Role of tumor necrosis factor-α in the regulation of keratinocyte cell cycle and DNA repair after ultraviolet-B radiation

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The present PhD thesis is based upon the following publications. These will be referred to by their roman numerals:

I. Faurschou A, Gniadecki R, Calay D, Wulf HC. TNF-α impairs the S-G2/M cell cycle checkpoint and cyclobutane pyrimidine dimer repair: Role of the PI3K-Akt pathway. Journal of Investigative Dermatology 2008; 128(8): 2069-77.

II. Faurschou A, Gniadecki R, Wulf HC. Infliximab inhibits DNA repair in ultraviolet B-irradiated premalignant keratinocytes. Experimental Dermatology 2008; 17(11): 933-8.

III. Faurschou A, Gniadecki R. TNF-α stimulates Akt by a distinct aPKC-dependent pathway in premalignant keratinocytes. Experimental Dermatology 2008; 17: 992-7.

BACKGROUND

Tumor necrosis factor-α (TNF-α)

Tumor necrosis factor-α (TNF-α) is a proinflammatory cytokine crucially involved in innate and adaptive immunity. The primary cell sources for TNF-α synthesis are macrophages and lymphocytes [1-3]. Keratinocytes also produce TNF-α and upon exposure to ultraviolet-B (UVB) radiation, the level of TNF-α has been shown to rapidly increase in the epidermis [4-5].

TNF-α is active as a membrane-bound and soluble form. Newly synthesized TNF-α is expressed on the plasma membrane as a 233 amino acid transmembrane protein (26 kDa) mediating its effect via cell-to-cell contact. Membrane-bound TNF-α may undergo proteolytic cleavage by TNF-α-converting enzyme (TACE) to a 157 amino acid soluble protein (17 kDa). Both membrane-bound and soluble TNF-α form non-covalently linked homotrimers, which are biologically active.

TNF-α exerts its effects by binding to two distinct cell surface receptors, TNFR1 (p55/60) and TNFR2 (p75/80). Keratinocytes lack TNFR2 expression and only TNFR1 is distributed throughout the viable layers of epidermis [6]. Binding of TNF-α to TNFR1 is believed to initiate opposing signaling pathways to promote or inhibit apoptosis. TNFR1 activation results in the formation of the signaling complex of TRADD (TNFR1-associated death domain protein)-RIP (receptor-interacting protein)-TRAF2 (TNFR-associated factor 2), which leads to activation of the prosurvival transcription factor NFκB [7].
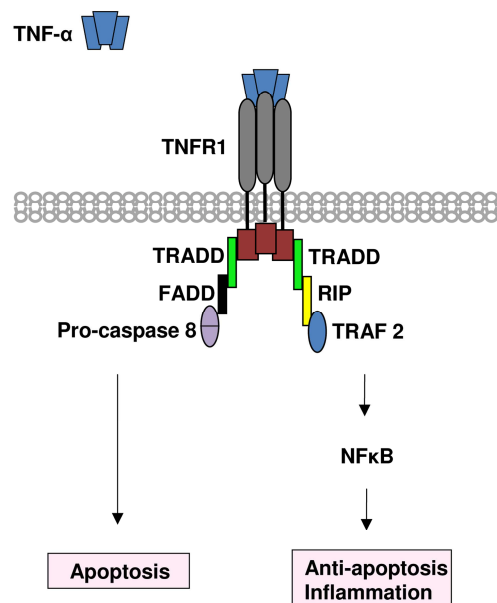

Figure 1. The two signaling pathways of TNF-α.

The result of NFκB activation is inhibition of apoptosis and promotion of inflammation [8]. This signaling pathway is competitive to the signaling route, wherein TRADD recruits FADD (Fas-associated-death domain protein) and caspase 8 leading to cell death.

Substantial evidence documents a crucial role of TNF-α in the pathogenesis of inflammatory skin diseases such as psoriasis and pyoderma gangrenosum [9-11]. Little is however known about the role of TNF-α in skin carcinogenesis. High doses of locally administered TNF-α can cause haemorrhagic necrosis of tumors via selective destruction of tumor blood vessels and generation of specific T cell anti-tumor immunity [1,12]. Integration and expression of the TNF-α gene in murine sarcomas cause regression of the tumors in a dose-dependent way [13]. In keratinocytes, soluble TNF-α has been demonstrated to increase apoptosis and the lack of TNFR1 in keratinocytes and gene-targeted knockout mice results in diminished apoptosis [14-15].

In view of these findings, it is surprising that intact TNF-α-signaling is required for induction of skin tumors. Thus, TNF-α and TNFR1 knockout mice are protected against squamous cell carcinoma [16-18]. The mechanisms by which TNF-α promotes skin carcinogenesis are not fully understood but the involvement of NFκB has been suggested. In normal keratinocytes NFκB signaling seems to protect against squamous cell carcinoma development whereas increased and deregulated NFκB activity is found in carcinomas and confers the anti-apoptotic, mitogenic signal [19-23]. In head and neck squamous cell carcinomas, constitutive activation of NFκB is mediated through TNF-α and protein kinase B/Akt signaling [22]. Altered response to NFκB probably occurs very early in carcinogenesis since TNF-α-induced NFκB inhibits anoikis in immortalized non-tumorigenic HaCaT keratinocytes [24]. It is also conceivable that in skin carcinogenesis, similarly to other cancers, NFκB inhibits the apoptotic response by transcriptional activation of prosurvival genes (bcl-xL, TRAF1, inhibitor of apoptosis, IAPs) [25-27], induction of the cyclin dependent kinase inhibitor p21Cip1/WAF1 [28], and inhibition the tumor suppressor gene PTEN (phosphatase and tensin homologue deleted from chromosome 10) [29-30]. The latter finding is interesting since PTEN is the most potent of known inhibitors of the Akt kinase signaling pathway, which is crucially involved in cell survival and metabolism. Studies have shown that TNF-α is able to activate Akt in several cell types including keratinocytes [31-33].

The question of the role of TNF-α in skin carcinogenesis is especially relevant at present in view of the increased use of anti-TNF-α approaches in the therapy of inflammatory diseases. Pharmacological deactivation of TNF-α by antibodies (adalimumab, infliximab) or antibody-receptor hybrid molecules (etanercept) has a marked therapeutic effect in psoriasis, psoriatic arthritis and pyoderma gangrenosum [4,9-10]. There is some evidence that the incidence of non-melanoma skin cancer is higher in patients who have received anti-TNF-α therapy [34]. Intense immunosuppressive therapy is well known to increase the risk of skin cancer [35-37]. Since non-melanoma skin cancer is highly prevalent among patients with psoriasis due to the use of carcinogenic phototherapies and excessive exposure to solar radiation, current recommendations therefore prescribe that skin photodamage and the presence of multiple pre-cancerous lesions such as actinic keratoses pose relative contraindications to anti-TNF-α treatment due to a theoretical risk of accelerating cancer development by immunosuppression. However, the role of anti-TNF-α treatment in skin carcinogenesis is not clear, in particular in view of the proposed need for TNF-α in the development of skin cancer.

