribosomes and large macromolecules. Only the first and the second fractions showed enzymatic activity. For optimizing assay conditions the second fraction (17500 rpm pellet) was used to avoid contamination of undisrupted cells, which can generate false or misleading results.

Different buffer conditions and salts were tested for their ability to improve the *in vitro* conversion of Vitamin A1 to A2. The optimal buffer for sonic disruption was found to be potassium phosphate pH 7.25 containing 280 mM sucrose and 10% glycerol, and the optimal buffer for assay was found to be 100 mM Tris-HCl pH 7.4 containing 2 mM $CaCl_2$, 1.5 mM $MgCl_2$, and 2 mM NADPH using 100-200 mg protein per 200 µl reaction. *In vitro* Vitamin A2 synthesis using the 17500 rpm fraction from SCC13 is shown in Figure I – 4a. This fraction was able to convert almost 50% of the substrate into Vitamin A2 during the 1-h incubation period (Figure I – 4a, A). No conversion was seen in the boiled control (Figure I – 4a, B). The retinoids identified by retention time are numbered 1-11 and are identified in Table I – 2. The ratio of A2/A1 in comparable cultures of SCC13 was 1.7 – 2.0. Reactions for *in vitro* conversion of Vitamin A1 to A2 were also carried out with the 17500 rpm fraction of sonically disrupted NHEK-O (Figure I – 4b, A) and sonically disrupted normal human dermal fibroblasts (Figure I – 4b, B). The latter serves as control since fibroblasts do not catalyze this reaction (88). Similar to findings in culture, the 17500 rpm fraction of NHEK converted less Vitamin A1 to A2 (13%) than did SCC13 and the 17500 rpm fraction of normal dermal fibroblast fraction did not convert any Vitamin A1 to A2.

In order to confirm the identity of the synthesized Vitamin A2 products spectral matching was carried out. For this purpose retinoids extracted from ten pooled reactions with 17500 rpm fraction of SCC13 sonicates were separated by HPLC (Figure I – 5a) and peaks with the retention times of Vitamin A2 alcohol and Vitamin A2 esters were analyzed with photodiode array detection (Figure I – 5b). The spectra of the *in vitro* synthesized A2 products matched the spectra and the retention time of authentic standards.

Characterization of the activity catalyzing conversion of Vitamin A1 to A2

To confirm that the conversion was catalyzed enzymatically, substrate saturation and protein dependency was evaluated. SCC13 grown in 3 – 1 medium to one week post-confluence was harvested, sonically disrupted and a 17500 rpm fraction was isolated for the *in vitro* reactions. ^{3}H-Vitamin A1 alcohol at 0.58 to 74 nM was used as substrate in 200 µl reactions containing 1 mg protein/ml. As shown in Figure 1 – 6A, the reaction is linear up to 4.6 nM and approaches V_{max} about 80 nM. (Figure I – 6a). The dependence of the *in vitro* reaction on the enzyme concentration was also confirmed by evaluating

reactions using increasing amounts (0.12 – 0.78 mg/200 µl: reaction volume) of the 17500 rpm fraction. The reaction was linear up to 0.8 mg/200 µl: reaction volume (Figure I – 6b). As the reaction is heat sensitive, can be substrate saturated and is protein dependent, the conversion is enzyme catalyzed.

Vitamin A1 to A2 converting activity associates with plasma membrane

In an attempt to identify the subcellular location of the converting activity fractions were prepared from cells sonically disrupted and from cell disrupted using a homogenizer. As shown in Table I – 3 A, in the sonically disrupted cells about 50% of the activity was recovered in the first fraction (3000 rpm pellet). By microscopic observation this fraction comprised large fragments of plasma membrane and non-fragmented cells. The second fraction (17500 rpm pellet) contained only 15.85% of the activity and had the highest specific activity. By microscopic observation this fraction comprised membrane fragments. The supernatant which contains microsomes and cytosol had the lowest specific activity. However, this fraction has about 60% of the total cell protein and by calculation has about 23% of the converting activity. Whether this fraction also contains contaminating plasma membrane fragments was not determined.

In the homogenized preparations, almost 96% of the activity was recovered into the first fraction (3000 rpm pellet) (Table I – 3 B), suggesting that the activity may be associated with the plasma membrane. Therefore plasma membrane ghosts were prepared from one week post-confluent cultures of SCC13 using procedures described by Atkinson and Summers (8). Four fractions were assayed. Fraction 1 is the homogenate that is prepared after cells are osmotically swollen and disrupted with a Dounce homogenizer. Fraction 2 is the diluted homogenate from which nuclei and unbroken cells have been removed by centrifugation. Fractions 3 and 4 were obtained after zonal centrifugation of fraction 2 through a discontinuous sucrose gradient. Fraction 3 is the material which did not move through the 30% sucrose solution and Fraction 4 is the material that was recovered from the interface of the 30% and 45% sucrose solutions. It is Fraction 4 that contains the plasma membrane ghosts (Figure I – 7).

As shown in Figure I – 7 A, Fraction 1 converted about 50% of the ^{3}H-Vitamin A1 alcohol to ^{3}H-Vitamin A2 alcohol and ester. The specific activity (SA) of the Vitamin A1 to A2 converting enzyme(s) was 1.028 pmol/h/mg protein. Fraction 2 had a SA of only 0.256 pmol/h/mg protein, although its ability to generate ^{3}H-Vitamin A1 esters remained high (Figure I – 7 B) and Fraction 3 had the same SA (0.278 pmol/h/mg protein) and its ability to generate ^{3}H-Vitamin A1 esters was retained (Figure I – 7 C). Only Fraction 4 with an SA of 1.235 pmol/h/mg protein showed enrichment of the conversion activity with concomitant reduction in retinoid esterification activity (Figure 1 – 8 D). The data suggests that at least one Vitamin A1 to A2 conversion activity is associated with the plasma membrane and that its location is distinct from the enzyme(s) responsible for Vitamin A1 and A2 esterification. Plasma membrane ghosts stained with trypan blue are shown in Figure I – 8.

Ketoconazole, a known inhibitor of p450 enzymes, inhibits Vitamin A2 synthesis *in vivo* and *in vitro*

Torma (121) previously demonstrated that ketoconazole treatment of skin organ culture reduced its ability to synthesize Vitamin A2 from Vitamin A1 alcohol. The concomitant reduction in substrate Vitamin A1 alcohol confounded interpretation of this result. However, in light of the role of cytochrome p450 enzymes in a number of other desaturase reactions, it seemed likely that the reduction in Vitamin A2 synthesis was due to reduced enzyme activity rather than reduced substrate availability (103, 89, 78). Therefore, in the next set of experiments the impact of ketoconazole on Vitamin A1 to A2 conversion was evaluated in NHEK-O and SCC13 cultures and in sonicates prepared from these cultures where substrate availability is not limiting.

In cultures of NHEK-O incubated with 0.5 μM ketoconazole the level of ^{3}H-Vitamin A2 in NHEK-O was reduced about 60%; no further reductions were detected in cultures exposed to 5 or 10 μM ketoconazole (Figure I – 9a). What were observed at the higher ketoconazole concentrations were increases in ^{3}H-Vitamin A1 retinoids most of which were esters. As the control cultures (0 μM ketoconazole) contain 20.81 (± 1.61) pmol/mg ^{3}H-Vitamin A1 and 3.54 (± 0.29) pmol/mg ^{3}H-Vitamin A2, the increase in ^{3}H-Vitamin A1cannot be accounted for the reduction in ^{3}H-Vitamin A2. *In vitro* analysis of NHEK-O sonicates also demonstrated that ketoconazole inhibited ^{3}H-Vitamin A2 synthesis (Figure I – 9b). *In vitro,* inhibition was concentration dependent and increased from about 15% inhibition at 0.5 μM ketoconazole to > 90% at 10 μM ketoconazole. In contrast to the *in vivo* results, ketoconazole (0.5-10 μM) reduced rather than increased ^{3}H-Vitamin A1 ester levels by 15-30% during the reaction. This difference is not unexpected as many enzymes catalyze Vitamin A1/A2 alcohol esterification and Vitamin A1/A2 ester hydrolysis not all of which are likely to be active in the *in vitro* assay.

In cultures of SCC13 incubated with 0.5 μM ketoconazole the level of ^{3}H-Vitamin A2 was reduced by about 75%. At 5-10 μM ketoconazole ^{3}H-Vitamin A2 levels were reduced by about 90% (Figure I – 10a). Similar to the results obtained with NHEK-O, ^{3}H-Vitamin A1 levels increased. However, in SCC13, the increase may be largely due to the inhibition of ^{3}H-Vitamin A1 conversion to ^{3}H-Vitamin A2. Control cultures contain 4.79 (± 1.11) pmol/mg ^{3}H-Vitamin A1 and 7.36 (± 0.41) pmol/mg ^{3}H-Vitamin A2 of which 7.1 (± 0.48) pmol/mg are esters. By inhibiting conversion 90%, the A1 ester content should increase to about 11.9 pmol/mg and be detected as a 2.4 fold increase in ^{3}H-Vitamin A1. *In vitro*, 0.5 μM ketoconazole inhibited ^{3}H-Vitamin A1 conversion to ^{3}H-Vitamin A2 by about 75% and 5-10 μM ketoconazole conversion inhibited conversion by about 80% (Figure I – 10b). Again *in vivo* ketoconazole treatment resulted in a reduction in ^{3}H-Vitamin A1 esters. The reduction was somewhat greater (40-50%) than that observed with NHEK-O (15-30%) perhaps reflecting differences between NHEK-O and SCC13 expressed enzymes.

Taken together the data support the postulate that p450 enzymes contribute to Vitamin A1 conversion to Vitamin A2. The data also suggests that ketoconazole impacts Vitamin A1/A2 alcohol esterification and/or Vitamin A1/A2 ester hydrolysis.

Conversion of Vitamin A1 to A2 increases with progression of culture confluence

In preliminary experiments with SCC13 the cellular content of Vitamin A2 was found to increase in post-confluent cultures. To determine whether this is a general property of epidermal keratinocytes, the impact of culture confluence on Vitamin A2 was studied using two normal epidermal keratinocyte strains (NHEK-O and D033) and two transformed cell lines (SCC12b and SCC13). Cultures were grown in 3 – 1 medium to 1, 3 or 5 days post-confluence at which time media was supplemented with ^{3}H vitamin A1 alcohol. After incubation for 24h retinoids were analyzed. As shown in Figure I – 11, the ^{3}H-Vitamin A2 levels increased with post-confluence in all cultures. In NHEK-O, D033, and SCC13, ^{3}H-Vitamin A2 levels increased from 3-4 pmol/mg at one day post-confluence to 6-7 pmol/mg at five days post-confluence, and in SCC12b, ^{3}H-Vitamin A2 levels increased from 0.5 pmol/mg to 2.1 pmol/mg. An increase in the ratio of ^{3}H-Vitamin A2: ^{3}H-Vitamin A1 with culture confluence was observed for both normal and transformed cell; the fractional increase was less in NHEK due to the abundance of ^{3}H-Vitamin A1 esters.

To investigate if changes in Vitamin A2 levels were the result of increased conversion activity, *in vitro* assays were carried out with sonicates prepared from one to five day post-confluent cultures of NHEK-O and SCC13. As shown in Figures I – 12 a linear increase in the rate of ^{3}H-Vitamin A2 synthesis was observed for using NHEK-O sonicates resulting in a 7 fold increase in the A2 retinoids. As substrate Vitamin A2 alcohol levels increased, more Vitamin A2 ester was synthesized. Vitamin A1 ester synthesis did not change. Similar results were obtained with SCC13 (Figure I – 13). Namely, a linear increase in the rate of ^{3}H-Vitamin A2 synthesis was observed resulting in a 5 fold increase in the A2 retinoids. Synthesis of the Vitamin A2 esters also increased with increasing levels of Vitamin A2 alcohol. In contrast to NHEK-O, in SCC13 enhanced esterification activity was noted in cultures grown to 5 and 7 days post confluence.

Taking into account the results obtained using *in vivo* and *in vitro* assessments, it is clear that the growth stage of the human keratinocytes plays a major role in the A1 to A2 conversion. It suggests that one or more enzymes participating in the desaturation of the β-ionone ring of Vitamin A1 is under differentiation control.

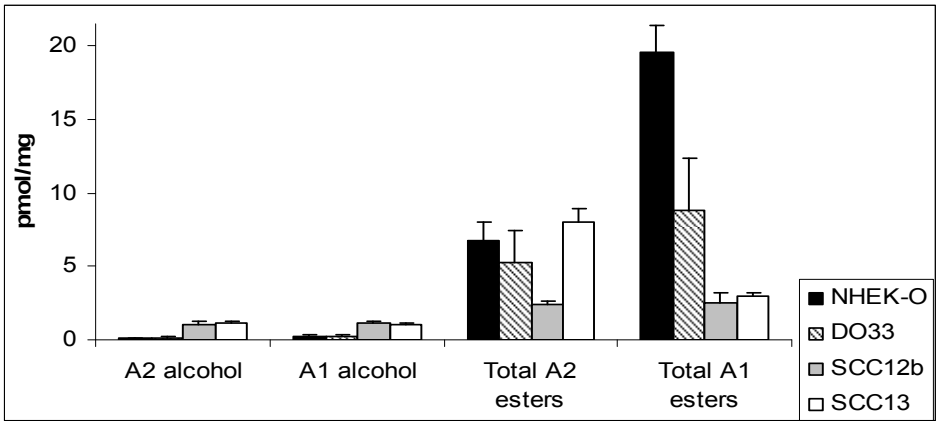
SUMMARY/CONCLUSIONS

- An *in vitro* assay of the Vitamin A1 conversion to Vitamin A2 catalyzed by keratinocyte extracts was developed and was used to determine the plasma membrane association of the involved enzymes.

- Similar to other desaturases, NADPH and P450 linked enzymes appear to promote the desaturation of the β-ionone ring of Vitamin A1.

- Vitamin A2 synthesis is enhanced in differentiated cultures suggesting a critical role in differentiating keratinocytes.

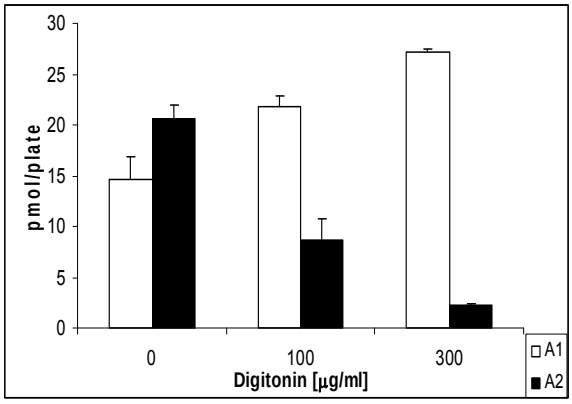
FIGURES FOR CHAPTER ONE



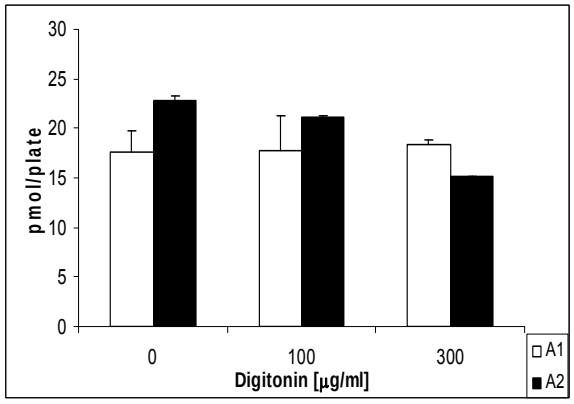
	NHEK-O		DO33		SCC12b		SCC13	
Vitamin A	pmol/mg	stdev	pmol/mg	stdev	pmol/mg	stdev	pmol/mg	stdev
A2 alcohol	**0.06**	0.04	**0.17**	0.07	**1.02**	0.08	**1.20**	0.04
A1 alcohol	**0.28**	0.09	**0.28**	0.11	**1.14**	0.04	**1.08**	0.06
A1 aldehyde	**0.00**	0.00	**0.00**	0.00	**0.00**	0.00	**0.12**	0.14
A2 ester (16:1; 14:0)	**1.91**	0.26	**1.46**	0.60	**0.53**	0.01	**0.82**	0.07
A1 ester (16:1; 14:0)	**5.48**	0.49	**2.52**	1.00	**0.52**	0.04	**0.42**	0.08
A2 ester (18:1; 16:0)	**4.53**	0.88	**3.43**	1.41	**1.78**	0.10	**6.16**	0.75
A1 ester (18:1; 16:0)	**12.85**	1.22	**6.19**	2.47	**1.90**	0.22	**2.19**	0.14
A2 ester (18:0)	**0.31**	0.09	**0.39**	0.15	**0.07**	0.00	**1.07**	0.04
A1 ester (18:0)	**1.19**	0.20	**0.16**	0.08	**0.10**	0.02	**0.39**	0.02
Total A2 esters	**6.76**	1.19	**5.28**	2.16	**2.37**	0.11	**8.05**	0.86
Total A1 esters	**19.53**	1.88	**8.86**	3.54	**2.53**	0.27	**3.00**	0.23
Vitamin A2	**6.82**	1.23	**5.44**	3.36	**3.75**	0.03	**9.25**	0.83
Vitamin A1	**19.80**	1.94	**9.20**	2.23	**3.39**	0.31	**4.21**	0.32
A2/A1	**0.34**	0.05	**0.59**	0.03	**0.92**	0.11	**2.20**	0.03
% conversion into A2	**25.62**		**37.16**		**47.69**		**68.71**	

Fig I - 1: Metabolism of Vitamin A1 alcohol in cultured NHEK and SCC cell lines
Cultures were grown in 3 – 1 medium until one week post-confluence, and incubated for 24h with 25 nM ^{3}H-Vitamin A1 alcohol. Cellular ^{3}H-retinoids were extracted, separated by reverse-phase HPLC and identified by retention time with in-line scintillation counting. Shown in the bar graph are the ^{3}H-Vitamin A1 and A2 alcohols and esters in the different cell lines. Shown in the table is cellular content of the ^{3}H-Vitamin A1 and A2 retinoids. Data represent the average ± standard deviation (stdev) for two - three experiments each with duplicate samples (n = 4-6).

A



C



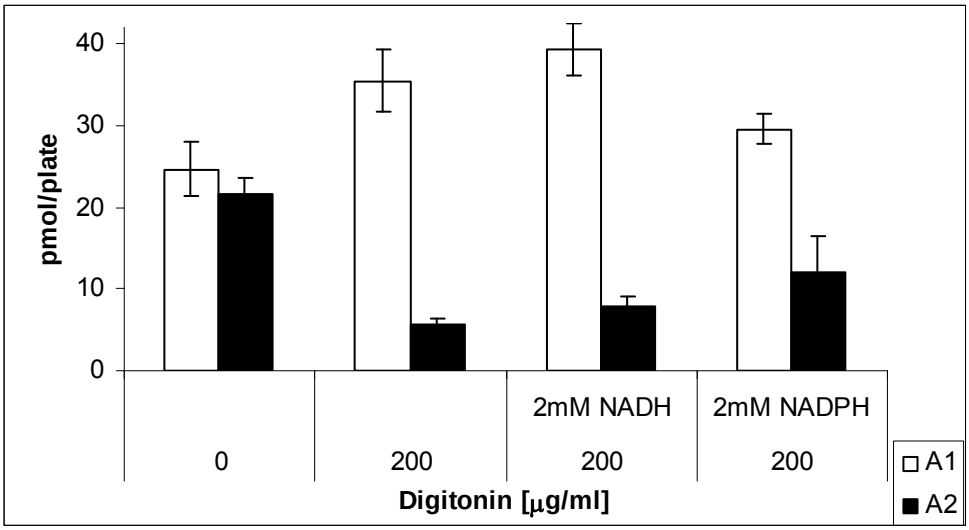
B

Digitonin [μg/ml]	0		100		300	
Vitamin A [pmol/plate]	mean	stdev	mean	stdev	mean	stdev
A2 alcohol	**5.5**	0.28	**1.9**	1.71	**0.6**	0.11
A1 alcohol	**3.9**	0.20	**4.9**	2.50	**9.2**	0.23
A1aldehyde	**0.2**	0.01	**3.8**	4.20	**6.0**	0.55
A2 esters	**16.6**	0.83	**6.8**	0.34	**1.7**	0.03
A1 esters	**12.5**	0.63	**13.1**	0.67	**12.0**	0.42
Total Vitamin A	**38.7**	1.94	**30.5**	1.02	**29.4**	0.23

D

Digitonin [μg/ml]	0		100		300	
Vitamin A [pmol/plate]	mean	stdev	mean	stdev	mean	stdev
A2 alcohol	**5.6**	0.39	**4.7**	0.62	**2.1**	0.26
A1 alcohol	**4.0**	0.02	**3.9**	0.38	**3.7**	0.12
A1aldehyde	**0.0**	0.00	**0.0**	0.00	**1.3**	0.06
A2 esters	**17.2**	0.77	**16.4**	0.76	**13.0**	0.21
A1 esters	**13.6**	2.02	**13.9**	3.17	**13.3**	0.60
Total Vitamin A	**40.5**	2.43	**38.8**	3.69	**33.4**	0.48

Fig I - 2: Effect of digitonin on the conversion of Vitamin A1 to A2 in SCC13
One week post-confluent cultures of SCC13 were treated with digitonin and then pulsed with ^{3}H-Vitamin A1 alcohol in 3 – 1 medium for 1h (A, B) or pulsed with ^{3}H-Vitamin A1 alcohol in 3 – 1 medium for 1h and then treated with digitonin (C,D). Cellular ^{3}H-retinoids were extracted, resolved by reverse-phase HPLC and identified by retention time with in-line scintillation counting. Shown in the bar graphs are the total content of ^{3}H-Vitamin A1 (open bars) and ^{3}H-Vitamin A2 retinoids (closed bars). The content of ^{3}H-Vitamin A1 and its metabolites are listed in the Table. Data represent the average ± standard deviation for two experiments each with duplicates samples (n = 4).



Digitonin [μg/ml]	0		200		200		200	
Cofactor [2mM]					NADH		NADPH	
Vitamin A [pmol/plate]	mean	stdev	mean	stdev	mean	stdev	mean	stdev
A2 alcohol	**9.07**	1.90	**1.91**	0.10	**2.94**	0.06	**4.47**	1.27
A1 alcohol	**8.60**	1.19	**14.13**	0.91	**15.27**	2.29	**11.08**	1.80
A1 aldehyde	**4.23**	3.42	**6.05**	0.80	**8.54**	3.86	**4.47**	1.37
A2 esters	**12.66**	3.63	**3.81**	0.76	**5.01**	0.99	**7.65**	3.02
A1 esters	**11.78**	1.03	**15.22**	3.69	**15.40**	4.77	**14.00**	1.42
Total Vitamin A	**46.35**	5.00	**41.12**	4.46	**47.16**	4.25	**41.66**	6.13

Fig I - 3: Effect of NAD(P)H on the conversion of Vitamin A1 to A2 in digitonin treated cultures of SCC13

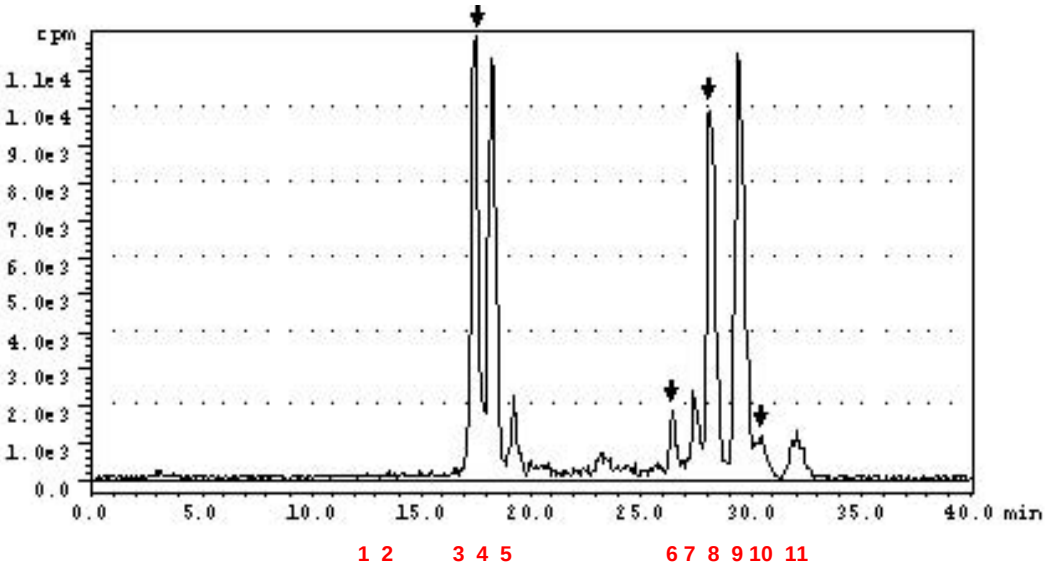
Five days post-confluent cultures were treated with digitonin and then pulsed with ^{3}H-Vitamin A1 alcohol in 3 – 1 medium for 1h in presence or absence of 2mM NADH or NADPH. Cellular retinoids were extracted, resolved by reverse-phase HPLC, and identified by retention time with in-line scintillation counting. Shown in the bar graphs are the total cellular content of ^{3}H-Vitamin A1 (open bars) and ^{3}H-Vitamin A2 retinoids (closed bars). Shown in the tables are cellular content of each metabolite of ^{3}H-Vitamin A1. Data represent the average ± standard deviation for two – three experiments each with duplicate samples, (n = 4 – 6).

NADPH (mM)	0	0	2
Digitonin (mg/ml)	0	200	200
NAD (nmol/10mg wet cells)	0.692	0.299	0.348
NADH (nmol/10mg wet cells)	0.035	0.012	0.007
NADP (nmol/10mg wet cells)	0.046	0.051	0.187
NADPH (nmol/10mg wet cells)	0.132	0.017	0.050

Table I - 1: Cofactors levels after treatment with digitonin in presence or absence of medium NADPH

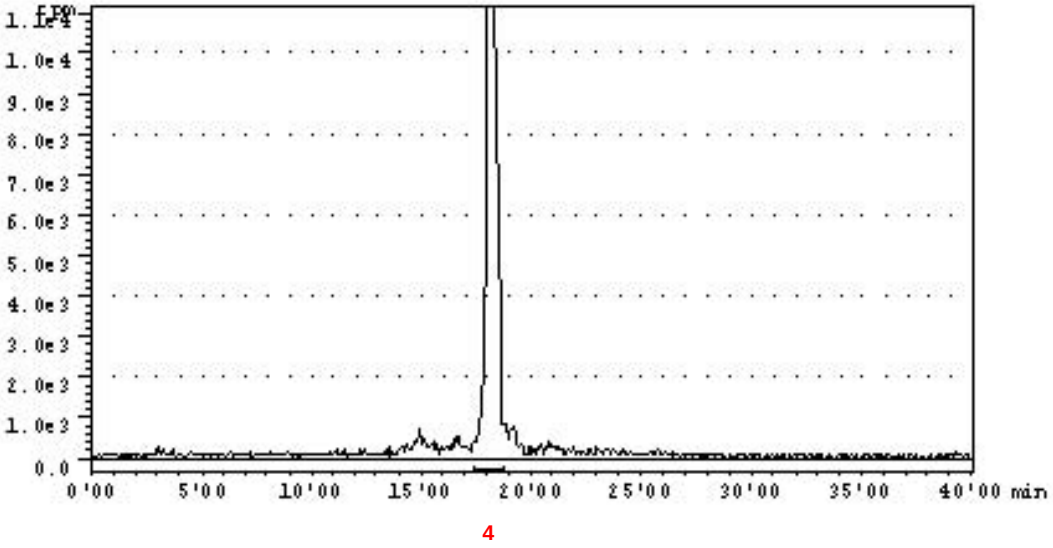
Five days post-confluent cultures were treated with digitonin incubated for 1h with 3 – 1 medium in presence or absence of 2mM NADPH. After the incubation period the cultures were harvested and the internal content of each cofactor was measured.

