Retinoic Acid Increases the Expression of p53 and Proapoptotic Caspases and Sensitizes Keratinocytes to Apoptosis: A Possible Explanation for Tumor Preventive Action of Retinoids

Paul Mrass,1 Michael Rendl,1 Michael Mildner,1 Florian Gruber,1 Barbara Lengauer,1 Claudia Ballaun,1 Leopold Eckhart,1 and Erwin Tschachler1,2

1Department of Dermatology, Medical University of Vienna, Vienna, Austria; and 2Centre de Recherches et d’Investigation Epidermiques et Sensorielles, Neuilly, France

ABSTRACT

Retinoids influence growth and differentiation of keratinocytes (KCs) and are widely used for the management of skin diseases and for prevention of nonmelanoma skin cancer (NMSC) in predisposed patients. Here we investigated the effect of all-trans-retinoic acid (ATRA) on KC apoptosis. When KCs were cultured in confluent monolayers for several days, they acquired resistance against UVB-induced apoptosis. In contrast, when the cells were treated with 1 μmol/L ATRA for 6 days and subsequently irradiated with different doses of UVB, they underwent massive apoptosis as assessed by morphology, expression of activated caspase-3, and DNA fragmentation. The same effect was observed when doxorubicin was used instead of UVB. Analysis by real-time PCR and Western blot revealed that ATRA treatment strongly increased the mRNA and protein expression of p53 and caspase-3, -6, -7, and -9, which are key regulators of apoptosis. UVB irradiation of ATRA-treated cells but not of control cells led to the accumulation of p53 protein and of its target gene Noxa. Inhibition of p53 and caspases with α-piﬁthrin and z-Val-Ala-Asp-ﬂuoromethyl ketone, respectively, blocked UVB- and doxorubicin-induced apoptosis in ATRA-treated KCs. Analogous to the observed ATRA effects in monolayer cultures, in vitro-generated organotypic skin cultures reacted with up-regulation of p53 and proapoptotic caspases and display increased sensitivity to UVB-induced apoptosis. The ability of retinoic acid to regulate the expression of proapoptotic genes and to sensitize KCs to apoptosis may play a role in their prevention of NMSC in transplant patients and patients with DNA-repair deﬁciencies.

INTRODUCTION

Keratinocytes are powerful modulators of cell proliferation, differentiation, and apoptosis and a mainstay therapy for a wide range of dermatologic diseases (1). In addition to their use in psoriasis and acne therapy (2), retinoids effectively prevent the development of nonmelanoma skin cancer (NMSC) in patients with defects of DNA repair, such as xeroderma pigmentosum (3), and in transplant patients receiving immune-suppressive therapies (4). Despite their use for many years, their mode of action in the prevention of NMSC still is unclear (5). Contrary to what would be expected of an anticancer drug, retinoids affect proliferation of keratinocytes (KCs) only moderately (6) and even induce KC proliferation when applied topically (7). In contrast to KCs, leukemic cell lines and cells derived from patients with acute promyelocytic leukemia differentiate and can undergo apoptosis when exposed to retinoids (1). Apoptosis in this setting has been attributed to the induction of tumor necrosis factor-related apoptosis-inducing ligand expression by retinoids and its paracrine effect on tumor cells (1).

In normal cells, DNA damage leads to the activation of protective mechanisms (e.g., DNA repair) that prevent the accumulation of mutations and emergence of neoplastic cells. An important step in DNA repair is a cell-cycle arrest mediated by the tumor suppressor protein p53 (8). The occurrence of DNA lesions as a consequence of UVB irradiation or treatment with cytotoxic agents, such as doxorubicin, leads to post-translational modifications of p53 (9), resulting in p53 accumulation and increased activity (10) and subsequently in transcriptional activation of p21 (11) and GADD45 (12), leading to cell-cycle arrest. If DNA damage is irreparable, p53 action leads to apoptosis (8), which is, at least in part, mediated by transcriptional up-regulation of proapoptotic members of the bcl-2 family, such as Bax (13), Noxa (14), and PUMA (15). In contrast, MDM2, another p53-dependent gene, inhibits p53 activity (9), thereby establishing an autoregulatory negative feedback loop (16). Mutations of p53, as in Li Fraumeni syndrome, and deletion of p53 in experimental animals predispose to the development of neoplastic tumors (17, 18). Overexpression of p53 by itself does not induce apoptosis; rather, it lowers the apoptotic threshold in response to DNA damage (19, 20). Importantly, experimental evidence indicates that the proapoptotic activity and the chemopreventive effect of retinoids depend on the presence of wild-type p53 (21–23). However, p53 by itself is not sufficient for apoptosis induction by retinoids. The synthetic retinoid CD437 is able to induce apoptosis in human lung cancer cells only when they contain functional p53, whereas primary lung epithelial cells are not susceptible (21, 24).