Ultraviolet radiation, DNA damage and DNA repair

The ultraviolet spectrum of solar radiation is divided into three regions: short-wave (UVC, 200-280 nm); mid-wave (UVB, 280-320 nm); and long-wave (UVA, 320-400 nm). Wavelengths shorter than 295 nm are absorbed by stratospheric ozone and the spectrum of ultraviolet radiation that reaches the earth's surface consists of 90-99% of UVA and 1-10% UVB.

Besides its well-known advantageous role in the biosynthesis of vitamin D, ultraviolet radiation has deleterious effects on human skin by inducing inflammation, immunosuppression, premature skin aging and carcinogenesis. UVB radiation is the most energetic component of solar radiation and is considered the major skin carcinogen. UVB is directly absorbed by DNA and causes DNA damage. The most frequent DNA lesions are bond formation between two adjacent pyrimidines within one strand leading to cyclobutane pyrimidine dimers (CPD) and pyrimidine (6-4) pyrimidone photoproducts ((6-4)-PP) (Figure 2) [38].

In sunlight, the (6-4)-PP may be converted to its Dewar valence isomer by absorption of UVA. The (6-4)-PP and its Dewar valence isomer are quickly repaired [38] while CPD are repaired more slowly and accumulate over time [39-40]. There is increasing evidence that CPD formation leads to the generation of mutations and immunosuppression [41-42]. In humans, more than 90% of squamous cell carcinomas have mutations in the p53 tumor suppressor gene, which stem from the erroneous repair of UVB-induced CPD lesions [43]. Animal studies indicate that immunosuppression plays an important role in allowing the transformed cells to escape tumor surveillance mechanisms and develop into skin cancer [41].

Several protective mechanisms exist in human cells to minimize the risk of mutations (Figure 3). Nucleotide excision repair (NER) is the main repair system for DNA damage caused by ultraviolet radiation. Removal of DNA damage can be carried out by two subpathways of NER: transcription coupled repair (TCR) that quickly removes damage present in actively transcribed genes,
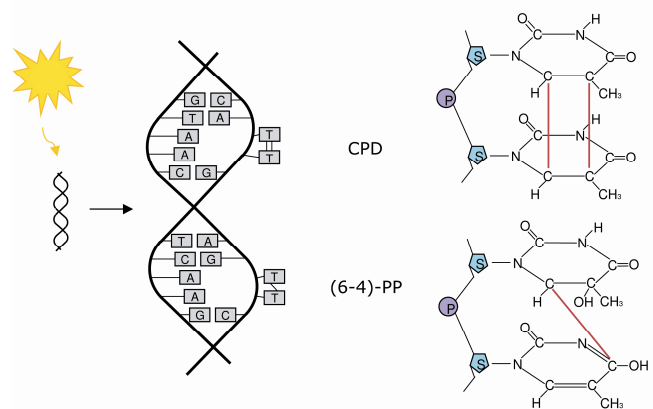

Figure 2. The most frequent DNA lesions formed by irradiation with UVB.

T and global genome repair (GGR) that works slower throughout the whole genome [44].The damaged bases are recognized by their abnormal phosphodiester backbone conformations and are removed by the "excision nuclease", a multisubunit enzyme system consisting of 6 repair factors (RPA, XPA, XPC, TFIIH, XPG and XPF•ERCC1) [44]. After local unwinding of the DNA duplex by approximately 25 bp, dual incisions are made bracketing the lesion in the damaged strand. The excised oligomer is released and repair synthesis fills the gap followed by a ligation. An intact NER is essential for preventing skin cancer formation as exemplified by patients with the photosensitivity syndrome xeroderma pigmentosum, in which defects in the DNA repair machinery results in a high frequency of skin cancer [45]. Similarly, subtle differences in DNA repair efficacy in otherwise healthy patients may increase the rate of non-melanoma skin cancer [46].

A second protective mechanism is the cell cycle checkpoints (G1/S and G2/M) that enable cell cycle arrest, prevent replication and provide more time for DNA repair. Following exposure to ultraviolet radiation, the PI3K-like kinase sensor protein ATR acts as a signal transducer and leads to activation of a web of mediator kinases and effector proteins resulting in inhibition of DNA replication and mitosis [47-48]. The tumor suppressor protein p53 is crucial for maintenance of the G1/S arrest [47] whereas the MAP kinase p38 has been demonstrated to be of critical importance for initiation of the G2/M checkpoint in response to ultraviolet radiation [49]. Moreover, studies have shown that protein kinase B/Akt is centrally involved in the regulation of cell cycle progression [50]. Akt activation can overcome cell cycle arrest at the G1/S and G2/M checkpoints induced by DNA damage and the cells may continue to divide and accumulate mutations [51-52]. This effect may be mediated through deactivation of the forkhead transcription factor FoxO3a [53]. Activated FoxO3a is located in the nucleus and positively affects DNA repair via activation of p53 and G1/S and G2/M cell cycle arrest [54-57]. FoxO3a is able to block cell proliferation, diminish glucose metabolism resulting in atrophy [53,58], and induce cell death via bim [59] and inhibition of bcl-xL [60]. Akt phophorylates FoxO3a that is translocated from the nucleus to the cytosol [61].

Finally, persistence of DNA damage blocks replication and leads to cell elimination by programmed death (apoptosis). Excessive exposure to ultraviolet radiation induces the formation of characteristic sunburn cells, which are DNA-damaged cells in the epidermis committed to apoptotic death [62-63]. Apoptosis can be initiated by the extrinsic pathway through binding of death ligands such as TNF-α, Fas L and TRAIL to their specific death receptor on the cell surface (Fas, TNFR1, DR3, TRAIL-R1, TRAIL-R2 and DR6) followed by caspase 8 and 3 activation, and/or the intrinsic mitochondrial pathway through proapoptotic Bcl-2 family members, cytochrome c release and caspase 9 and 3 activation [64]. UVB irradiation induces apoptosis through both intrinsic and extrinsic pathways in HaCaT cells [65]. Akt regulates apoptosis by multiple means including inactivation of FoxO3a and the proapoptotic Bcl-2 family member Bad [66]. Bad prevents bcl-xL–dependent cell survival [67].
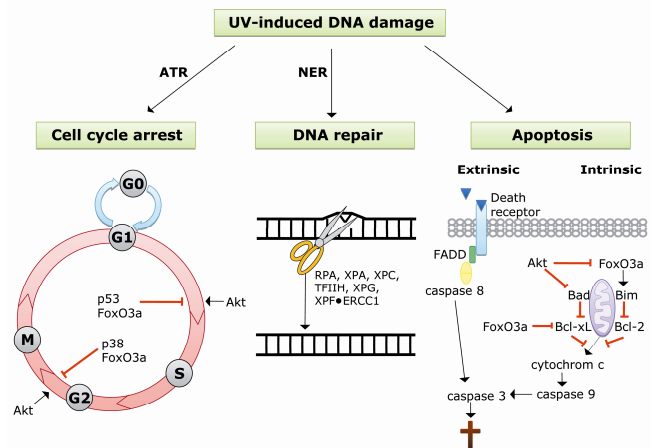

Figure 3.The protective mechanisms of the cells in response to UVB-induced DNA damage.