A



SCC13

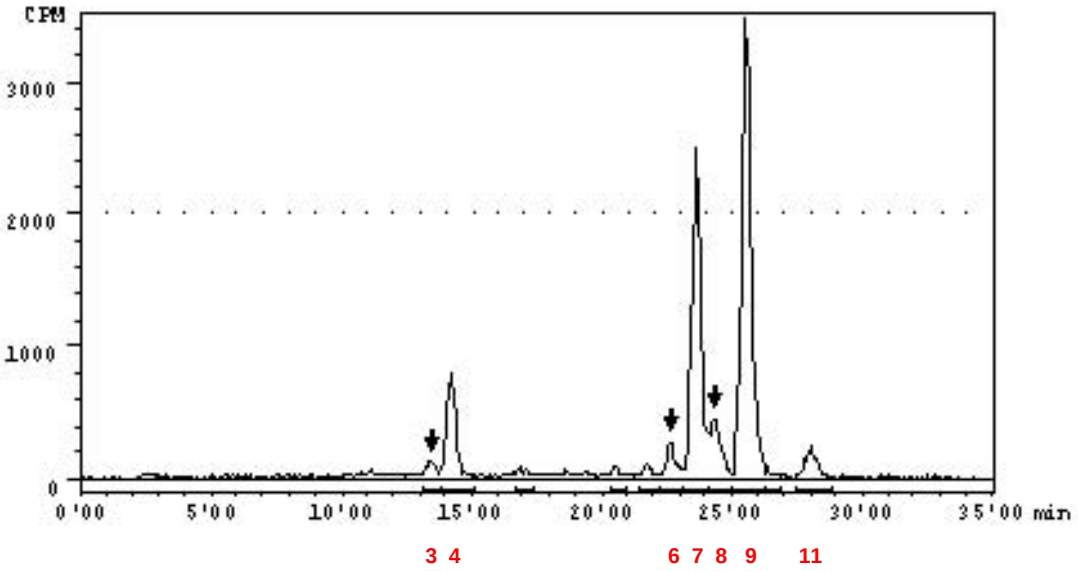
B



Boiled control

Fig I - 4a: *In vitro* Vitamin A1 to A2 conversion using sonicates of SCC13
One week post-confluent SCC13 cells grown in 3 – 1 medium were harvested, sonically disrupted and assayed using the 17500 rpm fraction (A) as described in the Methods. Retinoids were extracted, resolved by reverse-phase HPLC and identified by retention time with in-line scintillation counting. The arrows indicate Vitamin A2 metabolites and the red numbers indicate the retention times of authentic standards listed in Table – 2. The boiled control (B) was prepared by heating a part of the 17500 rpm fraction at 100°C for 5 minutes. Peak number 4 is the substrate Vitamin A1 alcohol.

A



B

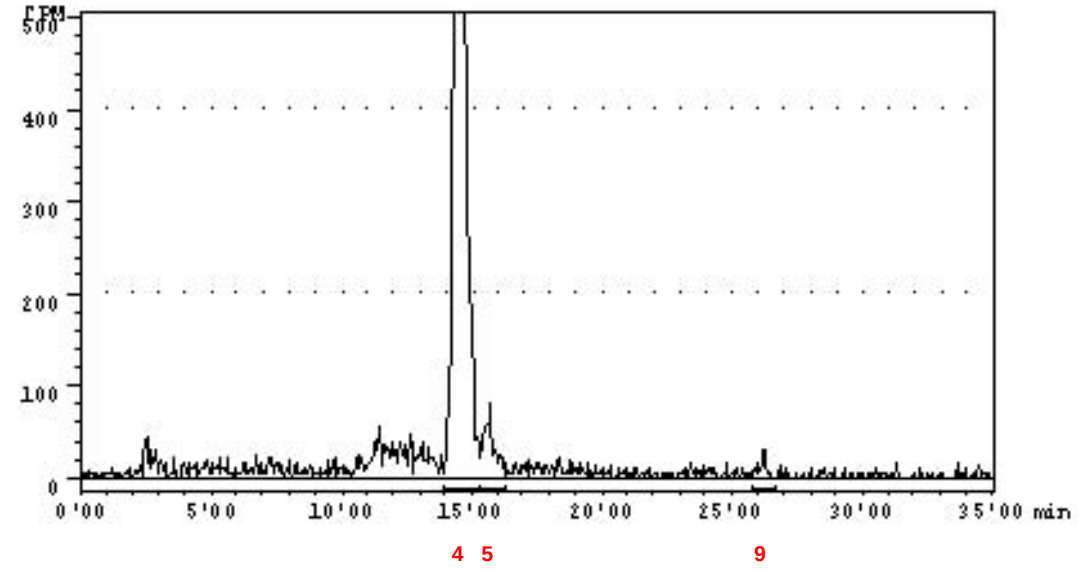


Fig I - 4b: *In vitro* Vitamin A1 to A2 conversion using DO33 and human fibroblasts
One week post-confluent cultures of DO33 grown in 3 – 1 medium (A) and human fibroblasts grown in DMEM supplemented with 10% FBS (B) were harvested, sonically disrupted and the 17,500 rpm fraction used for *in vitro* assay of A1 to A2 conversion. Retinoid were extracted, resolved by reverse-phase HPLC and identified by retention time with in-line scintillation counting. The arrows indicate Vitamin A2 metabolites and the red numbers indicate the retention times of authentic standards listed in Table – 2. Peak number 4 corresponds to substrate Vitamin A1 alcohol.

1	Vitamin A2 acid	3, 4 didehydroretinoic acid
2	Vitamin A1 acid	retinoic acid
3	Vitamin A2 alcohol	3, 4 didehydroretinol
4	Vitamin A1 alcohol	retinol
5	Vitamin A1 aldehyde	retinaldehyde
6	Vitamin A2 ester (16:1; 14:0)	3, 4 didehydroretinyl palmitoleate and 3, 4 didehydroretinyl myristate
7	Vitamin A1 ester (16:1; 14:0)	retinyl palmitoleate and retinyl myristate
8	Vitamin A2 ester (18:1; 16:0)	3, 4 didehydroretinyl oleate and 3, 4 didehydroretinyl palmitate
9	Vitamin A1 ester (18:1; 16:0)	retinyl oleate and retinyl palmitate
10	Vitamin A2 ester (18:0)	3, 4 didehydroretinyl stearate
11	Vitamin A1 ester (18:0)	retinyl stearate

Table I - 2: The numbered peaks which correspond to the retention times for authentic retinoid standards

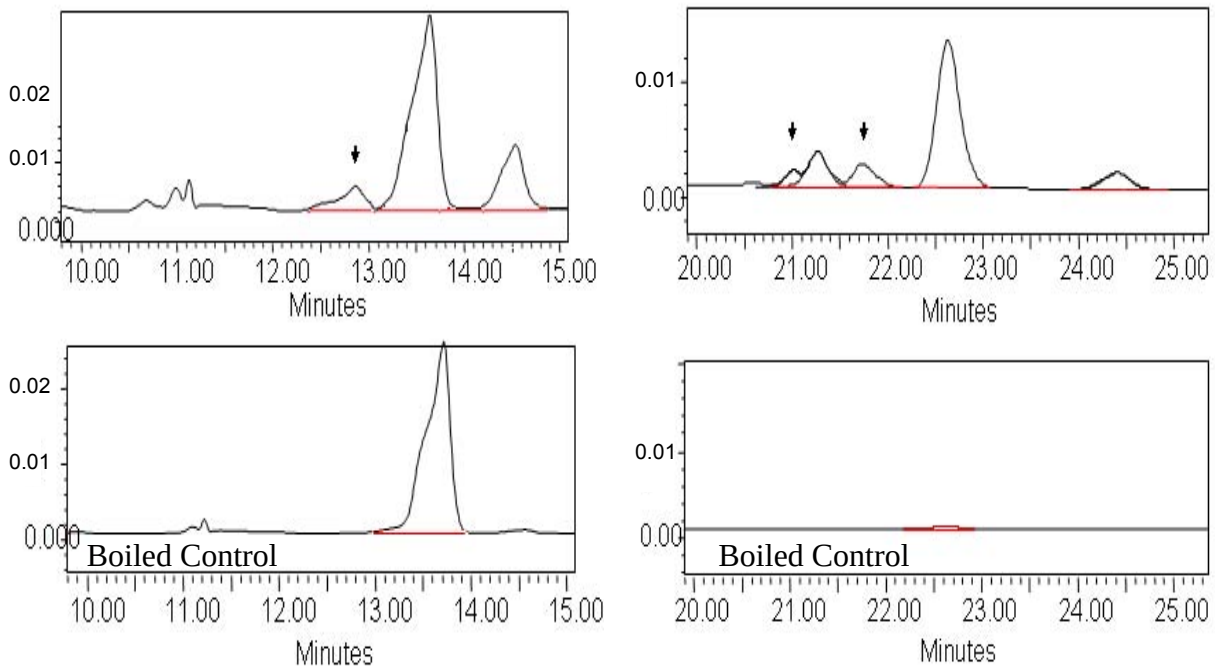
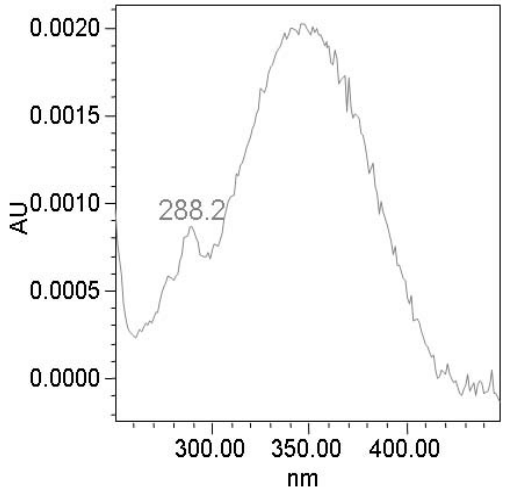


Fig I – 5a: The identity of A2 products was confirmed by retention time upon HPLC separation

One week post-confluent SCC13 cells grown in 3 – 1 medium were harvested, sonically disrupted and *in vitro* assayed using 17500 rpm fraction as described in the Methods. Ten individual reactions were combined and the retinoids were extracted and analyzed by reverse-phase HPLC. Noted by the black arrows are the correct retention times of Vitamin A2 metabolites (alcohol – left panel and esters – right panel).

Vitamin A2 alcohol



Vitamin A2 esters

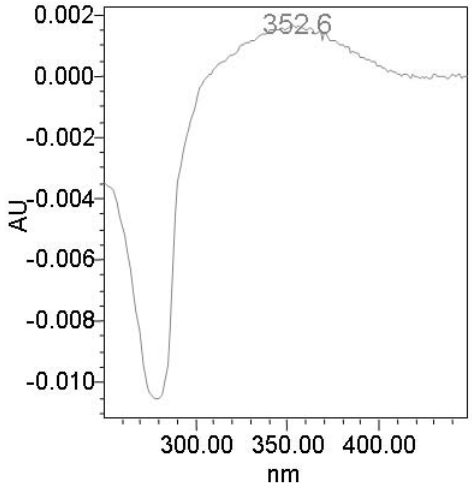
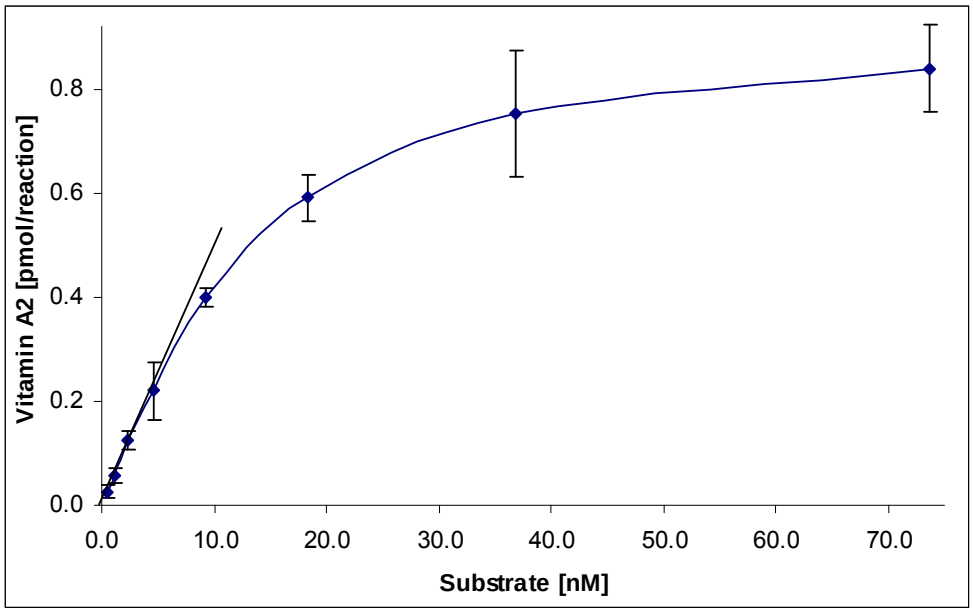


Fig I – 5b: Spectral matching of metabolites identified as A2 retinoids
The spectra of retinoids identified as Vitamin A2 alcohol and Vitamin A2 esters were analyzed by photodiode array. The correct absorption spectrum for Vitamin A2 alcohol (upper panel) and ester (lower panel) confirms identification.

A



B

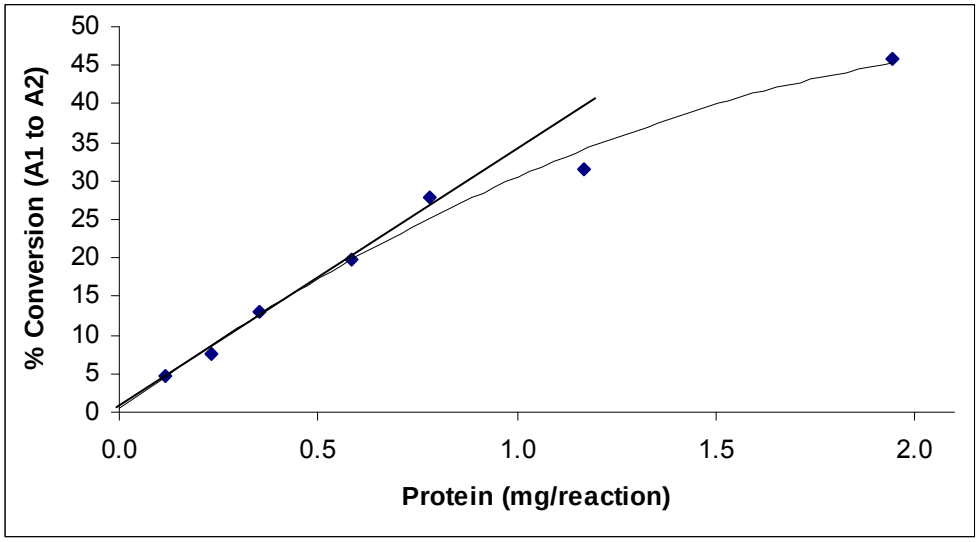


Fig I - 6: Vitamin A1 to A2 converting activity is a substrate a) and protein b) concentration dependent

One week post-confluent SCC13 cells grown in 3 – 1 medium were harvested, sonically disrupted and *in vitro* assayed using 17500 rpm fraction as described in the Methods. The *in vitro* reactions were performed with varying substrate (A) and the protein (B) concentrations. The retinoid metabolites were extracted and analyzed by reverse-phase HPLC. Data represent the average for two experiments each with duplicate samples (n = 4).

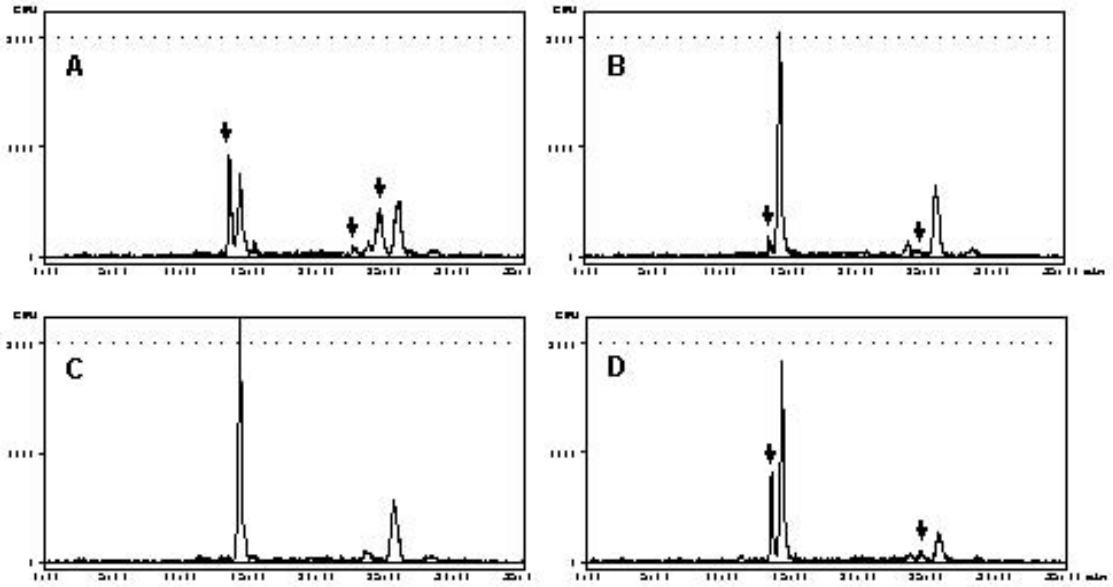
A

	total protein (mg)	pmol A2/ mg protein	total A2 (pmol)	Recovery (%)
Cell sonicate	104.19	0.46	47.63	100
3000 rpm	32.98	0.77	25.31	53.14
17,500 rpm	6.06	1.25	7.55	15.85
17500 rpm supernatant	63.43	0.17	10.90	22.89

B

	total protein (mg)	pmol A2/ mg protein	total A2 (pmol)	Recovery (%)
Cell homogenate	129.00	0.39	50.31	100.00
3000 rpm	109.76	0.42	45.67	95.88
17,500 rpm	5.34	0.21	1.14	2.39
17500 rpm supernatant	37.80	0.04	1.62	3.41

Table I - 3: Partial purification of the Vitamin A1 to A2 converting activity
One week post-confluent SCC13 cells grown in 3 – 1 medium were harvested and either sonically disrupted (A) or homogenized (B). Fractions were prepared by centrifugation and *in vitro* assayed as described in the Methods. The retinoid metabolites were extracted, separated by reverse-phase HPLC, and detected by retention time with in-line scintillation counting. Data represent the average ± standard deviation for two experiments each with duplicate samples, (n = 4).



		total mg protein	µg pr/reac-tion	pmol A2/h/mg protein	Units pmol A2/assay	pmol esters/h/ mg protein
Fraction 1	Homogenate	**140.65**	**293.14**	**1.028**	**301.53**	**1.01**
	stdev	6.31	4.13	0.066	23.52	0.16
Fraction 2	Homogenate without whole cells and nuclei	**67.88**	**169.70**	**0.256**	**41.37**	**1.35**
	stdev	30.90	77.24	0.052	10.96	0.93
Fraction 3	Layer over 30% sucrose	**4.88**	**40.70**	**0.278**	**11.39**	**4.18**
	stdev	0.62	5.20	0.028	2.58	2.78
Fraction 4	Layer containing plasma membrane ghosts over 45% sucrose	**1.48**	**123.02**	**1.235**	**150.74**	**0.48**
	stdev	0.19	16.22	0.141	2.71	0.68

Fig I - 7: Vitamin A1 to A2 *in vitro* conversion activity in plasma membrane ghosts
Ten days post-confluent cultures of SCC13 grown in 3 – 1 medium were harvested, disrupted by homogenization, and fractionated. *In vitro* A1 to A2 conversion assays were carried out with homogenate prior to removal of unbroken cells and nuclei (Panel A, Fraction 1), homogenate after removal of unbroken cells and nuclei (Panel B, Fraction 2), and the two fractions obtained from centrifugation through a sucrose step gradient. Fraction 3 (Panel C) is four times enriched in Vitamin A1 transacetylation activity. Fraction 2 (Panel D) contains plasma membrane ghosts with Vitamin A1 to A2 converting activity with partial enrichment of specific activity. Arrows indicate Vitamin A2 metabolites. Data in the table represent the average ± standard deviation of two experiments each with duplicate samples, (n = 4).

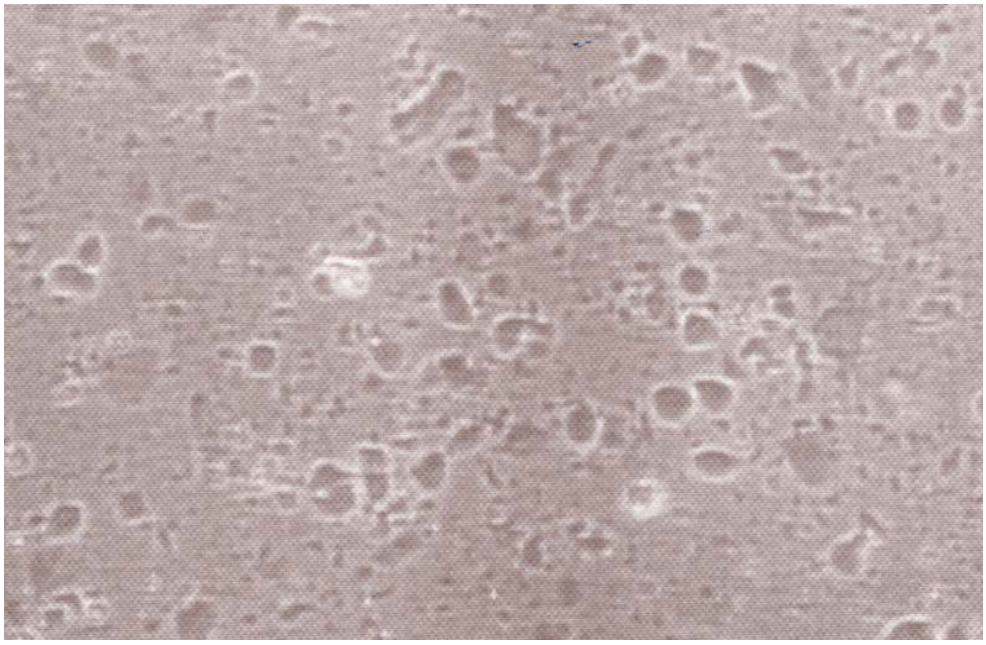


Fig I - 8: Plasma membrane ghosts stained with trypan blue
10 μl aliquot of Fraction 4 (Shown in the Figure – 7, Panel D) was taken and stained with trypan blue for microscopic observation as described in Methods.

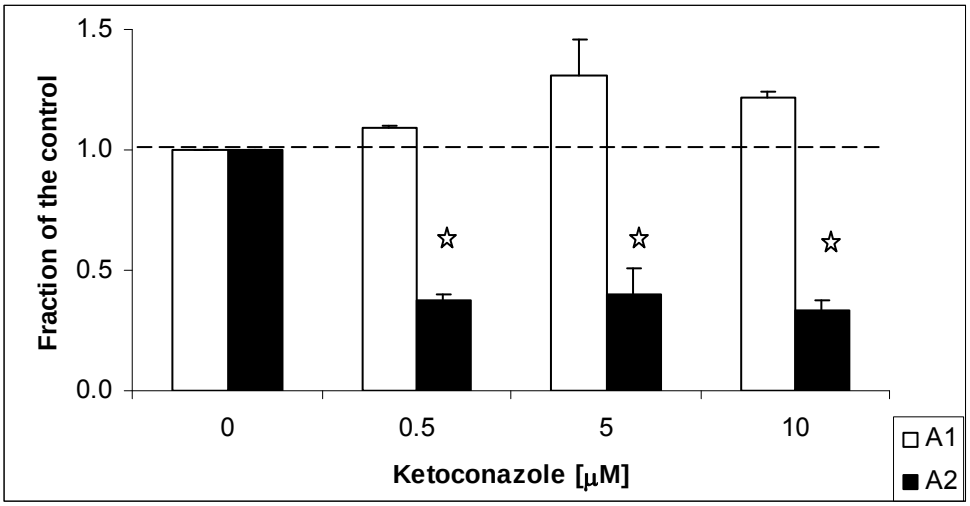


Fig I – 9a: Effect of ketoconazole on Vitamin A2 accumulation *in vivo* in NHEK-O
Two day post-confluent cultures of NHEK-O were pretreated with the indicated concentrations of ketoconazole for 1h and then incubated for an additional 24h with 25 nM ^{3}H-Vitamin A1 alcohol in presence of increasing concentrations of ketoconazole. Cellular ^{3}H-retinoids were extracted, resolved by reverse-phase HPLC and identified by retention time with in-line scintillation counting. The A1 content at 0 μM ketoconazole was 20.67 (±1.6), and the A2 content at 0 μM ketoconazole was 3.55 (±0.3). Data represent the average ± standard deviation for two experiments each with duplicate samples, (n = 4). Ketoconazole significantly (☆) inhibited ^{3}H-Vitamin A2 synthesis ($p<0.05$).

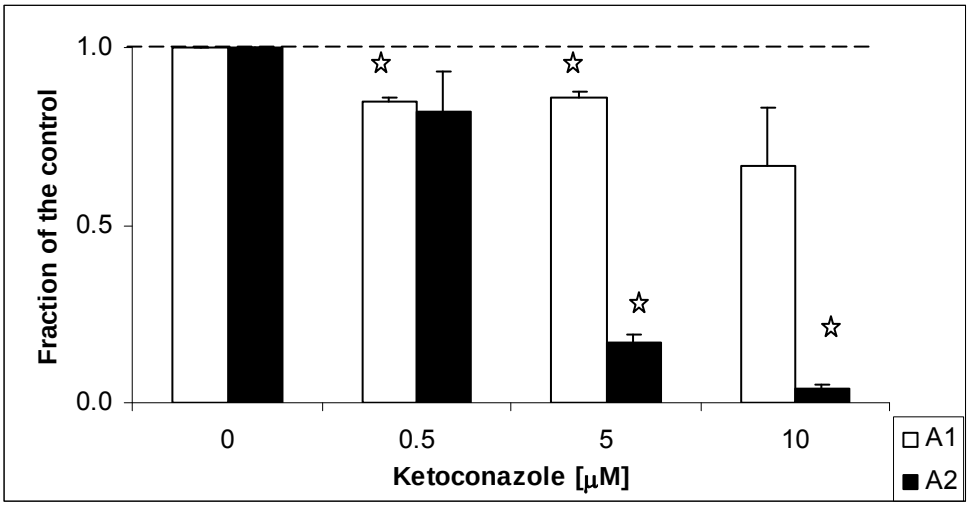


Fig I – 9b: Effect of ketoconazole on Vitamin A2 *in vitro* synthesis in NHEK-O
Cells were grown in 3 – 1 medium until two days post-confluence, harvested by trypsinisation, sonically disrupted and *in vitro* assayed with increasing concentrations of ketoconazole as described in the Methods. The retinoid metabolites were extracted and analyzed by reverse-phase HPLC. Shown are of ^{3}H-Vitamin A1 and A2 metabolites. The control that equals to 1 has Vitamin A2 alcohol - 0.03±0.02, A2 esters - 0.14±0.01, and A1 esters - 1.71±0.01 pmol/mg protein. Data represents the average ±standard deviation for two experiments, (n = 4). Ketoconazole (5 and 10 μM) significantly (☆) inhibited ^{3}H-Vitamin A2 synthesis ($p<0.05$).

