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Activation of the Aryl Hydrocarbon Receptor by TCDD Inhibits Senescence: A Tumor Promoting Event?

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Abstract

Activation of the aryl hydrocarbon receptor (AHR) 1 by the agonist, 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD) has been shown to promote tumor formation in both liver and skin. In the liver, but not the skin, the AHR-mediated events that contribute to TCDD's tumor promoting activities have been studied in some detail and are thought to involve perturbation of cell fate processes. However, studies performed using cultured cells have often resulted in apparent contradictory results indicating that the impact of TCDD on cell fate processes may be cell context dependent. We and others have shown that in primary cultured keratinocytes TCDD increases post-confluent proliferation and increases late differentiation. Further, our studies performed in these cells indicate that TCDD can also inhibit culture-induced senescence. While senescence, a permanent cell cycle arrest, is emerging as an important process regulated by oncogenes and considered to be of therapeutic importance, its role with respect to TCDD/AHR mediated tumor promotion has not been fully considered. The intent of this article is to focus primarily on senescence as a cell process relevant to skin tumorigenesis and explore the idea that the inhibition of senescence by TCDD could be an important mechanism by which it may exert its tumor promoting effects in the skin.

Keywords

Aryl hydrocarbon receptor; keratinocytes; 2,3,7,8 tetrachlorodibenzo-p-dioxin; senescence; tumorigenesis

1. Introduction

In the human population, systemic exposure to high levels of TCDD results in chloracne, a non-inflammatory skin condition that involves hyperkeratinization, hyperplasia and metaplasia [1] that ultimately resolves into disfiguring scarring [2]. Both the National Toxicology Program and the International Agency for Research on Cancer have classified TCDD as a known human carcinogen [3]. This classification is based largely on studies performed using laboratory animals which have shown that TCDD promotes the formation of neoplastic lesions in the

¹Abbreviations: AHR, aryl hydrocarbon receptor; MNF, 3'-methoxy-4'-nitroflavone; TCDD, 2,3,7,8 tetrachlorodibenzo-p-dioxin; ROS, reactive oxygen species; TGF- β , transforming growth factor beta; UVB, ultraviolet light B

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liver, lung, oral mucosa and the skin [4,5]. The importance of understanding how environmental agents that activate the AHR contribute to skin cancers is illustrated by the observation that cancers caused by environmental agents frequently occur in tissues with the greatest surface exposure to these agents [6]. Using the well-studied mouse skin model, exposure to tumor promoters is typically associated with an induction of cell proliferation and hyperplasia, indicating that these cells have undergone a change in gene regulation that incurs a proliferative advantage [7,8]. In considering tumor promoting events that may occur in the skin versus the liver, it should also be kept in mind that tumors within differing somatic environments have evolved in a tissue-dependent manner [9]. A significant difference between the skin and liver is the ability of the skin to shed putative initiated cells by committing these cells to terminal differentiation and death. Thus, continued proliferation of epithelial cells within the skin tissue most likely represents a bypass of the death pathways; terminal differentiation, apoptosis and senescence.

2. Characteristics of Differentiation and Apoptosis

Proper homeostasis of the skin requires that the delicate balance between proliferation and cell death (terminal differentiation, apoptosis and senescence) be appropriately maintained [10, 11]. Given the extensive recent reviews of differentiation [10,12–15] and apoptosis [16–18], these processes will be described only in brief. Terminal differentiation (also referred to as cornification) of keratinocytes is a carefully orchestrated process in which the proliferative keratinocytes within the basal layer enter a non-replicative state, migrate upwards and progressively differentiate into the dead, flattened cells of the uppermost cornified layer [14]. The terminal differentiation process is relatively slow and typically encompasses a several week period. Key regulators of differentiation include growth factors, nuclear receptors and calcium [13]. In the laboratory setting, calcium is most frequently employed to induce differentiation of nearly confluent proliferating keratinocytes. Underlying molecular mediators of terminal differentiation involve those that suppress proliferation (i.e., p27, p21, Rb, etc.), are induced by loss of adhesion (i.e., β -1 integrins, Wnt) and activate the undifferentiated/differentiated switch (i.e., Notch and p63)[12,14]. It is important to note, however, that cell cycle inhibitors (i.e., p16, p21 and 27) are not thought to play a general role in controlling the onset of differentiation [15].

