Effect of a New Retinoid, Arotinoid (Ro 13-6298), on in vitro Keratinocyte Proliferation and Differentiation

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In preliminary clinical trials, a new retinoid, Arotinoid Ro 13-6298, was found highly effective in hyperproliferation and keratotic disorders. The aim of this investigation was to study the effect of Arotinoid on in vitro neonatal mouse keratinocyte proliferation and differentiation. Five doses of Arotinoid were tested. [^{3}H]-Tdr labeling techniques and autoradiography were used to quantitate proliferation. Differentiation was assessed using one-dimensional polyacrylamide gel electrophoresis and autoradiography of six specific protein fractions extracted from the cells using a series of buffers. Our results indicate that Arotinoid is an extremely potent inhibitor of keratinocyte proliferation. Arotinoid significantly decreases the amount and synthesis of non-covalently and disulfide cross-linked keratins and cell envelopes while increasing the quantity of keratohyalin granule-associated proteins. No qualitative change in keratinocyte proteins was observed. *Key words: Arotinoid; Keratinocyte; Culture; Growth; Differentiation.* (Received January 25, 1984.)

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Vitamin A is an important modulator of normal epithelial differentiation. Most recently the use of a number of synthetic derivatives, the retinoids, as cutaneous drugs has substantially improved the treatment of hyperproliferative and keratinizing disorders. The aromatic retinoid (Ro 10-9359, Tigason®) and 13-cis retinoic acid (Ro 4-3780, Accutane®) are the most extensively used retinoids in cutaneous disorders (1, 2). Each drug has been used clinically: Tigason® for psoriasis, Accutane® for acne, and both drugs or keratinizing disorders (1, 2).

A new retinoid analog, Arotinoid Ro 13-6298 has recently become available for clinical testing. This new compound, which differs considerably from Tigason in its chemical structure, has been shown in vitro to be 1 000-fold more potent than the aromatic retinoid (3). This new retinoid appeared to be extremely efficacious in the treatment of keratinizing and hyperproliferative disorders, including psoriasis (3, 3 a).

It has recently been reported that the aromatic retinoid and 13-cis retinoic acid inhibit epidermal keratinocyte proliferation and specific differentiation processes (4). We have used the same in vitro systems to test the effect of the new Arotinoid drug on keratinocyte proliferation and differentiation (5). Our aim was to compare the in vitro potency of this drug and all-trans retinoic acid to that of Tigason and of Accutane.

MATERIALS AND METHODS

Reagents and animals

Neonatal mice were obtained from a Balb/c colony housed in the University of Michigan Medical School Dermatology Department. All reagents for tissue culture preparation are in Refs. 5, 7 and 9.
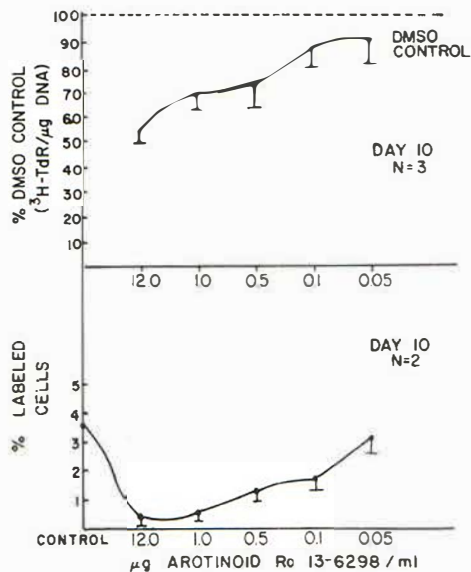
Fig. 1. Effect of Ro 13-6298 on epidermal keratino-
cyte proliferation. To quantitate keratinocyte pro-
liferation [^{3}H]-Tdr incorporation into DNA and
autoradiography were done. The results are ex-
pressed as cpm of [^{3}H]-Tdr label per/μg DNA. The
data are presented as percent of the DMSO vehicle
control (retinoid-treated culture cpm/μg DNA di-
vided by 0.2% DMSO cpm/μg DNA × 100). For
autoradiography the data are expressed as % la-
beled cells. The labeled nuclei in a minimum of 60
fields are counted using a Zeiss phase microscope.
Bars represent ± SD.

Arotinoid Ro 13-6298 (Arotinoid-ethylester, MW=376) was a generous gift from Hoffmann–La
Roche, Inc., Basel, Switzerland. All-trans vitamin A acid (retinoic acid) was purchased from Sigma
(St. Louis, Mo.).

Keratinocyte cultures

The procedure for isolating neonatal mouse basal cells from full thickness skin was as previously
described (5).