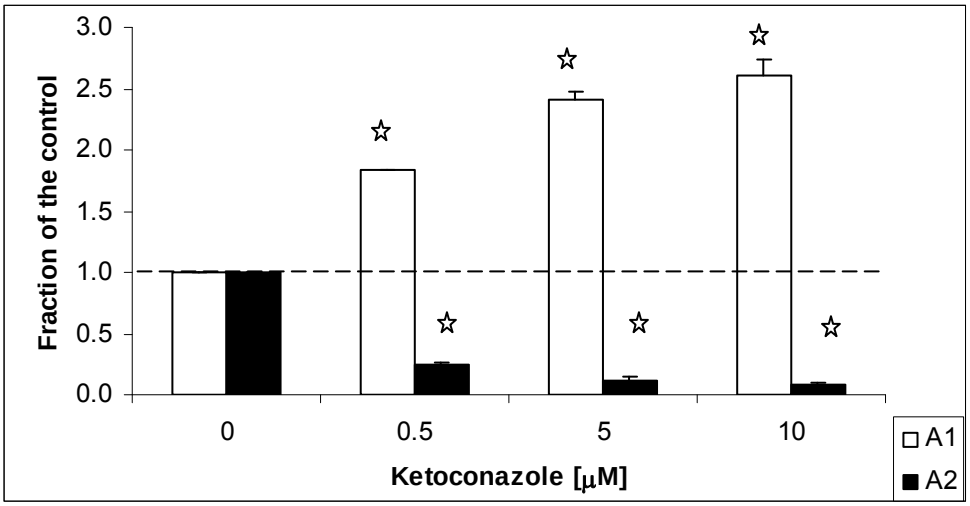


Fig I – 10a: Effect of ketoconazole on Vitamin A2 accumulation *in vivo* in SCC13
Two day post-confluent cultures of SCC13 were pretreated with the indicated concentrations of ketoconazole for 1h and then incubated for an additional 24h with 25 nM ^{3}H-Vitamin A1 alcohol in presence of increasing concentrations of ketoconazole. Cellular ^{3}H-retinoids were extracted, resolved by reverse-phase HPLC and identified by retention time with in-line scintillation counting. The A1 content at 0 μM ketoconazole was 4.57 (±1.11), and the A2 content at 0 μM ketoconazole was 7.37 (±0.42). Data represents the average ± standard deviation for two experiments each with duplicate samples, (n = 4). Ketoconazole significantly (☆) inhibited ^{3}H-Vitamin A2 synthesis ($p<0.05$).

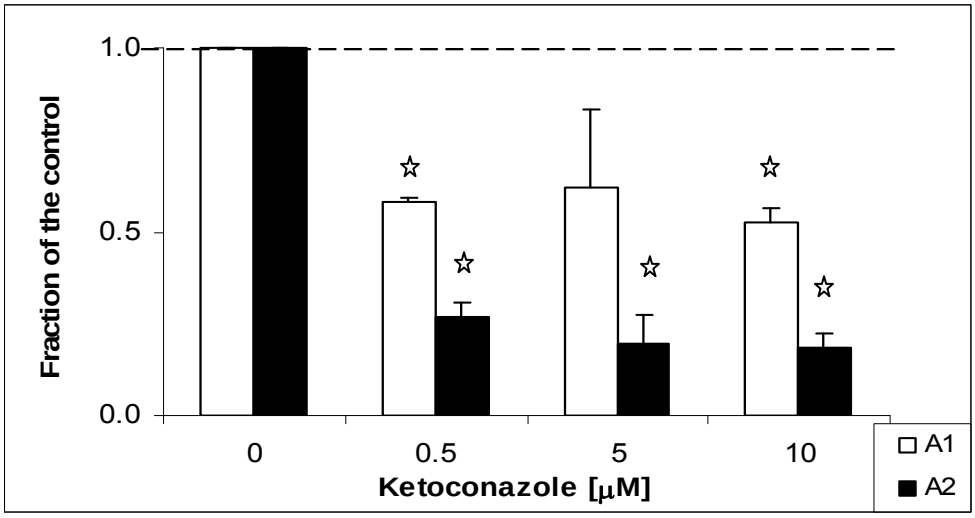
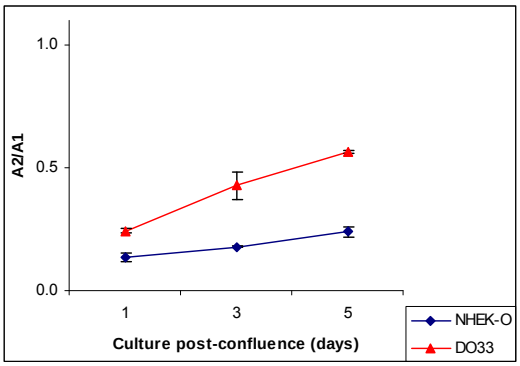
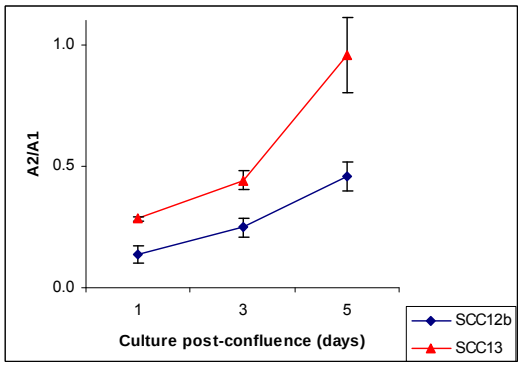


Fig I – 10b: Effect of ketoconazole on Vitamin A2 *in vitro* synthesis in SCC13
Cells were grown in 3 – 1 medium until two days post-confluence, harvested by trypsinisation, sonically disrupted and *in vitro* assayed with increasing concentrations of ketoconazole as described in the Methods. The retinoid metabolites were extracted and analyzed by reverse-phase HPLC. Shown are of ^{3}H-Vitamin A1 and A2 metabolites. The control that equals to 1 has Vitamin A2 alcohol- 0.05±0.01, A2 esters - 0.06±0.003, and A1 esters - 0.40±0.005 pmol/mg protein. Data represents the average ± standard deviation for two experiments each with duplicate samples, (n = 4). Ketoconazole significantly (☆) inhibited ^{3}H-Vitamin A2 synthesis ($p<0.05$).

A



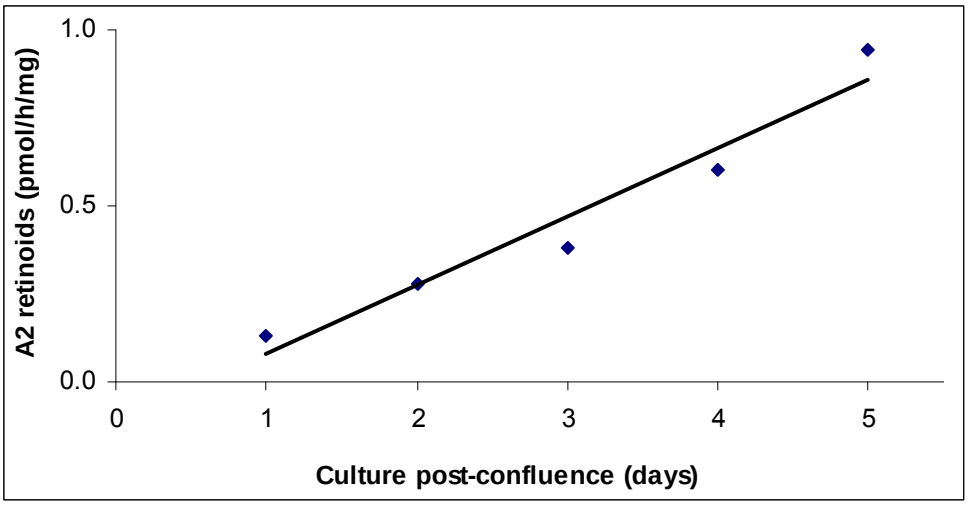
B



	days post-confluence	**1**		**3**		**5**	
		pmol/mg	stdev	**pmol/mg**	stdev	**pmol/mg**	stdev
NHEK-O	A2 alcohol	**0.01**	0.02	**0.05**	0.02	**0.03**	0.04
	A1 alcohol	**0.13**	0.12	**0.22**	0.04	**0.21**	0.18
	Total A2 esters	**3.85**	1.09	**4.97**	1.15	**6.31**	1.98
	Total A1 esters	**28.45**	9.46	**28.07**	6.28	**26.36**	6.10
	% convercion	**11.91**		**15.06**		**19.27**	
DO33	A2 alcohol	**0.10**	0.04	**0.10**	0.02	**0.26**	0.01
	A1 alcohol	**0.37**	0.09	**0.14**	0.03	**0.39**	0.03
	Total A2 esters	**2.87**	1.12	**3.61**	0.57	**6.36**	0.45
	Total A1 esters	**11.77**	4.28	**8.55**	0.83	**11.23**	0.52
	% convercion	**19.61**		**29.92**		**36.15**	
SCC12b	A2 alcohol	**0.05**	0.03	**0.16**	0.17	**0.72**	0.18
	A1 alcohol	**0.70**	0.46	**0.73**	0.73	**1.90**	0.33
	Total A2 esters	**0.48**	0.14	**1.72**	0.38	**1.36**	0.59
	Total A1 esters	**3.13**	0.54	**6.75**	0.67	**2.68**	1.60
	% convercion	**11.98**		**19.80**		**31.42**	
SCC13	A2 alcohol	**0.38**	0.01	**0.50**	0.04	**0.66**	0.06
	A1 alcohol	**1.23**	0.09	**1.16**	0.10	**0.70**	0.02
	Total A2 esters	**2.89**	0.27	**4.88**	0.44	**5.77**	0.68
	Total A1 esters	**10.27**	1.20	**11.00**	0.99	**5.90**	1.75
	% convercion	**22.17**		**30.66**		**48.92**	

Fig I - 11: Influence of culture confluence on Vitamin A2/A1 ratio *in vivo* in cultured NHEK (A) and SCC (B) cell lines

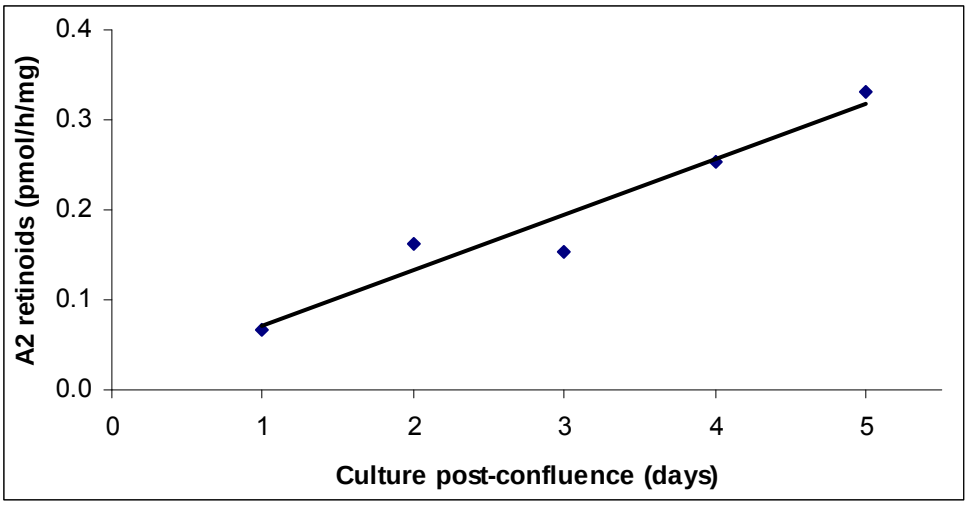
Cultures were grown in 3 – 1 medium until 1, 3 or 5 days post-confluence, incubated for 24h with 25nM ^{3}H-Vitamin A1 alcohol. Cellular ^{3}H-retinoids were extracted, resolved by HPLC and identified by retention time and in-line scintillation counting. Shown in the figures is increased Vitamin A2/A1 ratio with advancement of culture confluence. Shown in the table are cellular content of different types of ^{3}H-Vitamin A1 and A2 retinoids in the different cell lines. Data represents the average ± standard deviation for two – three experiments each with duplicate samples, (n = 4 – 6).



Days post-confluence	1		2		3		4		5	
	pmol/h/mg	stdev	pmol/h/mg	stdev	pmol/h/mg	stdev	pmol/h/mg	stdev	pmol/h/mg	stdev
Vitamin A2 alcohol	**0.009**	0.001	**0.039**	0.008	**0.068**	0.020	**0.137**	0.022	**0.217**	0.100
Vitamin A2 esters	**0.121**	0.012	**0.240**	0.022	**0.310**	0.029	**0.466**	0.032	**0.724**	0.207
Vitamin A1 esters	**1.687**	0.169	**2.229**	0.029	**1.567**	0.056	**1.598**	0.132	**1.868**	0.371
Vitamin A1	**1.687**	0.169	**2.229**	0.608	**1.567**	0.029	**1.598**	0.074	**1.868**	0.209
Vitamin A2	**0.130**	0.013	**0.279**	0.308	**0.378**	0.030	**0.604**	0.049	**0.941**	0.054
% conversion	**7.13**		**11.13**		**19.45**		**27.41**		**33.51**	

Fig I - 12: Influence of culture confluence on *in vitro* Vitamin A1 to A2 conversion by NHEK-O

NHEK-O was grown in 3 – 1 medium and harvested at post-confluence as indicated. Cells were then sonically disrupted and *in vitro* assayed as described in the Methods, and cellular ^{3}H-retinoids were extracted, resolved by HPLC and identified by retention time and in-line scintillation counting. Shown in the figure is increase in Vitamin A2 as a function of culture confluence. The level of ^{3}H-Vitamin A1 metabolites are given in the table. Data represents the average ± standard deviation for two – three experiments each with duplicate samples, (n = 4 – 6).



Days post-confluence	1		2		3		4		5	
	pmol/h/mg	stdev	pmol/h/mg	stdev	pmol/h/mg	stdev	pmol/h/mg	stdev	pmol/h/mg	stdev
Vitamin A2 alcohol	**0.031**	0.000	**0.088**	0.009	**0.072**	0.009	**0.038**	0.016	**0.196**	0.021
Vitamin A2 esters	**0.037**	0.002	**0.075**	0.007	**0.082**	0.009	**0.215**	0.019	**0.135**	0.001
Vitamin A1 esters	**0.334**	0.035	**0.343**	0.034	**0.344**	0.032	**0.785**	0.109	**0.568**	0.033
Vitamin A1	**0.334**	0.05	**0.343**	0.08	**0.344**	0.06	**0.785**	0.09	**0.568**	0.00
Vitamin A2	**0.068**	0.00	**0.162**	0.02	**0.153**	0.02	**0.253**	0.04	**0.331**	0.02
% conversion	**16.85**		**32.11**		**30.85**		**24.38**		**36.85**	

Fig I - 13: Influence of culture confluence on *in vitro* Vitamin A1 to A2 conversion by SCC13

SCC13 was grown in 3 – 1 medium and harvested at post-confluence as indicated. Cells were then sonically disrupted and *in vitro* assayed as described in the Methods, and cellular ^{3}H-retinoids were extracted, resolved by HPLC and identified by retention time and in-line scintillation counting. Shown in the figure is the increase in Vitamin A2 as a function of culture confluence. The level of ^{3}H-Vitamin A1 metabolites are given in the table. Data represents the average ± standard deviation for two – three experiments each with duplicate samples, (n = 4 – 6).

CHAPTER 2

THE EFFECT OF UVA AND UVB RADIATION ON VITAMIN A2 AND THE ABILITY OF VITAMIN A2 TO PROTECT CELLS FROM UV-INDUCED APOPTOSIS

Vitamin A2 has been proposed to serve as a better filter against UV radiation than Vitamin A1 and to be more resistant to UV-induced modifications. To verify differences in the stability of Vitamin A1 and A2 within the microenvironment of the keratinocyte, and to determine whether Vitamin A2 better protects keratinocytes against UV damage, the impact of UV on retinoid status and the impact of Vitamin A2 on UV-induced apoptosis were measured.

Vitamin A2 esters are more resistant than Vitamin A1 esters to UVA or UVB radiation

The stability of Vitamin A1 and A2 esters was compared in cultured keratinocytes irradiated with different doses of UVA or UVB. Keratinocytes (NHEK-O) were grown in 3 – 1 medium to 3-4 days post confluence and then incubated with ^{3}H-Vitamin A1 alcohol for 24 hours. Cultures were then rinsed with PBS, irradiated either with UVA (0, 1.25, 2.5, and 5 J/cm^2) or UVB (0, 0.12, 0.24, 0.3, and 0.4 J/cm^2) and then immediately harvested. After extraction, retinoids were resolved by HPLC and analyzed spectrophotometrically and by in-line scintillation counting. At the doses chosen no difference in viability was detected by staining with trypan blue or propidium iodide (data not shown).

As shown in Figure II – 1a A, B, increasing doses of UVA resulted in a loss in both Vitamin A1 and A2 esters. The loss of the A1 esters was greater and at only 1.25 J/cm^2, there was a 50% reduction of each A1 ester and only a 19% reduction of each A2 ester. The chromatogram shown in Figure II – 1b B, shows the remaining retinoids after a 5.0 J/cm^2 dose of UVA in which the levels of A1 and A2 esters were comparable but low. Similar to the results with UVA, decreases in ester mass were obtained with increasing doses of UVB (Figure II – 2a A, B). At 0.12 J/cm^2 there was a 50% reduction of each A1 ester, which compares to a 25% reduction of the A2 ester peak comprising oleate and palmitate esters and a 14% reduction of the A2 ester peak comprising palmitoleate and myristate esters. The chromatogram shown in Figure II – 2b B, shows the remaining retinoids after a 0.4 J/cm^2 dose of UVB in

which the levels of the A1 and A2 esters are comparable. Non-irradiated cultures served as control and their retinoid content is shown for comparison.

The newly synthesized, ^{3}H-retinoid pools also decreased with increasing doses of UVA (Figure II – 3a) and UVB (Figure II – 3b). As expected, controls demonstrate that during this time the cells took up and metabolized ^{3}H-Vitamin A1 alcohol mostly to Vitamin A1 (Peaks 7, 9, 11) and A2 esters (Peaks 6, 8) with little substrate ^{3}H-retinol (Peak 4) remaining. In all treatments, the irradiated cultures generated a retinoid with same retention time as Vitamin A2 alcohol (Peak 3), and in cultures irradiated with 0.4 J/cm^2 UVB, a retinoid with the retention time of Vitamin A2 stearate (Peak 10) was found. This retinoid was not detected in the control cells or in the cells irradiated with the lower two doses. In addition in UVB irradiated cultures after all doses a small peak with retention time corresponding to that of Vitamin A1 acid was detected (Peak 2). The quantity of the peaks with retention times of Vitamin A2 alcohol, and Vitamin A1 acid was too low to verify identity by spectral matching. Even higher doses of UVB (0.6-0.65 J/cm^2), that were found to lead to cell shrinkage and presumably apoptosis did not destroy completely the A2 esters (data not shown).

Restoration of Vitamin A1 and A2 ester pools post-UV exposure

The purpose of the next experiment was to determine the time required to restore internal pools of Vitamin A1 and Vitamin A2 esters after UVA or UVB irradiation of cultured keratinocytes. Keratinocytes (NEHK-O) were again grown in 3 – 1 medium till 3 - 4 days post confluence. The cells were washed with PBS and irradiated with a single dose of 15 J/cm^2 UVA or 0.4 J/cm^2 UVB radiation. Cultures were fed with 3 – 1 medium and harvested after 1, 2, 3, 4 and 6 days with feeding every other day. Retinoids were extracted, resolved by HPLC and identified by retention time and spectral matching.

After exposure to 15 J/cm^2 of UVA, Vitamin A2 ester pool recovered more slowly than Vitamin A1 ester pool (Fig II – 4 A). More than six days to completely restore the internal ester pools. After UVB treatment (0.4 J/cm^2) Vitamin A1 and A2 ester pools reach control levels by day 3 – 4 post-irradiation, most likely due to the esters remaining after irradiation (Fig II – 4 B). Initially Vitamin A1 esters recover with faster rate than A2 esters, possibly reflecting the requirement for substrate Vitamin A1 for biosynthesis of Vitamin A2 and its esters. The cells continue to accumulate esters and by the 6th day they have about 50% more Vitamin A1 and Vitamin A2 esters.

Determination of the effect of UVA (15 J/cm^2) or UVB (0.4 J/cm^2) irradiation on the *in vitro* conversion of Vitamin A1 to A2 in cultured NHEK cells

The impact of UVA and UVB on the *in vitro* conversion of Vitamin A1 to A2 was next measured. Cultures were grown as described above and were again irradiated with a single dose of UVA (15 J/cm^2) or UVB (0.4 J/cm^2). After irradiation cultures were fed with fresh 3 – 1 medium and incubated for 4 hours, 24 hours and 48 hours after the exposure. The *in vitro* Vitamin A1 to A2 conversion assay was

performed with the sonically disrupted cell prepared from irradiated or non-irradiated cultures. The *in vitro* reactions were performed for 1 hour as described in the "Methods" section after which retinoids were extracted and analyzed by HPLC with in-line scintillation counting.

As shown in Figure II – 5 A, the rate of Vitamin A2 synthesis was 22 – 33% greater in extracts prepared from UVA irradiated cultures compared to control. The increase in activity was seen as early as 4 hours after UVA exposure. An increase in conversion activity was also observed in extracts prepared from UVB treated cultures. The 4 hours, 24 hours, and 48 hours extracts were 30%, 83% and 36% greater than control (Figure II – 5 B). These extracts also catalyzed more esterification of both Vitamin A1 alcohol and Vitamin A2 alcohol, indicating a broader impact on retinoid metabolism. For analysis 24 hours post UV exposure was used for experiments that follow.

UV exposure increases the *in vivo* and *in vitro* synthesis of Vitamin A2

The next phase in my study was designed to determine whether the effect of UVA and UVB on the *in vitro* conversion of Vitamin A1 to A2 was also observed *in vivo*. Three to four days post confluent cultures of NHEK-O were treated as before with a single dose of 15 J/cm^2 UVA or 0.4 J/cm^2 UVB. For the *in vivo* experiments, immediately after the irradiation the cultures were incubated in 3 – 1 medium containing ^{3}H-Vitamin A1 alcohol. After 24 hours the retinoids were extracted, resolved by HPLC and identified by retention time with in-line scintillation counting. For the *in vitro* experiments, immediately after the irradiation the cultures were incubated in 3 –1 medium, grown for 24 hours and harvested. Sonically disrupted cells (0.2 mg/reaction) prepared from irradiated or non-irradiated cultures were used for the *in vitro* assay. The reactions were performed for 1 hour as described in Methods section and retinoids were extracted and analyzed.

Cultured NHEK-O showed a 32% increase in accumulation of Vitamin A2 retinoids 24 hours after irradiation with 15 J/cm^2 of UVA compared to the non-irradiated control. Vitamin A2 alcohol and A2 esters increased 36% and 33%, respectively. No change in the accumulation of Vitamin A1 retinoids was detected (Fig II – 6 A). A greater increase (46%) in Vitamin A2 retinoid synthesis was seen using the *in vitro* conversion assay; both Vitamin A2 alcohol and ester increased (Figure II – 6 B). Similar to the results obtained with UVA treatment, cultures exposed to 0.4 J/cm^2 UVB accumulated 30% more Vitamin A2 retinoids. Compared to control, Vitamin A2 alcohol and ester increased 40% and 29%, respectively. No change in the accumulation of the Vitamin A1 retinoids was detected (Figure II – 7A). Again, a greater increase (56%) in Vitamin A2 retinoid synthesis was seen using the *in vitro* conversion assay and again increases in both alcohol and ester were found. In contrast to the *in vivo* results, a 25% increase in the Vitamin A1 esters was found *in vitro*. Because of this discrepancy, the experiment was repeated using an only 0.24 J/cm^2 UVB. Under these conditions, no increase in the ^{3}H-Vitamin A1 esters were seen *in vivo* or *in vitro*, but increases in ^{3}H-Vitamin A2 production remained (34% *in vivo* and 44% *in vitro*) (Figure II – 7 B).

The observed increases in A2 esters *in vivo* and *in vitro* are most likely driven by increasing substrate – A2 alcohol production. The identity of the activity responsible for increased A1 esters in sonicates from UVB (0.4 J/cm^2) irradiated cultures is unknown.

Vitamin A2 limits UV-induced apoptosis more than does Vitamin A1

To determine whether Vitamin A2 esters more effectively reduce UV damage, in the next phase of my study I compared their ability to protect cells from UVA and UVB induced apoptosis. Normal foreskin keratinocytes (NHEK-F) depleted of their internal retinoids by passing them for four times in KGM were used for these experiments; attempts to deplete retinoids from strain NHEK-O resulted in a loss of viability. Cultures grown to one day post confluence in KGM in 6 well plates were supplemented with 0.1 μM Vitamin A1 alcohol, 0.1 μM Vitamin A2 alcohol, or BSA as a control in media containing 1.5 mM $CaCl_2$ and 0.5 μM ketoconazole to limit Vitamin A2 conversion from Vitamin A1. After 24 hours at 37^{o}C, cultures were washed once with PBS and exposed to two different doses of UVA (35 J/cm^2, 40 J/cm^2) or UVB (0.5 J/cm^2 and 0.6 J/cm^2). After the exposure the PBS was replaced with the KGM containing the corresponding Vitamin A1 or A2 alcohol or BSA all in presence of 1.5 mM $CaCl_2$ and 0.5 μM ketoconazole. At 16 hours post UV exposure the cells were harvested and 5000 cells from each sample were assayed for apoptosis by detecting the enrichment of nucleosomes in the cytoplasm using Cell Death Detection ELISA^{plus} kit. Non-irradiated replicate cultures were harvested to determine cellular retinoid content.

Based on retention time (Figure II – 8, A) and spectral matching (Figure II – 8, B) of HPLC resolved retinoids, cultures supplemented with Vitamin A1 alcohol produced only Vitamin A1 esters and cultures supplemented with Vitamin A2 alcohol produced only Vitamin A2 esters. The BSA treated cultures had no detectable retinoids. The cultures incubated with Vitamin A1 alcohol accumulated about 4 – 5 times the ester than did cultures incubated with Vitamin A2 alcohol (Figure II – 8, C). Cells cultured in addition of Vitamin A1 and A2 alcohol in the medium have decreased rate of apoptosis compared to those not supplemented with retinoids.

A dose dependent increase in apoptosis was observed in retinoid free cultures exposed to UVA radiation (Figure II – 9, A, B). Vitamin A1 and A2 supplementation reduced the number of apoptotic cells in about equal extend at the low dose of UVA irradiation. At the higher dose Vitamin A2 offered significant protection ($p=0.007$). Reduction of the apoptotic cells after UVA (40J/cm^2) irradiation in Vitamin A1 and A2 supplemented cultures was 45 and 74% respectively (Figure II – 9 C).

UVB induced a more robust apoptotic response (Figure II – 10, A, B). The apoptotic response induced by 0.5 J/cm^2 was reduced in cultures supplemented with either Vitamin A1 alcohol (64%) or Vitamin A2 alcohol (66%). After irradiation with 0.6 J/cm^2, again only supplementation with Vitamin A2 alcohol significantly ($p=0.02$) reduced UVB-induced apoptosis – 65%. Vitamin A1 also reduced apoptosis after irradiation with 0.6 J/cm^2 but only 31 % (Figure II – 10 C).

SUMMARY/CONCLUSIONS

- UVA exposure of keratinocyte cultures causes the preferential loss of Vitamin A1 retinoids in a dose dependent manner. However, UVA exposure will cause the loss of both Vitamin A1 and Vitamin A2 and will do so at doses (15 J/cm^2) that do not induce apoptosis.

- UVB also causes the preferential loss of Vitamin A1 retinoids in a dose dependent manner. However, in contrast to UVA, cell viability is lost before retinoid depletion.

