

Review article

Molecular mechanisms of UV-induced apoptosis

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Sunburn cells, single standing cells with typical morphologic features occurring in UV-exposed skin, have been recognized as keratinocytes undergoing apoptosis following UV irradiation. Induction of apoptosis following UV exposure appears to be a protective mechanism, getting rid off severely damaged cells that bear the risk of malignant transformation. UV-mediated apoptosis is a highly complex process in which different molecular pathways are involved. These include DNA damage, activation of the tumor suppressor gene p53, triggering of cell death receptors

either directly by UV or by autocrine release of death ligands, mitochondrial damage and cytochrome C release. Detailed knowledge about the interplay between these pathways will increase our understanding of photocarcinogenesis. This review briefly discusses recent findings concerning the molecular mechanisms underlying UV-induced apoptosis.

Key words: UVB; sunburn cells; apoptosis; death receptors; DNA damage.

One of the hallmark events of exposing skin to solar radiation is the formation of sunburn cells (SC) (1). These cells were originally described many decades ago as single standing cells with peculiar morphologic features (1). Although the major action spectrum inducing the formation of SC is in the ultraviolet (UV)-B range (290–320 nm), SC formation is also found after irradiation with UVC (200–290 nm) or high doses of UVA (320–400 nm), though to a much lesser extent (2, 3). Time course studies revealed that SC are detectable as early as 8 h after UV exposure, are maximally expressed after 24–48 h and disappear after 60–72 h (4). SC show typical morphologic features, including a shrunken eosinophilic cytoplasm with a condensed, pyknotic nucleus, which make them easily recognizable in H & E sections (5). Nevertheless, several issues have remained puzzling for quite a long time. One is that SC are always single standing cells and are never accompanied by an inflammatory infiltrate. In addition, the functional relevance of SC has been unclear until recently.

Sunburn cells are apoptotic keratinocytes

The microscopic and ultrastructural characteristics of SC were found to be in accordance with those described for

an apoptotic cell, starting with cell shrinkage and membrane blebbing and followed by chromatin condensation and genomic DNA fragmentation (6). Hence, by applying morphologic criteria, SC were recognized as keratinocytes undergoing apoptosis following UV exposure. Two major types of cell death are known: apoptosis and necrosis. Necrosis is a passive process mostly induced by a severe external insult which causes cytoplasmic swelling and disruption of the cell membrane. As a consequence, lysosomal enzymes are released, usually inducing an inflammatory reaction. In contrast, apoptosis is an active process in which a single cell initiates an inherent suicide program, resulting in the successive fragmentation of the cell. While necrosis is characterized by cellular swelling associated with membrane disintegrity, apoptotic cells start to shrink, finally breaking up into membrane enclosed fragments (apoptotic bodies) that are phagocytosed by neighboring cells, mostly macrophages. Because there is no release of inflammatory mediators, the apoptotic process is rarely associated with an inflammatory reaction. In addition, while necrosis always affects groups of cells, apoptosis is mostly seen in single standing cells (7).

Execution of the suicide program is a highly complex process in which a variety of biochemical pathways are involved, the most important being the activation of catabolic proteases, called caspases (8) (see section on “Death

Abbreviations: SC, sunburn cells; TNF, tumor necrosis factor; UV, ultraviolet.

receptors on the cell membrane” below). There are many ways to induce apoptosis, the three most important being the following:

- The apoptotic program is initiated by the cell itself when the time has come to die, e.g. due to aging; this is programmed cell death *sensu strictu* since the fate of all cells is determined (“programmed”) in this way.
- Induction of apoptosis can be the consequence of cell damage.
- Apoptosis can be induced via activation of the various death receptors that are expressed on the surface of almost any cell.

The morphologic recognition of SC as being apoptotic keratinocytes has explained why SC are always found individually scattered and are not associated with an inflammatory infiltrate, but did not give any insight into the biological or functional relevance of these cells. Since a correlation exists between the UV dose delivered and the number of SC, for a long time they were simply regarded as a marker for severity of sun damage (5).

