INVESTIGATIVE REPORT

Retinoids Reduce Formation of Keratin Aggregates in Heat-stressed Immortalized Keratinocytes from an Epidermolytic Ichthyosis Patient with a KRT10 Mutation*

Hao LI and Hans TÖRMÄ
Department of Medical Sciences/Dermatology, Uppsala University, Uppsala, Sweden

Epidermolytic ichthyosis (EI) is an autosomal dominant epidermal skin fragility disorder caused by mutations in keratin 1 and 10 (K1 and K10) genes. Mutated keratins form characteristic aggregates in vivo and in vitro. Some patients benefit from retinoid therapy, although the mechanism is not fully understood. Our aim was to demonstrate whether retinoids affect the formation of keratin aggregates in immortalized EI cells in vitro. EI keratinocytes were seeded on cover slips, pre-treated or not with retinoids, heat-stressed, and keratin aggregate formation monitored. K10 aggregates were detected in 5% of cells in the resting state, whereas heat stress increased this proportion to 25%. When cells were pre-incubated with all-trans-retinoic acid (ATRA) or retinoic acid receptor (RAR)-α agonists the aggregates decreased in a dose-dependent manner. Furthermore, ATRA decreased the KRT10 transcripts 200-fold as well as diminished the ratio of mutant to wild-type transcripts from 0.41 to 0.35, thus providing a plausible rational for retinoid therapy of EI due to K10 mutations. Key words: keratin mutations; retinoic acid receptors; keratin aggregates; cleft formation; epidermolytic hyperkeratosis.

(Accepted February 29, 2012.)


Hans Törmä, Department of Medical Sciences, Dermatology and Venereology, University Hospital, Uppsala University, SE-751 85 Uppsala, Sweden. E-mail: hans.torma@medsci.uu.se

*The Editor-in-Chief has not had responsibility for this article (which originates from his own department). The article has been handled fully by one of the Associate Editors, who also made the decision to accept it.

MATERIALS AND METHODS

Cell culture of immortalized epidermolytic ichthyosis keratinocytes

The polyclonal HPV16 E6/E7-immortalized keratinocytes (EH21) derived from a male patient presenting a moderate pheno-
Heat stress assay and pre-treatment with retinoic acid receptor agonists

Cells seeded on 12-mm glass cover slips in 24-well tissue culture plates were induced to differentiate. Retinoic acid receptor agonists and antagonists (from Sigma-Aldrich, Stockholm, Sweden: pan-RAR agonist all-trans-retinoic acid (ATRA); from Galderma R&D, Biot, France: RARβ agonist CD235 (Am580) and CD2081, RARγ agonist CD437, pan-RAR agonist CD367, pan-retinoid X receptor (RXR) agonist CD2425 (AGN191701), a retinoid with anti AP-1 activity (CD2409) pan-RAR-antagonist CD3106, and RARα antagonist CD2503), as well as the proteasome inhibitor MG132 (Sigma-Aldrich) were dissolved in dimethyl sulphoxide (DMSO) and added to the cells for 4–24 h. Subsequently, keratinocytes were subjected to a heat stress assay, performed as described elsewhere (14). Briefly, cells growing on glass cover slips in 24-well plates were placed in a pre-heated closed water bath at 43°C (the measured temperature inside the wells) for 30 min and subsequently allowed to recover in the CO2 incubator for 15 min.

Immunocytochemistry and immunofluorescence microscopy

The cells were harvested by 2 brief washes with phosphate-buffered saline (PBS) buffer solution and fixed in ice-cold methanol/acetic acid 1:1 for 5 min at –20°C. Unspecific binding epitopes were blocked with blocking sniper solution (Biocare Medical, Concord, CA, USA). Specific primary monoclonal antibodies recognizing K10 (VIK-10; Santa Cruz Biotechnologies, Santa Cruz, CA, USA) were added in a dilution of 1:200 for 1 h at +37°C. Alexa fluorophore-conjugated secondary antibodies (Invitrogen AB) were used for detection. The slides were visualized and analysed for staining by immunofluorescence using a digital Zeiss Axiohot (F1 imager) microscope (Carl Zeiss AB, Stockholm, Sweden) equipped with an Apo-Tome, Axiocam MRm digital camera and Axiovision software. In each cover slip the total number of cells expressing K10, together with the number of cells showing keratin aggregates, was evaluated in 30 viewing fields (40x magnification) and analysed as described (14, 22). A mean of > 500 differentiated EH21 cells were analysed per treatment.