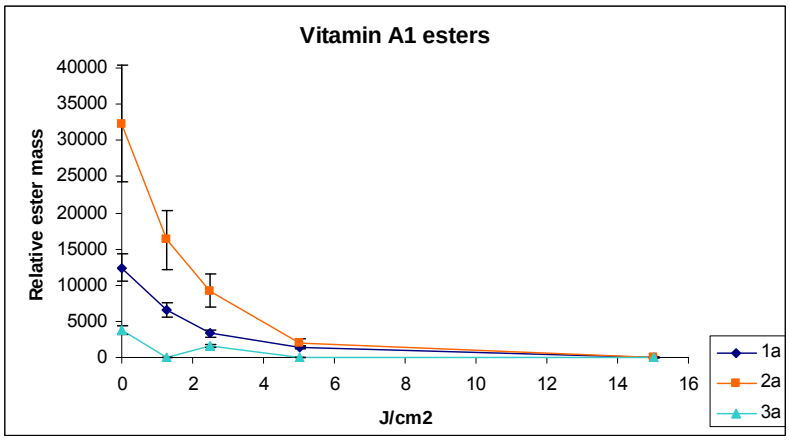
- Cultures exposed to UVA or UVB are induced to synthesize more Vitamin A2.

- Vitamin A2 retinoids limit UVA and UVB induced apoptosis in cultured epidermal keratinocytes more effectively than do Vitamin A1 retinoids.

- The stability of Vitamin A2 and its enhanced synthesis post-irradiation suggests that these retinoids serve a protective role in UV-exposed keratinocytes.

FIGURES FOR CHAPTER TWO

A



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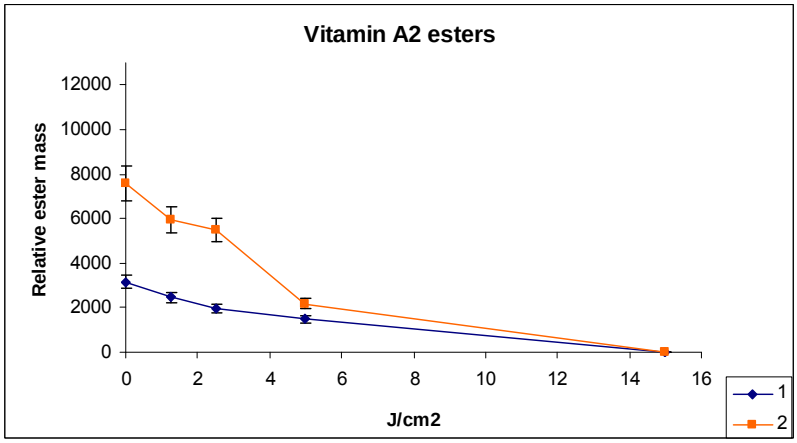
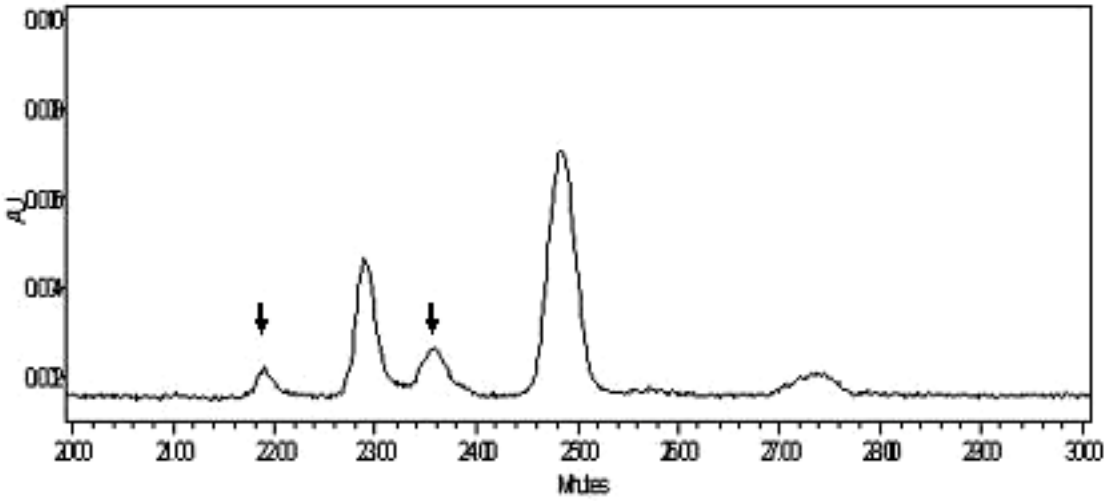


Fig II – 1a: Vitamin A1 and A2 esters of cultured NHEK-O after exposure to UVA radiation

The internal ester content of the cells irradiated with different doses UV was determined spectrophotometrically. Shown in the graphs are the dose dependent decreases of the internal Vitamin A1 (A) and A2 (B) esters. Data represent the average ± standard deviation for two experiments each with duplicate samples, (n = 4).

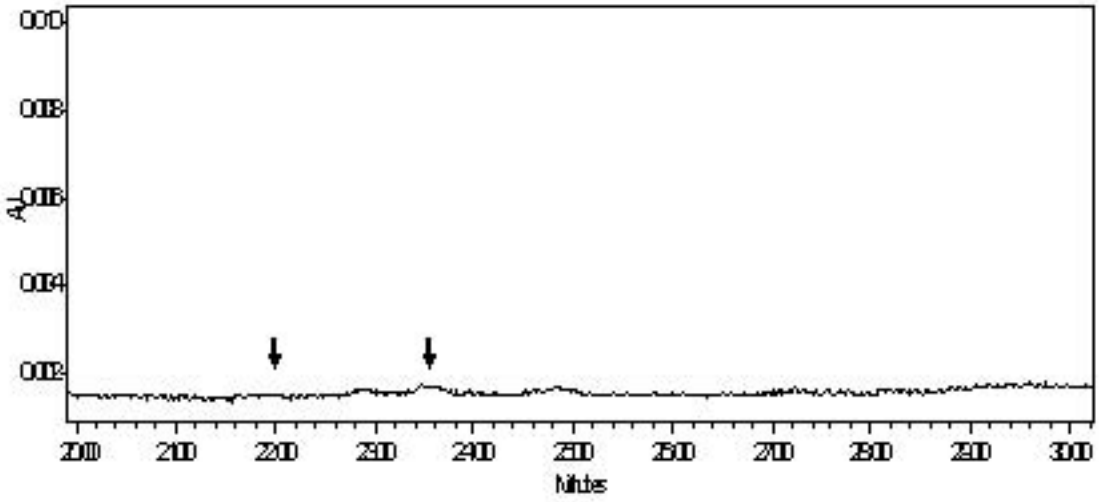
1 - Vitamin A2 ester (16:1, 14:0)
1a - Vitamin A1 ester (16:1, 14:0)
2 - Vitamin A2 ester (18:1, 16:0)
2a - Vitamin A1 ester (18:1, 16:0)
3 - Vitamin A2 ester (18:0)
3a - Vitamin A1 ester (18:0)

A



Control cells

B

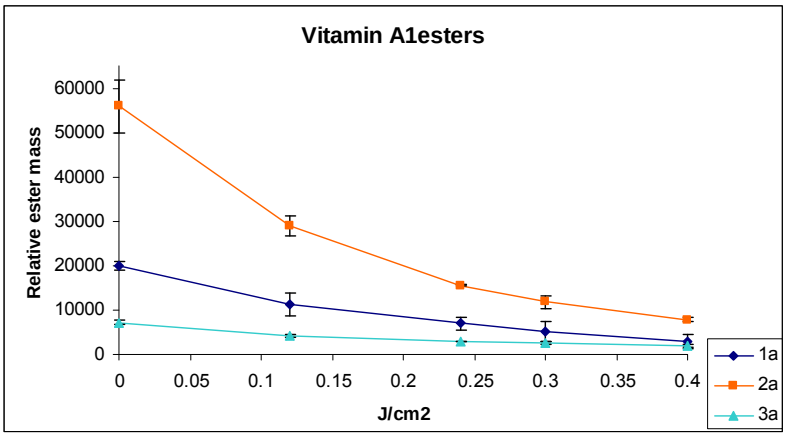


Cells after UVA irradiation (15 J/cm^2)

Fig II – 1b: Vitamin A1 and A2 esters of NHEK-O before and immediately after UVA (15 J/cm^2) exposure

Cultures were grown in 3 – 1 medium until one week post-confluence and then UV irradiated, as described in the Methods section. Immediately after irradiation the cells were harvested, and cellular retinoid content was analyzed by reverse-phase HPLC. The chromatograms show the internal Vitamin A1 and A2 esters in non-irradiated (A) and irradiated (B) cells. Noted by the black arrows are the locations of Vitamin A2 esters.

A



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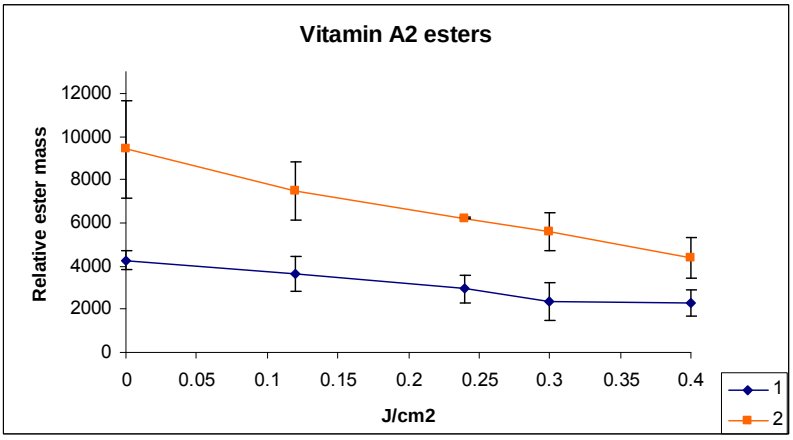
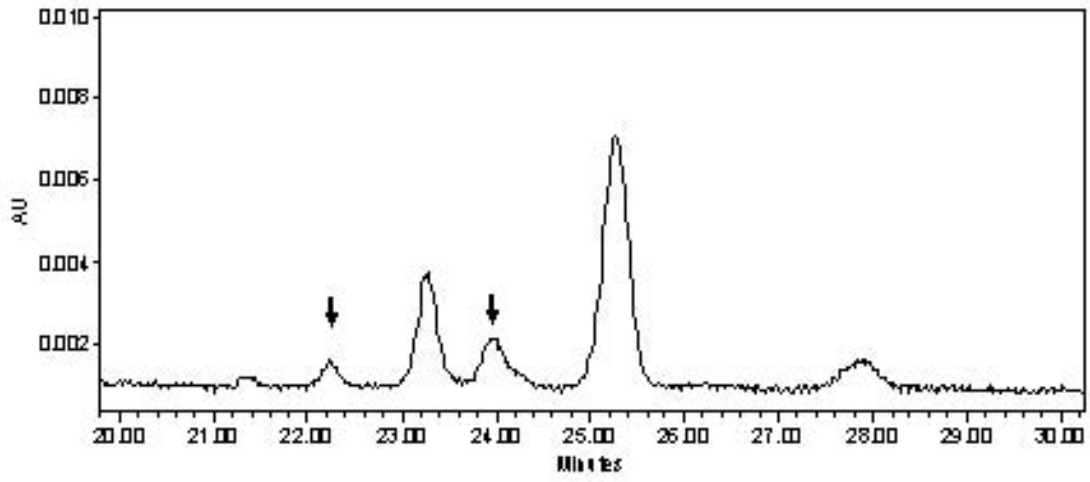


Fig II – 2a: Vitamin A1 and A2 esters of cultured NHEK-O after exposure to UVB radiation

The internal ester content of the cells irradiated with different doses UV was determined spectrophotometrically. Shown in the graphs are the dose dependent decreases of the internal Vitamin A1 (A) and A2 (B) esters. Data represent the average ± standard deviation for two experiments each with duplicate samples, (n = 4).

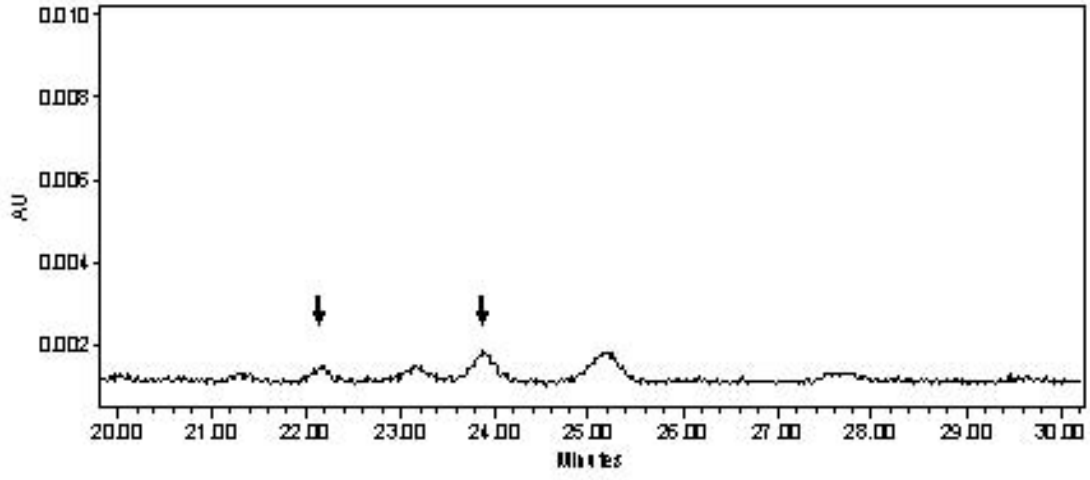
1	-	Vitamin A2 ester (16:1, 14:0)
1a	-	Vitamin A1 ester (16:1, 14:0)
2	-	Vitamin A2 ester (18:1, 16:0)
2a	-	Vitamin A1 ester (18:1, 16:0)
3	-	Vitamin A2 ester (18:0)
3a	-	Vitamin A1 ester (18:0)

A



Control cells

B



Cells after UVB irradiation (0.4 J/cm^2)

Fig II - 2b: Vitamin A1 and A2 esters of NHEK-O before and immediately after UVB (0.4 J/cm^2) exposure

Cultures were grown in 3 – 1 medium until one week post-confluence and then UV irradiated, as described in the Methods. Immediately after irradiation the cells were harvested, and cellular retinoid content was analyzed by reverse-phase HPLC. The chromatograms show the internal Vitamin A1 and A2 esters in non-irradiated (A) and irradiated (B) cells. Noted by the black arrows are the locations of Vitamin A2 esters.

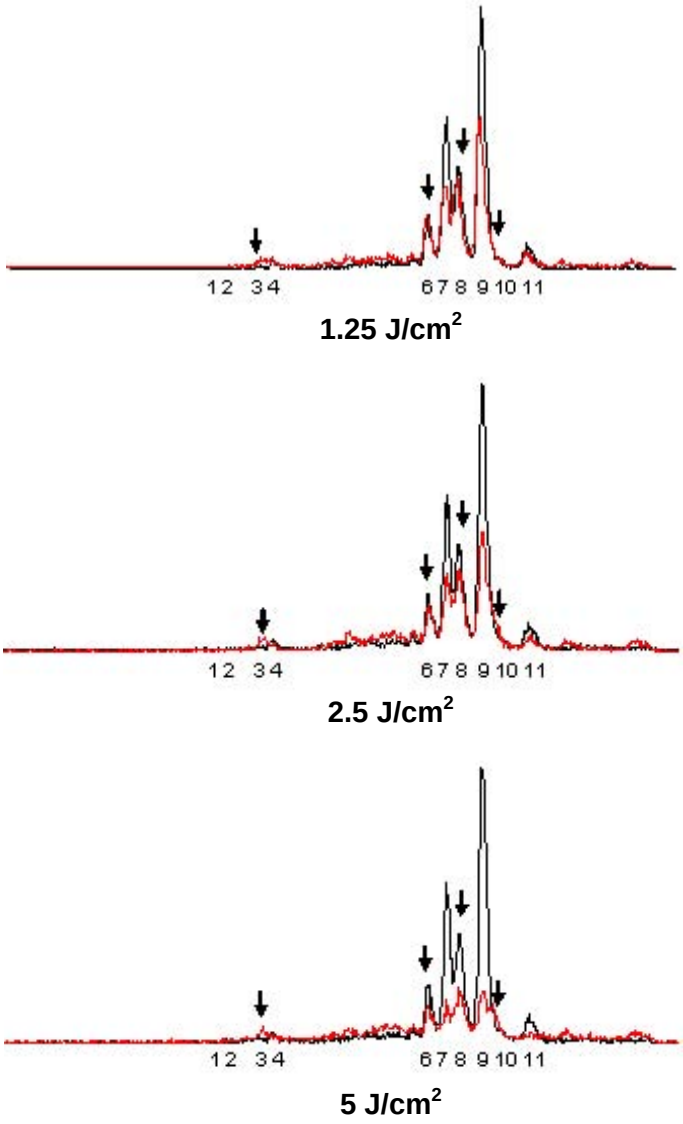


Fig II - 3a: Destruction of ^{3}H-prelabeled Vitamin A1 and A2 esters in NHEK-O by UVA radiation

The cultures were grown in 3 – 1 medium until one week post-confluence, incubated for 24h with 25nM ^{3}H-Vitamin A1 alcohol and then UV irradiated with three different doses as described in the Methods. Immediately after irradiation the cells were harvested, and cellular retinoid content was analyzed by reverse-phase HPLC. The chromatograms show the internal ^{3}H-Vitamin A1 and A2 metabolites in non-irradiated (black) and irradiated (red) cells. Noted by the black arrows are the peaks of Vitamin A2 metabolites. Numbers correspond to the retention times of authentic standards listed in Table I – 2.

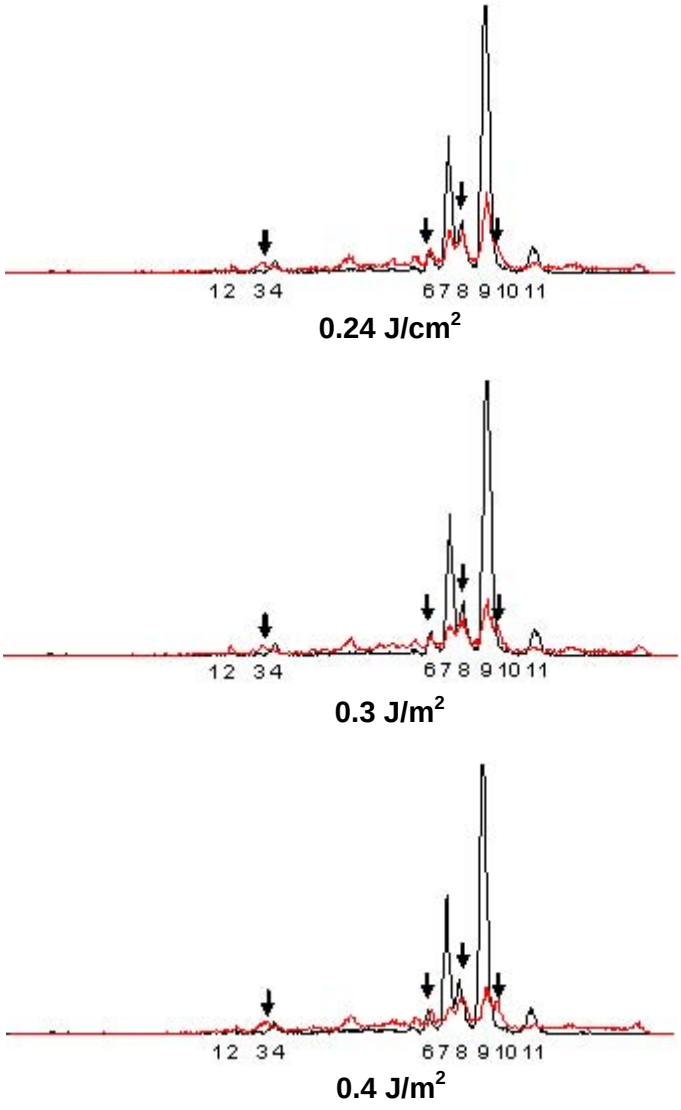
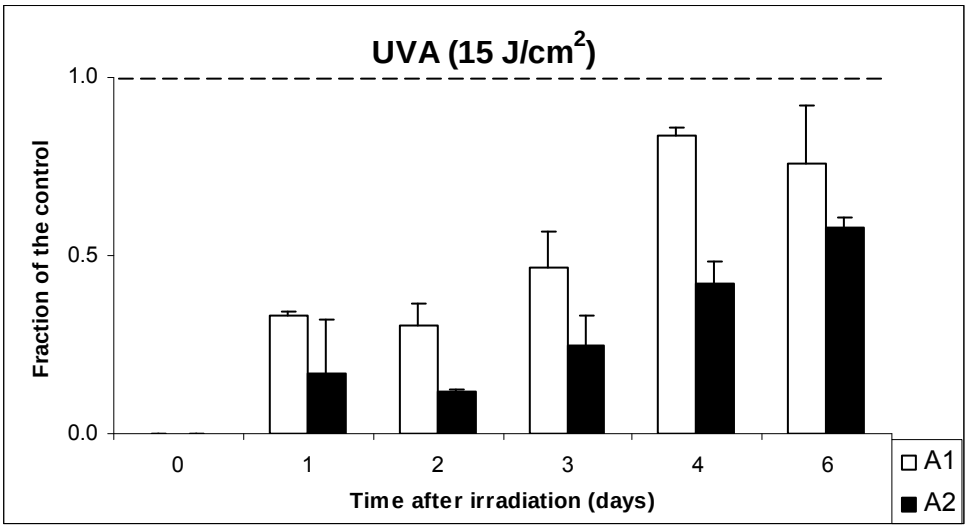


Fig 3b: Destruction of ^{3}H-prelabeled Vitamin A1 and A2 esters in NHEK-O by UVB radiation

The cultures were grown in 3 – 1 medium until one week post-confluence, incubated for 24h with 25nM ^{3}H-Vitamin A1 alcohol and then UV irradiated with three different doses as described in the Methods. Immediately after irradiation the cells were harvested, and cellular retinoid content was analyzed by reverse-phase HPLC. The chromatograms show the internal ^{3}H-Vitamin A1 and A2 metabolites in non-irradiated (black) and irradiated (red) cells. Noted by the black arrows are the peaks of Vitamin A2 metabolites. Numbers correspond to the retention times of authentic standards listed in Table I – 2.

A



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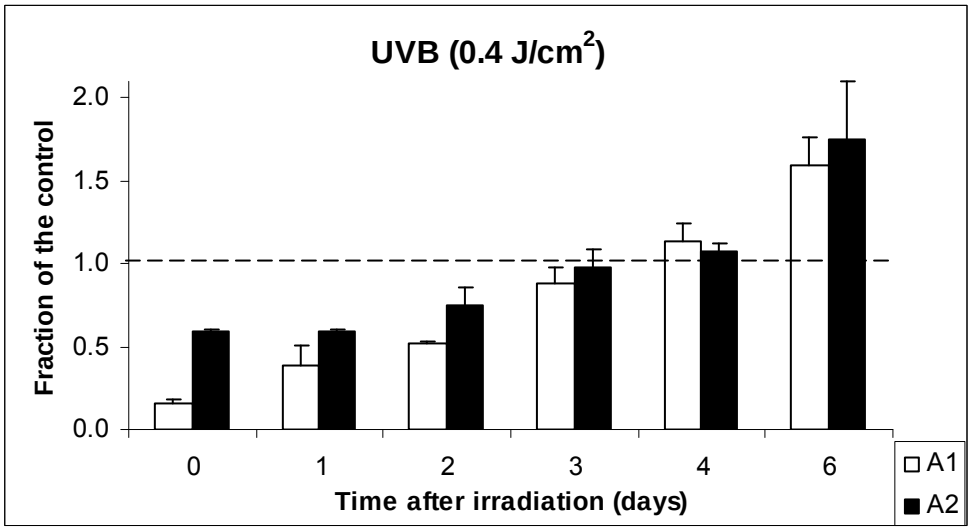
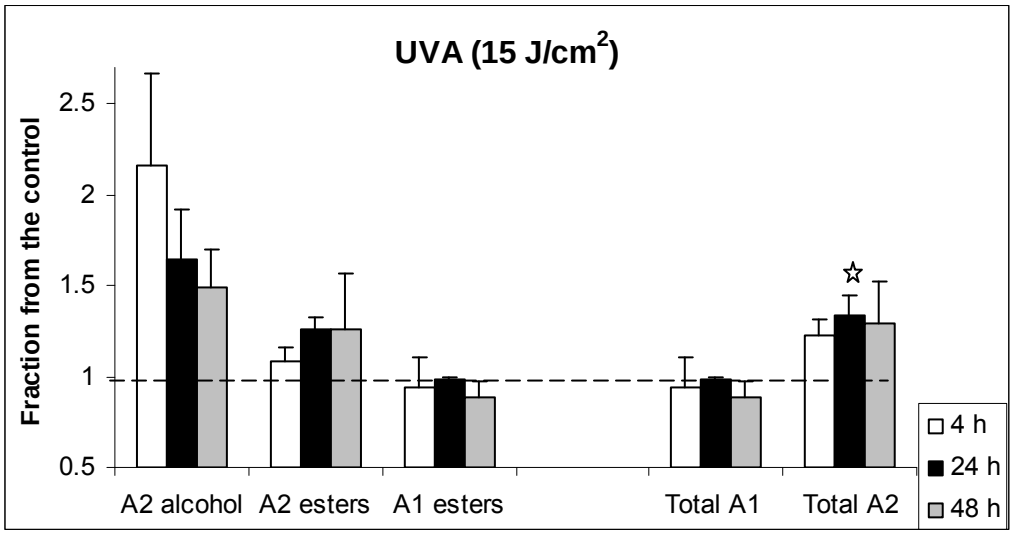


Fig II - 4: Restoration of NHEK-O Vitamin A1 to A2 esters pool pot-UV exposure
The cultures were grown in 3 – 1 medium until two days post-confluence and then UV irradiated as described in the Methods section. After irradiation, the cells were supplemented with 3 – 1 medium until the days post-exposure as indicated and cellular retinoids ester content was analyzed by reverse-phase HPLC. The cellular Vitamin A1 and A2 retinoids was determined spectrophotometrically. Time 0 equals immediately after irradiation. Shown in the figure are Vitamin A1 and A2 metabolites presented as fraction of the control (retinoid amount of irradiated cells/amount of none irradiated control cells). Data represent the average ± standard deviation for two experiments each with duplicate samples (n = 4).