Addition of vitamin A analogs

All the vitamin A analogs were dissolved in DMSO (Sigma, St. Louis, Mo.) and kept in small volumes
with minimal to no airspace at −30°C or −70°C until used. The retinoids were always protected from
light and were added to the basal cell monolayers on day 1 after plating, and with each subsequent
medium change. 0.2% DMSO in growth medium was the vehicle control.

[^{3}H]-Tdr labeling of DNA and autoradiography

Keratinocyte cultures are terminally pulse-labeled for 6 h with 1 μCi/ml of [^{3}H]-Tdr and the DNA
extracted and counted as previously described (5). Autoradiography was done as described in
reference 5.

Labeling of epidermal keratinocytes with tritiated histidine

Sixty Petri dishes were pulse-labeled for 2 h at 37°C with 10 μCi/ml of [^{3}H]-his in M-199 diluted 1:5
with PBS pH 7.2 on day 12 after adding the retinoids (9). After a 2 h cold chase with growth medium at
32°C, the cultures were washed twice with cold PBS containing 5 μg/ml PMSF, a protease inhibitor.
The cultures were then extracted at −70°C or stored until extraction.

Extraction of epidermal keratinocyte protein

The cultures were serially extracted using 4 buffers to yield 6 fractions (9).

The fractions were: (*a*) Keratohyalin granule (KG) fractions (macroaggregates and high salt soluble
proteins) (10); (*b*) SDS soluble proteins; (*c*) non-covalently cross-linked fibrous proteins; (*d*) disulfide
cross-linked fibrous proteins, and (*e*) cell envelopes.

All the supernates were dialyzed at 4°C against distilled water containing PMSF and then lyophy-
lized. All fractions were then redissolved as previously described (9).

10 μl triplicate aliquots of each fraction were assayed by a modified Lowry technique for protein
concentration using serum albumin as a standard (12). The radioactivity for fraction was determined
by liquid scintillation counting. Aliquots containing 25 to 150 μg of protein were analyzed using SDS
polyacrylamide gel electrophoresis (SDS-PAGE).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis and fluorography

SDS-PAGE was performed according to the method of Laemmli at pH 8.8 (13) as previously described by us (9).

RESULTS

Effect of Arotinoid on epidermal keratinocyte proliferation—time- and dose-response study

Fig. 1 shows a study done 9 days after adding the retinoid. The doses of Ro 13-6298 were from 12 µg/ml (32 µM) to 0.05 µg/ml (0.13 µM). After 9 days of growth in the drug (Fig. 1), keratinocyte [^{3}H]-Tdr incorporation into DNA was inhibited 50% by the 12 µg/ml dose; 1.0 and 0.5 µg/ml Arotinoid were also inhibitory.

Autoradiography showed that the percentage labeled cells on day 10 was greatly decreased (usually less than 25% the control values) by all the doses studied except the 0.05 µg dose. The 12 µg/ml dose of Ro 13-6298 was cytotoxic to the cultures (extreme loss of protein, DNA and destruction of the monolayer). At all other doses and time points, Arotinoid slightly decreased the total protein per culture, and the total µg of DNA per dish was equal to or slightly less than the control value (data not presented). In other experiments, doses as low as 0.001 µg (2.6 nM) and 0.0005 µg (1.3 nM) were inhibitory 14 days on drug (data not presented).

Effect of Arotinoid and all-trans retinoic acid on the protein distribution and the incorporation of [^{3}H]-histidine by epidermal keratinocytes

The cells were grown for 11 days in 0.5 µg/ml Arotinoid and 12 µg/ml all-trans retinoic acid. The cultures were then pulse-labeled with 2 µCi/ml of [^{3}H]-histidine and were serially extracted. As presented in Table I, all-trans retinoic acid and Arotinoid both decreased the

Table I. *Effect of Arotinoid and all-trans retinoic acid on the total protein/fraction and the incorporation of ^{3}H-histidine by epidermal keratinocytes*

Fraction	µg protein/fraction				
	Control[a]	All-trans R.A.	% of control	Arotinoid	% of control
KG–supernate	92.4±5.2	85.99±6.1	6.93 ↓	127.84±5.8	38.35 ↑
KG–macroaggregate	326.58±17.4	255.11±12.0	21.88 ↓	325.22±15.3	0.42 –
SDS	651.56±44.3	750.45±17.1	15.17 ↑	783.31±21.3	20.22 ↑
Fibrous (non-covalently + disulfide cross-linked)	763.74±33.1	280.18±20.8	63.3 ↓	155.43±8.0	79.6 ↓
Envelope	280.15±25.4	58.99±9.6	78.9 ↓	72.17±10.4	74.2 ↓
	Change from control value of ^{3}H-His/fraction (%)				
KG–supernate	25.2 ↓			9.7 ↑	
KG–macroaggregate	21.1 ↓			58.8 ↑	
SDS	0.3			23.9 ↑	
Fibrous	56.1 ↓			44.3 ↓	
Envelope	33.6 ↑			28.8 ↑	