In situ proximity ligation assay

Using the Duolink proximity ligation assay (PLA) kit and Duolink detection kit (Olink AB, Uppsala, Sweden) we tested whether K10 co-localizes with the C terminus of Hsc70-70 interacting protein (CHIP), ubiquitin or phosphorylated p38 in heat-stressed EI keratinocytes. The signals are formed by ligation and circulation of the proximity-bound probes, and synthesis of DNA by rolling circle amplification of the circular DNA template, followed by hybridization of fluorescence-labelled oligonucleotides to the amplified DNA (23). A positive co-localization is detected as a “red blob”. Maximum distance between the 2 proteins is < 30 nm (24). Briefly, cells on cover slips were fixed in methanol/acetic acid and exposed to blocking solution for 30 min at 37°C. Mouse monoclonal anti-K10 (1:200; Santa Cruz Biotechnologies) and rabbit polyclonal anti-CHIP (1:200; GeneTex, Irvine, CA, USA), rabbit polyclonal anti-ubiquitin (1:200; GeneTex) or rabbit polyclonal anti-p38 (1:100; Cell Signaling, Danvers, MA, USA) were added to the cover slips, followed by incubation at 4°C overnight. Anti-mouse probe PLUS and anti-rabbit probe MINUS were diluted 1:10 and incubated at 37°C for 2 h. Antibodies and probes were diluted in antibody diluent, provided in the Duolink detection kit. Hybridization, ligation, and the amplification and detection step were performed according to the manufacturers’ instructions. Cover slips were mounted on slides with Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) (Vector Labs, Burlingame, USA) before being examined.

Analyses of allele-specific mRNA expression

Total KRT10 expression in complementary DNA (cDNA) from retinoid-exposed cells was compared using the comparative cycle threshold (CT) (2ΔΔCT) method, based on the assumption that the PCR amplification efficiencies of the target and the reference gene (PPIA) are similar to each other and approximately close to 100% (25). The expression of KRT10 was normalized to the reference gene.

Allele-specific expression was analysed by kinetic PCR. Two parallel PCR reactions with primer pairs amplifying the wild-type and the mutated alleles were performed using Fast SYBR Green Master Mix (Applied Biosystems, Stockholm, Sweden) as previously described (14). Triplicate reactions were set up, and placed in a 7500 Fast (Applied Biosystems) programmed as follows: 9 min at 95°C followed by 40 cycles of 15 s at 95°C and 60 s at 59°C. The Ct was set to similar values for the 2 parallel reactions and the difference in Ct values between the reactions was proportional to the difference in frequency between the 2 alleles. The relative frequency of the mutated allele was determined on the basis of the ΔCt using the equation: frequency of allele = (2^ΔCt – 1) / 2ΔCt.

Statistical analysis

Data analysis was performed by one-way analysis of variance (ANOVA) test with Bonferroni’s multiple comparison test using GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA). Results are expressed as means ± standard deviation (SD). Statistical significance was denoted * if p < 0.05, ** if p < 0.01 and *** if p < 0.001.

RESULTS

Keratin aggregation is reduced by all-trans-retinoic acid via retinoic acid receptor activation but without involving repression of activator protein-1

Calcium-induced differentiation of the immortalized EI keratinocytes EH21 resulted in expression of K10 (Fig. 1a). In response to heat stress (43°C for 30 min), aggregates consisting of mutant K10 appeared in the periphery of the K1F network of EI keratinocytes that were stained by a K10 antibody (Fig. 1b and c). The aggregates markedly reduced within 4 h after termination of heat stress (data not shown). Since the patient studied here benefited from oral acitretin therapy (patient #9 in Virtanen et al. (2001) (8)), we examined whether retinoids interfere with the formation of keratin aggregates.

