Coordinate Control by Vitamin A of Keratin Gene Expression in Human Keratinocytes

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In the present study, we examine the effects of vitamin A on keratin protein and mRNA levels in human keratinocytes. In epidermal keratinocytes, the levels of keratins 5, 6, 14, and 17 decrease and keratins 13 and 19 increase in response to increasing concentrations of a potent synthetic trans-retinoic acid analog, arotinoid Ro 13-6298. In tracheal keratinocytes, a similar suppression is observed for keratins 5, 6, 14, 17, and 18 and an increase in keratin 19. Both induction and suppression responses show identical kinetics and both processes are half-maximal at 5 nM arotinoid and maximal at 10 nM. Utilizing cDNAs specific for keratins 5, 6, 13, and 19, we demonstrate that the mRNA levels for these keratins change coordinately with the corresponding amount of keratin protein, indicating that the control of keratin protein expression most likely resides at the level of mRNA synthesis and/or degradation. The identical kinetics for all of the responses, both inductive and suppressive, suggests that a common mechanism controls the expression of these genes. These results indicate that vitamin A produces more sweeping changes in keratinocyte function than previously appreciated in that many and perhaps all keratins are modulated by vitamin A. Moreover, these responses are 10- to 100-fold less sensitive to retinoid than the process of envelope formation, suggesting that at least two sets of processes with different sensitivities to vitamin A are present in keratinocytes.

Vitamin A produces various effects in numerous cell types (Strickland and Mahdavi, 1978; Lotan, 1980; Wolf, 1980; Elias and Williams, 1981), usually altering the state of differentiation. In human keratinocytes, the vitamin has been shown to produce morphological and biochemical changes. These changes include production of a characteristic swirled phenotype (Fuchs and Green, 1981); increased levels of keratin 19 (Mr = 40,000), and decreased levels of keratin 1 (Mr = 67,000); increased levels of mRNA for keratins 19 and 13 (Mr = 52,000) (Fuchs and Green, 1981; Eckert and Green, 1984); and suppression of envelope formation (Green and Watt, 1982; Gilfix and Green, 1984).

In the present study, we describe results which indicate that vitamin A causes even more sweeping changes in keratinocyte function and may alter the levels of nearly every keratin. Dose response and time course studies indicate that all of the vitamin A-responsive keratins respond at the same concentration of vitamin A.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Human keratinocytes derived from foreskin epidermis or fetal or adult trachea were grown in the presence of lethally irradiated 3T3 cells (Rheinwald and Green, 1975). The growth medium and the preparation of vitamin A-free cultures was as previously described (Eckert and Green, 1984). In the present experiments, a potent synthetic trans-retinoic acid analog, arotinoid Ro 13-6298 (Hoffman-La Roche, Inc.), was utilized. The synthesis and structure of this compound is described by Loeliger et al., 1980. Ro 13-6298 was used to produce all of the responses in cultured keratinocytes produced by naturally occurring retinoids, but at a lower concentration (Gilfix and Green, 1984).

**Protein Methods**—To assess the levels of the various keratin proteins, cells from 1/10 of a confluent 50-cm² dish were collected by scraping, pelleted for 5 s at 15,000 × g, and washed three times with phosphate-buffered saline to remove serum. The final pellet was resuspended in 0.25 ml of 20% dithiothreitol containing 2% sodium dodecyl sulfate, boiled 20 min, and stored at −20 °C. Protein concentration in extracts was determined by the method of Hughes et al., 1981.

Samples were fractionated by electrophoresing equal amounts of protein on 6 or 8.5% denaturing acrylamide gels (Laemmli, 1970). The gels were stained with Coomassie Blue and destained exactly as described (Hoffman-La Roche, Inc., 1971) and dried onto transparent cellophane sheets (Bio-Rad) as suggested by the manufacturer for transmission densitometry. Coomassie staining per unit molecular weight was determined by dividing the peak area observed for each keratin by its molecular weight. This value was then divided by the identical value determined for actin to give the molar ratio of keratin/actin. Actin content was used to normalize keratin changes, since its level is not affected by retinoid treatment. Keratins were identified on the basis of molecular weight and pI as determined from two dimensional electrophoresis (O'Farrell et al., 1977). Keratin nomenclature followed that of Moll et al., 1982.

**Nucleic Acid Methods**—After harvesting cells for determination of keratin content, the remaining cells were harvested for preparation of mRNA by the guanidine/CsCl gradient method (Maniatis et al., 1982). Poly(A)+ RNA was prepared using oligo(dT)-cellulose as previously described (Aviv and Leder, 1972).