Keratinocytes harbor all of the components necessary for apoptosis [16]. In contrast to terminal differentiation, the apoptotic process is rapid and accomplished within a few hours. Characteristic cellular features of an apoptotic cell include membrane blebbing and nuclear fragmentation. Apoptosis can be induced by either intrinsic (mediated by the mitochondria) or extrinsic (mediated by death receptors) pathways [17]. Of the many stimuli of apoptosis in keratinocytes, perhaps the most important is UVB irradiation due to its known role in skin carcinogenesis [19]. Induction of apoptosis by UVB is initiated following DNA damage which activates the intrinsic apoptotic pathway and requires p53. Regardless of how apoptosis is initiated, the final stages of the apoptotic process involves activation of the executioner caspases and phagocytosis of the dying cell.

Comparison and contrast between apoptosis and terminal differentiation in keratinocytes reveal the following. Similarities include the fact that both processes are metabolically active and are accompanied by a number of dramatic cellular changes [10]. An additional feature shared by apoptosis and terminal differentiation is the indispensable role of protease activity [16]. While apoptosis, like terminal differentiation is controlled by extracellular matrix interactions, it does not appear to occur spontaneously in the epidermis when cells detach from the basement membrane. However, despite the presence of several common features, the majority of evidence indicates that apoptosis and terminal differentiation occur via distinct molecular pathways [10,11,16]. First, p53 plays a critical role in apoptosis, but not differentiation. Second,

caspsases are involved in both the initiation and execution of apoptosis, but do not appear to be involved in terminal keratinocyte differentiation. Finally, it appears that protecting the cell from apoptosis may be a necessary feature of successful terminal keratinocyte differentiation.

3. Characteristics of Senescence

The term “cellular senescence” was initially used to describe primary cells that during prolonged culture reached a critical point at which they were no longer able to divide [20]. When examined morphologically these cells were described as relatively large with a flattened appearance that proved to be capable of staining positive for β -galactosidase activity [21]. The increase in β -galactosidase activity is thought to arise from lysosomal β -galactosidase and reflect the increase in lysosomal biogenesis that typically occurs during senescence [20]. These senescent cells also accumulated foci of heterochromatin and acquired a granular appearance within the cytoplasm [22]. It is these morphological features, in addition to lack of membrane blebbing and chromatin condensation that distinguished senescent cells from those undergoing apoptosis. The senescent cells also appeared to have reached an irreversible quiescent state of G1 growth arrest wherein they were resistant to mitogenic and many apoptotic stimuli. Although senescence was initially thought to occur only in primary cultured cells, a number of cultured tumor cells have more recently been found to retain their ability to attain a senescence-arrest typically in G2 or S phase of the cell cycle.

Senescence can be initiated by either telomere-dependent or telomere-independent mechanisms. Telomere-dependent or replicative senescence involves a shortening of the telomeres that upon reaching a critical length trigger a DNA damage response and cell cycle arrest [20]. Telomere-independent premature senescence can be induced by agents that induce formation of reactive oxygen species (ROS), induce DNA damage, or that alter chromatin structure. Senescence can also be induced by certain cell culture conditions, overexpression of oncogenes and the presence of anti-proliferative cytokines, in particular, TGF- β [20]. “Culture-shock” senescence appears to occur primarily in cultured human epithelial cells, such as keratinocytes [23] and those isolated from mammary tissue [24] and is likely due to the stress associated with inappropriate growth conditions. While the stress response appears to be a critical mediator of “culture-shock” induced senescence, multiple mechanisms likely underlie oncogene-induced senescence [25]. At this point, senescence induced by oncogenes including those of the Ras signaling pathway (i.e., Ras, RAF, MEK, MOS and BraF) and appears to be triggered by accumulation of DNA damage and ROS, activation of the p38 kinase pathway and formation of ‘senescence-associated heterochromatic foci’. Amongst the cytokines capable of inducing senescence, perhaps the best characterized thus far is TGF- β which appears to exert its senescence-inducing effect by inducing G1 arrest (via upregulation of p15 and p21) [26, 27] and expression of telomerase reverse transcriptase [29].