Formation of sunburn cells is a consequence of UV-induced DNA damage

To exert its biological effects, UV must first be absorbed by a cellular chromophore, which transfers the energy into a biochemical signal. Since the wavelength dependency of some UVB effects matches that for DNA absorption, for years genomic DNA has been regarded as the major and even the only chromophore for UVB within the cell. UVB radiation induces DNA damage by formation of cyclobutane pyrimidine dimers and <6-4> photoproducts (9). Most of these photoproducts are removed in normal cells by DNA excision repair (10, 11). Hence, cells from xeroderma pigmentosum (XP) patients, who are defective in DNA nucleotide excision repair, are hypersensitive to induction of mutations in their DNA by UV radiation (9, 12). In addition, UV-induced DNA damage was recognized to play an important role in mediating a variety of biological effects of UVB. It was found that UVB-mediated immunosuppression, surface molecule expression and cytokine release could be reduced and prevented by applying T4N5 endonuclease, a bacteria-derived DNA repair enzyme (13–15). Penetration of T4N5 into cells was enabled by incorporation into liposomes, a route which allows T4N5 delivery both *in vitro* and *in vivo* (16). Similar observations were made with another repair enzyme, photolyase, which reverses cyclobutane pyrimidine dimers specifically upon illumination with photoreactivating light, a repair process called photoreactivation (17). Taken together, these findings strongly supported the concept that any UVB-response is mediated via DNA damage.

A major advance in the understanding of the functional role of SC was the discovery of the link between SC formation and p53. p53 is a tumor suppressor gene involved in many cellular functions, including cell cycle inhibition, regulation of differentiation, transcription and DNA repair (18). Irradiation with UV is known to arrest cells during the G1 phase in a p53-dependent manner to accomplish DNA repair prior to DNA synthesis (19), thereby reducing the DNA mutation rate. It was recognized that p53 is critically involved in the formation of SC since mice lacking functional p53 (p53 knock out mice) revealed significantly fewer SC upon UV-exposure than did UV-exposed wild type mice (20). Since p53 becomes activated by DNA damage, it was concluded that the formation of SC is linked to the severity of UV-induced DNA damage. This led to the assumption that UV-damaged keratinocytes that have failed to repair their DNA damage will die as SC, thus escaping the risk of becoming malignant. Therefore, the formation of SC was proposed as a scavenging phenomenon controlled by the p53 gene, which protects the individual from developing UV-induced skin cancer (21). Consequently, keratinocytes with mutated p53 should be more susceptible to the tumor-promoting effects of UV radiation. Due to diminished p53-mediated apoptotic cell death, these cells should survive, whereas surrounding cells carrying damaged DNA but wild type p53 are eliminated by apoptosis (21, 22). Since UV radiation preferentially mutates p53 (23), it may exert a selective pressure for the mutated, damage-resistant keratinocytes, thereby allowing these cells to clonally expand and to form actinic keratosis, the prestage of skin cancer (20). Accordingly, p53 knock out mice exposed to chronic UV radiation revealed a significantly increased susceptibility to skin cancer induction when compared to UV-exposed wild type control mice (24).

The demonstration of the importance of p53 for the formation of SC indicated that DNA damage is crucial in mediating UV-induced apoptosis. However, though plausible, evidence for this was rather indirect than direct. Direct proof has recently been obtained in several studies performed both *in vitro* and *in vivo*. Kulms et al. postulated that if DNA damage is causally related to UV-mediated apoptosis, an increase in DNA repair should result in reduction of the apoptotic response after UV exposure (25). Therefore, the epithelial cell line HeLa was exposed to UV radiation and, subsequently, DNA repair enhanced by adding the repair enzyme photolyase via liposome delivery. Subsequent exposure to photoreactivating light caused a significant reduction of cyclobutane pyrimidine dimers. Accordingly, the rate of apoptosis was remarkably reduced (25). In addition, *in vivo* studies showed that enhancement of DNA repair by topical application of the repair enzyme T4 endonuclease V in liposomes reduces

the number of SC (26). Similar observations were made in opossums following photoreactivation (27). Furthermore, photoreactivation of UV-irradiated fish cells that have endogenous photolyase reduces apoptosis (28).