Equal quantities (2–10 µg) of poly(A)+ RNA, as determined by [³H]polyuridylic acid hybridization, was electrophoresed on 1.25% agarose denaturing gels (Boedtker, 1971). The fractionated mRNA was transferred to Biodyne A (Pall), covalently fixed to the membrane as previously described (Southern, 1975; Church and Gilbert, 1984), and probed with keratin- or actin-specific cDNAs nick-translated with [³²P]dCTP to 2 × 10⁶ cpm/µg (Rigby et al., 1977). Blots were hybridized for 10 h at 66 °C as described (Church and Gilbert, 1984) except that the hybridization mixture contained denatured salmon sperm DNA at 100 µg/ml (Eckert and Green, 1984), washed 3 times for 5 min each with 40% sodium phosphate containing 1 mM EDTA and 1% sodium dodecyl sulfate preheated to 65 °C (Church and Gilbert, 1984), and exposed on preflashed Kodak XAR-5 film (Laskey and Mills, 1975). Band intensity was quantified by laser densitometry.

**RESULTS**

Arotinoid Regulation of Keratins—An interesting phenomenon of keratin suppression was observed. Fig. 1 shows a plot...
of the molar ratio of total keratin normalized to actin versus concentration of arotinoid; total keratin content was the sum of the individual molar ratios of all keratins present (see legend for a listing of specific keratins). Shown are the results of two experiments using a fetal tracheal keratinocyte strain, Tr-2; a second fetal tracheal strain, Tr-1; an adult tracheal strain, Tr-V; and an epidermal strain, Epi-AR. In each case, the level of total keratin decreased with increasing levels of arotinoid. This decrease occurred even though the level of keratin 19 increased. Suppression was half-maximal at 5 × 10⁻⁶ M and maximal at 1 × 10⁻⁷ M.

Fig. 2 shows the changes in levels of individual keratins in response to increasing concentrations of arotinoid. In adult tracheals the levels of keratins 5, 6, 14, 17, and 18 declined while the level of keratin 19 increased. In human epidermal cells, keratins 5, 6, 14, and 17 declined while the level of keratin 19 increased. In all cases, suppression was half-maximal at 5 × 10⁻⁶ M arotinoid and maximal at 1 × 10⁻⁸ M; moreover, the level of keratin 19 increased with an identical dose dependence. The level of keratin 13, which is expected to increase based on its mRNA increase (see below), is not shown because it is difficult to quantify due to the presence of other proteins of similar molecular weight.

**Arotinoid Regulation of Keratin mRNA**—To determine whether this change in keratin concentration could be explained by a change in message content, mRNA was isolated from fetal tracheal keratinocytes, electrophoresed on denaturing gels, blotted to Biodyne A, and probed with nick-translated cDNA probes specific for each keratin and with an actin cDNA probe (Eckert and Green, 1984) (Fig. 3). The level of message for keratins 5 and 6 declined, while that of keratin 19 increased; actin mRNA levels remained stable. Although there was a marked dose-dependent retinoid stimulation of mRNA corresponding to plasmid pK13-1 in three other tracheal strains examined (data not shown), very little hybridizing message was detected in tracheal strain Tr-2. It is likely that this represents a strain difference. A plot of results from laser densitometric scans of these gels is shown in Fig. 4. All keratin message levels change with an identical dose dependence; suppression and induction are half-maximal at 5 × 10⁻⁹ M and maximal at 1 × 10⁻⁸ M. These mRNA response profiles agree well with those observed for the corresponding keratin proteins (Fig. 2). The exception is the increase in mRNA for keratins 5 and 6 in cells treated with 1 × 10⁻¹¹ M to 1 × 10⁻¹⁰ M arotinoid as compared to the untreated cells. This may be due to the greater stratification of untreated cells (see "Discussion").

**Time Dependence of Arotinoid-promoted Changes**—To assess the time dependence of the changes in keratin mRNA levels, human fetal tracheal keratinocytes were treated with 2 × 10⁻⁶ M arotinoid Ro 13-6298 for various periods of time. Messenger RNA isolated at 0, 1, 2, 3, 4, and 5 days was electrophoresed on a 1.25% agarose gel containing formaldehyde, transferred to Biodyne A membrane, and hybridized with plasmids pK5-1, pK6-1, pK19-1, or pA-1. Autoradiographs were scanned with a laser densitometer and plotted relative to the signal observed in control cultures (Fig. 5). Message levels for keratins 5 and 6 decrease with a similar time course to the increase in mRNA encoding keratin 19. The level of actin mRNA is stable. Although very little
message corresponding to pK13-1 was detected in tracheal
strain Tr-2, message corresponding to pK13-1 increased in a
coordinate manner to message hybridizing to pK19-1 (40-kDa
keratin) in other tracheal strains and in epidermal keratino-
cyte strain Epi-AR. Suppression of mRNA corresponding to
pK5-1 and pK6-1 was also observed in these strains.