A



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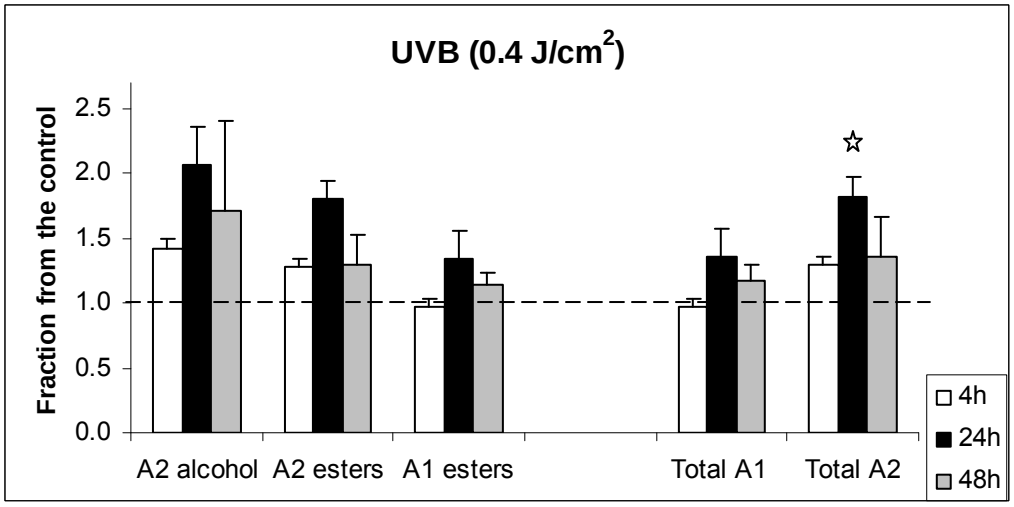
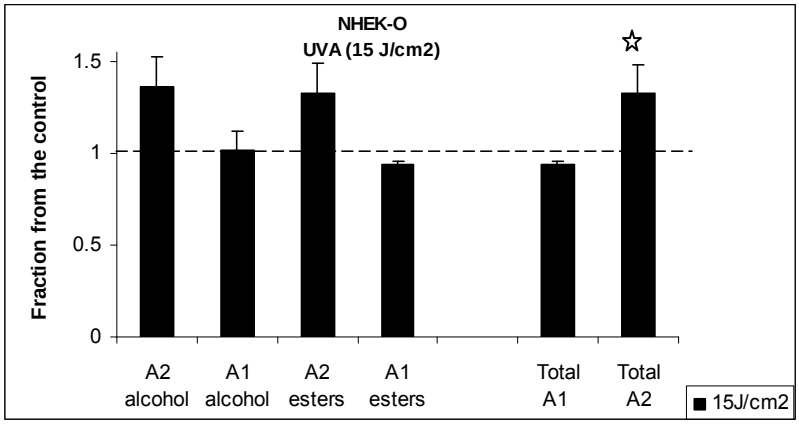


Fig II - 5: *In vitro* conversion of Vitamin A1 to A2 in cultured NHEK-O after different time post exposure to UV radiation

The cultures were grown in 3 – 1 medium until three - four days post-confluence and then UV irradiated. At different time post exposure as indicated the cells were harvested by trypsinisation, sonically disrupted and *in vitro* assayed as described in the Methods section. The retinoid metabolites were extracted and analyzed by reverse-phase HPLC. Shown is upregulated Vitamin A1 and A2 conversion. Data represent the average ± standard deviation for two experiments each with duplicate samples (n = 4). UV radiation significantly increased Vitamin A2 synthesis (☆) 24 h post-exposure. Significance is defined as $p<0.05$.

A



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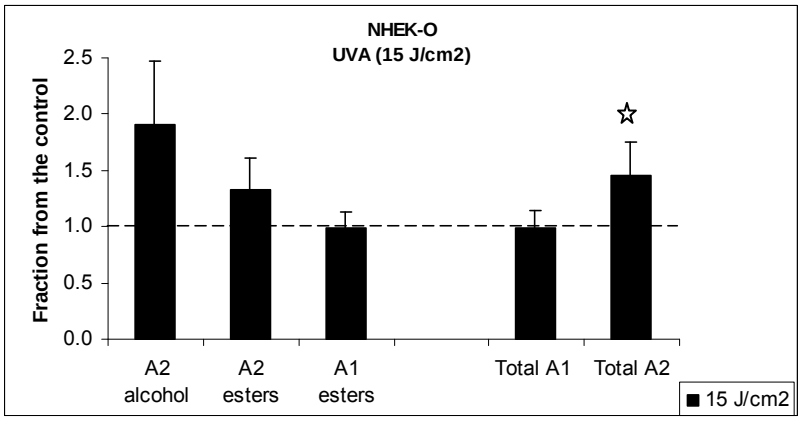
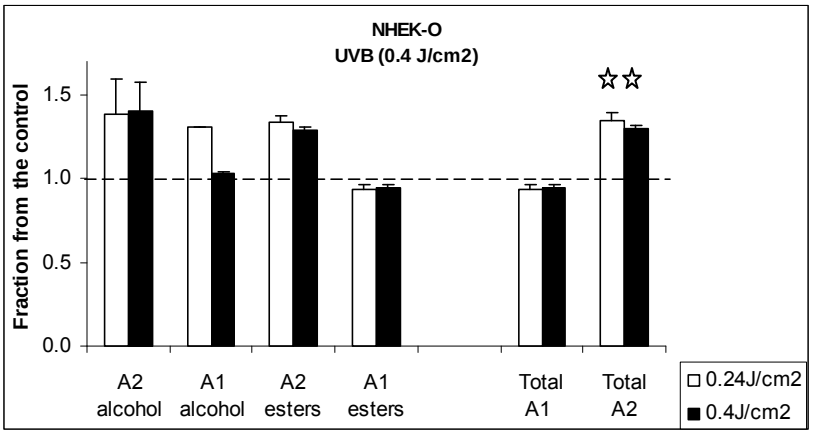


Fig II - 6: Effect of UVA radiation on Vitamin A1 to A2 conversion
The cultures were grown in 3 – 1 medium until three - four days post-confluence and then UV irradiated. Immediately after the exposure the cells were incubated for 24h with or without 25nM ^{3}H-Vitamin A1 alcohol. The labeled cells were harvested, and cellular retinoid content was analyzed by reverse-phase HPLC (A). Data represent the average ± standard deviation for five experiments each with duplicate samples (n = 10). Non-labeled cells were harvested by trypsinisation, sonically disrupted and *in vitro* assayed. Retinoid metabolites were extracted and analyzed by reverse-phase HPLC. Data represent the average ± standard deviation for ten experiments each with duplicate samples (n = 20). Shown is Vitamin A2 accumulation *in vivo* (A) and upregulated synthesis *in vitro* (B). UVA radiation significantly increased Vitamin A2 synthesis (☆) *in vivo* and *in vitro*. Significance is defined as $p<0.05$.

A



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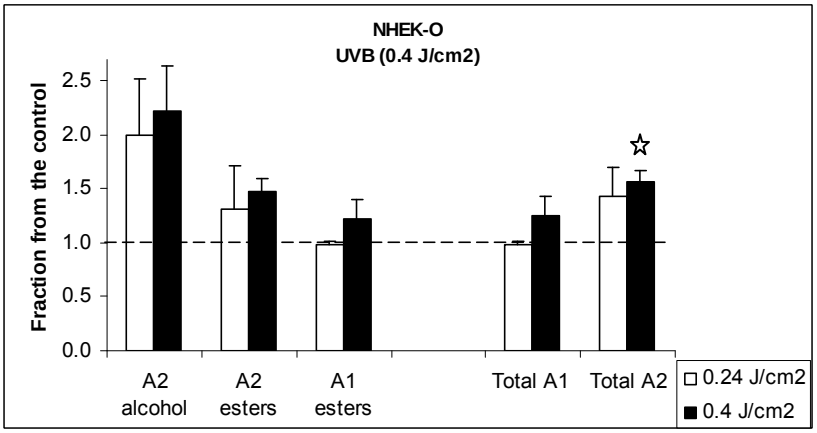
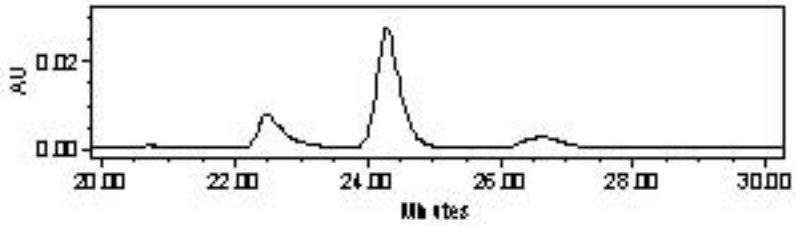


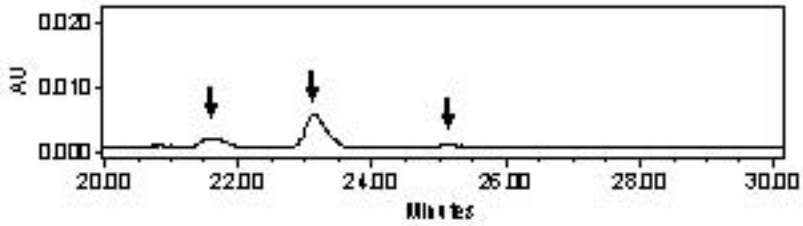
Fig II - 7: Effect of UVB radiation on Vitamin A1 to A2 conversion
The cultures were grown in 3 – 1 medium until three - four days post-confluence and then UV irradiated. Immediately after the exposure the cells were incubated for 24h with or without 25 nM ^{3}H-Vitamin A1 alcohol. The labeled cells were harvested, and cellular retinoid content was analyzed by reverse-phase HPLC (A). Data represent the average ± standard deviation for two experiments each with duplicate samples (n = 4). Non-labeled cells were harvested by trypsinisation, sonically disrupted and *in vitro* assayed. Retinoid metabolites were extracted and analyzed by reverse-phase HPLC. Data represent the average ± standard deviation for two – three experiments each with duplicate samples (n = 4 – 6). Shown is Vitamin A2 accumulation *in vivo* (A) and upregulated synthesis *in vitro* (B). UVB radiation significantly increased Vitamin A2 synthesis (☆) *in vivo* and *in vitro*. Significance is defined as $p<0.05$.

A

Vitamin A1 esters

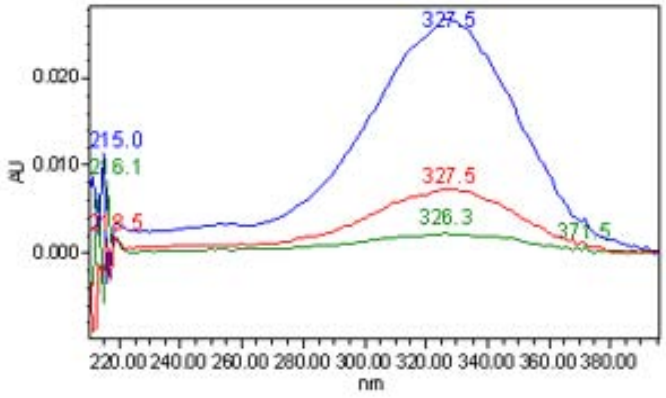


Vitamin A2 esters

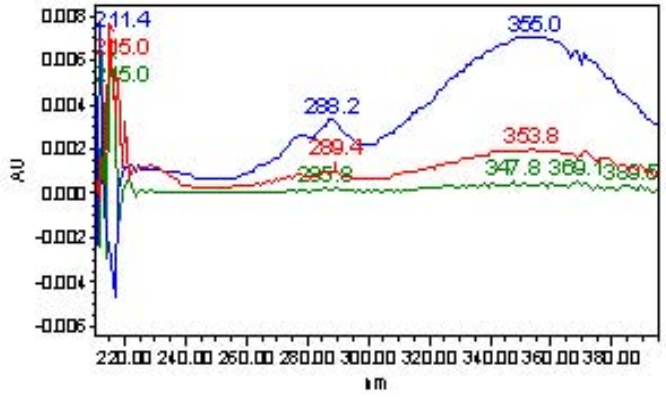


B

Spectra of Vitamin A1 esters



Spectra of Vitamin A2 esters



C

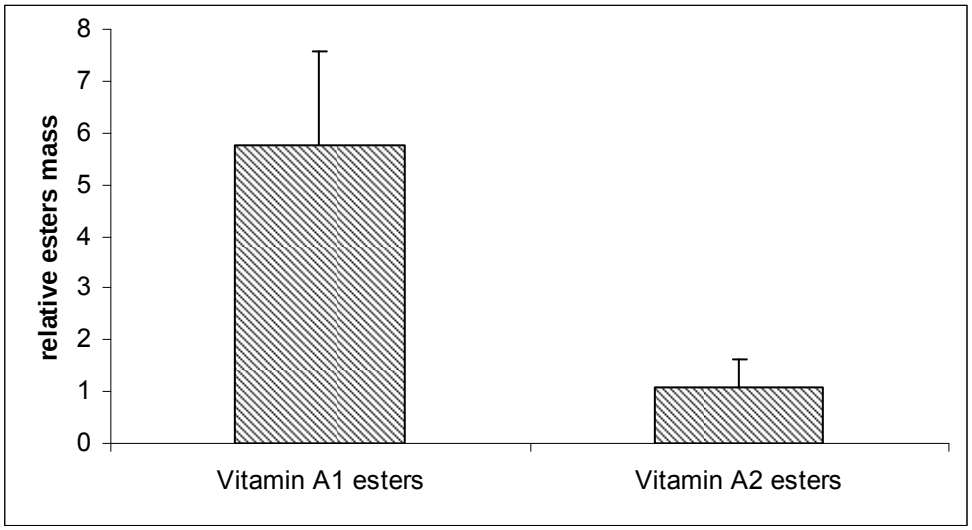
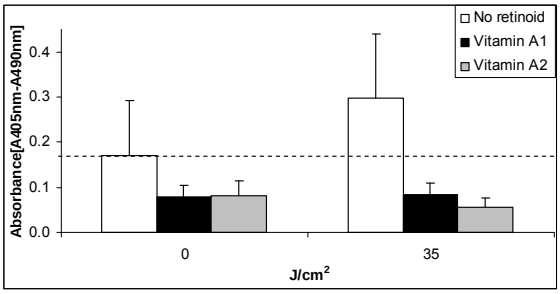


Fig II - 8: Accumulation of Vitamin A1 and A2 esters for 24h in cultured NHEK-F
Cells depleted from the internal retinoid content were grown in KGM medium until three – four days post-confluence and supplemented with Vitamin A1 (0.1 μM) or A2 (0.1 μM) alcohol or BSA (control). The medium contained also 1.5 mM Ca^{++} and 0.5 μM ketoconazole. 24h post-treatment with Vitamin A1 and A2 alcohol the cells were harvested and cellular retinoids content was analyzed by reverse-phase HPLC. Cells that were supplemented with Vitamin A1 (0.1 μM) alcohol accumulate only A1 esters (A – upper panel) and cells that were supplemented with Vitamin A2 (0.1 μM) alcohol accumulate only A2 esters (A – lower panel). Noted by the black arrows are the correct retention times of Vitamin A2 esters. This was confirmed by obtaining the correct absorption spectrum for Vitamin A1 esters (B – upper panel) and A2 esters (B – lower panel). Shown in the bar graph is the cellular content of Vitamin A1 and A2 esters accumulated for 24h by the cells (C).

A



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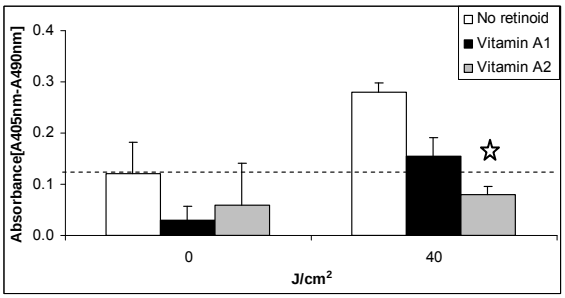


C

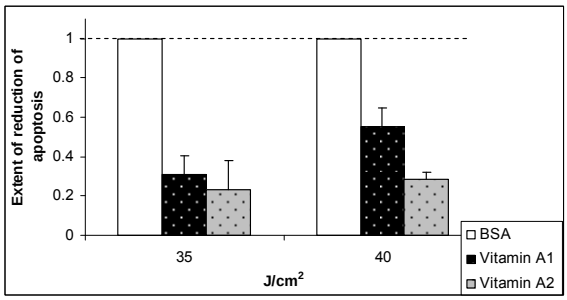
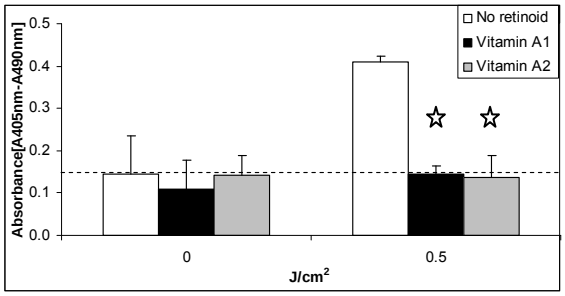
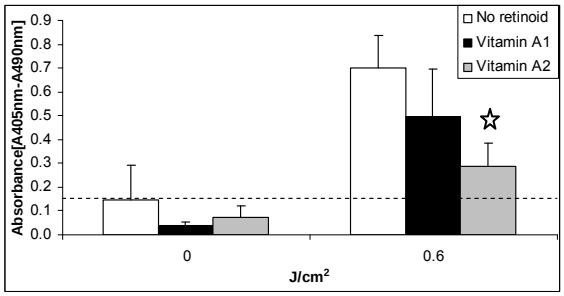


Fig II - 9: Detection of nucleosomes in cytoplasmic fractions of cultured NHEK-F after UVA irradiation Cells with depleted internal retinoid content were grown in KGM medium until post-confluence and supplemented for 24h with Vitamin A1 (0.1 μM), A2 (0.1 μM) alcohol or BSA (control). The medium also contained 1.5 mM Ca^{++} and 0.5 μM ketoconazole. After the incubation period the cells were irradiated with a single dose of UVA radiation – 30 J/cm^2 (A) or 40 J/cm^2 (B). After irradiation the cells were allowed to recover in the corresponding medium for 16 hours and then harvested and subjected to cell death detection ELISA plus assay. The level of apoptotic cells measured by the absorbance for the two UVA treatments is shown in Panels A and B. The dashed line is set at the level of the no retinoid, 0 J/cm^2 control. The protective function of the retinoids is shown in Panel C (Extent of reduction of apoptosis = absorbance of the UV irradiated sample (supplemented with retinoids)/absorbance of the UV irradiated control (no retinoids)). At 40 J/cm^2 only Vitamin A2 significantly reduced apoptosis, compared to the no retinoid control (☆). Significance is defined as $p<0.05$.

A



B



C

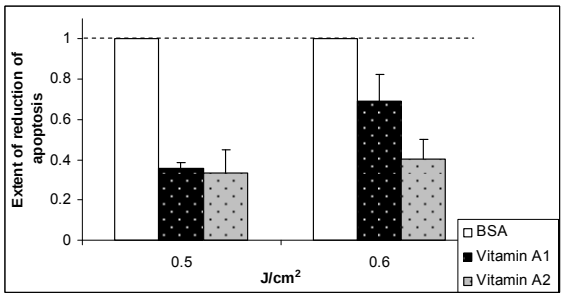


Fig II - 10: Detection of nucleosomes in cytoplasmic fractions of cultured NHEK-F after UVB irradiation Cells with depleted internal retinoid content were grown in KGM medium until post-confluence and supplemented for 24h with Vitamin A1 (0.1 μM), A2 (0.1 μM) alcohol or BSA (control). The medium also contained 1.5 mM Ca^{++} and 0.5 μM ketoconazole. After the incubation period the cells were irradiated with a single dose of UVB radiation – 0.5 J/cm^2(A) or 0.6 J/cm^2(B). After irradiation the cells were allowed to recover in the corresponding medium for 16 hours and then harvested and subjected to cell death detection ELISA plus assay. The level of apoptotic cells measured by the absorbance for the two UVB treatments is shown in Panels A and B. The dashed line is set at the level of the no retinoid, 0 J/cm^2 control. The protective function of the retinoids is shown in Panel C (Extent of reduction of apoptosis = absorbance of the UV irradiated sample (supplemented with retinoids)/absorbance of the UV irradiated control (no retinoids)). At 0.5 J/cm^2 both Vitamin A1 and A2 significantly reduced apoptosis compared to the no retinoid control (☆). However at 0.6 J/cm^2 only Vitamin A2 significantly reduced apoptosis (☆). Significance is defined as $p<0.05$.

CHAPTER 3

ANALYSIS OF THE CAPABILITIES OF VITAMIN A1 AND A2 ACIDS TO ALTER EXPRESSION OF GENES PARTICIPATING IN p53 MEDIATED UV RESPONSE

The data presented in Chapter 2 suggested that Vitamin A2 esters could serve a protective function against UV damage. It was therefore of interest to determine whether Vitamin A2 acid also alters the response to UV. Vitamin A1 acid ameliorates UVB damage through altering gene transcription (27, 24). Presumably the effect of Vitamin A2 acid would also involve its role as a transcription factor ligand (2, 119). To screen for this effect and to determine whether Vitamin A1 and A2 acids elicit different transcriptional responses to UV, gene expression profiling was carried out on 84 genes related to the p53 signaling pathway. P53 responsive genes were chosen due to the documented role that p53 plays in the cellular response to UV damage (24). Vitamin A2 acid and ^{3}H-Vitamin A2 acid used for these experiments was synthesized by Prof. Kathleen Parker and Erik Stolarzewicz, Department of Chemistry, SUNY at Stony Brook.

Purification of newly synthesized Vitamin A2

The newly synthesized Vitamin A2 acid was first extracted (15), dried under argon and purified by reverse phase HPLC on a semi-preparative NovaPakC18 column. A 15 minutes linear gradient from 10 nM ammonium acetate in methanol:water (68:32) to methanol:dichloromethane (4:1) followed by a 15 minutes hold at a flow rate of 4 ml/minute was used for the separation. As shown in Figure III –1 this chromatography yielded five peaks with absorbance at 326 nm. Peaks 1, 2, and 3 had spectra matching Vitamin A2 alcohol. Peak 3, which contained the most mass, was collected, dried under argon and further purified by reverse phase HPLC using a SunFire C18 column and the same mobile gradient system, but with a flow rate of 1 ml/minute. Two peaks were eluted both of which contained material with spectra matching Vitamin A2 alcohol (Figure III –2). The major peak of pure Vitamin A2 acid was collected dried under argon and an aliquot was subjected to reverse phase HPLC using an analytical NovaPakC18 column with absorbance detection at 326 nm. The only peak detected had the retention time (11.15 minutes) and spectra (Figure III – 3 A) of Vitamin A2 acid. The absorption

spectrum was confirmed using a separate spectrophotometer (Fig. III – 3 B). The retention time was 1 minute less than the retention time of Vitamin A1 acid (12.23 minutes, data not shown), which corresponds to previous observations.

The identity of newly synthesized Vitamin A2 acid was confirmed by mass spectrometry and the isomer as all-*trans* was confirmed by Nuclear Magnetic Resonance (data not shown). These analyses were performed by Prof. Kathleen Parker and Erik Stolarzewicz in the Department of Chemistry, SUNY at Stony Brook.

Newly synthesized and purified Vitamin A2 acid is functionally active

To ensure that the purified Vitamin A2 acid is free of any contaminants that might inhibit its function as a transcription factor ligand, HaCaT cells were transfected with vectors containing retinioic acid response elements (RARE) driving CAT expression. The RARE used were those containing direct repeats with half sites separated by one (DR1), two (DR2) or five (DR5) nucleotides, each of which is found to regulate different target genes (64). The sequence containing the DR1 and DR2 RAREs were introduced into pBLCAT2 (Fig. III – 4) by cloning between the HindIII and XbaI restriction sites. Dr. N. Markova from our laboratory kindly provided the construct harboring DR5 RARE also in pBLCAT2 and cloned into the HindIII and XbaI restriction sites. Insert orientation was verified by DNA sequencing carried out by the DNA sequencing facility at Stony Brook University.

Each plasmid DNA was introduced into HaCaT cells by transfection; these cells transfect at high efficiency even when depleted of internal retinoid stores. After glycerol shock, PBS was replaced with KGM supplemented with 10 or 100 nM Vitamin A1 acid, or with 10 or 100 nM Vitamin A2 acid. Cultures grown with 1 mg/ml BSA were used as control. After 40 hours incubation at 37^{o}C, cell extracts were prepared and assayed for CAT activity. Extracts from nontransfected cells were used to measure background which should be equal to the activity observed with extracts prepared from transfectants grown with 1 mg/ml BSA. This was observed in cells transfected with the DR1 and DR5 RARE constructs, but not with the DR2 RARE constructs. The DR2 RARE showed a 68% loss of CAT activity compared to background confounding interpretation of data from this arm of the experiment. Vitamin A1 acid positively regulated activity only in cells transfected with the DR1 RARE construct with significance reached using 100 nM concentrations (Figure III – 5). Vitamin A2 acid also positively regulated the CAT activity driven by the DR1 RARE and also required 100 nM concentrations to reach significance. In contrast to Vitamin A1 acid, the A2 acid also drove CAT activity off of the DR5 RARE constructs when supplied at 100 nM.

As Vitamin A2 acid drove transcription off of exogenous promoters, the presence of transcription inhibiting contaminants is unlikely and the newly synthesized retinoids were then used for the series of experiments leading to and including gene expression profiling. The ability of Vitamin A2 acid but not Vitamin A1 acid to drive transcription off of the DR5 RARE supports the notion that dependent upon promoter context and transcription factor expression Vitamin A1 and A2 acids may differentially regulate the transcriptome. Contributing to the observed difference

may be the expression of RARβ which in HaCaT cells are expressed in addition to RARα, RARγ and RXRα normally found in NHEK (120).

Retinoid depleted NHEK take up and accumulate different levels of Vitamin A1 and A2 acids

Keratinocytes grown in 3 – 1 medium have previously been shown to take up and concentrate Vitamin A1 acid 50-100 X over media concentrations and then to rapidly metabolize it (89). Because it is unlikely that the media concentrations of the retinoid acids will be identical to the cellular concentrations, prior to gene expression profiling, the uptake and metabolism of ^{3}H-Vitamin A1 acid and ^{3}H-Vitamin A2 acid was compared.

For these experiments, ^{3}H-Vitamin A2 acid was synthesized Prof. Kathleen Parker and Erik Stolarzewicz, Department of Chemistry, SUNY at Stony Brook and then purified using the protocol developed for nonlabeled Vitamin A2 acid. ^{3}H-Vitamin A1 acid was purchased from PerkinElmer Life and Analytical Sciences. The purity of each of the retinoid acids was verified by retention time on reverse phase HPLC with in-line scintillation (Fig III – 6). Cultures were depleted of retinoids by passage in KGM and loss of residual retinoid stores was verified by HPLC analysis of extracted lipids (data not shown). Because specific activity of newly synthesized ^{3}H-Vitamin A2 acid was low (30 dpm/pmol), ^{3}H-Vitamin A1 acid was diluted with nonlabeled Vitamin A1 acid to achieve same specific activity for both acids and μM concentrations of each radiolabeled acid was used to monitor cellular concentrations.

Replicate plates of retinoid depleted normal human keratinocytes (strain NHEK-F) were cultured in KGM to one day post-confluence, and then switched to KGM supplemented with 1.5 mM $CaCl_2$ for 24 hours. As expected, the addition of 1.5 mM $CaCl_2$, resulted in an increased cell:cell contact which microscopically mimics that observed in cultures grown in 3 – 1 medium (Figure III – 7) and promotes differentiation as monitored by involucrin and the formation of suprabasal keratinocytes (Figure III – 8). Cultures were then pulsed with ^{3}H Vitamin A1 (1μM or 0.25μM) or ^{3}H Vitamin A2 (1μM) acids in high calcium KGM medium; non-labeled cultures were used as control. At 2, 6, and 24 hours media samples were taken and cells were harvested for retinoid extraction and analysis by HPLC with in-line scintillation counting. Even at 1 μM of the retinoid acids, cultures appeared normal microscopically with the predicted formation of suprabasal layers and increased cell:cell contact (Figure III – 9).

Typical chromatograms of the extracted retinoids are shown in Figure III – 10 and the calculated intracellular concentration of the acids is shown in Figure III – 11. As previously observed in keratinocytes cultured in 3 – 1 medium, KGM cultured keratinocytes rapidly concentrated medium retinoid acids and then lost these acids presumably due to oxidative metabolism. Incubation with 1 μM and 0.25 μM ^{3}H-Vitamin A1 acid resulted in the accumulation of 100 μM and 27 μM ^{3}H-Vitamin A1 acid intracellularly at the 2 hours time point. A reduction of 30% in the intracellular concentration of the A1 acid was observed at 6 hours and further reductions were noted at 24 hours. Similar results were obtained in incubations with

1 μM ^{3}H-Vitamin A2 acid. However, the concentration effect was 25-fold not 100-fold. At 2 hours the intracellular concentration of ^{3} H-Vitamin A2 acids was about 25 μM. This acid was also reduced about 40% during the incubation period. Because the cultured keratinocytes concentrate these retinoids and do so to different extents, in order to achieve physiologic levels intracellularly (~10 nM), Vitamin A1 and A2 acid should be added at 0.25 nM and 1.0 nM, respectively. Because Vitamin A1 retinoids are typically 3 – 4 folds more abundant, comparisons using 1.0 nM Vitamin A1 acid are also warranted.