These data indicated that DNA damage is crucially involved in UV-induced apoptosis. This was in accordance with the conventional concept that any biological UV effect is mediated via DNA damage. However, several issues remained to be clarified. First, in their *in vitro* system, Kulms et al. (25) were not able to prevent UV-induced apoptosis completely, even by increasing the concentration of photolyase, implying that pathways other than DNA damage are also involved. This assumption is also supported by a clinical observation. Up to 4% of keratinocytes of normal appearing sun-exposed skin cells carry p53 mutations and thus should be protected from apoptosis, but far fewer than that develop into actinic keratoses or cancer. Thus, the majority of cells have to undergo squamous differentiation or apoptosis induced by other pathways than p53 and DNA damage (22, 29). This is also supported by the observation that cells carrying p53 mutations like the spontaneously transformed human keratinocyte cell line HaCaT (30, 31) undergo apoptosis upon UV exposure (32, 33).

Death receptors on the cell membrane are involved in UV-induced apoptosis

Death receptors belong to the tumor necrosis factor (TNF) receptor gene superfamily, which is defined by similar, cysteine-rich extracellular domains and a homologous cytoplasmic sequence termed "death domain" (34). Among the many death receptors (including TNF-receptor-1, TRAIL-receptor-1, TRAIL-receptor-2 and death receptor-3 (DR-3)) CD95, also called Fas or Apo-1, is one of the most potent transducers of apoptosis (35). Triggering of the CD95 molecule either by agonistic antibodies or by the natural ligand, CD95L (FasL), induces apoptosis. Ligand binding causes trimerization of CD95 and the trimerized cytoplasmic region (death domain) then transduces the signal by recruiting the adapter molecule FADD (Fas-associating protein with death domain) (35). The N-terminal region of FADD is responsible for downstream signal transduction, inducing the activation of a cascade of cysteine proteases, called caspases. All caspases are constitutively synthesized as proenzymes, which become proteolytically processed to form active heterodimeric enzymes, some of them bearing the capacity to autoactivate (36). Upstream activated caspases then specifically trigger cytoplasmic cascades of caspases, resulting in cleavage of specific substrates, which ultimately execute apoptosis. The most upstream caspase involved in CD95 signaling is caspase-8, which binds to FADD. Activated caspase-8 then triggers a cascade of dif-

ferent downstream caspases, including caspase-3, finally resulting in cleavage of various nucleoproteins, followed by DNA-fragmentation and apoptotic cell death. The death promoting TRAIL receptors, TRAIL-R1 and TRAIL-R2, utilize exactly the same signaling pathway as CD95 (37).

The discussion about whether death receptors are involved in UV-induced apoptosis arose when it was recognized that keratinocytes express the CD95 receptor (38, 39). Accordingly, UVC radiation was found to upregulate both CD95 and its natural ligand CD95L in primary keratinocytes (40). In addition, application of a neutralizing antibody against CD95L to the cells *in vitro* reduced the UVC-induced apoptosis rate. Similar observations were made when exposing freshly isolated peripheral blood lymphocytes or lymphocyte cell lines to UV (41).

Upregulation of CD95L, but not of CD95, was also detected on T helper cells after exposure to UVA1 (340–400 nm), consequently enhancing UVA1-induced apoptosis. Incubation of cells with an antagonistic antibody against CD95 reduced UVA1-induced apoptosis partially, implying that upregulation of CD95L induced by UVA1 is functionally relevant for UVA1-mediated apoptosis (42). In addition, UVA1-induced upregulation of CD95L could be blocked by the electron quencher sodium azide, implying that the formation of singlet oxygen species is involved. This observation might be of clinical relevance, since UVA1 is known to improve atopic dermatitis, a T cell-mediated disease (43). Enhanced T cell apoptosis was detected *in situ* in skin samples obtained from atopic patients undergoing UVA1 phototherapy (42).