Caspases are cysteine-dependent aspartic proteases that are divided into proapoptotic (caspase-2, -3, -6, -7, -8, -9, and -10), and proinflammatory (caspase-1, -4, and -5) members (25). They are expressed as inactive proenzymes that are activated by proteolytic cleavage into large and small subunits and form tetrameric complexes (25). Pro-apoptotic caspases are grouped into initiator (caspase-8, -9, and -10) and executor (caspase-3, -6, and -7) caspases (25). Although caspase activity is regulated primarily at the post-translational level, overexpression of caspases sensitizes cells for apoptosis (26, 27).

We recently have shown that retinoids down-regulate the epidermis-specific caspase-14, which appears not to be involved in classical apoptosis but is thought to play a role during terminal KC differentiation (28). In contrast to caspase-14, the proapoptotic caspase-3 was up-regulated, and caspase-8 expression remained unchanged, indicating differential regulation of caspases by retinoids (29). Furthermore, we observed spontaneous retinoid-induced apoptosis of KCs cultured under differentiating conditions (29). In the present report, we further investigate this observation and show that retinoids induce the expression of p53 and several proapoptotic caspases, leading to increased sensitivity to UVB- and doxorubicin-induced apoptosis.

MATERIALS AND METHODS

Antibodies and Reagents. Mouse monoclonal antibodies against the following proteins were used in the present study: caspase-3 (Santa Cruz Biotechnology, Santa Cruz, CA), caspase-6 (Medical & Biological Laboratories, Nagoya, Japan), caspase-7 (NeoMarker, Fremont, CA), caspase-9 (Oncogene, Boston, MA), p53 (Immunotech, Marseille, France), and p63 (NeoMarker). A rabbit antisera directed against cleaved caspase-3 (R&D Systems, Minnea-
oligo (MWG Biotech, Ebersberg, Germany): caspase-3_f (5'-aggaagagcatcaagttgac-3'), caspase-3_r (5'-atcagccgactaattgagagca-3'), caspase-6_f (5'-cattagccgagcacaataa-3'), caspase-6_r (5'-aatcagccgactaattgagagca-3'), caspase-7_f (5'-gggagccggagcacaataa-3'), caspase-7_r (5'-aatcagccgactaattgagagca-3'), caspase-9_f (5'-aatcagccgactaattgagagca-3'), caspase-9_r (5'-aatcagccgactaattgagagca-3'), p53_f (5'-gcgcacagaggaagagaatc-3'), p53_r (5'-tctcggcacaaaggac-3'), MDM2_f (5'-tggtagcgtgagcttggttc-3'), MDM2_r (5'-ctcggagcttgcgtgctt-3'), Noxa_f (5'-ctcggagcttgcgtgctt-3'), and Noxa_r (5'-tctcggagcttgcgtgctt-3'). In all of the experiments, the results were normalized to the expression of the housekeeping gene GAPDH (GenBank accession no. X01381) using the method described by Pfaffl et al. (32). The following primers were generated using a mathematical model described by Pfaffl et al. (32). The following primers were generated (MWG Biotech, Ebersberg, Germany): caspase-3_f (5'-aggaagagcatcaagttgagac-3'), caspase-3_r (5'-atcagccgactaattgagagca-3'), caspase-6_f (5'-cattagccgagcacaataa-3'), caspase-6_r (5'-aatcagccgactaattgagagca-3'), caspase-7_f (5'-gggagccggagcacaataa-3'), caspase-7_r (5'-aatcagccgactaattgagagca-3'), caspase-9_f (5'-aatcagccgactaattgagagca-3'), caspase-9_r (5'-aatcagccgactaattgagagca-3'), p53_f (5'-gcgcacagaggaagagaatc-3'), p53_r (5'-tctcggcacaaaggac-3'), MDM2_f (5'-tggtagcgtgagcttggttc-3'), MDM2_r (5'-ctcggagcttgcgtgctt-3'), Noxa_f (5'-ctcggagcttgcgtgctt-3'), and Noxa_r (5'-tctcggagcttgcgtgctt-3'). In all of the experiments, the results were normalized to the expression of the housekeeping gene GAPDH (GenBank accession no. X01381) using the method described by Pfaffl et al. (32).
gene 5-aminolevulinic acid synthase, using the following primers: alas_f (5'-ccactggaagagctgtgtga-3') and alas_r (5' -accctccaacacaacaaang-3').