The cells were pre-treated for 24 h with 2 different concentrations (0.1 and 1 μM) of ATRA prior to heat stress and reduced keratin aggregation was noticed...
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However, the fraction of cells stained with the keratin 10 antibody was reduced (Fig. S1; available from http://www.medicaljournals.se/acta/content/?doi=10.2340/00015555-1368). Keratin clumping was evaluated in a semi-quantitative manner (see Materials and Methods). At resting state, 2–5% of the EH21 cells showed keratin aggregates. Upon heat stress, this percentage increased to 25%. When pre-exposing the cells to ATRA at 0.1 µM and 1 µM the cells containing keratin aggregates decreased to 10% and 7%, respectively (Fig. 2a). To examine whether the effect of ATRA is a result of RAR activation, a pan-RAR antagonist, CD3106, was added together with ATRA at pre-incubation. The addition of CD3106 did not significantly affect the reduction in keratin aggregate formation obtained by addition of ATRA (Fig. 2b).

We also examined whether the protective effect of ATRA is a result of its anti-AP-1 activity. A synthetic retinoid with anti-AP-1 effect, but devoid of RAR activation properties (CD2409) was added to the cells together with ATRA. We found that CD2409 alone had no effect on keratin aggregate formation, and no beneficial effect was observed when combined with ATRA (Fig. 2b).

Retinoic acid receptor-α agonists, but not -β or -γ agonists, affect keratin aggregation

We next examined the effect of specific RAR subtype agonists. The keratin aggregate formation was analysed in cells pre-exposed to various synthetic retinoids with affinity for specific RARs. The RARα agonist CD336 at 1 µM reduced the number of aggregate-containing cells, while the same concentration of retinol (the precursor of ATRA), CD2314 (a RARβ agonist) and CD437 (a RARγ agonist) had no effect (Fig. 2c). To confirm the selectivity of this inhibition, we examined the effects of a second RARα agonist (CD2081). This compound decreased keratin aggregates with similar potency as CD336 in a dose-dependent manner (Fig. 2d). Furthermore, addition of CD2503 (a selective RARα antagonist) showed a tendency to reduce the effect of CD336 (data not shown).

Fig. 1. Keratin 10 (K10) aggregates appear after heat stress in differentiated epidermolytic ichthyosis (EI) keratinocytes. Keratin aggregates were analysed in EI cells by immunofluorescence in unstressed cells (a) and in stressed cells (b, c and d). In unstressed EI cells the filament structure appeared to be normal (a), whereas in heat-stressed EI cells, i.e. after a 30-min heat stress period followed by a 15-min recovery period, aggregates (arrows) were formed on the edge and the border between cells (b and c), while pre-incubation with 1 µM retinoic acid reduced the keratin aggregation (d). Scale bars denote 20 µm.

Fig. 2. All-trans-retinoic acid (ATRA) counteracts the formation of keratin aggregates in heat-stressed cells, but this is not due to its anti-activator protein 1 (anti-AP-1) activity. Epidermolytic ichthyosis (EI) keratinocytes were exposed to ATRA or vehicle (a) ethanol or (b and c) dimethyl sulphoxide for 24 h prior to heat stress. Keratin aggregates were analysed after a 30-min heat stress period followed by a 15-min recovery period. In untreated EI keratinocytes, 2% of the cells expressed keratin aggregates, which increased to 25% upon heat stress. (a) The addition of ATRA reduced the formation of keratin aggregates in heat-stressed cells in a dose-dependent manner. (b) When treated with 0.1 µM ATRA together with 1 µM CD3106 (a pan-retinoic acid receptor (RAR) antagonist), they revealed a tendency of higher keratin aggregate exhibition. (b) Pre-treatment with 0.1 µM CD2409 (a compound with anti-AP-1 activity but lacking RAR activation activity) did not affect keratin aggregate formation. Agonists of RARα, RARβ and RARγ at 0.1 µM were added to the cells 24 h prior to heat stress. (c) The RARα agonist CD336 decreased the heat stress-induced keratin aggregation, while retinol (an ATRA precursor), CD2314 (RARβ) and CD437 (RARγ) had no positive effect on keratin aggregation. (d) A second RARα agonist, CD2081, cytoskeletal-protective effects at 0.1 and 1 µM, respectively. At least 500 cells being keratin 10+ were counted in each treatment group. The data are expressed as aggregate-containing cells/keratin 10+ cells (mean ± standard deviation, n = 3), NS: non-significant, *p < 0.05, **p < 0.01 and ***p < 0.001.
**Effect of all-trans-retinoic acid treatment on keratin 10 gene expression**