Involvement of the CD95/CD95L system in UVB-induced apoptosis, however, was also demonstrated *in vivo*. In murine skin, CD95 and CD95L expression was observed to be induced upon UVB-exposure (44). In addition, SC formation was found to depend on CD95L expression, since SC were significantly reduced in *gld*-mice, which lack functional CD95L. Furthermore, following chronic UV-exposure, an accumulation of p53 mutations was found in *gld*-mice; this led to the hypothesis that in UV-exposed mice, CD95L-induced apoptosis is required to efficiently eliminate DNA-damaged keratinocytes and that a disturbance of CD95/CD95L interactions may contribute to the development of UV-induced skin cancer (44).

But the CD95/CD95L system does not appear to be the only death receptor/ligand system involved in UV-induced apoptosis. Schwarz et al. have already reported that auto-crine release of TNF α might play a role in UV-mediated apoptosis (33). Neutralization of TNF α by blocking antibodies or inhibition of release by pentoxifylline was found to partially inhibit UV-mediated cell death (33, 45).

Together, these data indicate that the activation of death receptors by their cognate ligands is involved in UV-induced cell death.

However, there also exists evidence of a direct interaction between UV radiation and membrane-associated death receptors. Rosette & Karin observed that UV light or osmotic shock activates multiple growth factor and cytokine receptors, consequently activating the jun-kinase cascade. From this, they identified a new cell membrane-associated pathway by which UV can mediate its effects (46). Confocal laser scanning microscopy studies revealed that exposure of HeLa cells to UV or osmotic shock induced clustering and internalization of the cell surface receptors for epidermal growth factor, TNF and interleukin-1 (46). Studies, based on these findings and on the prediction that this phenomenon might be applicable to any surface receptor which requires oligomerization for its activation, have examined whether, through this pathway, UV can also activate the apoptosis-related CD95 receptor and whether activation of this receptor may be functionally relevant for UV-mediated apoptosis. Indeed, confocal laser scanning microscopy revealed UV-induced but CD95L-independent clustering of CD95 on the surface of cells from the keratinocyte cell line HaCaT (32). Involvement of CD95 activation in UV-induced apoptosis was further supported by transfection of cells with a dominant-negative mutant of FADD, showing a significantly reduced apoptosis rate after UV irradiation (32, 47). In addition, CD95 death receptor clustering could be prevented by exposing cells to UV at low temperature (4–10°C). This was accompanied by a partial reduction in the apoptosis rate induced by UV, indicating that UV-induced activation of CD95 is functionally relevant (32). Similar to CD95L-induced apoptosis, activation of the upstream caspase-8 and downstream caspase-3 was observed following UV-irradiation, further suggesting that activation of the CD95 pathway is also involved in UV-induced apoptosis. Since prevention of UV-induced CD95 clustering was associated with a partial reduction of apoptosis, these data suggested that UV-induced apoptosis can also be initiated at the cell membrane (32). Accordingly, Sheikh et al. showed that the TNF receptor-1 becomes aggregated by UV but not by drugs damaging DNA only (48). These data challenged the concept that UV-induced apoptosis is exclusively dependent on DNA damage.

Both nuclear and membrane effects contribute to UV-induced apoptosis

To answer the question as to whether UV-induced apoptosis is exclusively dependent on genomic DNA-damage, or whether UV triggers apoptosis mainly via direct activation of membrane bound cell death receptors, Kulms et al. recently attempted to measure the relative contribution of nuclear and membrane effects in UV-

induced apoptosis (25). UV irradiation of HeLa cells at 4°C, which prevents death receptor clustering, caused partial reduction of apoptosis. Enhancement of DNA repair by photoreactivation resulted in a more pronounced inhibition of UV-induced apoptosis. However, neither of these approaches alone was able to prevent apoptosis completely. Only when receptor clustering was blocked (by keeping the cells at low temperature) followed by photoreactivation (removing DNA damage) was complete reduction of apoptosis observed. Although under these experimental conditions activation of death receptors and DNA damage are induced by the same stimulus, i.e. UV, they represent independent events and are not biochemically linked. Since inhibition of both events results in an additive reduction of apoptosis, this observation indicated that death receptor activation and DNA damage contribute independently to UV-induced apoptosis. In addition, inhibition of caspase-3, the downstream protease in the CD95 signaling pathway, blocked both CD95L- and UV-induced apoptosis, while blockade of caspase-8, the most proximal caspase, inhibited CD95L-mediated apoptosis completely, but UV-induced apoptosis only partially. This implies that apoptosis induced by UV-mediated DNA damage is independent of caspase-8. Although, according to the findings of Kulms et al. (25), nuclear effects appeared to be more effective in mediating UV-induced apoptosis than membrane events, both seem to be necessary for the complete apoptotic response. Thus, this study showed that nuclear and membrane effects are not mutually exclusive and that both components contribute independently to a complete response to UV.