AIM OF THE STUDY

Since TNF-α has been implicated in the promotion phase of skin carcinogenesis [16-18], the aim of the study was to investigate how TNF-α modulates the physiologic response of premalignant keratinocytes to UVB radiation. In particular, we were interested to study the effect of TNF-α on DNA repair, cell cycle progression, and apoptosis in response to UVB and to examine the possible impact of anti-TNF-α treatment.

RESULTS AND DISCUSSION

Effect of TNF-α on DNA repair and apoptosis

Since cyclobutane pyrimidine dimers (CPD) are considered the most important DNA lesions induced by ultraviolet radiation and responsible for the vast majority of carcinogenic mutations, we studied the effect of TNF-α on the repair of UVB-induced CPD.

A previous study on HaCaT cells has demonstrated that doses of UVB from 0-15 mJ/cm^2 result in no significant apoptosis after 24 hours indicating that the CPD are fully repaired. On the contrary, UVB in the range 20-50 mJ/cm^2 leads to significant apoptosis increasing to 80% 24 hours after exposure to 50 mJ/cm^2 [65]. As a consequence, we used UVB irradiation in the range 0-50 mJ/cm^2 for our experiments with the main part conducted in the range of 0-20 mJ/cm^2 due to excessive apoptosis following irradiations with 50 mJ/cm^2.

The amount of CPD was measured by indirect ELISA using purified DNA from irradiated cells and a specific antibody against CPD. In HaCaT cells, it has been shown that the kinetics of recognition and incision of UVB-induced DNA lesions slow with increasing dose of UVB and the majority of CPD are repaired within the first 24 hours. A study reported that 74-85% of CPD are repaired within 24 hours after exposure to 5 mJ/cm^2 UVB while only 20-31% of CPD are repaired at 24 hours after exposure to 50 mJ/cm2 [38]. Our results support these findings. Measurement of the UVB-induced CPD, as described in paper I and II, showed that 90-99% CPD were repaired within 24 hours after exposure to 10 mJ/cm^2 whereas only 48-65% of the CPD were repaired following

Table 1.

Percent CPD repaired in UVB-irradiated HaCaT cells with or without TNF-α treatment 24 and 48 hours after irradiation. Shown are means and standard deviation of one experiment representative of three independent experiments. The results were compared using unpaired t test.

	10 mJ/cm² UVB			20 mJ/cm² UVB		
	Control	TNF-α	P-value	Control	TNF-α	P-value
24 hours	99% (1%)	78% (3%)	0.0003	65% (13%)	48% (8%)	0.126
48 hours	100% (1%)	76% (3%)	0.0002	85% (5%)	73% (8%)	0.092

exposure to 20 mJ/cm² with only a slight further repair at 48 hours (Table 1 and 2).

After treatment with TNF-α, the repair of CPD in HaCaT cells was significantly decreased both at 24 and 48 hours after irradiation with 10 mJ/cm² compared to controls. Thus after 24 hours, only 78% of CPD in TNF-α-treated cells had been repaired following exposure to 10 mJ/cm² UVB compared to 99% of CPD in control cells. The CPD level in TNF-α-treated cells irradiated with 20 mJ/cm² UVB was also slightly elevated 24 and 48 hours after irradiation compared with irradiated control cells (Table 1) but did not reach statistical significance, which may be due to increased apoptosis after this dose of irradiation. The effect was not found in normal human keratinocytes (Paper I) indicating a role for TNF-α in promotion rather than initiation of skin cancer.

Laser scanning cytometry (LSC) and confocal microscopy was employed to visualise the distribution of CPD-containing HaCaT cells in the phases of the cell cycle and to investigate whether these cells were mitotically active. Propidium iodide (PI) is a red-fluorescent molecule that is intercalated between bases of DNA and can be used to stain DNA for cell cycle analysis. By double labelling the cells with anti-CPD antibody and PI, we found the CPD-containing cells in all phases of the cell cycle with an increased number after TNF-α treatment (Figure 4). To measure whether the CPD-containing cells were mitotically quiescent, as it could be expected, we stained the cells with a specific antibody against CPD and pulse-labelled the cultures with bromodeoxyuridine (BrdU) that is incorporated into DNA during the S-phase of the cell cycle. Surprisingly, we found that some CPD-containing cells were also labelled by BrdU indicating that nuclear CPD were able to enter S phase. The proportion of these cells was significantly higher in the TNF-α treated cultures (Paper I).

The increased amount of CPD in TNF-α treated cells could be due to diminished DNA repair or apoptosis. To investigate this issue, we labelled HaCaT cells with PI and BrdU and visualised the cell cycle with laser scanning cytometry. 10-20 mJ/cm² UVB caused a dose-dependent decrease in G1 phase cells and increase in S- and G2 cells, both 24 and 48 hours after irradiation in HaCaT cells. In comparison to the control cells, TNF-α caused a marked increase in the proportion of actively cycling, BrdU-incorporating HaCaT cells in S-phase and decreased the proportion of G2/M cells after UVB irradiation indicating an impaired G2/M checkpoint (Figure 5).