Gene expression profiling of Vitamin A1 and A2 acid treated keratinocytes

These sets of experiments were based on the use of the RT Profiler PCR Array System and examined the gene expression profile of 84 genes that are transcriptionally regulated by the p53 protein. This system was chosen because it combines the advantage of the real time PCR sensitivity with the ability of microarrays to detect the expression of many genes simultaneously.

Retinoid depleted normal human keratinocytes (strain NHEK-F) were again cultured in KGM to one day post-confluence, and switched to KGM supplemented with 1.5 mM $CaCl_2$ for 24 hours. Replicate cultures were then treated for 4 hours with 0.25 nM and 1.0 nM Vitamin A1 acid and 1.0 nM Vitamin A2 acid to allow expression of early retinoid target genes. Cells were then harvested and total RNA was extracted. RNA quality was confirmed by agarose gel electrophoresis with ethidium bromide staining, which showed sharp rRNA band and by an absorption ratio of 260/280 which was 2.0 (Figure III – 12). Next the RNA preps were reverse transcribed to cDNA and used for PCR - Based Gene Expression Profiling. The analysis of the data was performed using the free software provided by the manufacturer. This software uses the delta-delta CT method, which is based on the assumption that the efficiency of the primers for the different genes is identical. That is why this method is approximate. Only genes demonstrating about two fold differences were further evaluated.

Shown in Figure III – 13a-c are scatter plots of the relative change in genes expressed in retinoid-treated versus non-treated control cultures. The graphs were generated using the delta-delta CT method software and are the average of two independent experiments. Treatment with 1 nM Vitamin A1 acid resulted in a relative increase in the mRNA levels of FASLG, E2F1, E2F3 and CDK4 and a decrease in the expression of IFNb. Treatment with 0.25 nM Vitamin A1 acid resulted in an increase in the mRNA levels of E2F1 and TP73 and a decrease in IFNb, and treatment with 1 nM Vitamin A2 acid resulted in an increase in FASLG and E2F1 relative to controls.

Dependent upon the expression levels of each gene, this screening can generate false positives and false negatives. Therefore, the expression levels of these genes and several others (Tables III – 1) that showed 1.5 or 0.5 fold differences in the mRNA expression were measured using real time RT-PCR. Eleven of the twelve genes evaluated showed less than two fold differences compared to control (Fig III – 14 a). The expression of BAI1, BCL2A1 and E2F3 were enhanced ~1.5 fold in the 1 nM Vitamin A1 acid treatment group, the

expression of ESR1 was enhanced 25 - 50% in each treatment group and the expression of IL6 was reduced ~ 40% in each treatment group.

The expression of one gene, FASLG, was increased ~ 9 fold in cultures incubated with 0.25 nM or 1.0 nM Vitamin A1 acid. Fas ligand and the Fas receptor (FasR) are transmembrane proteins. When Fas ligand binds to the FasR of the target cell, the receptor trimerizes, leading to binding of FADD and procaspase 8 which then autocatalyzes leading to a series of proteolytic events and cell death. This pathway is used by cytotoxic T lymphocytes in cell mediated immunity and alternatively is used to generate immune privilege in cornea.

FAS/FASLG driven apoptosis is active in mouse and human skin. The Vitamin A1 acid mediated increase in FASLG is consistent with earlier reports using immortalized and newborn mouse keratinocytes (134) and mouse dermal fibroblasts (99). The negative regulation of FASLG by Vitamin A1 acid in thymocytes was only found at concentrations $\geq$ 100 nM (53, 58, 126) and were postulated to be the result of suppression of AP-1 and NFAT transcriptional activation. In normal human keratinocytes grown in 3 – 1 medium, both Fas and FASLG were shown to be up regulated within 4 hours of UV irradiation (59), suggesting their physiological relevance and their participation eliminating “sunburn cells”. In this regard it is noteworthy that PUVA treatment (a combination of 8-methoxypsoralen and UVA radiation) has been used to eliminate highly proliferative cells in the epidermis and that data from FASLG-null mice demonstrate that PUVA induced apoptosis requires Fas/FasL (101).

In contrast to the results obtained with Vitamin A1 acid, treatment with Vitamin A2 acid did not significantly enhance FASLG expression. Thus, while both acids positively regulated some of the tested genes, their function is non-identical. This is not entirely surprising. It is well known that the transcriptional activity of retinoids in general is dependent upon their chemistry and conformation. This dictates binding affinity to and conformational changes in the nuclear receptors with which the retinoids interact and which can lead to differences in the proteins recruited to promoters. Irrespective of the mechanism, the data supports the notion that Vitamin A2 acid has a unique function.

SUMMARY/CONCLUSIONS

- In cultured NHEK, the uptake and accumulation of Vitamin A2 acid is 4 fold less than Vitamin A1 acid.

- Vitamin A2 acid serves as a transcription factor ligand which uniquely modulates gene transcription; some but not all genes are positively or negatively regulated similarly to Vitamin A1 acid

- The Vitamin A2 and A1 families appear to cooperate in regulating apoptosis. The Vitamin A2 family of retinoids appears to protect keratinocytes against apoptosis, while the Vitamin A1 family of retinoid appears to promote apoptosis.

    - Cells treated with Vitamin A1 acid increase FASLG, while cells treated with Vitamin A2 do not.
    - Cells treated with Vitamin A2 alcohol are more resistant to UV-induced apoptosis.

FIGURES FOR CHAPTER THREE

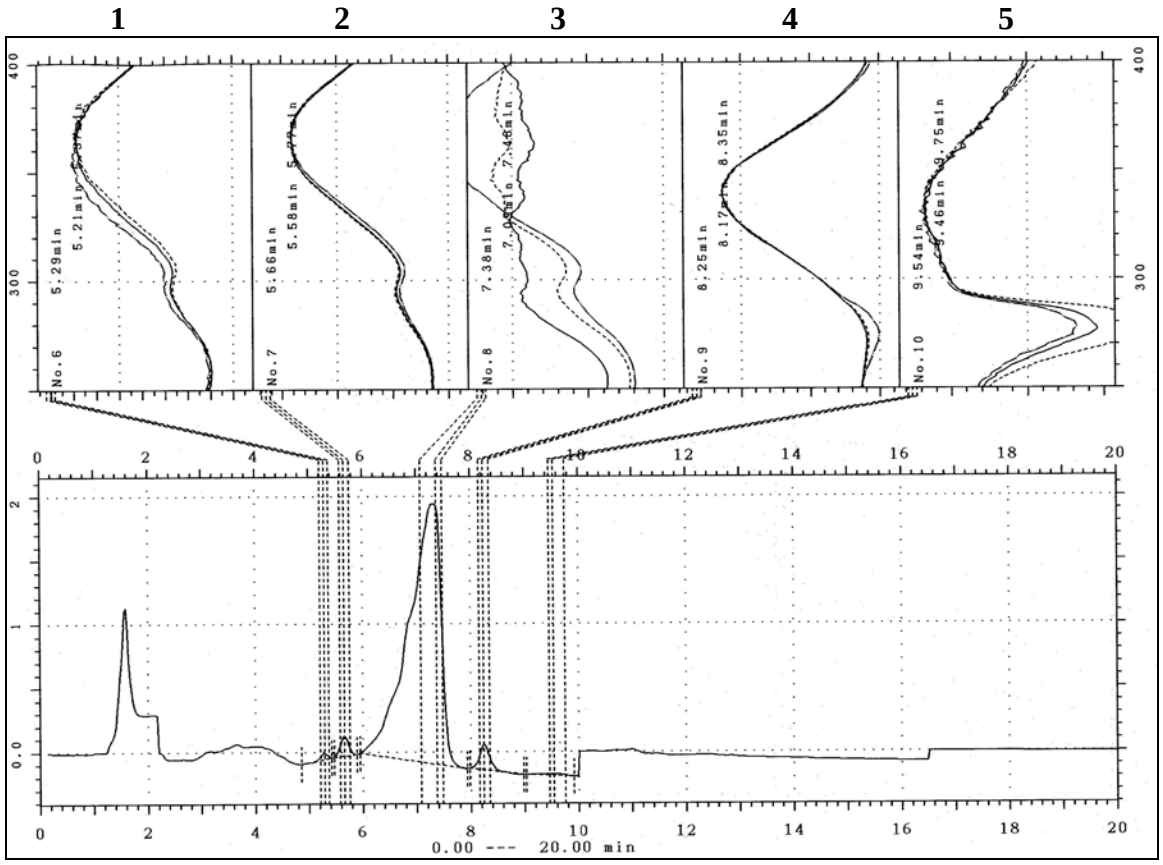


Fig III - 1: Reverse- phase gradient HPLC resolution of the newly synthesized Vitamin A2 acid

The crude preparation of newly synthesized Vitamin A2 acid was extracted by using Bligh and Dyer extraction procedure dried and resolve on reverse phase semi-preparative NovaPackC18 column. Peaks 1, 2, 3 have retinoid spectrally matching Vitamin A2 acid.

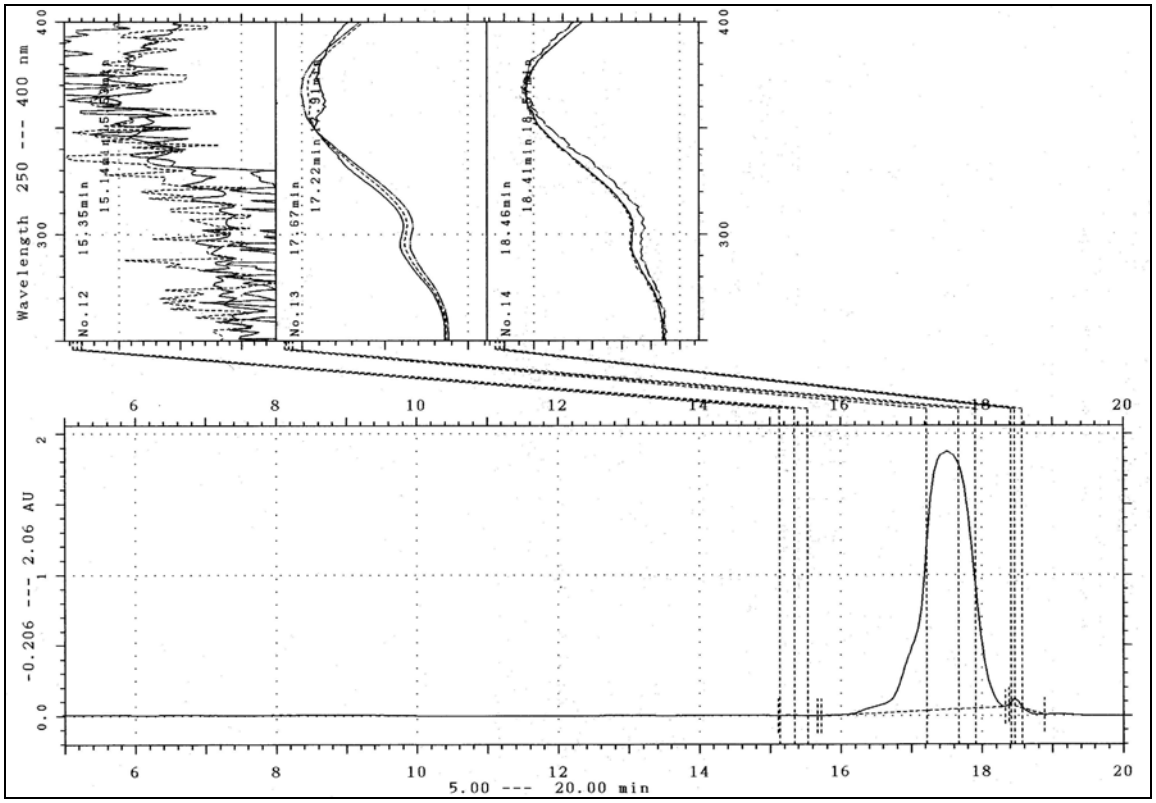
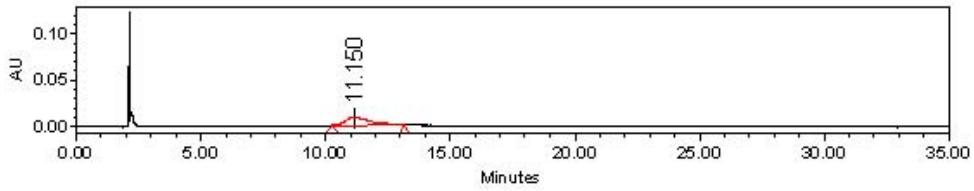
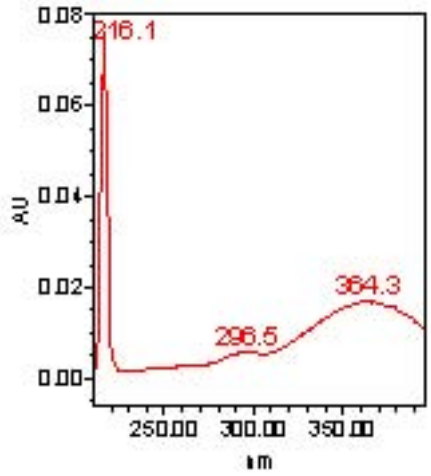


Fig III - 2: Reverse-phase gradient HPLC resolution of partially purified Vitamin A2 acid peak 3 from the previous separation on NovaPackC18 column

Peak 3 (Figure III – 1) was collected and further purified by reverse phase HPLC using SunFireC18 column. The major peak with retention time between 17.22 – 17.91 minutes corresponds to Vitamin A2 acid.

A





B

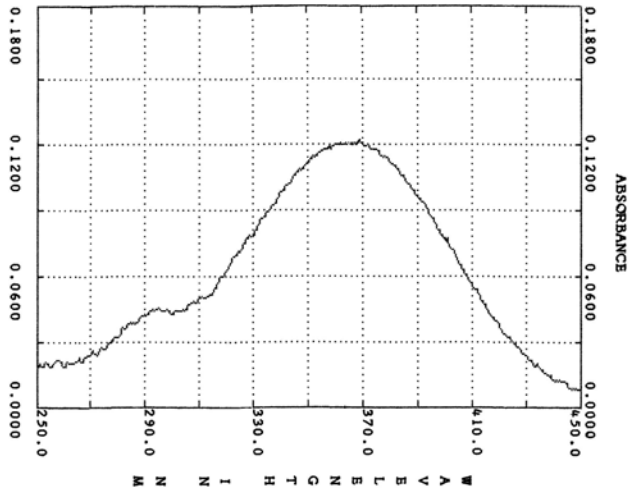


Fig III - 3: The purified Vitamin A2 acid was next resolved by HPLC using Nova-Pak HR C18 reverse phase column and identified as pure Vitamin A2 acid by retention time and spectral matching (A, B)

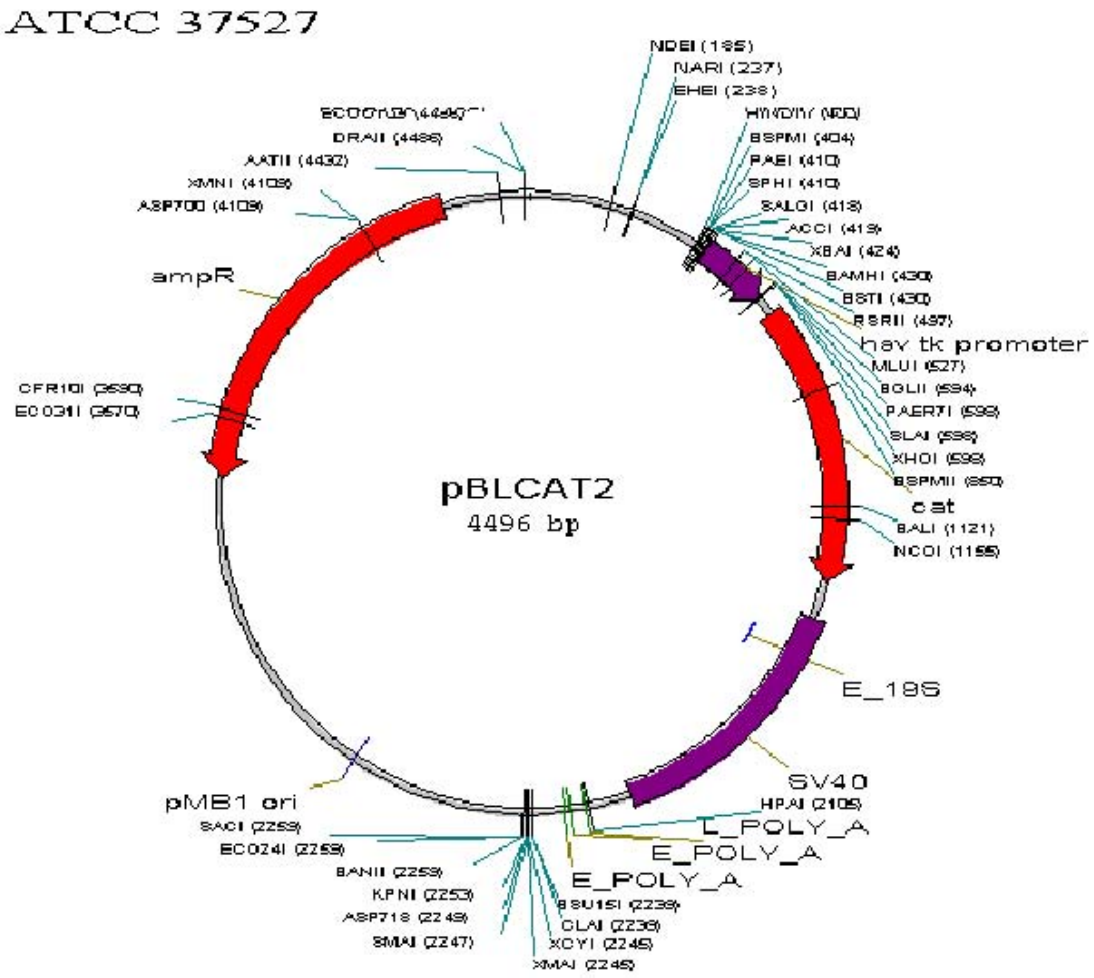


Fig III - 4: Plasmid pBLCAT2
This plasmid was used to clone RARE (DR1, or DR2) sequences between Hind III and Xba I restriction enzyme sites in front of a Δ tk promoter.

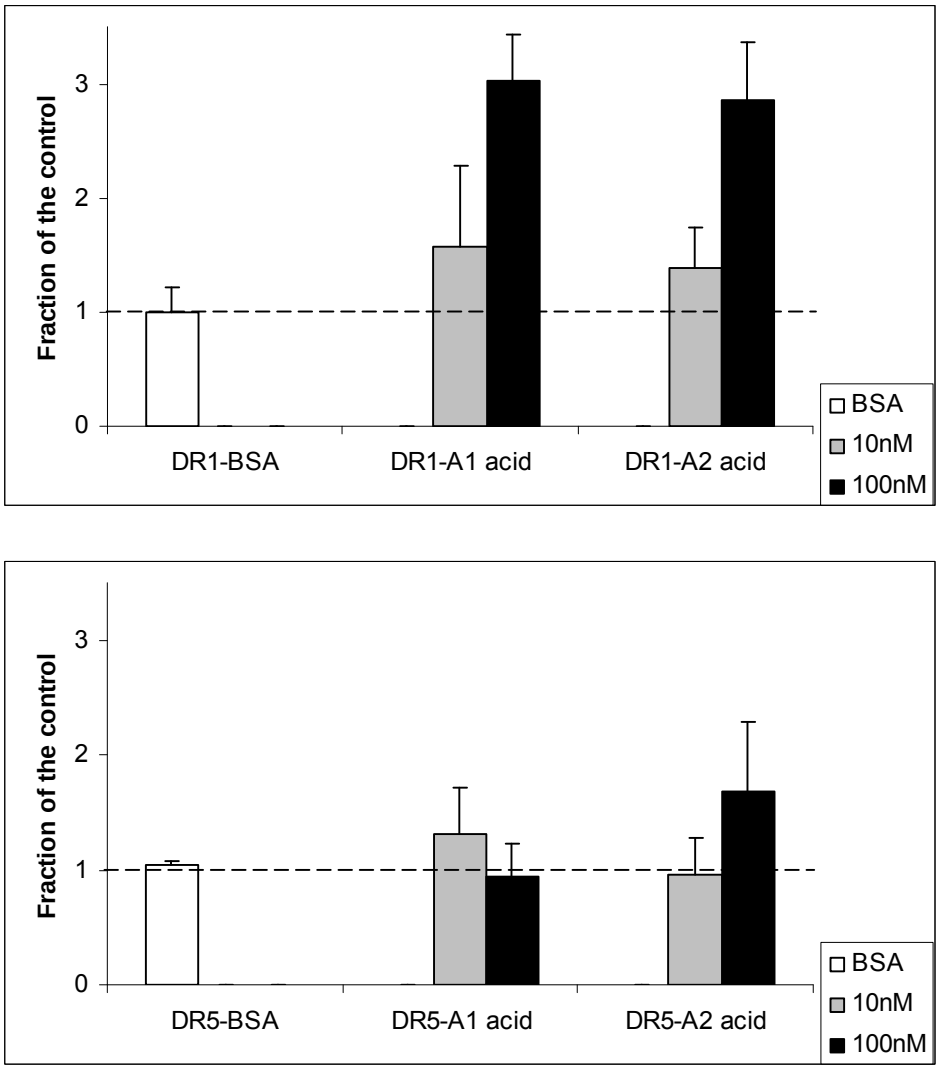


Fig III - 5: Capabilities of vitamin A1 or A2 acids to promote transcription through DR1 regulatory sequences

HaCaT cells depleted of retinoids and transfected with pBLCAT2 plasmid carrying DR1 or DR5 RARE were supplemented with 10 nM or 100 nM Vitamin A1 acid; 10 nM or 100 nM Vitamin A2 acid or 1 mg/ml BSA. After 40h of incubation the cultures were analyzed using CAT reporter assay. Shown in the bar graphs is the relative change in expression compared to non-transfected control. Data represent the average ± standard deviation for two experiments each with duplicate samples, (n = 4).

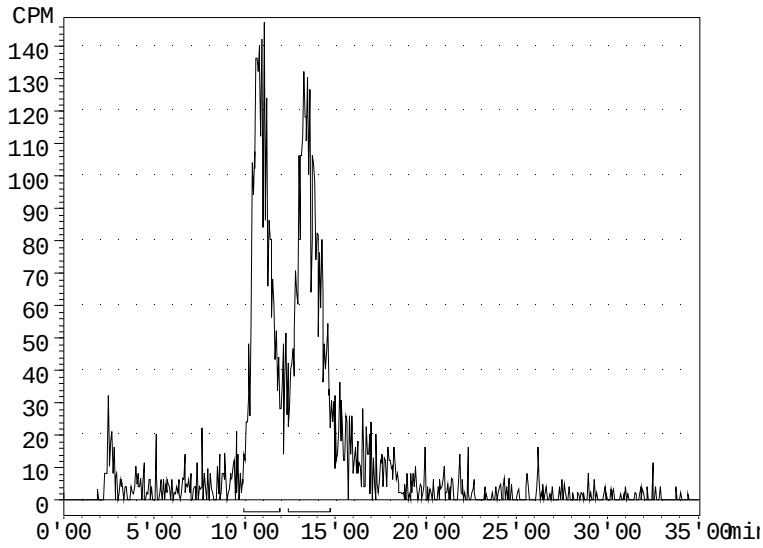
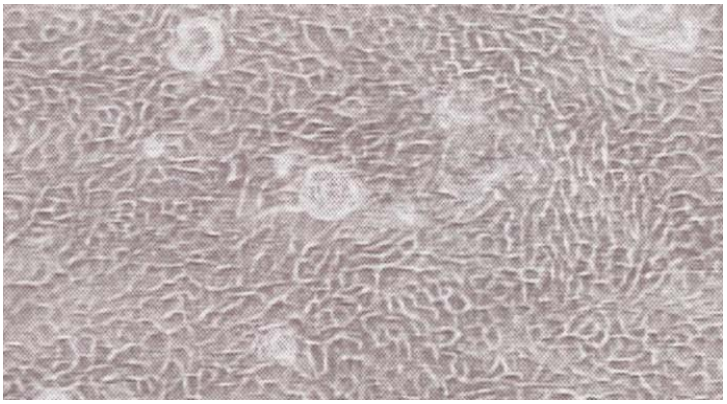
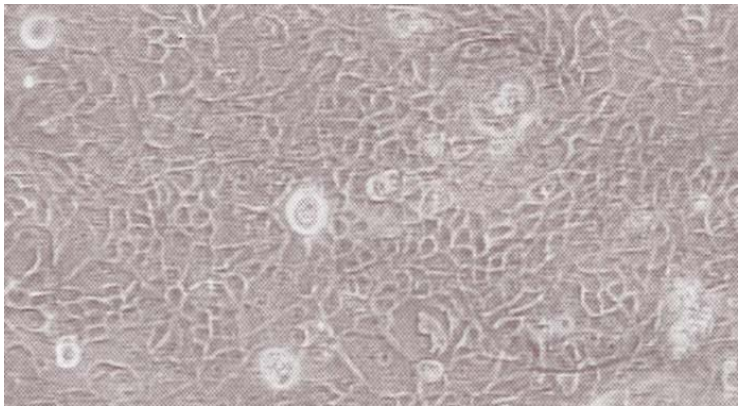


Fig III - 6: Reverse- phase gradient HPLC resolution of ^{3}H-Vitamin A1 and A2 acids
^{3}H-Vitamin A2 acid (1) and Vitamin A1 acid (2) standards resolve on NovaPack HR C18 reverse phase column.

3 – 1 medium



KGM with 1.5 mM Ca^{++}



KGM without Ca^{++}

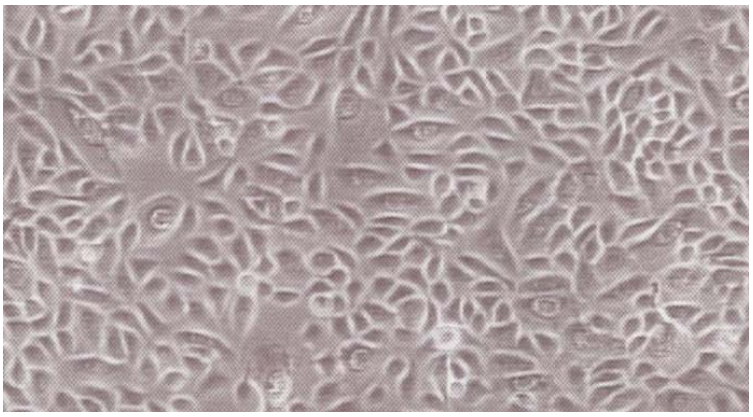


Fig III - 7: Normal human epidermal foreskin keratinocytes
Cells were grown in the three indicated media until two days post-confluence.