The concept that different pathways contribute to UV-induced apoptosis is in accordance with previous findings that have reported that UV radiation triggers at least two apoptotic pathways in human keratinocytes, one being p53-dependent, the other p53-independent (49). The p53-independent pathway could be blocked by integrin-mediated cell attachment. This observation gave rise to the speculation that the integrin-sensitive pathway may serve as a safety mechanism, removing transformed anchorage-independent cells regardless of their p53 status. In addition, Godar showed that UV can induce both immediate and delayed apoptosis (50). Immediate apoptosis appears to be protein synthesis independent, using constitutive (preprogrammed) mechanisms, while delayed death is dependent on inducible apoptotic mechanisms being protein synthesis dependent.

Participation of the Bcl-2 protein family and mitochondrial cytochrome C release in UV-induced apoptosis

The oncogenic potential of Bcl-2 has been attributed to its ability to inhibit apoptosis induced by growth factor

withdrawal, application of chemotherapeutic agents, heat shock, activation of cell death receptors and UV irradiation in a variety of cells (7). The Bcl-2 apoptosis regulatory protein family consists of antiapoptotic (Bcl-2, Bcl-X_L, Bcl-w, Bfl-1, Bcl-2L1, Mcl-1, A1) and proapoptotic (Bax, Bak, Bcl-X_S, Bad, Bid, Bik, Hrk) members (51), whereas their balance within the cell determines whether apoptosis is induced or prevented. Antiapoptotic Bcl-2 and its homologs are anchored to contact sites between the outer and the inner mitochondrial membranes, where they presumably create selective ion channels to stabilize the transmembrane potential ($\Delta\psi_m$) (52). Initiation of cell death leads to mitochondrial permeability transition, resulting in “megapore” formation followed by release of cytochrome C as well as of oxygen free radicals into the cytosol (52). Cytosolic cytochrome C associates with Apaf-1 and procaspase-9, causing ATP-dependent activation of caspase-9, followed by activation of downstream caspases consequently inducing apoptosis (53). The mechanism by which the mitochondrial pathway is triggered during UV-induced apoptosis is still the subject of debate but its participation is indisputable.

Overexpression of Bcl-2 in marsupial cells (PtK2) was found to delay but not to prevent UVC-induced apoptosis (54), while Bcl-2 overexpression in a human T lymphoblastoid cell line (CEM) prevented UV-induced cytochrome C release (55). Furthermore, it could be demonstrated that nitric oxide protects keratinocytes and endothelial cells from UVA-induced apoptosis, probably via upregulation of Bcl-2 (56). Similarly, extracellular matrix-mediated protection of retinal pigment epithelial cells from UVA-induced apoptosis was found to correlate with Bcl-2 protein expression (57). In addition, skin explants from Bcl-2 deficient mice exhibited a higher number of SC following exposure to UVB (58). On the other hand, UV itself seems to be able to modulate Bcl-2 expression; UV was found to downregulate Bcl-2 expression in normal skin as well as in DNA repair-deficient fibroblasts (59, 60).