Arotinoid was used at a 0.5 µg/ml dose. The concentration of all-trans retinoic acid was 12 µg/ml; Arotinoid was slightly cytotoxic at the 12 µg/ml dose. $N=2$. Control contained 0.2% DMSO.
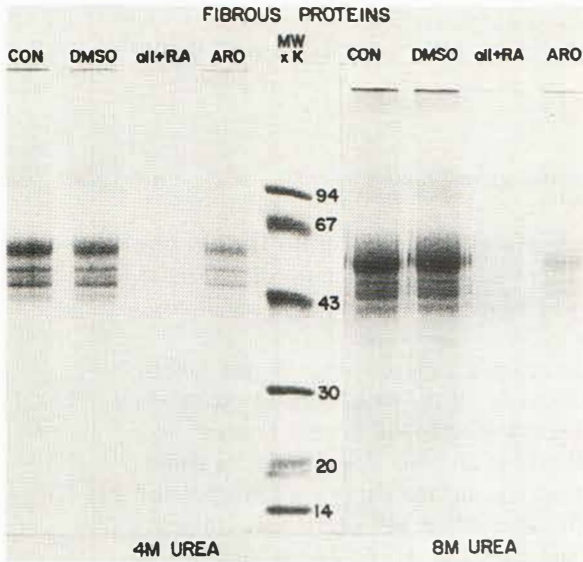[a] Mean ± SD of duplicates.

Fig. 2. SDS-PAGE of fibrous proteins extracted from all-trans retinoic acid and Arotinoid-treated cultures. Proteins serially extracted from cultures with identical cell numbers (5×10^6 cells per 60 mm Petri dish) using 4 M urea are non-covalently cross-linked keratin polypeptides ranging in relative MW from 46 to 68 daltons. When these polypeptides are processed intracellularly, they become insoluble in 4 M urea+buffers, but can be solubilized by the subsequent buffer containing 8 M urea+buffers+reducing agents (9, 5).

amount and synthesis of fibrous keratin proteins and cell envelope proteins in the cultures. Arotinoid was effective at a much lower dose, 0.5 µg. Arotinoid was observed to increase the amount and synthesis of KG-associated proteins in the cells, whereas all-trans retinoic acid slightly decreased the amount and synthesis of KG-associated proteins; Arotinoid and all-trans retinoic acid also slightly stimulated the synthesis of SDS-soluble proteins.

SDS-PAGE of fibrous proteins extracted from all-trans retinoic acid and arotinoid-treated cultures

Coomassie blue-stained gels of the fibrous keratin proteins extracted from cultures treated with 12 µg/ml all-trans retinoic acid and 0.5 µg Arotinoid are presented in Fig. 2. The data showed that both retinoids inhibited the amount (and synthesis; autoradiograms are not shown) of the keratin polypeptides by the cultures (4 M urea buffer extractable). The 8 M urea+buffer+reducing agent extraction showed that both retinoids greatly decreased the amount of disulfide-cross-linked keratins. Although almost total inhibition of the disulfide-cross-linking of the keratin polypeptides by the 0.5 µg/ml dose of Arotinoid was seen, only a partial inhibition of keratin polypeptide synthesis was caused by Arotinoid.

SDS-PAGE of KG-associated macroaggregated proteins extracted from retinoid-treated cultures

As presented in Fig. 3, the amount of KG-associated macroaggregated proteins was decreased by all-trans retinoic acid. The Arotinoid-induced increases in KG proteins were seen. The arrows indicate the bands where the amount of protein was most obviously increased. All the runs are layered with the same amount of protein.