RESULTS

ATRA Sensitizes KCs to UVB-Induced Apoptosis. When normal human epidermal KCs are grown in SE culture and are maintained for 6 days at the air-liquid interface, a multilayered epidermis is formed (ref. 30; Fig. 1A, a). Addition of 1 μmol/L ATRA during the 6-day differentiation period, a concentration which is achieved in the course of topical retinoid therapy (33), leads to epidermal thickening with impaired stratification and cornocyte layer formation (Fig. 1A, b) and to spontaneous apoptosis of 10 to 20% of KCs (ref. 29; Fig. 1A, d). To investigate whether KCs in ATRA-treated SEs also are less resistant to a standard proapoptotic stimulus, we irradiated SEs with UVB. In control SEs exposed to UVB, only few basal cells displayed apoptotic morphology with nuclear condensation and expression of activated caspase-3 (Fig. 1A, e and g). In contrast, a dramatic apoptotic effect on virtually all of the suprabasal KCs was observed in the ATRA-treated sample (Fig. 1A, f and h). These data show that in addition to increasing spontaneous KC apoptosis, ATRA treatment of SEs sensitizes KCs to UVB-induced apoptosis.

To study the basic mechanisms of ATRA on the apoptotic threshold observed in SEs, we went back to the simpler model of KCs grown in monolayer culture in the absence of fibroblasts. KCs grown under differentiating conditions (34) in 0.15 mmol/L calcium at confluence for several days were resistant to UVB-mediated apoptosis (Fig. 1B, e and f). In contrast, ATRA-treated KCs became highly apoptotic after UVB exposure as shown by morphology (Fig. 1B, g), active caspase-3 staining (Fig. 1B, h), and DNA fragmentation (Fig. 1C, Lanes 5 and 7).

ATRA Inhibits Down-Regulation of p53 and Increases the Expression of Caspases in Confluent KCs. When KCs were grown for 4 days at confluence, expression of p53 mRNA (Fig. 2A) and protein (Fig. 2B) strongly decreased. However, addition of ATRA to the culture medium completely inhibited down-regulation of p53 (Fig. 2A and B). The expression of p63, a p53 family member that has anti-apoptotic effects in KCs (36), also was reduced by retinoid treatment, although much less than that of p53 (Fig. 2B). The increased expression of p53 was paralleled by an induction of MDM2 (Fig. 2C) but not of the proapoptotic Noxa (Fig. 2C). These data show that ATRA treatment of KCs prevents confluence-associated down-regulation of the tumor suppressor protein p53 and leads to an increased transcription of MDM2, a p53 target gene that counterbalances p53 activity.

Next we focused on the effect of UVB irradiation on p53 expres-
sion and activity. After UV irradiation, mRNA levels for p53 mRNA remained elevated in ATRA-treated as compared with nontreated KCs but showed no further increase (Fig. 3A). In contrast, p53 protein levels strongly increased after UVB exposure of ATRA-treated but not of nontreated KCs (Fig. 3B), showing p53 protein accumulation. Simultaneously, a rapid decrease of MDM2 mRNA paralleled by a steep increase of Noxa, which was much more pronounced in ATRA-treated cells, was observed (Fig. 3C).

Next we analyzed caspase-3, -6, -7, and -9 mRNA and protein levels. Caspase mRNA expression was increased already after 2 days of ATRA treatment and reached a maximum induction after 6 days (Fig. 4A). Western blot analysis confirmed that caspase proteins also were strongly up-regulated after 4 and 6 days of the initiation of ATRA treatment (Fig. 4B).

**Chemical Inhibition of p53 or Caspases Prevents UV-Induced Apoptosis of ATRA-Treated KCs.** To test the involvement of p53 and caspases in the UVB-induced cell death, the pharmacologic inhibitors α-pifithrin (37) and z-VAD-fmk, respectively, were added to the KC cultures immediately after UVB treatment. Although not completely preserving normal cell morphology, z-VAD-fmk abolished detachment of KCs (Fig. 5A), blocked activation of caspase-3 (Fig. 5B), and prevented DNA fragmentation (Fig. 5C). α-Pifithrin also reduced cell detachment (Fig. 5A), caspase-3 activation, and chromatin condensation (Fig. 5B) and strongly but not completely reduced DNA fragmentation (Fig. 5C). These data confirm that p53 and caspases are involved in UVB-mediated apoptosis of confluent ATRA-treated KCs.