As observed for other apoptotic stimuli, including ionizing irradiation, oxidative stress or death receptor activation, mitochondria are also involved in UV-mediated apoptosis. Cytochrome C release, a hallmark event in apoptosis, was observed as an early event in UV-induced cell death. Since UV-induced cytochrome C release could not be prevented by caspase inhibitors, it was postulated to occur upstream of caspase activation and prior to $\Delta\psi_m$ depolarization (61). This hypothesis was recently supported by immunohistochemistry and time-lapse confocal microscopy in HeLa cells. On a single cell level, UV-induced cytochrome C release was demonstrated to be an event of a few minutes occurring prior to phosphatidylserine exposure at the outer cell membrane and prior

to the loss of plasma membrane integrity, both regarded as early events in apoptosis (62). Variation of the strength of the apoptotic stimuli altered only the onset, not the duration, of cytochrome C release, which was completed within 5 min. These observations are in contrast to some other apoptosis models, e.g. ionizing irradiation, in which caspase-3 dependent cleavage of Bcl-2 promotes release of mitochondrial cytochrome C (63). Hence, in this system a positive feedback loop encouraging further caspase activation is suggested as a tool to complete apoptosis. Furthermore, caspase-8 dependent initiation of cytochrome C release is involved in death-receptor induced apoptosis, and it is proposed that caspase-8 activates the proapoptotic protein Bid which heterodimerizes with the antiapoptotic proteins Bcl-2 or Bcl-X_L, resulting in mitochondrial megapore formation followed by cytochrome C release (64). If activation of death receptors is an essential component of UV-induced apoptosis, as indicated by several observations (25, 32, 33, 40–42, 44, 45, 47, 48) (see section on “Death receptors on the cell membrane” above), this pathway should be involved in UV-mediated cell death as well. It still remains to be determined how receptor activation, DNA damage, Bcl-2 deactivation and cytochrome C release relate to each other in UV-induced apoptosis.

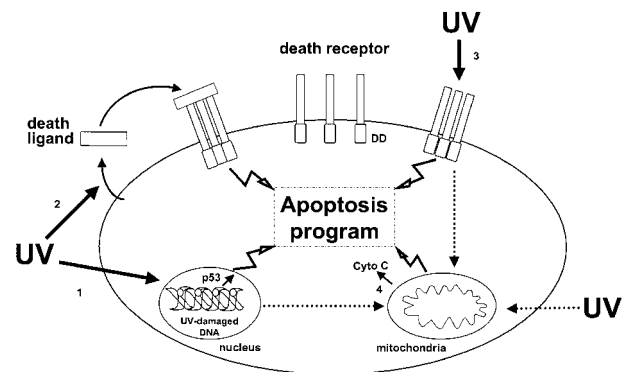


Fig. 1. Pathways involved in UV-induced apoptosis. UV induces nuclear DNA damage which induces apoptosis via activation of p53 (1). In addition, UV activates death receptors expressed on the cell surface via induction of release or upregulation of death ligands (2). UV directly activates death receptors in a ligand-independent way by inducing receptor clustering (3). Activated death receptors trimerize and transduce the apoptotic signal via their intracytoplasmic death domain (DD). UV induces the release of cytochrome C from mitochondria (4). Whether UV affects the mitochondria directly or whether this is a consequence of DNA damage or receptor activation remains to be determined. This graph represents a simplified cartoon, since for the sake of clarity many details mentioned in the text have been omitted.

Conclusion

Major advances have been made in recent years in understanding the phenomenon of SC formation. There is unanimous agreement that UV-induced apoptosis of keratinocytes represents a scavenging mechanism, getting rid of DNA-damaged cells and thereby reducing the risk of malignant transformation. Hence, disturbances in the apoptotic machinery may increase the risk of skin cancer. UV-induced apoptosis is a complex event involving different pathways (Fig. 1). DNA damage appears to be the predominant factor determining whether a cell undergoes apoptosis, but it is definitely not the only factor. Activation of death receptors on the cell surface also contributes to apoptosis. Death receptors can be activated either directly by UV or by the respective ligands whose release is induced by UV in an autocrine or paracrine manner. Mitochondrial changes and cytochrome C release are also involved in UV-mediated apoptosis; however, it is not yet clear whether these changes are a direct consequence of UV exposure or are related to the membrane and DNA changes, respectively. In addition, the role of oxidative stress in UV-induced apoptosis remains to be determined (65). Further elucidation of the interplay between the different pathways will significantly increase our understanding of photocarcinogenesis and will demonstrate whether enhanced risk of skin cancer is associated with alterations in the apoptotic response.

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