Treatment with colcemid was used to depolymerise microtubules leading to metaphase arrest of the cells, thereby visualising BrdU-

labelled cells in mitosis by confocal microscropy. The effect of TNF-α was further supported by a 24h growth assay showing that TNF-α had a small, but significant growth-enhancing effect. In normal keratinocytes, TNF-α did not stimulate DNA synthesis and cell cycle progression (Paper I). Taken together, TNF-α seemed to increase the transition through the cell cycle and to attenuate the S-G2 checkpoint imposed by UVB in HaCaT cells.
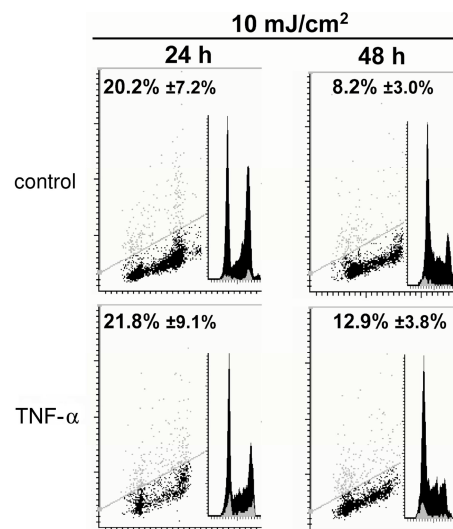

*Figure 4. The distribution and number of CPD-containing cells (grey) in the cell cycle. Histograms represent one of three independent experiments. Data are given as means with standard deviation and were compared by unpaired t test. * P<0.05 compared with irradiated control cells.*
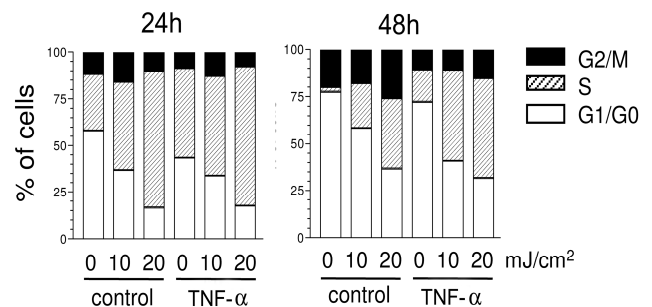

Figure 5. Proportion of sham and UVB-irradiated HaCaT cells in G0/G1, S and G2/M phases with or without TNF-α treatment. Shown is one experiment representative of three experiments.

Following exposure to ultraviolet radiation, apoptotic HaCaT cells have been reported to appear after 8 hours [65]. We therefore measured apoptosis at 4, 24 and 48 hours after irradiation using the apoptosis-specific caspase 3/7 activity assay. The caspase 3/7 activity assay provides a proluminescent caspase-3/7 DEVD-aminoluciferin substrate and a luciferase. Following cell lysis, caspase cleavage of the substrate generates a luminescent signal proportional to the amount of caspase activity present. Our results confirmed that apoptosis is not significantly increased in HaCaT cells after exposure to 10 mJ/cm2 4, 24, and 48 hours after irradiation but is increased 24 and 48 hours after exposure to 20 mJ/cm^2 UVB. We also confirmed that HaCaT cells are more susceptible to UV-induced apoptosis than normal keratinocytes by showing that exposure to UVB resulted in significantly less apoptotic cells among normal keratinocytes than among HaCaT cells (Figure 6) [68].

Earlier studies identified TNF-α as an accelerator of UVB-induced apoptosis [15]. We obtained similar results. TNF-α significantly enhanced apoptosis in response to UVB in HaCaT cells at all time points after irradiation with both 10 and 20 mJ/cm^2 compared with irradiated control cells (P<0.05; Figure 6). TNF-α also caused a significant increase in unirradiated controls. In NHK, TNF-α did only slightly increase apoptosis 48 hours after irradiation with 20 mJ/cm^2 UVB.

From these experiments we concluded that the increased CPD content in HaCaT cells treated with TNF-α was caused by diminished DNA repair and not by impaired removal of irreversibly damaged cells.

Effect of infliximab on DNA repair and apoptosis

To address the putative involvement of anti-TNF-α therapy in the early stages of skin carcinogenesis, we measured CPD in irradiated HaCaT cells treated with the potent TNF-α-neutralizing antibody infliximab. Infliximab is a chimeric monoclonal antibody with murine variable regions and human immunoglobulin (Ig) G1 constant regions. Cells treated with infliximab had significantly decreased DNA repair compared with irradiated untreated cells both 24 and 48 hours after irradiation with 10 mJ/cm^2 UVB. Thus, only 42% of CPD were repaired in infliximab-treated cells 24 hours after exposure to 10 mJ/cm^2 UVB compared with 90% of control cells (Table 2). The CPD level in cells irradiated with 20 mJ/cm2 UVB was also slightly elevated after 24 and 48 hours (Table 2) but did not reach statistical significance.
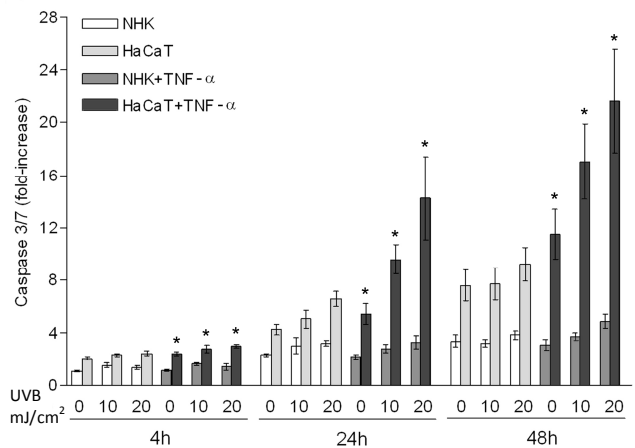

Figure 6. Apoptosis of sham and UVB-irradiated HaCaT cells and NHK with or without TNF-α treatment measured 4, 24, and 48 hours after irradiation. Data represent means with standard deviation of four replicates in one experiment representative of three independent experiments. Data were analysed by unpaired t test. * P<0.05 compared with sham or UVB-irradiated control HaCaT cells at the same time point

We examined the possibility that the increased level of CPD in infliximab-treated cells was due to diminished DNA repair and/or attenuated apoptosis. However, infliximab markedly reduced the proportion of actively cycling, BrdU-incorporating cells in S-phase after UVB irradiation accumulating the cells in G0/G1 and G2/M (Figure 7). Also, infliximab treated cells demonstrated significantly increased apoptosis both 24 and 48 hours after sham-irradiation or irradiation with 10-20 mJ/cm^2 UVB (P<0.0001; Figure 8). Thus, infliximab stimulated both the G2/M checkpoint leaving more time for DNA repair and the apoptotic response. The latter was an unexpected finding since we previously showed that TNF-α Enhances apoptosis in HaCaT cells and others have shown that
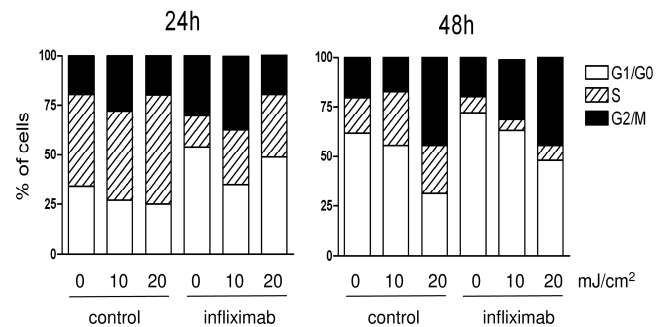
Figure 7. Proportion of sham and UVB-irradiated HaCaT cells in G0/G1, S and G2/M phases with or without infliximab treatment. Shown is one experiment representative of three experiments.