As mentioned above, regulation of the senescence program at the molecular level requires primarily the p53 and Rb pathways [28]. At the earliest stages of senescence, the p53 pathway is activated upon phosphorylation of the p53 protein by ATM/ATR, Chk1/Chk2 and p19ARF (a product of the p16 locus). Subsequent events may then proceed via either Rb and p21 or via an as yet, poorly defined Rb-independent manner. Activation of Rb typically requires either p21 or p16. Once the p53/Rb pathway has been upregulated and cell cycle progression has been inhibited, expression of additional gene pathways, such as those involved in tissue remodeling (i.e., cell adhesion, angiogenesis, cell-cell contact and extracellular matrix remodeling) and inflammation [31–33] are modulated. Altered expression of genes encoding cytoskeletal proteins such as vimentin most likely underlie many of the changes in senescence-induced cell morphology [29]. A comparison of the similarities and differences between senescence and either terminal differentiation or apoptosis in keratinocytes (Table 1) reveals

that while these processes share a number of features they can be distinguished in the laboratory using several molecular endpoints.

4. Relationship between senescence and cancer

Perhaps the first evidence that senescence represented an event that could occur *in vivo* was the demonstration that positive β -galactosidase staining, similar to that observed in cultured cells, was detected within the dermis and epidermis of adult skin and increased in frequency and intensity with respect to age [21]. A number of subsequent studies then demonstrated that senescence plays an important role in the carcinogenic process, in particular, in the transition from benign to malignant lesions. For example, using the RAS-induced tumorigenesis model, it was found that premalignant lung adenomas, but not malignant adenocarcinomas bore features of the senescence program [30]. Similarly, tumors initiated by the RAS family member, Braf progressed to adenocarcinomas only when combined with an inactivating genotype of either p16 or p53 [31] and in the skin progressed from benign papillomas to malignant carcinomas upon downregulation of the TGF- β pathway [32]. Finally, in a number of *in vivo* tumorigenesis models, restoration of the p53 pathway could be shown to be sufficient for induction of the senescence program and regression and/or suppression of tumorigenesis [33–35]. Interestingly, the ability of p53 to activate either apoptosis or senescence appears to vary in a tissue-dependent manner [34]. Collectively, these studies [30,32–35] supported the notion that senescence presents a barrier to tumorigenesis that must be overcome in order for a tumor to achieve its fully malignant form. While the overall impact of senescence appears to be primarily a tumor suppressive effect due to its inhibition of growth, a converse aspect, one of tumor progression should also be considered. In this paradigm, the senescent, growth arrested cell may promote tumorigenesis by secreting inflammatory cytokines and growth factors as well as enzymes that degrade the extracellular matrix resulting in disruption of the normal tissue architecture and promote the growth, migration and invasion of a neighboring tumor cell [20]. Thus, whether senescence represents a tumor inhibiting or tumor promoting mechanism may be dependent on the context of a particular cellular environment.

5. How does a cell choose between terminal differentiation, apoptosis and senescence?

The relationship between terminal differentiation, apoptosis and senescence is intertwined as collectively, they act to suppress proliferation and share common features [10]. Critical features that dictate how the cell will respond to a specific stimuli involves the cell type, the proliferative status of the cell and the relative expression of oncogenes/tumor suppressors. With respect to cell type, a molecule that can promote proliferation and apoptosis in other cell types will promote proliferation and differentiation in keratinocytes given their potential to differentiate. Similarly, a highly proliferative cell may exhibit a higher threshold to common upstream signaling pathways than a quiescent cell and may translate this signal differently [11]. Additionally, the relative intensity of the oncogenic stimuli with respect to the absence or presence of tumor suppressor proteins (i.e., p53) has also been shown to be important [41]. For example, *in vivo* studies have shown that low levels of aberrant Ras activation could result in tumor formation whereas high levels resulted in irreversible senescent growth arrest. Given the key role that p53 plays in the apoptotic and senescent responses, it has been defined as a “master” regulator that guides the decisions between apoptosis and senescence [36]. The relative timing of exposure to apoptotic/senescence-inducing stimuli should also be considered as studies have indicated that oncogene-expressing cancer cells that have been able to bypass senescence appear to be more susceptible to the induction of apoptosis [35]. A bypass of tumor cells from the apoptotic process initiated by chemotherapeutic drugs to the premature senescent process has important implications with respect to a patient’s response to drug therapy and recurrence of the primary cancer [37]. Thus, while it is currently unclear what specifically

determines a cell's ability to embark down a senescent pathway rather than one leading to apoptosis or differentiation, further exploration is currently underway to address this issue.