DISCUSSION

Retinoids have been shown to increase the levels of keratins
13 and 19, decrease the level of keratin 1, produce morpholog-
ical changes, and suppress envelope formation in human
Fetal Tracheals (Tr-2) mRNA

![Fetal Tracheals (Tr-2) mRNA](image)

FIG. 3. Arotinoid modulation of keratin mRNA levels in
fetal tracheal keratinocytes. Human fetal tracheal keratinocytes
were cultured in vitamin A-free medium and treated with various
concentrations of arotinoid Ro 13-6285 for 6 days. The mRNA was
isolated and blotted to Biodyne A membrane. The blots were hybrid-
ized with plasmids pK5-1 (58 kDa), pK6-1 (56 kDa), pK19-1 (40
kDa), or pA-1 (actin), each nick translated to 10^6 dpm/µg of DNA.
The blots were then washed and exposed on preflashed x-ray film.
The 40 kDa, 56 kDa, and 58 kDa designations identify the keratins
corresponding to the mRNA encoded by each cDNA. The numbers
in parentheses are the keratin designations from the catalog of Moll
et al., 1982. The message sizes are actin, 2100; 50 kDa, 1580; 56 kDa
and 58 kDa, 2350 bases in close agreement with previous determina-
tions (Eckert and Green, 1984).

![Fetal Tracheals (Tr-2) Poly(A)^{+} RNA](image)

FIG. 4. Quantitation of retinoid effects on keratin and actin message
levels in tracheal keratinocytes. Autoradiographs of the RNA blots shown
in Fig. 5 were scanned by laser densitom-
etry and the results are expressed as per-
cent of control. The label on each curve corresponds to the label on each band in
Fig. 5.
It has previously been shown that the suppression of envelope formation is more sensitive to the retinoid than the keratin responses described here (Gilfix and Green, 1984). This suggests that in part, mechanistically distinct from the route through which keratin levels are modulated.

It appears that a significant component of the control of keratin gene activation or suppression by vitamin A involves control of the mRNA level. This is supported by data, Figs. 2 and 4, which demonstrate that changes in mRNA content parallel changes in protein content; therefore, control of keratin gene expression probably results from a change in mRNA synthesis and/or degradation. An exception is the relatively low level of mRNA coding for keratins 5 and 6 relative to the level of keratin proteins 5 and 6 present in control cultures (compare Figs. 2 and 4). It is clear that there is a slight stimulation of the level of mRNA encoding keratins 5 and 6 at low levels of retinoid (Fig. 3 and 4). It is possible that the lower level of mRNA for these keratins in control cultures is due to a vitamin A-deficient state. The most likely reason why the level of the corresponding keratins is not reduced is that they are accumulating in metabolically inactive cells residing in the upper stratified layers of the control cultures. Cells in these differentiated layers accumulate a disulfide-stabilized matrix of keratin proteins (Green, 1977), but do not contain RNA (Morrissey and Green, 1979).

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**Fig. 5.** Time course of changes in keratin mRNA content in human fetal tracheal keratinocytes.

Cells of human fetal tracheal keratinocyte strain Tr-2 were cultured in vitamin A-free medium as described in the legend to Fig. 1. One day prior to confluence, retinoid Ro 1-26298 was added at 20 nm and culture was continued for the number of days indicated. Fresh medium plus retinoid was added every 2-3 days. At 0, 1, 2, 3, 4, and 5 days, two 50-cm² dishes were harvested for preparation of poly(A)⁺ RNA. The mRNA (8 μg/lane) was fractionated on a 1.25% agarose gel containing formaldehyde and transferred to Biodyne A. Blots were hybridized with plasmids pK19-1 (40 kDa), pK5-1 (58 kDa), pK6-1 (56 kDa), and PA-1 (actin) each nick-translated to 10¹⁰ dpm/pg of DNA. Autoradiographs of the blots were scanned with a laser densitometer and the results were plotted as a percent of the signal observed in control cultures. The no retinoid signal (0 day) was identical whether the cells from this group were harvested on the first or last day of the time course.

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