Table 2.

Percent CPD repaired in UVB-irradiated HaCaT cells with or without infliximab treatment 24 and 48 hours after irradiation. Shown are means and standard deviation of one experiment representative of three independent experiments. The results were compared using unpaired t test.

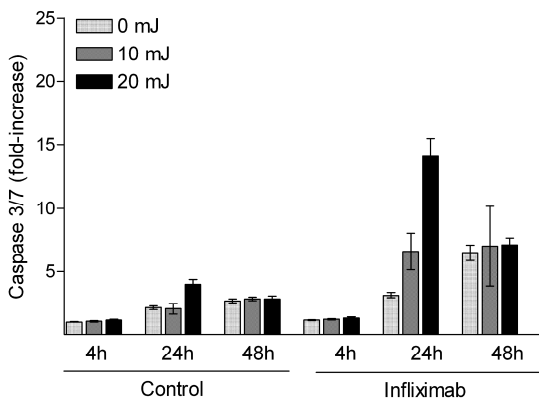|	10 mJ/cm^2 UVB			20 mJ/cm^2 UVB		
	Control	**Infliximab**	**P-value**	**Control**	**Infliximab**	**P-value**
24 hours	90% (18%)	42% (6%)	0.0119	48% (10%)	34% (12%)	0.1955
48 hours	91% (10%)	42% (4%)	0.0039	63% (8%)	48% (4%)	0.0833

Figure 8. Apoptosis of sham and UVB-irradiated HaCaT cells with or without infliximab treatment measured 4, 24, and 48 hours after irradiation. Data represent means with standard deviation of four replicates in one experiment representative of two independent experiments. Data were analysed by unpaired t test.
*P<0.05 compared with sham or UVB-irradiated control HaCaT cells at the same time point.

the lack of TNFR1 or treatment with polyclonal rabbit anti-TNF-α antibodies result in less UVB-induced apoptosis in HaCaT cells or murine skin (Paper I) [15].

The reason for our findings remains unknown but one of the causes could be outside-inside (reverse) signaling via membrane-bound TNF-α (mTNF-α). Antibodies contain two binding sites and are able to cross link (cap) membrane-bound proteins, including mTNF-α, and may act as a ligand. In this situation mTNF-α serves as a receptor transmitting signals through the cytoplasmic tail of mTNF-α into the mTNF-α-bearing cell itself (reverse signaling).

It has been demonstrated that infliximab is able to bind to transmembrane TNF-α and initiate reverse signaling. In a human T cell line, infliximab increased apoptosis and cell cycle arrest through reverse signaling [69]. Moreover, stimulation of monocytes and activated lamina propria T lymphocytes by infliximab activates caspase-3 resulting in increased apoptosis [70-72}. Reverse signaling by infliximab has also been shown to increase the proportion of G1 cells and decrease the number in S phase in infliximab treated T lymphocytes similar to what we found [69]. Another study showed that an anti-TNF-α antibody inhibited the proliferation of two different head and neck squamous cell carcinoma cell lines, which could reflect the consequence of decreased cell cycle progression [22].

In conclusion, infliximab stimulates apoptosis and the G2/M checkpoint in UVB-irradiated premalignant keratinocytes. Despite this, the repair of CPD is impaired.

Akt signaling as a mediator of the effects of TNF-α

TNF-α has been shown to modulate the activity of protein kinase B/Akt in keratinocytes [31]. Abnormal activation of Akt is found in human and murine squamous cell carcinomas and sensitizes keratinocytes to spontaneous and mutagen-induced transformation [73-74]. Since hyperactivation of Akt seems to be an important step facilitating tumor development and progression in a majority of cancers [75], we decided to investigate the

involvement of this signaling pathway in the response of HaCaT cells to TNF-α.

Protein kinase B/Akt comprises a family of serine/threonine kinases that include three different isoforms, Akt1, Akt2 and Akt3 [66]. Four sites have been identified on Akt1 that are phosphorylated in vivo (Ser-124, Thr-308, Thr-450, Ser-473). Ser-124 and Thr-450 are constitutively phosphorylated whereas phosphorylation at Ser-473 and Thr-308 depends on activation by the phosphatidylinositol 3-kinase (PI3K). PI3K consists of a regulatory subunit (p85) that binds to an activated growth f actor/cytokine receptor and undergoes phosphorylation, which results in the activation of its catalytic subunit (p110). PI3K catalyzes the conversion of phosphatidylinositol 4,5-biphosphate (PIP2) to phosphatidylinositol 3,4,5-triphosphate (PIP3) that facilitates the recruitment of Akt to the plasma membrane. Full activation of Akt requires phosphorylation of Thr-308 by phosphatidylinositol-dependent kinase-1 (PDK1) [76]followed by phosphorylation at Ser-473 (Figure 9). The tumor suppressor protein PTEN counteracts PI3K activity by dephosphorylating PIP3 [77].

The involvement of Akt in TNF-α signaling was studied by western blotting using the antibodies specific for the activatory phosphorylated sites at Thr-308 and Ser-473 and by employing different pharmacologicals blockers of PI3K (LY294002 and wortmannin). Treatment with TNF-α resulted in a double phosphorylation of Ser-473 and Thr-308 in Akt in HaCaT cells (Figure 10). This effect could be reproduced also in normal human keratinocytes and the transformed cell line A431(Paper I). LY294002 and wortmannin blocked the TNF-α-induced activation of Akt (Paper I).

UVB (10-20 mJ/cm^2) induced Akt activity in HaCaT cells in accordance with previous data [78]. When UVB irradiation was administered to the TNF-α-treated HaCaT cells, the Akt phosphorylation was more pronounced on both phosphorylation sites (Paper I).
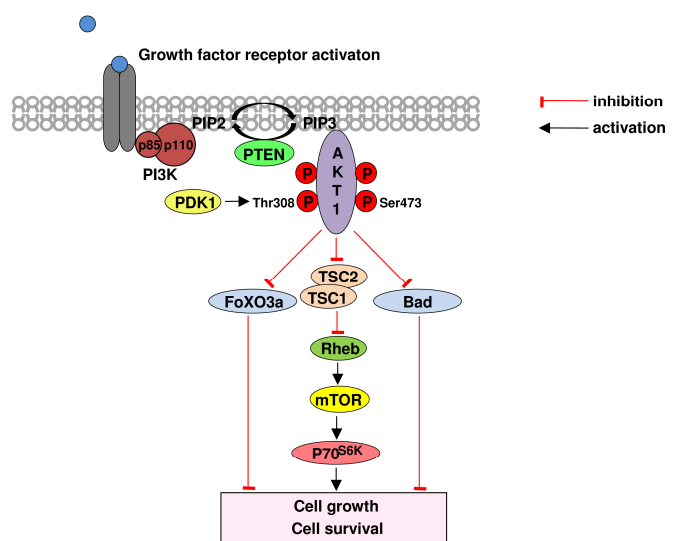

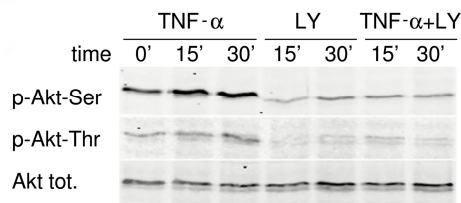Figure 9. Akt activation and downstream signaling.

time	TNF-α			LY		TNF-α+LY	
	0'	15'	30'	15'	30'	15'	30'
p-Akt-Ser							
p-Akt-Thr							
Akt tot.							