6. Impact of TCDD on terminal differentiation, apoptosis and senescence of keratinocytes

Given the substantial interest in understanding how the processes of terminal differentiation, apoptosis and senescence contribute to skin tumorigenesis, our laboratory has investigated the role of the TCDD/AHR signaling pathway in these processes. Studies performed in a number of laboratories have shown that the administration of TCDD to a variety of cultured cells has been shown to alter their ability to proliferate [38], migrate [39], apoptose [40–43], senesce [44,45] and differentiate [44,46–48]. While in many cases the response of cells subsequent to activation of the AHR pathway by TCDD may appear to be contradictory, the many factors involved in dictating a particular cell's response to a certain stimuli as discussed in the previous sections should be carefully considered. In light of this it would not be prudent to directly compare events observed in primary keratinocytes to those observed using established tumor cell lines. More importantly is whether or not any experiments performed in either cultured cells or using murine models appropriately mirror events observed in the human population?

As mentioned previously, appropriate terminal differentiation of keratinocytes is characterized by growth arrest followed by an orderly transition into differentiation that is detected by the expression of specific differentiation markers. It is here that TCDD appears to make its footprint. First, as reported by us and others, TCDD makes an impact on the initial step of growth arrest by increasing post-confluent growth [44,49]. Second, with respect to terminal differentiation, TCDD treatment appears to alter the orderly transition from early to late differentiation [46–48]. For example, treatment with TCDD has been shown to result in a decrease in early markers of differentiation (i.e., keratin 10) followed by an increase in the expression of late markers of differentiation (i.e., profilaggrin and transglutaminase) [47,48]. Consistent with reports from other laboratories [47], the keratinocytes cultured in our laboratory do not appear to be apoptotic when cultured in either the absence or presence of TCDD as determined by assaying for caspase 3 activity (Unpublished Results).

With respect to senescence, a role for the AHR was first noted when it was discovered that mouse embryonic fibroblasts derived from the AHR null mice ceased to divide and reached a senescent state more rapidly than those derived from the wild-type mice [50]. Additionally, it was observed that human primary keratinocytes continuously passaged with TCDD retained the ability to proliferate far longer than those cultured with the vehicle control (DMSO) [45]. Although telomere status was not determined in either of these studies [45,50], it is highly likely that both are examples of telomere-dependent replicative senescence and suggest that AHR content and/or constitutive activation can extend the proliferative lifespan of certain cultured primary cells. Putative mechanisms by which the AHR may alter telomere-dependent replicative senescence include activation of telomerase reverse transcriptase transcription similar to that described for other nuclear receptors such as the estrogen receptor [51]. A more important question, however, is whether these AHR-dependent activities observed *in vitro* have any relevance for *in vivo* replicative capacity?

TCDD can also impact telomere-independent senescence outcomes as well. We have shown that primary neo-natal human foreskin keratinocytes grown to confluence in a “standard” epidermal medium (serum negative, low calcium and defined growth supplements) and then switched to a serum positive/high calcium medium attain the hallmarks of a p16-dependent, telomere-independent senescent state within 6–8 days of culture [44,45]. As shown in Figure 1A, after two days of culture in the serum positive/high calcium medium, a few keratinocytes stained positive for β -galactosidase activity, a positive indicator of senescence (Table 1)