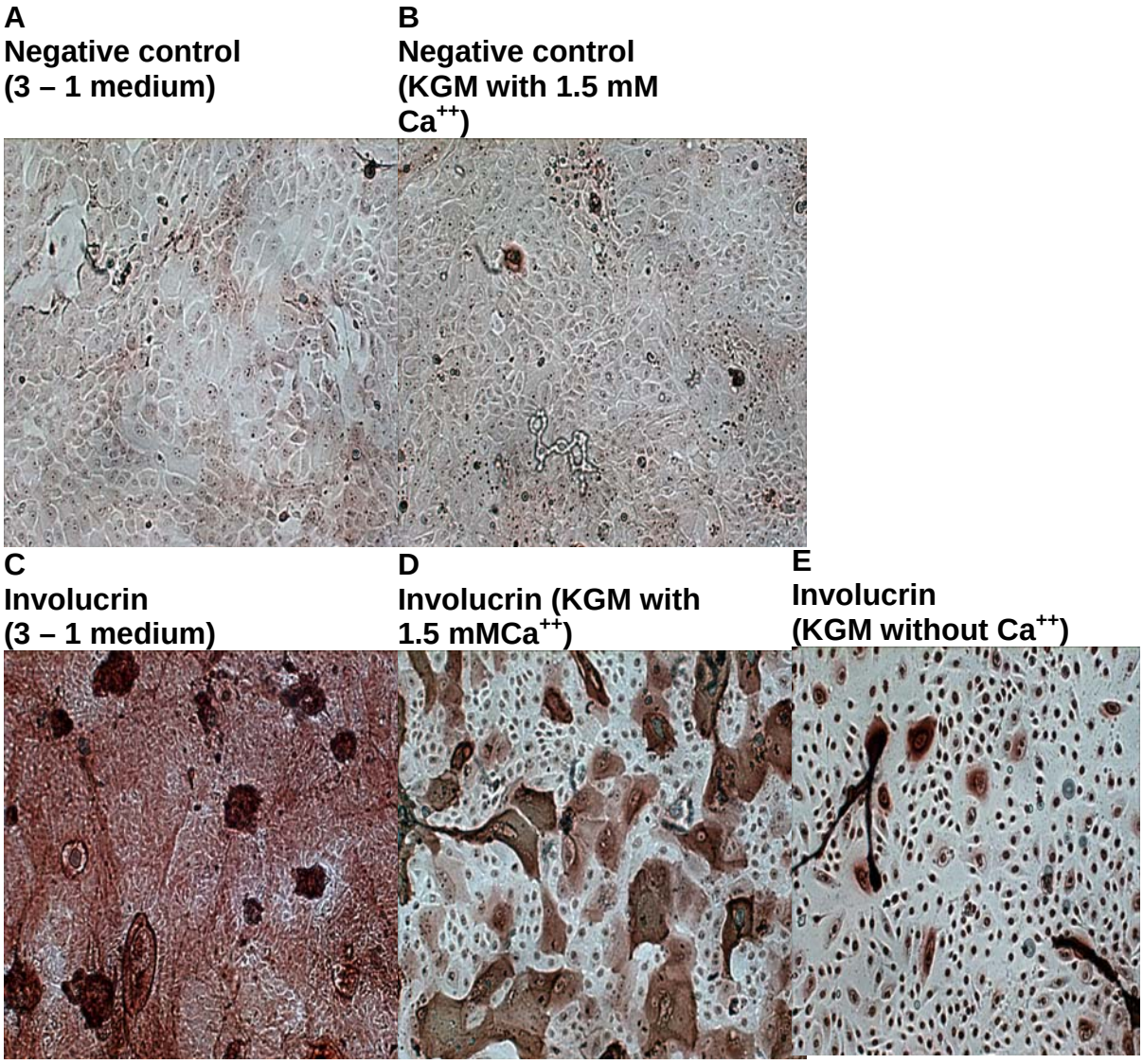


Fig III - 8: Involucrin expression of NHEK-F
Phase contrast microscopic appearance of two days post-confluent cells grown in 3 – 1 medium (Panel A and C), KGM supplemented with 1.5mM Ca^{++} (Panel B and D), and KGM low calcium (Panel E). The negative controls without primary antibody are shown in A and B.

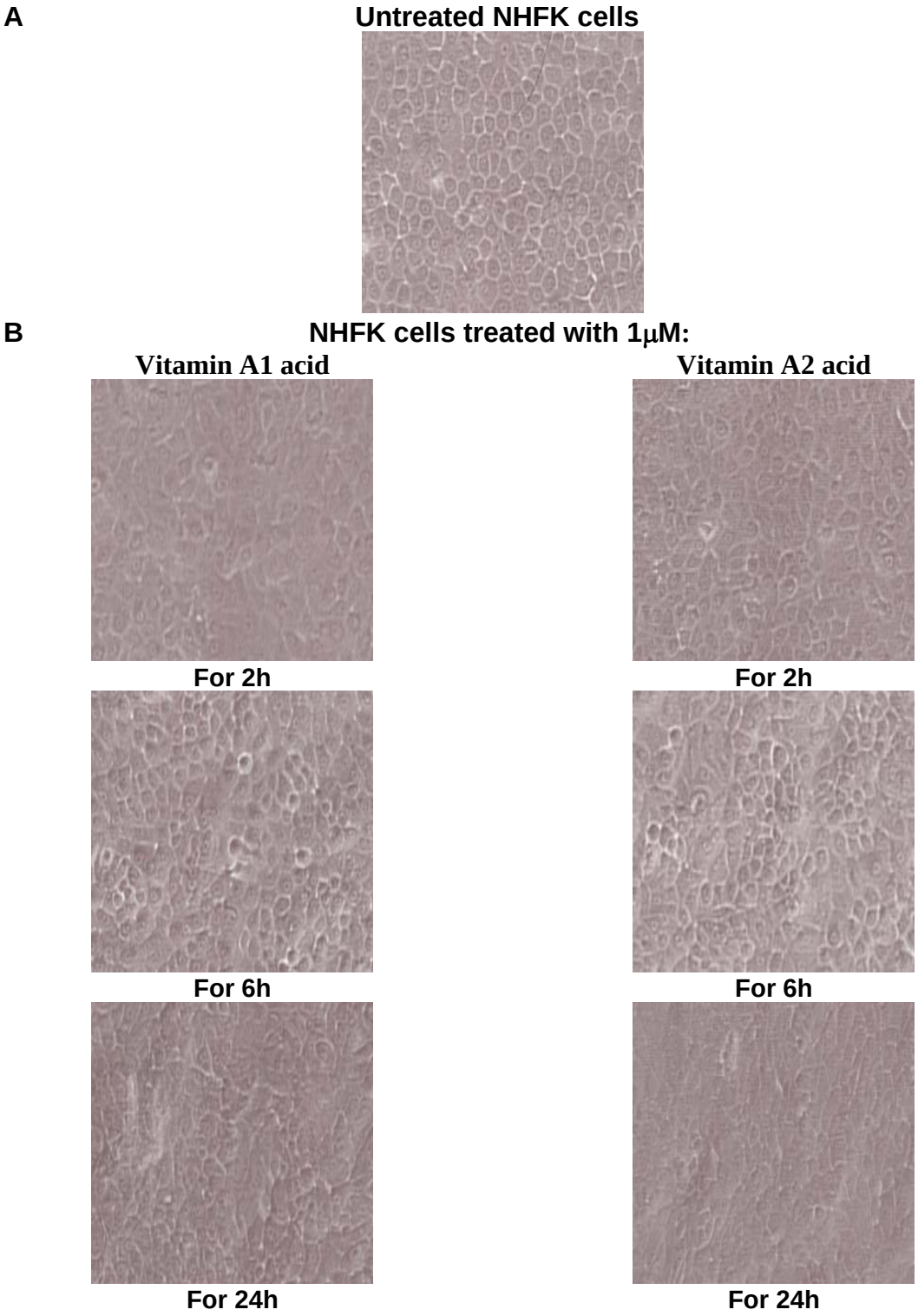


Fig III - 9: NHEK-F treated with Vitamin A1 or A2 acid for different periods of time
Cultures grown to one day postconfuence in KGM incubated for one day with KGM – high Ca^{++} and then supplied with Vitamin A1 or A2 acid for the indicated times.

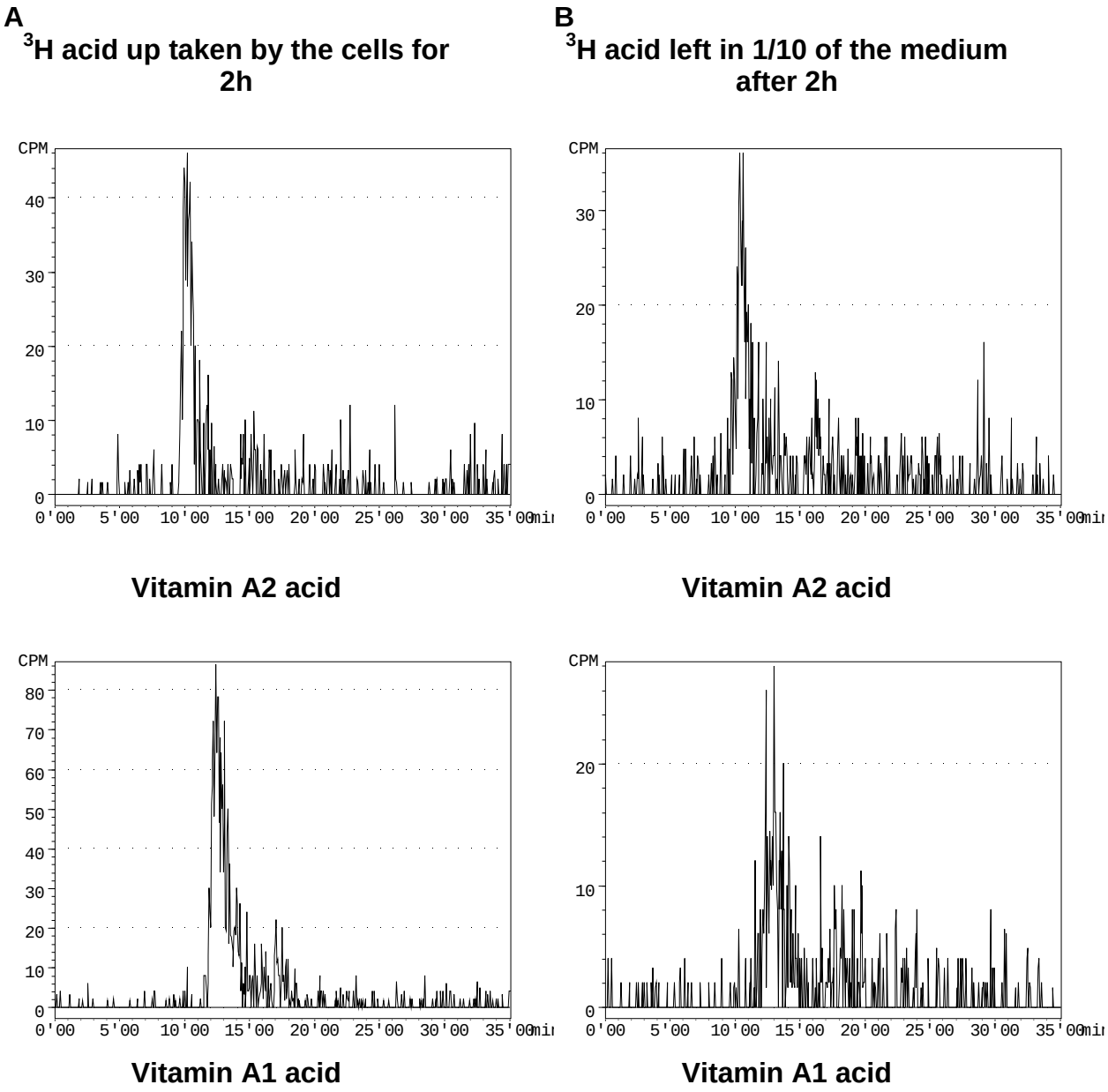


Fig III - 10: Reverse- phase gradient HPLC resolution of ^{3}H-cellular and medium retinoids
Two days post-confluent NHEK-K depleted of internal retinoid stores were supplemented with 1μM ^{3}H-Vitamin A1 (lower panels) or A2 (upper panels) acid for 2h and analyzed by reverse-phase HPLC. No oxidized metabolites were detected with cellular content (A) or medium (B).

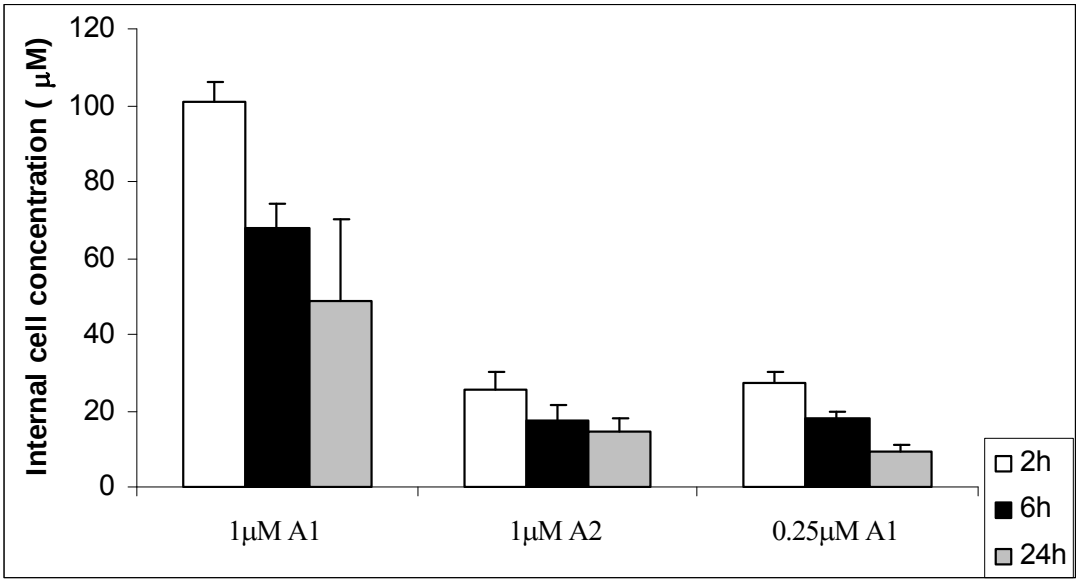


Fig III - 11: Uptake and metabolism of ^{3}H-Vitamin A1 and A2 acids by NHEK-F
Two days post-confluent cultures depleted of internal retinoids were supplemented with 1μM ^{3}H-Vitamin A1 or A2 acid, or 0.25μM Vitamin A1 acid for different periods of time and their cellular ^{3}H-retinoid content was analyzed by reverse-phase HPLC.

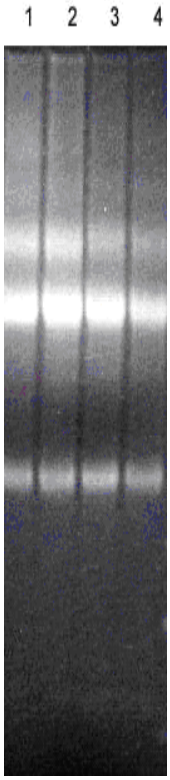


Fig III - 12: Determination of RNA integrity
Total RNA was isolated from NHEK-F grown in KGM to two days post-confluence and then incubated for 4 hours with:
Line 1 – cells treated with 1 nM Vitamin A1 acid
Line 2 – cells treated with 1 nM Vitamin A2 acid
Line 3 – cells treated with 0.25 nM Vitamin A1acid
Line 4 – control cells without treatment
RNA was electrophoretically separated in a 2% agarose gel.

Vitamin A1 acid [1nM]

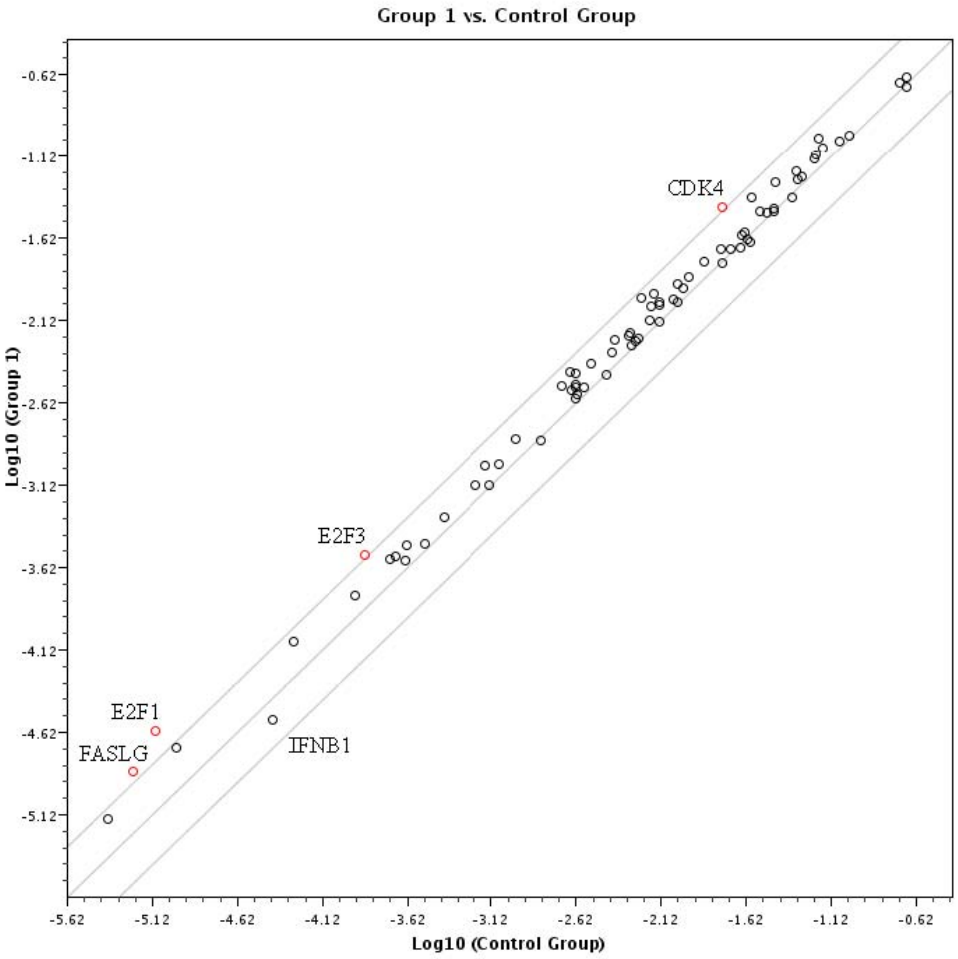


Fig III - 13a: Vitamin A1 acid (1 nM) regulated gene expression
Shown in the graph is relative change of gene expression in two days post confluent NHEK-F grown in KGM medium supplemented with 1.5 mM Ca^{++} in presence of 1nM Vitamin A1 acid (Group 1) for 4 h compared to the untreated control (Control group).

Vitamin A2 acid [1nM]

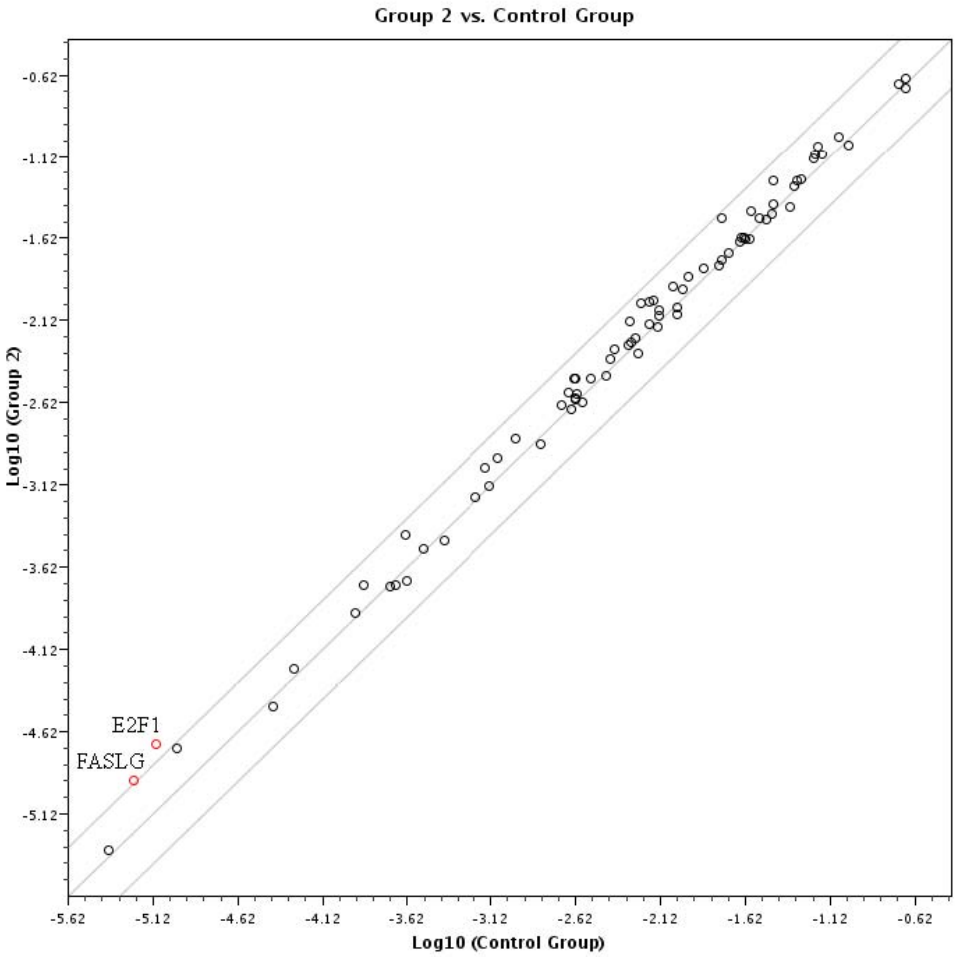


Fig III – 13b: Vitamin A2 acid (1 nM) regulated gene expression
Shown in the graph is relative change of gene expression in two days post confluent NHEK-F grown in KGM medium supplemented with 1.5 mM Ca^{++} in presence of 1nM Vitamin A2 acid (Group 2) for 4 h compared to the untreated control (Control group)

Vitamin A1 acid [0.25nM]

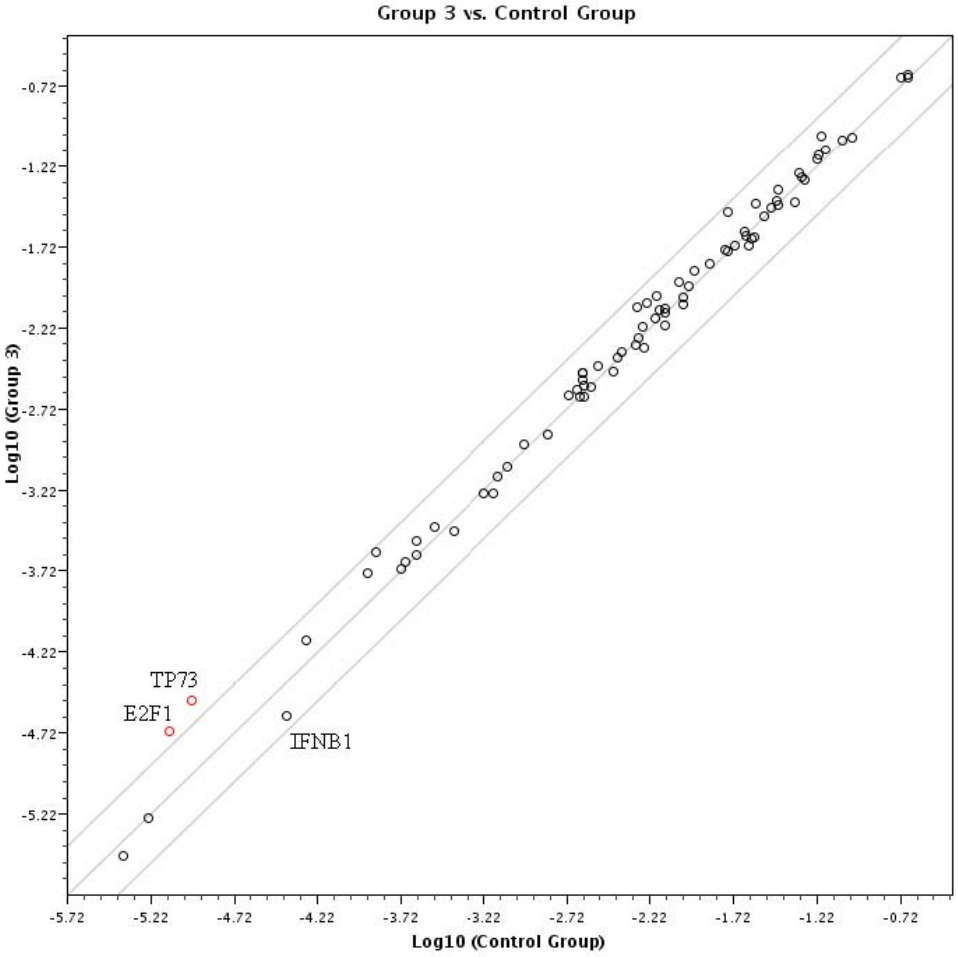


Fig III - 13c: Vitamin A1 acid (0.25 nM) regulated gene expression
Shown in the graph is relative change of gene expression in two days post confluent NHEK-F grown in KGM medium supplemented with 1.5 mM Ca^{++} in presence of 0.25 nM Vitamin A1 acid (Group 3) for 4 h compared to the untreated control (Control group).

A

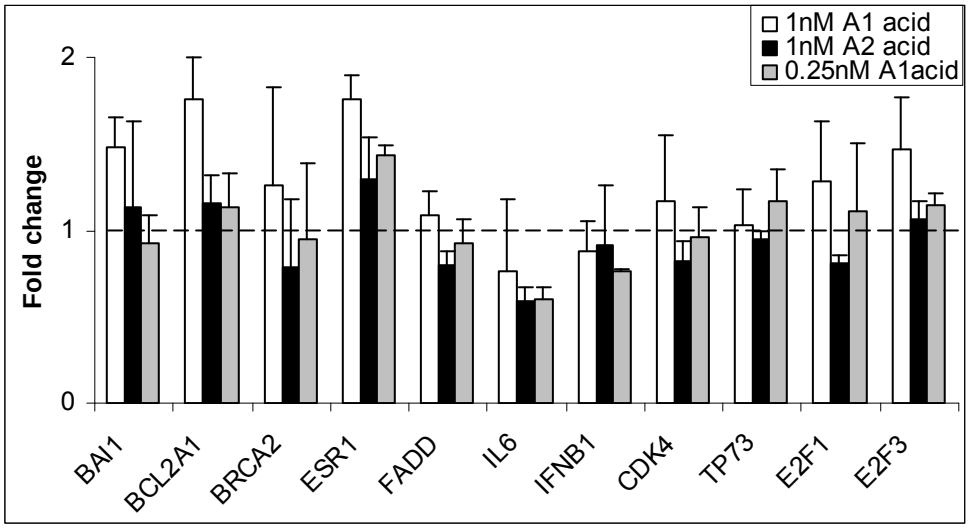
	Vitamin A1		Vitamin A2		Vitamin A1	
	[1 nM]	stdev	[1 nM]	stdev	[0.25 nM]	stdev
Gene symbol						
BAI1	**1.67**	0.20	**1.14**	0.26	**1.39**	0.28
BCL2A1	**1.37**	0.16	**1.43**	0.56	**1.12**	0.42
BRCA2	**1.41**	0.37	**1.17**	1.02	**1.16**	0.67
ESR1	**1.29**	0.88	**1.63**	0.18	**1.25**	0.07
FADD	**1.63**	0.46	**1.45**	0.20	**1.14**	0.26
IL6	**1.47**	0.77	**1.07**	0.09	**1.67**	0.80
IFNB1	**0.76**	0.27	**0.90**	0.26	**0.62**	0.05
CDK4	**2.19**	0.88	**2.05**	1.30	**1.94**	1.11
TP73	**1.85**	0.28	**1.84**	0.35	**3.01**	1.21
E2F1	**3.76**	2.99	**2.72**	1.31	**2.87**	2.05
E2F3	**2.17**	0.57	**1.46**	0.64	**1.85**	0.07
FASLG	**2.50**	1.07	**2.15**	0.65	**1.00**	0.22

B

Unigene	GeneBank	Gene Symbol	Description
Hs.194654	NM_001702	BAI1	Brain-specific angiogenesis inhibitor 1
Hs.227817	NM_004049	BCL2A1	BCL2-related protein A1
Hs.34012	NM_000059	BRCA2	Breast cancer 2, early onset
Hs.95577	NM_000075	CDK4	Cyclin-dependent kinase 4
Hs.654393	NM_005225	E2F1	E2F transcription factor 1
Hs.269408	NM_001949	E2F3	E2F transcription factor 3
Hs.208124	NM_000125	ESR1	Estrogen receptor 1
Hs.86131	NM_003824	FADD	Fas (TNFRSF6)-associated via death domain
Hs.2007	NM_000639	FASLG	Fas ligand (TNF superfamily, member 6)
Hs.93177	NM_002176	IFNB1	Interferon, beta 1, fibroblast
Hs.654458	NM_000600	IL6	Interleukin 6 (interferon, beta 2)
Hs.697294	NM_005427	TP73	Tumor protein p73

Table III - 1: Chosen genes to be assessed by real time RT-PCR
Shown in the tables are the relative change obtained by PCR - Based Gene Expression Profiling experiments (A), and gene description (B).