Figure 10. Western blot showing that TNF-α leads to Akt activation through PI3K in HaCaT cells.

Cell cycle analyses revealed that LY294002 retarded the cell cycle causing a G0/G1 arrest, restored the G2 cell cycle block, and abrogated the stimulatory effect of TNF-α on DNA synthesis and mitosis in sham- and UVB-irradiated HaCaT cells. LY294002 moreover caused a general decrease in the number of CPD cells. All effects of TNF-α could be counteracted by LY294002 indicating that Akt signaling is responsible for the effects of TNF-α (Paper I).

Infliximab also blocked Akt phosphorylation and, as previously mentioned, caused cell cycle arrest and increased apoptosis. The level of apoptosis induction was comparable to that detected after Akt blockade with an Akt specific inhibitor.

Based on our finding in paper I that TNF-α diminished DNA repair in HaCaT cells but not in NHK, we did more thorough investigation of the TNFα-Akt signaling pathway in HaCaT cells. Since TNF-α-induced activation of the transcription factor NFκB has been shown to inhibit the tumor suppressor PTEN, which is the most potent repressor of Akt, we examined the effect of blocking NFκB on Akt phosphorylation [30]. MG132 is a potent, reversible proteasome inhibitor, which prevents NFκB activation due to blockade of TNF-α induced IkB ubiquitin-proteasome degradation. In TNF-α-stimulated cells, activatory phopsphorylations of Akt were reduced 2-fold after treatment with MG132 indicating that NFκB contributes to Akt phosphorylation (Paper III).

We have previously shown that TNF-α-induced NFκB activation is mediated by atypical protein kinase C species (Lisby et al., 2006). The atypical protein kinase C species (aPKC) ζ and ι/λ constitute a subclass of the protein kinase C family of serine/threonine kinases. aPKC differ from other PKCs in that their catalytic activity is not dependent upon diacylglycerol, calcium or phorbol esters [80]. aPKC have been implicated in carcinogenesis and activated PKCζ is associated with malignant progression of squamous cell carcinoma of the head and neck. We therefore next used the PKCζ pseudopeptide inhibitor (PKCζPS) to block the activity of aPKC and investigate whether this would influence the TNF-α-Akt signaling. The effect of TNF-α on Akt was inhibited by blockade of aPKC (Figure 11).
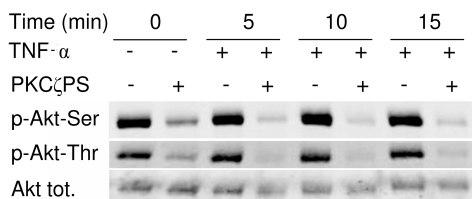

Time (min)	0		5		10		15	
TNF-α	-	-	+	+	+	+	+	+
PKCζPS	-	+	-	+	-	+	-	+
p-Akt-Ser								
p-Akt-Thr								
Akt tot.								

Figure 11. Western blot showing that TNF-α leads to Akt activation through aPKC in HaCaT cells.

The Akt signaling pathway counteracts the proapoptotic activity of TNF-α-related apoptosis-inducing ligand (TRAIL) [81-82]. Blocking the aPKC increased apoptosis significantly in HaCaT cells exposed to TRAIL compared to control HaCats (P<0.05; Figure 12). The level of apoptosis induction was comparable to that detected after Akt blockade with a specific inhibitor or PI3K blockade. Apoptosis in response to UVB measured as either caspase 3/7 activity or survival after 24 hours was however not significantly different between aPKC-blocked and control HaCaT cells (Paper III).

The mitogen-activated protein kinase p38 is critically involved in cell cycle arrest and apoptosis in response to cellular stress. It has been shown that TNF-α induces p38 in HaCaT cells [31], which we confirmed. We moreover showed that blockade of p38 signaling using SB202190 and PD169316 reduced TNF-α-induced Akt activation (Paper III). This suggests that also p38 mediates Akt phosphorylation by TNF-α. The PI3K blockers LY294002 and wortmannin did not affect phosphorylation of p38 but aPKC was shown to suppress p38 activation by TNF-α. These results indicate that p38 participates in Akt phosphorylation upstream PI3K and that p38 activation is suppressed by aPKC. In response to UVB and infliximab treatment, p38 activity was also increased but Akt activity was decreased.

Reactive oxygen species (ROS) function as important second messengers in several signaling pathways [83]. TNF-α enhances ROS production [84] and we therefore investigated the role of different ROS blockers in Akt phosphorylation. N-acetylcysteine (NAC) is a thiol compound that acts both as a precursor of reduced glutathione by providing sulfhydryl groups and as a direct scavenger of ROS including hydrogen peroxide (H_2O_2), hydroxyl radicals (•OH) and superoxid anion (O_2•-)[85-86]. NAC slightly decreased Akt phosphorylation in response to UVB and TNF-α. Tocopherol (vitamin E) is a lipid soluble molecule that tends to concentrate in membranes and quenches singlet oxygen ($^1O_2$), •OH and O_2•. Tocopherol slightly reduced activatory phophorylations of Akt in untreated cells but did not affect UV-induced Akt/p70 activation suggesting a partial involvement of the superoxide and H_2O_2.
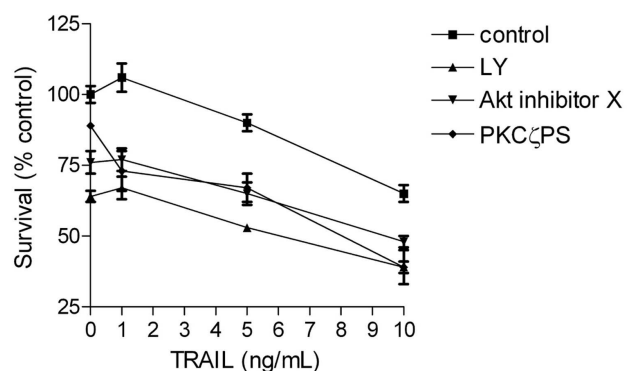

*Figure 12. The cell survival among HaCaT cells treated with TRAIL and different blocking agents. Shown are means with standard deviation of one experiment representative of two independent experiments conducted in four replicates each. Data were compared by unpaired t test. * P<0.05 compared with control cells treated with the same concentration of TRAIL.*

The iron chelator, desferroxamine [87] that blocks the Fenton reaction, the major pathway leading to the formation of •OH from H2O2 in keratinocytes, completely abolished phosphorylation of Akt at the baseline and in TNF-α-treated cells. This indicates a crucial role of •OH in Akt activation.