To further examine the mechanism of retinoid-induced reduction in keratin aggregates, the mRNA expression of KRT10 and the ratio of mutant to wild-type allele were analysed. The mRNA expression of KRT10 (including wild-type and mutant transcripts) decreased 200-fold upon exposure to 1 μM ATRA for 24 h (Fig. 3a). In untreated EI cells the expression ratio of the mutant allele was 0.48, which decreased to 0.41 in DMSO-treated EI cells. By contrast, in cells treated with ATRA for 24 h and 72 h the ratio of the mutant KRT10 allele decreased to 0.35 and 0.38, respectively (Fig. 3b).

**Keratin 10 aggregations after heat stress in the EH21 cell line is increased by a proteasome inhibitor**

The degradation of basal keratins (K5/K14) in epidermal keratinocytes (15) and of other keratins in liver cells is supposed to involve proteasome-mediated degradation of the ubiquitylated, mutated keratin (17). However, it has not been studied whether the suprabasal keratins K1 and K10 are degraded by the same mechanism. We therefore treated our cells with the proteasome inhibitor MG132 to evaluate keratin degradation in this *in vitro* system. The K10-positive cells exhibiting aggregates increased from 25% in the vehicle-treated controls to 35% in the MG132-treated cells (Fig. S2; available from: http://www.medicaljournals.se/acta/content/?doi=10.2340/00015555-1368).

**Co-localizations of K10 with C terminus of Hsc70-interacting protein, ubiquitin and mitogen-activated protein kinase p38 are not associated with K10 aggregates**

The degradation of wild-type and mutant keratins has previously been shown to involve the chaperone-dependent E3 ubiquitin ligase CHIP, mitogen-activated protein (MAP) kinase (MAPK) p38 and Hsp70 in a proteasome-mediated mechanism (18, 26). *In situ* PLA clearly showed co-localization of K10 with CHIP, ubiquitin and p-p38 in the cytoplasm (Fig. S3; available from: http://www.medicaljournals.se/acta/content/?doi=10.2340/00015555-1368). However, the co-localization of these proteins (shown by *in situ* PLA) was not enriched at the site of keratin aggregates (shown by K10 staining), nor did heat stress affect the co-localization pattern (Fig. S3).

**DISCUSSION**

Immortalized cell lines represent invaluable tools in the early stages of devising therapeutic strategies for genetic disorders, and several studies have demonstrated proof of principle for the rescue of such cells by genetic manipulation. Only 3 reports have described the establishment of immortalized cells from EI patients (13, 14, 27). With the exception of a positive effect by trimethylamine oxide (TMAO) and 4-phenylbutyrate (4-PBA) in the cell line EH31 (14), these immortalized cells have not been used further for establishing novel therapies of EI.

Retinoids have been used as a therapy for EI patients (8) and previous reports have shown that retinoids affect the expression of certain keratins *in vivo* (28). KRT2 is markedly down-regulated by ATRA and this could explain why EI patients with KRT1 mutations tolerate retinoids less well than those with KRT10 mutations (8), because K2 can partially compensate for the mutated K1. In a recent study it was found that RARα agonists were the most potent modulators of keratin expression in organotypic skin (29). Consequently, we examined the effect of isoform-specific retinoid receptor ligands on keratin aggregation in a cell line established from a patient with the relatively common p.Arg156Gly mutation in K10 and found that RARα agonists potentially affected the aggregation, which correlated with the effect on keratin expression in organotypic epidermis (29). The used immortalized EI cells are able to differentiate upon modulation of growth conditions and they form a stratified epidermis, although not fully differentiated, in organotypic cultures (14). Whether the lack of a complete differentiation programme affects the present results is not known. It also remains to be studied whether these effects can be reproduced in cells from EI patients with other KRT10 (and KRT7) mutations. However, from a clinical perspective, RARα
agonists are probably preferable to RARβ and RARY agonists, because skin irritation due to retinoids, is mediated by RARβ and RARY (30, 31).