**ATRA-Treated KCs Are Highly Sensitive to Doxorubicin-Induced Apoptosis.** The chemotherapeutic agent doxorubicin induces DNA damage (38) and leads to programmed cell death by a p53-dependent mechanism (39), and we found that doxorubicin treatment of confluent KCs caused apoptosis only if they were treated with ATRA as confirmed by morphologic criteria (data not shown) and DNA fragmentation (Fig. 5D). The apoptotic effects of doxorubicin also could be blocked by the addition of z-VAD-fmk and α-pifithrin (Fig. 5D). These results show that ATRA treatment also sensitizes KCs to non–UVB-dependent, p53/caspase-mediated apoptosis.

**ATRA Treatment Leads to Up-Regulation of p53 and Proapoptotic Caspases in KCs of SEs.** To test whether similar observations could be found in the more in vivo-like SEs, we analyzed the effect of retinoids in SEs and found in analogy to monolayer culture that ATRA treatment led to an increased expression of p53 but not of p63 (Fig. 6A) and to enhanced expression of caspases (Fig. 6B). Immunofluorescence staining revealed that p53 was strongly expressed in all of the layers after ATRA treatment (Fig. 6C, d), whereas in controls it was only detectable in basal cells (Fig. 6C, c). In contrast,
ATRA INCREASES UVB-INDUCED APOPTOSIS IN KC

DISCUSSION

Retinoids have been a mainstay of therapy for psoriasis for several decades (2). Retinoids more recently have been successfully used to prevent NMSC in high-risk individuals, such as patients with xeroderma pigmentosum (3) and transplant recipients receiving immune suppression (4). The observation that activator protein acts similarly in primary KCs and in squamous cell carcinoma lines (41), argues against an exclusive role of this transcription factor. Here we show for the first time that ATRA treatment sensitizes KCs to apoptosis induced by UVB and by the DNA-damaging agent doxorubicin. This proapoptotic effect is accompanied by induction of the tumor suppressor protein p53 and the induction of proapoptotic caspases.

The fact that despite the strong up-regulation of p53 and proapoptotic caspases no spontaneous apoptosis of retinoid-treated KCs was observed in monolayer cultures is in agreement with the previous observations that even strong overexpression of caspases or p53 does not necessarily cause apoptosis but rather sensitizes cells to apoptotic cell death (19, 20). This notion is supported at the molecular level by the observation that in the absence of apoptotic stimuli, the p53 target gene MDM2 is up-regulated, whereas protein accumulation of p53 and strong up-regulation of the proapoptotic target gene Noxa were present only after additional exposure to UVB. The parallel increase of p53 and several caspases, together with the previous observation that p53 can increase the transcription of caspases (27, 42), suggests a connection between these two events. However, the temporal pattern (i.e., up-regulation of caspases before up-regulation of p53) shows that in addition to p53, other pathways may be responsible for the increased expression of caspases.

The involvement of p53 and caspases in apoptosis of retinoid-treated KCs could be supported by the reduction or abrogation of apoptosis using the p53 inhibitor α-pifithrin or the caspase-inhibitor z-VAD-fmk. Unfortunately, it recently became evident that α-pifithrin is not an exclusive inhibitor of p53 but also is able to inhibit heat shock and glucocorticoid signaling pathways (43). Therefore, we cannot formally exclude that a p53-independent proapoptotic effect of α-pifithrin may play an additional role in our experimental system. Because of the difficulty of efficiently transfecting highly confluent primary KCs and the loss of inhibitory activity 4 days after transfection, we could not achieve transfer of specific RNA interference at acceptable efficiency in our setting, thus precluding the use of this specific gene knockdown approach.

The effect of retinoid treatment on the sensitivity of KCs to UVB-induced apoptosis and p53 expression has been addressed previously by Li et al. (44), who reported that no influence on either parameter was found. We found that the proapoptotic effect of retinoids is evident only in confluent but not in preconfluent KCs. Because Li et al. (44) do not detail their protocol for KC cultures, we can only assume that the dissenting data may be caused by different culture conditions.

With regard to a possible causal relationship between p53 increase and suppression of KC differentiation, it was shown in the past that retinoids influence p53 expression in several cell types (45, 46) and that this was associated with a modulation of differentiation. Because ATRA inhibition of terminal KC differentiation and the maintenance/increase of p53 expression occurred simultaneously, we cannot rule out at the moment that a causal link exists between these two events. Arguments against such a role come from studies that show that neither mice with deletions of the p53 gene (18) nor mice overexpressing a mutant activated form of p53 (47) show epidermal abnormalities. Conversely, the finding that overexpression of p53 in human KCs in vitro delays expression of differentiation-associated proteins argues for a direct link (48). A way to address this question in our experimental setting would be the silencing of p53 in KCs (e.g., by using

---

1 Unpublished observation.
RNA interference). For the reasons outlined previously, this is currently not practicable in long-term cultures of human KCs; therefore, the question of the role of p53 will have to be addressed in future studies.