Subsequently, we decided to investigate the activatory status of the mitogen-activated protein kinase p38, since it is the major sensor of free radical stress in many cells. TNF-α activated p38 in keratinocytes as earlier described [31], but the activatory p38 phosphorylation could be inhibited by NAC and tocopherol rather than by desferroxamine. This suggests that downstream signal transduction from TNFR1 to Akt and p38 involves different ROS species.

To investigate the physiological consequences of the TNF-α-induced Akt signaling, we examined the functional status of its three main targets: FoxO3a, Bad and the survival complex mTORC1. Moreover, we studied Erk 1/2 that is indirectly activated by Akt [88]. Erk1/2 provides a mitogenic signal necessary for tumor progression and is increasingly activated in human head and neck squamous cell carcinoma correlating with tumor proliferation index [89].

FoxO3a became rapidly translocated from the nucleus to the cytoplasm upon treatment with TNF-α. This effect could be inhibited by LY2944002. The rapamycin-sensitive mTOR complex 1 (mTORC1) is activated via phosphorylation and inhibition of the tuberous sclerosis proteins TSC1 and TSC2 and small GTPase Rheb [90] and promotes nuclear transcription by stimulation of cytoplasmic substrates including the serine/threonine kinase ribosomal p70 protein kinase (p70S6K). mTORC1-p70S6K is a positive regulator of cell metabolism, nutrient uptake and cell growth. Treatment with TNF-α resulted in phosphorylation of p70S6K confirming the activation of mTORC1 by this cytokine. We also observed that Bad became rapidly phosphorylated on Ser112 by TNF-α, which has previously been shown to mediate binding to 14-3-3 proteins and Bad inactivation [67]. Finally, Erk 1/2 activation by the TNF-α signaling axis was dependent on the activation of aPKC and could be blocked by desferroxamine.

Taken together, these data provide evidence that TNF-α causes a functional activation of Akt in premalignant keratinocytes.

GENERAL DISCUSSION AND PERSPECTIVES

The cellular model

The HaCaT cell line is a spontaneously transformed human epidermal cell line derived from adult skin keratinocytes. The cells were originally developed by Boukamp et al in 1988 from a histologically normal skin specimen obtained from the distant periphery of a melanoma on the upper back of a 62-year-old male patient [91]. Long-term growth in primary culture (without passaging) was optimal at low Ca2+ concentration and elevated temperature (38.5 C). The cell line was therefore designated HaCaT to denote its origin from human adult skin keratinocytes and its initial growth conditions.

HaCaT cells are highly comparative to cells of an early stage in skin carcinogenesis and may serve as a cellular model of

premalignant skin lesions [92-93]. This cell line has a transformed phenotype in vitro, is immortal (> 140 passages), is aneuploid and has an abnormal and dysfunctional NFκB signaling [24,94]. HaCaT cells exhibit mutations at typical UV hotspots in both alleles of the p53 gene that probably was present in the skin of the patient before excision. Also, increased telomerase activity may stabilize the immortal phenotype [93]. Studies indicate that HaCaT cells are defective in the G0/G1 checkpoint [95] but have an intact G2/M checkpoint.

HaCaT cells remain however non-tumorigenic and non-invasive when injected subcutaneously or grafted as surface transplants onto nude mice [91,96], and they have retained their capacity to form an orderly structured and differentiated epidermal tissue in vitro or after transplantation in mice.

The involvement of TNF-α in UVB-induced carcinogenesis

In the present PhD thesis, we identified several possible mechanisms by which TNF-α contributes to photocarcinogenesis. Exposure to ultraviolet radiation is known to be a potent inducer of TNF-α in the epidermis [4-5,97]. We were able to demonstrate that TNF-α had a deleterious effect on the UVB-induced G2/M checkpoint in premalignant keratinocytes allowing cells with unrepaired CPD to progress through the cell cycle. DNA repair was diminished in TNF-α-treated cells despite increased apoptosis. The effect of TNF-α was not found in normal human keratinocytes (NHK).

One of the possible mechanisms of action of TNF-α was via activation of protein kinase B/Akt (Paper I and III). The importance of Akt in the promotion phase of skin carcinogenesis is underscored by the observations of overexpression of Akt in human and murine squamous cell carcinomas [73-74]. Upregulation of Akt has been linked to tumor promotion in head and neck squamous cell carcinoma via constitutive activation of NFκB [22]. Akt enhances cell survival and metabolism via the prosurvival complex mTOR, kinases ERK1/2 and by its ability to block the proapoptotic proteins Bad and FoxO3a. Similar effects on these downstream signaling proteins were observed in our study (paper I). Both
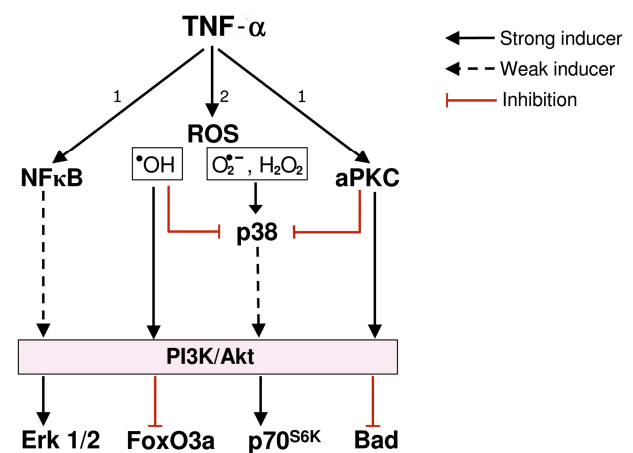

Figure 13. The model of TNF-α signaling proposed by this PhD thesis. 1 Lisby et al., 2006; 2 Jamaluddin et al., 2007.

Erk1/2 and FoxO3a have been shown to contribute to squamous cell carcinoma formation in mice and probably in humans [73,89].

In our model, the activation of Akt by TNF-α was dependent on the atypical PKC species (aPKC) (Paper III). aPKC have been associated with malignant progression of squamous cell carcinoma of the head and neck (SCCHN) [98]. aPKC have also been shown to convey mitogenic signals from the epidermal growth factor receptor (EGFR) through Erk 1/2 in normal keratinocytes and in several SCCHN cell lines [98].