A



B

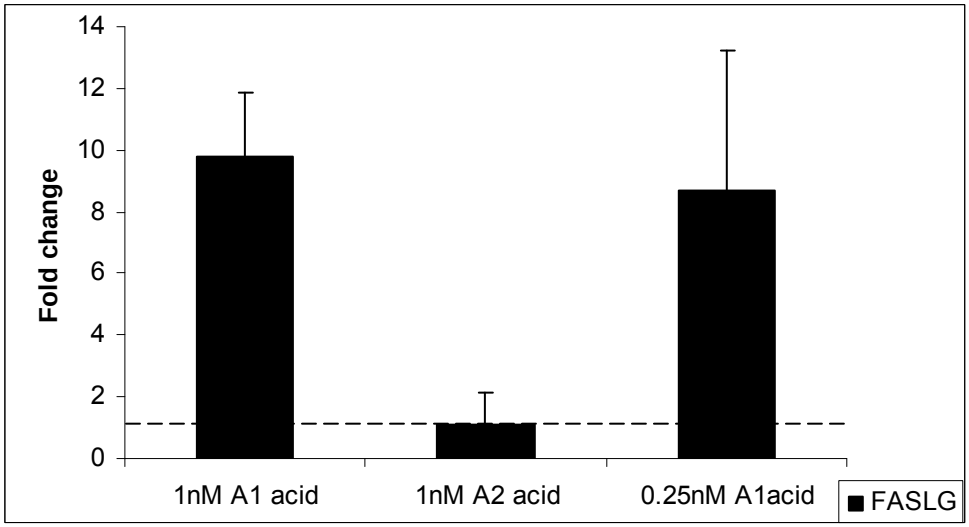


Fig III - 14: Gene expression in NHEK-F
Real time RT-PCR conformation of changes in gene expression due to treatment with Vitamin A1 or A2 acids. Shown in the bar graphs are genes with less than two fold change (A) and FASLG (B).

SUMMARY

Vitamin A and its derivatives play an important role in epidermal cell growth and differentiation. Keratinocytes have the ability to enzymatically convert Vitamin A1 alcohol to A2 alcohol. The two molecules differ by the addition of a double bond in the β-ionone ring of the Vitamin A2. The skin is one of the few tissues that produce Vitamin A2 naturally but the biological significance and functions of these retinoids in the skin is not well understood.

Previous work has shown that Vitamin A1 can be as effective as a sunscreen with SPF 20 at limiting ultraviolet (UV) radiation. At the same time it has also been shown that exposure of Vitamin A1 alcohol or ester to UV radiation generates numerous reactive oxygen species (ROS). These ROS have detrimental effect on the cells by damaging a number of cellular targets, including proteins, lipids and DNA. Furthermore *in vitro* experiments have shown that Vitamin A1 esters (retinyl palmitate) and its decomposition products can be photomutagenic agents. Vitamin A2 alcohol is more stable and is depleted more slowly than Vitamin A1 by UV irradiation. It is found in the eye lens of some lizard species where it binds to ι-crystallin and serves as a filter that absorbs short wave length radiation and protects the retina from the harmful UV radiation.

Numerous studies have shown that the active form of Vitamin A1 – the retinoic acid (Vitamin A1 acid) is a transcription factor ligand that participates in the regulation of a broad spectrum of cellular functions including the response to UV radiation. Vitamin A1 acid can bind to several different nuclear receptors and influence the expression of genes that, in many instances, have opposite functions dependent upon cell type and pattern of receptors expressed.

In vitro and *in vivo* studies have shown that Vitamin A2 acid is biologically active and has array of properties that are similar to Vitamin A1 acid. In the light, its stability to photodecomposition and because differences in retinoid chemistry predicts differences in transcriptional impact, our long term goal was to understand the biological function of the Vitamin A2. Our short term goals were to determine the impact of UV radiation on retinoid stability and biosynthesis within keratinocytes and to determine the differential impact of Vitamin A1 and A2 retinoids on the keratinocyte response to UV.

To achieve these goals [1] an *in vitro* assay for conversion of Vitamin A1 to A2 was developed, [2] the reciprocal effects of retinoid status on cellular response to UV were measured and [3] the impact of Vitamin A1 and A2 acid on p53 related gene expressed was assessed. The assay is the first to describe the conditions needed for *in vitro* synthesis of Vitamin A2 alcohol and to demonstrate the plasma membrane as a subcellular location for its biosynthesis. Similar to other desaturase complexes, the converting activity is NADPH dependant and inhibited by ketoconazole. Vitamin A1 to A2 converting activity is under

differentiation control and is upregulated following UV irradiation. The A2 retinoids limit UVA and UVB induced apoptosis more efficiently than the A1 retinoids. This result may be the sum of multiple differences between the retinoids including stability of Vitamin A1 and A2 esters, photodecomposition products and transcriptional changes consequent to A1 and A2 acid nuclear localization. Gene expression profiling of p53 related genes with confirmation by real time PCR demonstrated similarities in the transcriptional response to the two acids with one major difference. FASLG expression was differentially regulated by Vitamin A1 and A2 acids. Thus, the genes responsive to Vitamin A1 and Vitamin A2 acid are non-identical and suggest that Vitamin A2 acid has a unique and dedicated role in skin biology. For instance, under certain conditions Vitamin A2 acid can bind to nuclear retinoic acid receptor (RAR) and have similar effects on the gene expression as Vitamin A1 acid that could trigger cell growth arrest. Contrarily in a different setting it can bind the orphan nuclear receptor and inhibit the apoptosis and stimulate the growth of the cells (136).

In the cultured keratinocytes Vitamin A2 esters are more stable than Vitamin A1 esters after UV irradiation. Using “cell death detection ELISA plus assay” it was possible to show that Vitamin A2 esters provide better protection from UV induced apoptosis compared to Vitamin A1 esters.

DISCUSSION

The skin is the primary barrier to the environment. It is a specialized organ that protects the body from the harmful effects of different factors of physical, chemical and biological origin. To perform this complex function the skin has elaborated specialized architecture and complex signaling systems employing a wide array of molecular species. Among its numerous unique properties, the skin has developed the ability to convert Vitamin A1 to Vitamin A2. In studies with different species, these two families of retinoids have been shown to be able to perform a variety of functions; several of which enhance the protective quality of the skin. The biology and the function of Vitamin A1 in the skin has been the subject of numerous studies and is much better understood compared to Vitamin A2.

Vitamin A2 was first described in human skin by Vahlquist in 1980 (128). However, since that time there have been only a limited number of reports on this vitamin. From these reports we know that Vitamin A2 retinoids can bind with comparable affinities to nuclear receptors to transactivate genes (119). It has also been shown that Vitamin A2 acid but not Vitamin A1 acid inhibits the activity of retinol dehydrogenase-4 (RoDH-4) *in vitro* (54). In this dissertation an *in vitro* assay for conversion of Vitamin A1 alcohol to Vitamin A2 alcohol was described for the first time and functions important to skin biology were identified.

Critical for epidermal maintenance and differentiation are enzymes catalyzing fatty acid, ceramide and retinol desaturation. We believe that similar to fatty acid desaturases such as Δ 5- (22), Δ 6- (46) and Δ 9- (76) desaturases Vitamin A1 to A2 converting activity is a part of a membrane bound enzyme complex. The desaturase enzyme complexes may include NADH cytochrome *b*(5) reductase and cytochrome *b*(5) or may include p450 (CYP) enzymes. These desaturase complexes facilitate coupled reactions that transfer electrons from NAD(P)H to a terminal desaturase. The first component of these electron-transport chains is a cytochrome *b*(5) reductase, which accepts electrons from NAD(P)H and transports them to the cytochrome *b*(5) heme group. The next reaction transfers electrons from cytochrome *b*(5)to the terminal desaturase.

Desaturation that leads to changes in biological function is exemplified in ceramide formation. The biosynthesis of ceramide (*N*-acylsphingosine) which is a bioactive lipid involved in cell growth, differentiation and apoptosis has been shown to require the introduction of a 4,5-*trans*-double bond in dihydroceramide (*i.e. N*-acylsphinganine). This desaturation is necessary for its biological activity (38). The enzyme responsible for the double bond formation – dihydroceramide desaturase has a mechanism of action that resembles the mechanism of Δ 9-desaturase (stearoyl-CoA desaturase) and uses either NADH or NADPH as a cofactor (72).

Ketoconazole a known inhibitor of p450 enzymes inhibits Vitamin A2 biosynthesis in cultured cells (121) and inhibits the Vitamin A1 to A2 converting activity detected in homogenates, suggesting that p450 enzymes may be involved in the desaturation of the β-ionone ring of Vitamin A1. A possible participant in electron transport is NADPH:cytochrome p450 reductase. In plants NADPH:cytochrome p450 reductase catalyzes the transfer of electrons from NADPH to cytochrome *b*(5), which then donate reducing equivalents to desaturate lipids (17). There is 38% homology between the plant and the mammalian NADPH:cytochrome p450 reductase (105). Protein-protein interactions between mammalian NADPH-cytochrome p450 reductase and cytochrome *b*(5) enzymes as well as with some cytochrome p450 enzymes have also been demonstrated. The same work also shows that p450 enzymes can interact and form complexes with cytochrome *b*(5) enzymes suggesting that complexes of these proteins may be involved in desaturation (106).

p450 enzymes have been studied extensively. Several different p450 enzymes share properties with the Vitamin A1 to A2 converting activity including enhanced expression by UV radiation (CYP1A1, CYP1B, CYP4A11, CYP2S1), positive regulation by differentiation (CYP 2 family) and plasma membrane association (CYP2D6, CYP2C11, CYP2E1). It is not yet known whether these enzymes or other CYPs share all properties of the conversion activity, nor whether these enzymes would function similarly within the context of the epidermal keratinocyte. However, all enzymes are inhibited by ketoconazole as is Vitamin A2 biosynthesis in normal and transformed keratinocytes.

A group of p450 enzymes that are responsive to stress signals such as UVA and UVB radiation and may be involved in Vitamin A2 synthesis are CYP1A1, CYP1A2, and CYP1B. These enzymes are known to be upregulated through the aryl hydrocarbon receptor. The ability of this receptor to up regulate target genes increases in differentiated keratinocytes and it has also been shown that UV radiation impacts aryl hydrocarbon receptor signaling and causes induction of CYP1A1 and CYP1B1at the level of transcription (115). It also has been shown that CYP4A11 protein synthesis is up regulated after UV irradiation (41).

Another gene family that is expressed preferentially in extra hepatic tissues in humans and specifically in differentiated keratinocytes is the CYP 2 family of genes. Since CYP2S1 can convert Vitamin A1 acid to its 4-hydroxy and 5,6-epoxy form, it was suggested that it could play an important role in regulating epithelial differentiation and the production of molecules that affect the competency of the epidermal barrier. It was shown that cutaneous CYP2S1 gene expression also was induced by ultraviolet irradiation (31).

Our data demonstrate that Vitamin A1 to A2 conversion is under differentiation control. However, it should be noted that in culture a normal stratum granulosum does not develop. Therefore our data only indicate an increase in Vitamin A2 biosynthesis with differentiation in cells similar to those in the spinous layer of the epidermis. These data and the interpretation are in consistence with the finding of Torma and Vahlquist (124). Also cancers that originate from supra basal layers such as squamous cell carcinoma (SCC) have

upregulated synthesis of Vitamin A2, while, cancers that originate from basal layers such as basal cell carcinoma (BCC) do not show any up regulation of Vitamin A2 synthesis. The concentration of Vitamin A2 in keratoacanthoma and SCC is 6-7 times elevated compared to the normal skin levels (130).

We have found that at least one enzymatic activity for Vitamin A1 to A2 conversion is associated with the plasma membrane. Although most of the p450 enzymes are associated with the endoplasmic reticulum membrane, there is a flow of vesicles from these membranes to the plasma membrane via the Golgi apparatus. Some of the p450 enzymes that have been shown by immunofluorescence and immunoperoxidase labeling to reach the plasma membrane in rat and human hepatocytes are members of the CYP2D subfamily (62, 75), CYP2C11 (93), or CYP2E1 (33, 137). Also shown is NADPH-cytochrome c reductase activity associated with the plasma membrane (61).

In contrast to the Vitamin A1 to A2 converting activity, no other retinoid metabolic enzyme has been found associated with the plasma membrane compartment. For instance LRAT (52), ARAT (52, 97) and the Vitamin A1 alcohol dehydrogenases of the short-chain dehydrogenase/reductase (SDR) family (51, 65) are associated with the microsomal fraction, while alcohol dehydrogenases, (66) and the Vitamin A1 aldehyde dehydrogenases (63, 142) are found in the cytosol. This enzyme distribution makes direct competition for substrate (Vitamin A1 alcohol) unlikely and suggests a mechanism that controls substrate distribution.

Also in this work the biological function of Vitamin A2 was examined. First we explored the role of the Vitamin A2 retinoids as molecules capable of limiting UV-induced cell damage. The data showed that not only was Vitamin A2 more effective than Vitamin A1 at preventing UV-induced apoptosis, but that in UVA and in UVB irradiated cells Vitamin A2 synthesis was enhanced. One possible explanation for this effect is that the cells need Vitamin A2 as a protective molecule. We believe that the protection of the cells is provided by both, the greater ability of Vitamin A2 retinoids to absorb UV light without generating toxic decomposition products and by the signaling properties of Vitamin A2 acid.

Some studies have shown that Vitamin A1 retinoids offer protection against UV radiation by absorption (112). Vitamin A is a common example of a conjugated system, with alternating single and double bonds (48), which forms the basis of chromophores – the light absorbing part of a molecule. Molar absorptivity is used to determine and compare the relative strength of light absorption by different chromophores. The molar absorptivity (ε) is very large for strongly absorbing chromophores such as retinoids (>10,000) and is small for a weakly absorbing systems (10 to 100). Each additional double bond in the conjugated system shifts the absorption maximum about 30 nm to longer wavelengths. Vitamin A2 alcohol (λ_{max}=352nm) maximal absorption is 26 nm shifted to longer wavelength due to the additional double bond compared to Vitamin A1alcohol (λ_{max}=326nm). Also this additional double bond increases the molar absorptivity (132) of Vitamin A2 alcohol (ε_{352}=41322) compared to Vitamin A1 alcohol (ε_{352}=26240) (90).

In agreement with these findings we found that the A2 retinoids limited UVA and UVB-induced apoptosis more effectively than the A1 retinoids. Irradiation with UVA (35 or 40 J/cm^2) or UVB (0.5 or 0.6 J/cm^2) of cells devoid of retinoids caused an increase of apoptosis 16 hours after post-irradiation. This increase was significant with the higher UVA or UVB doses. With retinoid supplementation we observed two effects. First, both Vitamin A1 and Vitamin A2 reduced apoptosis in retinoid depleted control cells ($p<0.05$). Second, both Vitamin A1 and Vitamin A2 decreased the extent of UV-induced apoptosis. At the lower doses of UVA (35 J/cm^2) and UVB (0.5 J/cm^2) both Vitamin A1 and A2 reduced apoptosis .Vitamin A2 treated cultures reduced the number of apoptotic cells by 77% (UVA) and 66% (UVB) and Vitamin A1 reduction of the number of apoptotic cells was 69% and 64%, respectively. As predicted by its absorption maximum (352 nm), Vitamin A2 offered better protection against UVA than UVB; its maximal absorption spectrum coincides with the UVA wavelength. At the higher irradiations doses (40 J/cm^2 UVA and 0.6 J/cm^2 UVB) Vitamin A2 was able to provide protection, which was at the same significant extent ($p<0.02$) compared to the irradiated cultures without intracellular retinoids. At these higher doses Vitamin A1 also offers protection but to a much lesser extent (45% – UVA and 31% – UVB reduction of the number of apoptotic cells).

The fact that Vitamin A2 offered better protection may also be explained by the more rapid photodecomposition of the Vitamin A1 retinoids. Although the photodecomposition products were not the subject of our study, their presence and potential cytotoxicity should not be excluded. Photodecomposition of Vitamin A1 retinoids have been studied extensively because of their application in cosmetics (21) and as a UV blockers (6). Even though in some studies Vitamin A1 has been shown to have antioxidant activities (82) under certain conditions it can have pro-oxidant activity. This dual character has also been observed for carotenoids (143). Multiple studies have shown that Vitamin A1 alcohol and esters are subject to photodecomposition (21, 47, 138, 139). UVA irradiation (14 J/cm2) of retinyl palmitate in ethanol resulted in 14 identified photodecomposition products and formation of reactive oxygen species (36). Some of these decomposition products have been shown to be photomutagenic (68, 69).There is also discrepancy between the positive photomutagenicity results for retinyl palmitate in combination with UVA when tested in a mouse lymphoma assay (69) and the negative results obtained in S. typhimurium TA102 (21). This discrepancy may be explained by the mode of action of retinyl palmitate via a clastrogenic mechanism (36).

Also, studies in solution and animal models (111, 127) showed similarities in the photochemical behavior of Vitamin A1. However Vitamin A1alcohol was protected in animal models by binding to RBP or CRBP and this resulted in slower photdegradation compared to the unprotected retinyl esters (116).

Despite the abundance of Vitamin A1 data, little is known about the photophysics, photochemistry, and photobiology of Vitamin A2 alcohol and esters in solution. Although it is known that Vitamin A2 decreases less than A1 after UV irradiations (3, 12, 13), the identity of the Vitamin A2 decomposition products has not been reported. It has also been found that Vitamin A2 can function as an UV

filter (95, 102) and can act as an antioxidant due to its capabilities to quench oxygen, free radicals and singlet oxygen (102). These findings are in agreement with our results and indicate that Vitamin A2 offers better protection against UV irradiation compared to Vitamin A1. Most probably, Vitamin A2 retinoids offered better protection against UV due to both: its high absorptivity and its minimal cytotoxicity.

Because Vitamin A1 and A2 are found at a 4:1 molar ratio in human epidermis and in our cultures, in future studies of UV induced apoptosis it would be of interest to determine the effect of varying the intracellular concentrations of A1 and A2, and thereby to determine if the cytotoxic effect due to photodecomposition of Vitamin A1 after UV irradiation as a result of its higher intracellular concentration.

As both A1 and A2 acids can serve as a transcription factor ligands differences in their transcriptional abilities were evaluated. There are several methods that can be used. Microarrays accommodate an enormous dynamic range of gene expression in a single processing event. However, they yield many false positive and false negative results (26) and interpretation requires multiple experimental and biologic replicates. That is why quantitative real time RT-PCR is used routinely to verify changes in gene expression and this has been shown to be especially important for low abundance messages (94). These validation experiments have often shown quantitative differences between gene expression levels detected with the microarray analysis and quantitative RT-PCR. For example in microarray analyses of STAT gene expression in the rat hippocampus after focal cerebral ischaemia and reperfusion, changes in the gene expression of STAT2, STAT5b and SOCS4 were 5.8, 3.4 and 67 fold respectively. In contrast, the validation experiments using quantitative real time RT-PCR showed increases of 15, 20 and 20-fold, respectively (39). In other studies changes in expression found by microarray were actually altered in the opposite direction when evaluated by quantitative real time RT-PCR assessment (131).

Failures like those described, led to development of a gene expression screening system that is based on real time PCR. The RT Profiler PCR Array System is one of these systems and was used in this work. It combines the sensitivity of real time PCR with the ability to detect the expression of many genes simultaneously. Even though this system allows for more accurate determination of the expression of genes with different mRNA abundance simultaneously, it still has to manage the expression of multiple genes that have different priming efficiency. This array uses an approximation method (delta-delta Ct) to evaluate the fold change in gene expression and assumes that the efficiency with which the targets and the reference (housekeeping) gene transcripts anneal to its primers is the same. It also assumes that all the genes will be amplified with 100% efficiency; this is rarely the case. This is especially critical for genes that have very low mRNA abundance per cell. Due to the differences in the efficiency with which they are primed or amplified false positive or false negative results could arise. For instance in a study on the involvement of MAP kinase signaling in the degradation of extracellular matrix in osteoarthritis

with the RT Profiler PCR Array System, it was demonstrated that MAPK13 and MAPK14 gene expression increased 239 and 1783 fold, respectively. Validation of these results by quantitative real time RT-PCR showed increases of only 4 and 20 fold respectively (98).

Again, it is imperative to validate findings by quantitative real time RT-PCR that gives more accurate results by taking into account the efficiency of detection of the target and the reference (housekeeping) gene and use it in the formula for the calculation of the fold change in gene expression (84).

Using the RT Profiler PCR Array System as the initial screening device we found 12 genes whose expression increased more than 1.5-fold or decreased to less than 0.5 times that of control. The change in the expression of these genes was validated by quantitative real time RT-PCR and three different types of retinoid responding genes were detected: one in which A1 and A2 acids both up regulated gene expression, one in which A1 and A2 retinoids both down regulated gene expression, and one in which the A1 acid significantly up regulated gene expression, but the A2 acid failed to do that. This last group includes only one gene, FASLG, which was significantly ($p=0.03$) up regulated by 1nM Vitamin A1 acid (8.12±0.24, 11.26±0.34 fold) compared to 1nM Vitamin A2 acid (0.42±0.06, 1.82±0.04 fold). The positive regulation by Vitamin A1 acid is consistent with previous reports (134). As Vitamin A1 acid is concentrated 4-fold more than Vitamin A2 acid, its relatively high potency may be concentration driven. However, it is also possible that these two retinoids alter transcription differently. The data obtained from the treatment with 0.25 nM Vitamin A1 acid showed significant variation in the biological duplicates (2.21±0.19, 15.13±0.29 fold) and is confounding. It is not known whether these differences in FASLG expression are due to small changes in the intracellular Vitamin A1 acid concentration or to other transcriptional regulators sensitive to small differences in the culture confluence. Separate aspect of the gene expression analysis is the translation of the message. Not always more abundant mRNA translates into more protein. It will be important to examine in future studies by Western blotting analysis if the upregulated transcription of FASLG in cells treated with Vitamin A1acid leads also to upregulation of translation of FASLG protein.

It is not unlikely that Vitamin A1 and A2 acids have different abilities to promote transcription. The differences in their chemical structure alter their binding affinities for at least some of the RAR and RXR nuclear receptors. Although it has been shown that Vitamin A1 and A2 acids bind to the RARs with similar affinities and induce transcriptional activation with the same dose dependency (119) it has been shown also that Vitamin A2 acid binds to RXRα with higher affinity than Vitamin A1 acid. Also the ability of Vitamin A2 acid to activate transcription mediated by RXRα homodimers was two to three fold higher than that of Vitamin A1 acid and these authors showed also that Vitamin A2 acid transactivates at least one RXRα heterodimer more effectively than does Vitamin A1 acid (100). The differences in binding and transactivation could be due to conformational differences between the two families. However, other factors may be involved.

In attempt to develop agonists and antagonists of Vitamin A1 acid with higher affinity for the different nuclear receptors which in turn can be used as alternative drugs, it was discovered that smaller number of hydrophobic contacts confers preference for the RXR isotype selectivity over RAR (5). The formation of double bond in the β-ionone ring takes away two hydrogen atoms. This results in lower number of hydrophobic contacts that may contribute and explain the higher affinity of Vitamin A2 to RXR.

The complexity of the action of Vitamin A1 and A2 acids may be even more profound if we take into account other nuclear receptor besides RARs and RXRs through which retinoids could modulate gene expression. For example it has been recently shown that PPARβ/RXR heterodimers bind Vitamin A1 acid and transactivate genes. It is possible that differences in the binding of Vitamin A1 and Vitamin A2 acids to the nuclear receptors are responsible for the protection against UV induced damage by differential induction of transcription.

Binding of Vitamin A1 and A2 acids to RAR/RXR versus PPARγ/RXR are further regulated by the proteins that deliver retinoids to these transcription factors. In cells that express a high CRABP-II/FABP5 ratio, Vitamin A1 acid is directed to RAR/RXR resulting in growth inhibition. In cells that have a low CRABP-II/ FABP5 ratio Vitamin A1 acid is directed to PPARβ/δ, up-regulating survival pathways (107, 136, 71). Thus, it is also necessary to explore the relative binding of Vitamin A1 and A2 acids to CRABP-II and FABP5.

The differential transcriptional activity of Vitamin A1 and A2 acids may be further facilitated by nuclear receptor coregulators. They can act by remodeling the chromatin structure for example by acetylation (p160 family, CBP/p300), methylation (CARM1, PRMT) or at the level of the preinitiation complex (TATA box-binding protein associated factors, positive factors-PC, etc.) and facilitate gene transcription.

In summary dependent upon the expression of binding proteins, nuclear receptors, nuclear coactivators/corepresors the same ligand (for instance Vitamin A1 or A2 acid) can have different effects in different cell types. Because each of these proteins may be regulated by differentiation, the same ligand may have different effects in the same cell type but at different stage of differentiation.

Based on our findings we propose that Vitamin A2 retinoids protect cells from the damaging effect of UV radiation in two ways. First, they serve as a UV protective shield by absorbing the harmful radiation. This facilitates protection by lowering the effect of UV radiation on important molecules such as DNA. Vitamin A2 retinoids protect the basal and suprabasal layers of the skin. In the basal layer this helps prevent UV induced mutations in the stem cell compartment that can lead to malignant transformation. In addition Vitamin A2 decreases UV induced apoptosis and spares the cells required to maintain epidermal integrity and permeability barrier. Second, Vitamin A2 acid may act as transcription factor that modifies cell response to UV radiation.

In future studies it would be of interest to determine whether Vitamin A2 retinoids exposed to UVA/B radiation generate less or less toxic photodecomposition products than Vitamin A1 retinoids which may lessen the genotoxic UV damage. It would also be of interest to determine whether Vitamin

A2 acid blunts the effect of Vitamin A1 acid on gene transcription initially by evaluating its ability to suppress the observed Vitamin A1 acid enhanced transcription of FASLG. Ultimately the relative impact of Vitamin A2 retinoids on sun-protected and sun-exposed skin will be required to determine the protective and therapeutic potential of this retinoid family.

FUTURE STUDIES

It has been shown that compared to Vitamin A1 esters, Vitamin A2 esters more effectively protect cultured epidermal keratinocytes from undergoing apoptosis after UV radiation. The stability of Vitamin A2 and its enhanced synthesis post-irradiation further suggests that these retinoids serve a protective role in UV-exposed keratinocyte. Therefore it would be of interest to

- Determine whether Vitamin A2 esters prevent DNA strands break and thymidine dimer formation to lessen genotoxic UV damage.

- Determine whether Vitamin A2 esters exposed to UVA/B generate less or less toxic photodecomposition products than Vitamin A1 esters.

Furthermore, as Vitamin A2 acid is transcriptionally active and differs from Vitamin A1 acid in the up regulation of FASLG, it would be of interest to

- Determine whether Vitamin A2 acid blunts the effect of Vitamin A1 acid on gene transcription initially by evaluating its ability to suppress the observed Vitamin A1 acid enhanced transcription of FASLG

- Determine whether Vitamin A1 and A2 acid differentially alter the transcription of UVA or UVB exposed keratinocytes

- Expand gene expression profiling beyond that of p53 related genes to include genes encoding nuclear receptors and coregulators

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