As to the practical implications of our findings, we suggest that the chemopreventive effect of retinoids may, at least in part, be mediated by the apoptosis-sensitizing effect of retinoids. Xeroderma pigmentosum patients (49) and immune-suppressed transplant recipients (50) have a highly increased risk for the development of NMSC on sun-exposed skin. Both groups have an increased load of DNA damage, which is the result of either defects in the DNA repair machinery (49) or of introduction of DNA damage by immunosuppressive regimens, such as azathioprine, which besides its immunosuppressive action favors the occurrence of mutations (51). In both settings, lowering of the apoptotic threshold by retinoids could favor the elimination of mutated cells. It is of interest that cyclopasin A, another immunosuppressive agent that is frequently used in transplant patients, has an opposite effect on KCs to the one we observed with ATRA (i.e., it suppresses apoptosis and p53-dependent repair DNA synthesis after UVB irradiation; Ref. 52). Reduced apoptosis of UVB-damaged cells caused by cyclopasin A medication would favor survival of cells harboring mutations. Therefore, the prevention of NMSC by retinoids in these patients may be explained by counterbalancing the antiapoptotic effects of cyclopasin A. Whereas retinoids effectively prevent the appearance of new primary tumors, the growth of already-existing tumors is unaffected (40). Because NMSCs frequently harbor p55 mutations (53, 54), the lack of action of retinoids in existing tumors may relate to the fact that functional p53 is no longer available for the effects of retinoids. However, p55 mutations cannot be detected in all of the NMSCs (53, 54). Therefore, the question as to whether tumor cell lines and NMSCs containing wild-type p53 are susceptible to retinoid-mediated sensitization to apoptosis will be an interesting subject for future studies. If this were the case, this might serve as a basis for the development of novel strategies for the management of skin cancers involving retinoids. As to the participation of other factors involved in the regulation of apoptosis by retinoids, regulation of c-Jun has been extensively studied in the past, and retinoids have been shown to abrogate UVB-mediated up-regulation of this transcription factor (55). In this context, it is interesting that mice with a liver-specific c-Jun inactivation showed increased p53 activity associated with increased apoptosis of hepatocytes (56). Intriguingly, early stages of tumor development were strongly inhibited in these mice, whereas the growth of advanced tumors was not suppressed (56). It would be interesting to determine whether inhibition of UVB-mediated c-Jun up-regulation by retinoids (55) has a causal relationship with increased expression and activation of p53 described here.

A further scenario in which alteration of apoptosis by retinoids may be important is their teratogenic potential (57). Tissue growth and involution by apoptosis are central mechanisms of organogenesis and morphogenesis during embryonic development (58). Teratogenicity of retinoids results in abnormalities of craniofacial, cardiovascular, and thymic morphogenesis, among others (57). Increased apoptosis associated with aberrations in pharyngeal, thymic, and cardiovascular development occurs in FGFB hypomorphic mice (59); therefore, it is conceivable that the teratogenic effects of retinoids are caused by their action on the apoptotic machinery (i.e., to alterations of p53 and caspase expression). However, in-depth studies will be necessary to substantiate such a link.

In conclusion, we have shown that ATRA treatment up-regulates central elements of the proapoptotic machinery in human primary KCs and sensitizes them to apoptosis induced by DNA damage. On the basis of our data, we suggest that sensitization to apoptosis by retinoids may play a role in the prevention of UV-associated NMSC in patients with immunosuppressive therapies and DNA-repair defects.

REFERENCES

32. Duell EA, Astrom A, Griffiths CE, Chambon P, Voorhees JJ. Human skin levels of retinoic acid and cytochrome P-450-derived 4-hydroxyretinoic acid after topical application of retinoic acid in vivo compared to concentrations required to stimulate
Retinoic Acid Increases the Expression of p53 and Proapoptotic Caspases and Sensitizes Keratinocytes to Apoptosis: A Possible Explanation for Tumor Preventive Action of Retinoids

Paul Mrass, Michael Rendl, Michael Mildner, et al.

*Cancer Res* 2004;64:6542-6548.

Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/64/18/6542

Cited articles
This article cites 56 articles, 27 of which you can access for free at:
http://cancerres.aacrjournals.org/content/64/18/6542.full.html#ref-list-1

Citing articles
This article has been cited by 14 HighWire-hosted articles. Access the articles at:
/content/64/18/6542.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.