regardless of the presence of TCDD. However, at the latter time points (i.e., day 6), the number of positive staining cells was significantly increased in the DMSO but not TCDD treated cultures. To further define the status of these keratinocytes as senescent, we examined the protein expression levels of p14, p16, p21 and p53. As shown in Figure 1B, relative to β -actin, increases in the protein levels of p14 and p27 were first observed at day 2 in the DMSO control and were followed by increases in the protein levels of p16 and p53 at day 6. Taken together (the increase in detection of β -galactosidase activity coupled with the observed increase in p16 and p53 levels) these data (Figure 1 and [44,45]) indicate that at the day 6 time point, the majority of the keratinocytes cultured in the presence of the DMSO vehicle control can be considered to be senescent. The effect of TCDD was most pronounced when the expression levels of p14, p16 and p53 were analyzed at the day 6 and 8 time points. It should be noted that due to the confluent nature of these cultures, the enlarged, flattened appearance typical of senescent cells is difficult to observe. Additional unpublished work performed in our laboratory indicate that TCDD activation of the AHR does not appear to directly alter the senescence program but instead, exerts its effect by inhibiting an as yet poorly characterized inducer of senescence. At this time, it is also unclear whether or not TCDD may alter senescence via an epigenetic mechanism. We have also extended the findings shown in Figure 1 by demonstrating that AHR agonists present in cigarette smoke condensate are capable of inhibiting culture-induced senescence of human oral keratinocytes [52]. These latter findings may have implications in cigarette smoke-induced head and neck cancers [53].

Additional work performed in our laboratory focused on examining the effect of the AHR pathway on the ability of primary human keratinocytes to undergo culture-dependent population doublings [45]. Here, the primary human keratinocytes were allowed to proliferate in the constant presence of DMSO, TCDD or the AHR antagonist MNF [54,55] and were passaged as they reached approximately 70% confluency. As shown in Figure 2, the keratinocytes cultured under normal conditions (i.e., untreated) or with DMSO ceased dividing after approximately 27 population doublings. However, in the presence of TCDD, the cells continued to divide for at least 50 population doublings (i.e., > 80 total population doublings) beyond their normal limit/threshold. Treatment with MNF alone did not significantly alter the life span of these cells, but blocked the TCDD-induced bypass of senescence indicating a role of the AHR. Similarly, a role of the AHR in the TCDD-induced effects on the premature “culture-shock” senescence of confluent cells (i.e., Figure 1) that was observed in both epidermal and oral human keratinocytes is indicated by use of the AHR antagonist, MNF and siRNA approaches in these experiments [45,52](unpublished results). While these data [44, 45] together indicate that TCDD can inhibit “culture-shock”-induced senescence in human keratinocytes, it is not yet clear whether other inducers of telomere-independent senescence (for example, oxidative stress or oncogene activation) can be similarly affected by AHR activation.

7. Future Directions and Summary

Ever since Hayflicks' initial observation of replicative senescence *in vitro*, its physiological relevance has been questioned or been dismissed completely as an artifact of culturing by much of the scientific community. Today, this view has shifted considerably for several reasons. The first is the recognition that senescence is a cell fate decision that can be initiated by a number of different stimuli using potentially different effector pathways to converge upon the similar cellular phenotype of irreversible growth arrest. This broadening of the senescent response beyond its initially perceived role as simply a counter of accumulated cellular doublings to a more general one induced by various cellular abnormalities, many of which are proposed contributory to the tumorigenic process, has accordingly broadened its appeal as a potential therapeutic entry point. Secondly, over the past several years a number of studies [25,30,33, 56–58] have finally begun to show evidence of the senescent phenotype in *in vivo* models of

tumor promotion/progression. Particularly, they have provided evidence of senescent markers being lost in the transition from benign to malignant tumors suggesting that senescence is indeed a bona fide tumor suppressive mechanism that must be breached, at least for some cancers and perhaps all, for the disease to reach malignancy. As such, the study of any agent (s) that has been documented to have tumor promoting activities such as many of the ligands of the AHR cannot ignore a potential impact on senescence as a mechanism of their tumor promotion.

Our data [44,45] suggests that TCDD, in at least one cellular context, can inhibit the telomere-independent senescence response. Obviously, and we think importantly, this observation needs to be extended to a number of different models of premature senescence to test its universality. Can TCDD impact senescence induced in the classical activated oncogene or oxidative stress model systems? Is it limited to epithelial senescence? *In vitro* studies directed at these questions will begin to shed light on whether TCDD/AHR signaling has a more general effect on the senescent phenotype or is limited to the particular peculiarities of our culture system. Clearly though, the most important test will be evidence of TCDD manipulation of senescence in the *in vivo* models that have been established over the last three years [30,33,56–58]. An example of such *in vivo* study includes use of a mouse model containing the same Ras transgene as that of the TgAC mice [59], but would also include an additional genetic modification to facilitate acute re-expression of p53 and induction of senescence [34,35]. If the tumor promoting activities of TCDD require AHR-mediated inhibition of senescence, then the increased expression of p53 and subsequent induction of senescence would block TCDD-induced tumorigenesis. Finally, appropriately designed studies must be performed to address the ultimate question: Do any of these events occur in a meaningful manner in the human population that would alter either progression of epithelial cancers or response to chemotherapeutics?