It is known that TNF-α induces ROS in several cell types. ROS can function as signaling molecules and also act as mutagens by inducing oxidative DNA damage [99]. We determined that ROS mediate the activation of Akt by TNF-α in HaCaT cells. Our studies indicate that especially •OH plays an important role in this signaling pathway.

Taken together, we propose a model by which TNF-α activates Akt and impairs protective cellular responses to DNA damage in premalignant keratinocytes (Figure 13). Apart from PI3K/Akt itself, this model identifies two other important signaling intermediates that can serve as possible pharmacological targets in cancer prevention and treatment: aPKC and ROS (in particular •OH).

Obviously, the effect on DNA repair is not the only mechanism of action of TNF-α during carcinogenesis. Inflammation per se is associated with increased risk of carcinogenesis and the degree of risk seems to be greater with severity and persistence of inflammatory activity [100]. TNF-α may facilitate neoplastic development by sustaining a low-grade skin inflammation that eventually contributes to tissue changes and by impairing immune surveillance.

Does anti-TNF-α treatment influence skin carcinogenesis?

Based on the above data it was reasonable to predict that anti-TNF-α treatments would provide a novel anti-cancer strategy. However, unexpectedly HaCaT cells treated with the therapeutic anti-TNF-α antibody, infliximab, had significantly increased levels of CPD compared with irradiated untreated cells despite enhanced G2/M checkpoint arrest and apoptosis. It is possible that this effect is due to a paradoxical reverse TNF-α signaling (Paper II). To further elucidate this issue, studies must be carried out comparing the effects of different anti-TNF-α approaches, especially using the Fab portions of anti-TNF-α antibodies such as certolizumab.

We did not investigate the effect of infliximab in NHK since paper I showed that TNF-α only had deleterious effects on DNA repair in HaCaT cells. However, it could be interesting to explore whether the DNA repair is also decreased in NHK as our results surprisingly showed that this effect possibly is mediated through a non-TNFR1 and non-Akt dependent mechanism.

Our observation is in line with clinical data showing that anti-TNF-α treatment is associated with increased risk of malignancies, in particular lymphoma and skin cancer [11, 101-103]. Although impairment of the Th1 and Th17 responses is a major factor, the effect on DNA repair efficacy warrants further studies especially in view of the fact that phototherapies such as

PUVA and UVB radiation are commonly used for the treatment of inflammatory skin diseases.

CONCLUSIONS

1. TNF-α inhibits DNA repair and the G2/M cell cycle checkpoint in UVB-irradiated premalignant (HaCaT) keratinocytes.

2. Via atypical protein kinase C species and reactive oxygen species, TNF-α activates the Akt signaling cascade including activation of mTOR and inhibition of FoxO3a and Bad.

3. Akt signaling is responsible for the observed impaired cellular responses to UVB in TNF-α-treated HaCaT cells.

4. Infliximab, a TNF-α neutralizing antibody, inhibits DNA repair in HaCaT cells after UVB irradiation despite enhanced G2/M cell cycle checkpoint and apoptosis, and inhibition of Akt.

5. The diminished DNA repair and cell cycle arrest in UVB-irradiated, TNF-α-treated cells may be of relevance for promotion of squamous cell carcinoma.

ABBREVIATIONS AND DEFINITIONS

aPKC	Atypical protein kinase C species
BrdU	Bromodeoxyuridine
CPD	Cyclobutane pyrimidine dimers
DNA	Deoxyribonucleic acid
ELISA	Enzyme-Linked ImmunoSorbent Assay
h	Hours
GGR	Global genome repair
LSC	Laser scanning cytometry
MAPK	Mitogen-activated protein kinase
mJ	Millijoule = 10^{-3} joule
NER	Nucleotide excision repair
NFκB	Nuclear factor-kappa B
NHK	Normal human keratinocytes
nm	Nanometer = 10^{-9} meter
(6-4)-PP	Pyrimidine (6-4) pyrimidone photoproducts
PI	Propidium iodide
PKCζPS	PKCζ pseudopeptide inhibitor
PTEN	Phosphatase and tensin homologue deleted from chromosome 10
RIP	Receptor-interacting protein
SD	Standard deviation
TCR	Transcription coupled repair
TNF-α	Tumor necrosis factor-α
TNFR1	Tumor necrosis factor- α receptor 1 (p55/60)
TNFR2	Tumor necrosis factor- α receptor 2 (p75/p80)
TRADD	TNFR1-associated death domain protein
TRAF2	TNFR-associated factor 2
TRAIL	TNF-α-related apoptosis-inducing ligand
UVA	Ultraviolet-A radiation (320-400 nm)
UVB	Ultraviolet-B radiation (280-320 nm)

SUMMARY

Tumor necrosis factor-α (TNF-α) is a proinflammatory cytokine produced in the skin in response to ultraviolet B radiation (UVB). TNF-α facilitates UVB-induced apoptosis and probably contributes to removal of damaged cells. Surprisingly, murine TNF-α-knockout models have demonstrated that TNF-α is necessary for the early stages of skin carcinogenesis and development of squamous cell carcinoma.

In the present PhD thesis, we examined the effects of TNF-α on DNA repair and cell cycle regulation in UVB-irradiated keratinocytes. In the model of premalignant keratinocytes (HaCaT), TNF-α abolished the UVB-induced G2/M checkpoint and diminished the DNA repair despite induction of apoptosis. TNF-α activated the protein kinase B/Akt and regulation of its downstream targets, mTOR, Bad and FoxO3a. This effect was dependent on atypical protein kinase C species (aPKC) since a specific peptide blocking the activity of the PKCζ and ι/λ abrogated the activation of Akt by TNF-α. The aPKC-Akt axis was likely to be responsible for the TNF-α-induced decrease in DNA repair since blocking of Akt activity restored DNA repair.

Since anti-tumor necrosis factor-α (TNF-α) approaches are increasingly used in the therapy of autoimmune diseases and one of the safety concerns is the potential enhancement of skin carcinogenesis, we investigated the effect of the chimeric monoclonal anti-TNF-α antibody infliximab on UVB-irradiated HaCaT cells. Cells treated with infliximab had significantly increased levels of DNA damage despite enhanced G2/M checkpoint arrest, increased apoptosis and inhibition of Akt.

In conclusion, we identified a possible novel mechanism by which TNF-α promotes UVB-induced skin carcinogenesis. This depends on aPKC-Akt activation and inhibition of DNA repair.

TNF-α-treated cells are prone to escape checkpoint control and are possibly more likely to accumulate mutations, which may constitute a relevant mechanism enhancing tumor development. The effect of anti-TNF-α therapy on skin carcinogenesis warrants further investigation as our study indicates that, in contrast to what had been expected, infliximab may impair DNA repair.

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