It is not clear whether classic retinoic acid response element (RARE) activation is involved in retinoid-induced inhibition of keratin aggregate formation, since in our study pan-RAR and RARα antagonists only partially reduced the effects of the agonists. In lamellar ichthyosis patients, liarozole, a retinoic acid metabolism blocking agent (RAMBA), has been reported to mildly affect epidermal expression of retinoid-regulated genes, suggesting a retinoid-mediated effect of this drug (32). By contrast, in EI cells liarozole and rambazole did not affect keratin aggregation at 5 μM (unpublished observation), which speaks against a pure RARE-mediated effect. This discrepancy may also be due to different effects of RAMBAs in vivo and in vitro.

Retinoic acid treatment reduced the overall expression of KRT10 200-fold, but there was also a slight reduction in the ratio of mutant to wild-type mRNA. It is not known if this is enough to mitigate keratin aggregation in our EI cells, but it has previously been shown that a 2–3-fold reduction in the expression of the wild-type protein is enough to suppress the phenotype in mouse models of EI (33).

Somewhat to our surprise there was a discrepancy between the protein and mRNA levels of K10 (see Fig. 3 and Fig. S1). The mRNA levels were markedly reduced, whereas the protein was still expressed. This could be explained by the fact that the half-life of keratin filament has been reported to be in the range of 8–12 h (34, 35). However, although degradation and de novo biosynthesis is important, it seems that keratin recycling is at least as important. Keratin precursor particles integrate into the filament network in the periphery, and move toward the nucleus and form bundles. Some of them form a stable perinuclear network, which is anchored to desmosomes and hemidesmosomes. Others disassemble into soluble oligomers which are recycled and thus once again become available for integration into precursor particles in the periphery of the cells (36, 37). This opens up a hypothetical mechanism by which the formation of stress-induced aggregates results in degradation of the mutated keratins in direct relation to the aggregates, and that this removal of mutated keratins occurs repeatedly when keratin oligomers are recycled. If so, wild-type keratin would be enriched in the cell and thus favour formation of stable keratin filaments. During retinoid treatment this situation probably occurs more rapidly due to reduced expression of the mutated (and wild-type) proteins.

It has been shown recently that topical retinoic acid covalently binds to K10 in mouse skin (38), which raises the intriguing possibility that retinoylation of keratins may be involved in the retinoid-induced reduction of aggregation by yet unknown mechanisms. HaCaT cells, transiently transfected with a mutant pEGFP-K10 R156G construct, also show increased keratin aggregation upon heat-stress (Li et al., unpublished observation). Since ATRA had no effect on keratin aggregation in this model of EI (Li et al., unpublished observation), a direct interaction between retinoids and keratin protein is unlikely. Perhaps the above-mentioned mechanisms could be better explored by treating mice with tamoxifen-inducible K10 mutations, as a model of the EI disease (33), with RARα agonists and other retinoids.

As previously shown for K5 aggregates in cells from EBS patients (15), we also found that K10 aggregation is increased by an inhibitor of proteasome-mediated degradation in EI cells. It was also shown previously, by using in situ PLA, that K1 co-localizes with hsp70 in heat-stressed EI cells (14). Here we also demonstrate a co-localization of K10 with several members of the proteasome-ubiquitin-mediated degradation, e.g. CHIP, ubiquitin and p-p38. Unexpectedly, this co-localization in most cases did not overlap with the keratin aggregates, suggesting that other mechanisms may be involved in the elimination of the keratin aggregates. Although our results speak against ubiquitylation of keratins in the aggregates, it cannot be excluded that the detection of co-localization by in situ PLA technique is prevented by epitope masking.

In conclusion, we have used an immortalized keratinocyte cell line, EH21, from an EI patient with a KRT10 mutation to examine the effects of retinoids on keratin aggregate formation. This study shows that retinoids, especially RARα agonists, rescue the filament structure of EI keratinocytes. The mechanism of retinoid action is still obscure, but modification of expression of suprabasal keratins appears to be involved. Modulation of the ratio of wild-type to mutant protein may also be involved.

ACKNOWLEDGEMENTS

We would like to thank Professor Anders Vahlquist for valuable discussions and Mrs Inger Pihl-Lundin for skilful technical assistance. This study was supported by The Welander and Finsen Foundations, Foundation for Ichthyosis & Related Skin Types, and the Uppsala County Council.

The authors declare no conflicts of interest.

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