DISCUSSION

This study shows that the new retinoid Ro 13-6298 (Arotinoid) inhibited in vitro epidermal keratinocyte proliferation. These results confirmed some previous reports (3, 4, 14, 15) while contradicting other published studies done using a number of retinoids (16, 17). This
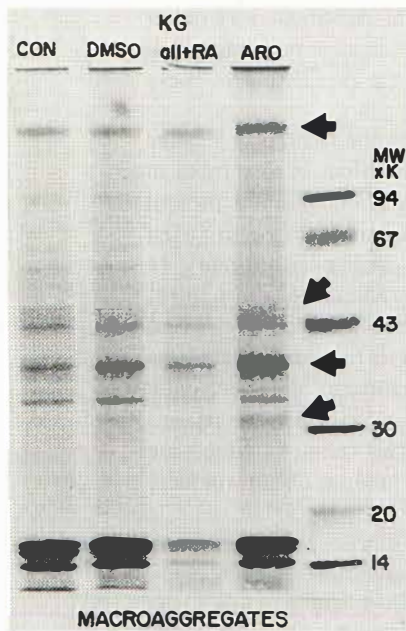
Fig. 3. SDS-PAGE of keratohyalin granule-associated proteins extracted from all-trans retinoic acid and Arotinoid-treated cultures with identical numbers of cells (5×10^6 cells per 60 mm Petri dish). 1 M KPO_4 buffer containing 0.22 M histidine preferentially extracts keratohyalin granule-associated proteins (KG) from keratinocyte cultures and whole epidermis (see Refs. 9 and 10 for details). Exhaustive dialysis of the KPO_4 buffer+proteins yields two fractions: a precipitate of macroaggregated KG proteins, and a supernate containing other KG-associated proteins.

difference in results probably stems from variations in technologies, cellular systems, and in the complex effects of the different retinoids themselves. One example of this was our observation that Arotinoid-induced [^{3}H]-Tdr pool changes masked the extensive inhibition of keratinocyte proliferation caused by low doses of Arotinoid when only the cpm/μg DNA data was assessed (Figs. 1 and 2). The autoradiography data showed that Arotinoid was an extremely potent inhibitor of epidermal keratinocyte proliferation, especially when compared to the other psoriasis therapeutic aromatic retinoid, Ro 10-9359 (4). Arotinoid was observed to be more inhibitory in less time than the aromatic retinoid.

The neonatal mouse epidermal keratinocytes used in these studies form a basal cell monolayer within 1 day after plating; the proliferating culture then stratifies and undergoes a number of differentiation events (5). Some of these events can be qualitatively and quantitatively studied (9). Thus the amount and synthesis of keratohyalin granule-associated proteins, viable cell proteins solubilized in SDS buffers, non-covalently cross-linked keratins, disulfide cross-linked keratins, and cell envelopes in cultures grown in Arotinoid were also studied. As was observed with 13-cis retinoic acid and the aromatic retinoid (4), Arotinoid and all-trans retinoic acid inhibited specific parts of the differentiation process. There was a slight increase in the SDS-soluble protein fraction; this is important to note, since cytotoxicity would decrease the amount of protein in this fraction (9). All-trans retinoic acid markedly inhibited the terminal differentiation of the cells. The amount of both non-covalently and disulfide cross-linking keratins, and cell envelope proteins were not affected. This data agreed with studies reported by Yuspa, Ben & Steinert (18), Yuspa, Lichti, Ben et al. (14) and Yaar, Stanley & Katz (19).

Arotinoid was an even stronger inhibitor of the terminal differentiation of the cultures, since effects similar to those caused by 12 μg/ml all-trans retinoic acid were induced by 0.5 μg/ml of the Arotinoid. The Arotinoid effect differed from the all-trans retinoic acid effect: this new retinoid increased the amount of proteins present in the keratohyalin granule macroaggregated protein fraction. The incorporation of [^{3}H]-histidine into this fraction

was also increased by this Arotinoid. These results suggest that Arotinoid was either increasing the synthesis of those keratohyalin granule proteins which have been identified to be rich in histidine (9, 10, 20, 21) and/or possibly inhibiting the processing of these proteins into cornified cell envelopes.

It has been reported that "histidine-rich proteins" are altered in the hyperproliferative disorder, psoriasis (21). It is possible that the potent therapeutic effect of Arotinoid in psoriasis (3, 3 a) is related to the observed increase in keratohyalin granule protein synthesis. Other evidence supports this premise. All-trans retinoic acid, which is not used to treat psoriasis, caused no change in the keratohyalin granule-associated proteins. In another study done in our laboratories (submitted for publication), 13-cis retinoic acid (12 μg/ml) (effective in acne and not in psoriasis) almost totally inhibited the synthesis and amount of proteins in the keratohyalin granule-associated protein fractions while also greatly decreasing keratin protein and cell envelope protein synthesis. The aromatic retinoid, Tigason®, which is highly effective in the treatment of psoriasis, increases keratohyalin granule-associated protein synthesis (4). Thus the two retinoids which are therapeutic in psoriasis share this one effect, i.e., increasing the amount of keratohyalin granule-associated proteins.

Thus this present study has demonstrated that Ro 13-6298, Arotinoid, is a very potent inhibitor of in vitro epidermal keratinocyte proliferation and differentiation. The relevancy of these in vitro effects in psoriasis and other cutaneous disorders still remains to be determined. However, the retinoid effect on very basic keratinocyte functions probably involves a number of mechanisms, some of which can be studied using this in vitro keratinocyte system.

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