The evidence supporting the idea that activation of the AHR by TCDD exerts its tumor promoting effects *in vivo* by modulating the senescence program is thus far, indirect. First, in most tumor-promotion studies performed in mouse skin the most commonly observed effect of TCDD is an increase in the rate of cellular proliferation and hyperplasia [59–61]. Second, TCDD-induced hyperplasia in these models appears to be mediated by the AHR [62]. Third, in skin tumor promotion studies, treatment with TCDD appeared to increase the formation of squamous cell carcinomas from papillomas which is consistent with a TCDD-induced suppression of senescence that would allow the tumor cell to overcome the senescence imposed barrier to malignant transformation [59]. Finally, in a murine lung tumor model, administration of TCDD increased the number of tumors initiated by N-nitrosodimethylamine in a manner that was accompanied by altered RAS, RAF and ERK signaling [63]. This latter study, together with findings that TCDD downregulates the expression of p53 [44,45,64–66] are supportive of a putative role of the TCDD/AHR pathway in the three-stage model of Ras-induced tumorigenesis that is initiated by an activating Ras mutation and resolves into evasion of the p53-p16-dependent senescence checkpoint [58]. It should be noted, however, that with respect to initiator/promoter studies, alternative explanations should also be considered and include the possibility that the immunosuppressant activities of both agents (i.e., N-nitrosodimethylamine and TCDD) act to increase tumor numbers by decreasing immune competence. Although none of these studies directly test TCDD/AHR signaling and senescence, they are encouraging enough, especially when coupled with the limited *in vitro* data to merit a more concerted effort to fully determine whether activation of the AHR can promote tumorigenesis by helping to bypass the senescent barrier to malignancy.

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Figure 1A

Figure 1B

Figure 1. Impact of TCDD on senescence of primary human keratinocytes

Preconfluent (80–90% confluent) primary human epidermal keratinocytes were induced to differentiate with the addition of high calcium (1.5 mM) in the presence of either TCDD (1 nM) or DMSO (0.01%). **A)** At the indicated time points, the cells were analyzed for senescence associated β-galactosidase activity. Following staining, the cells were photographed at 20X magnification using phase contrast microscopy. **B)** At the indicated time points, the cells were harvested, protein extracts were prepared and aliquots were subjected to western blot analyses (D, DMSO and T, TCDD). From [44].

Figure 2. Impact of TCDD and MNF on the replicative life span of proliferating normal human keratinocytes

Primary human epidermal keratinocytes were cultured in low Ca^{++} media [0.06mM, Epilife media] with either 0.01% DMSO or 1nM TCDD in presence or absence of 1 μ M MNF. The cells were passaged when approximately 70% confluent until they ceased to grow and cumulative population doublings were plotted against total time in culture (From [45]).

Table 1

Comparison of cell death processes in keratinocytes.

Cell Death Process	G1 Arrest Required?	Stimuli	Morphology	Molecular Markers
Terminal Differentiation	Yes[11]	Calcium[13,14]	Enlarged; Flattened; Anuclear[13,14]	Transglutaminase activation; Involucrin; Profilaggrin[13,14]
Apoptosis (Intrinsic)	Yes[11]	Oxidative stress; Activated oncogenes; DNA damage[17,18]	↓ Cell volume; Membrane blebbing; Chromatin condensation; Nuclear fragmentation[17, 18]	Caspase activation[17,18]
Senescence	Yes[11]	Oxidative stress; Activated oncogenes; DNA damage; Telomere shortening[18, 20]	Enlarged; Flattened; Senescence-associated heterochromatic foci; Granular cytoplasm[18,20]	Senescence- associated β -galactosidase activation; p15, p16 and p53 expression[18,20]

A summary of the characteristic features of terminal differentiation, apoptosis and senescence in keratinocytes.