Calcineurin Inhibitors Decrease DNA Repair and Apoptosis in Human Keratinocytes Following Ultraviolet B Irradiation

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The use of calcineurin inhibitors in solid organ transplantation results in an increased risk of skin cancer. We examined the effect of these drugs on DNA repair in normal human keratinocytes after ultraviolet B (UVB) irradiation. We found that both cyclosporine A (CsA) and ascomycin inhibited removal of cyclobutane pyrimidine dimers, and that they also inhibited UVB-induced apoptosis. We also observed that UVB induced nuclear localization of the transcription factor nuclear factor of activated T-cells (NFAT), and that this was blocked by CsA and ascomycin. These data suggest that the increased risk of skin cancer observed in organ-transplant patients may be as a result of not only systemic immune suppression but also the local inhibition of DNA repair and apoptosis in skin by calcineurin inhibitors. These findings may have implications for the use of topical calcineurin inhibitors in sun-exposed skin and eyes.

Key words: apoptosis/calcineurin/cyclosporine/DNA repair/pimecrolimus/skin cancer/tacrolimus
J Invest Dermatol 125:1020–1025, 2005

Calcineurin inhibitors such as cyclosporine A (CsA) and the ascomycin-related drugs tacrolimus and pimecrolimus are immunosuppressive drugs used systemically in organ transplantation and topically to treat atopic dermatitis and other dermatological conditions. These drugs bind to immunophilin class proteins (cyclophilins in the case of CsA, and FK Binding Protein (FKBP) in the case of ascomycin-type drugs) and these drug-immunophilin complexes then bind to calcineurin and inhibit its phosphatase activity (reviewed in Reynolds and Al-Daraji, 2002). Inhibition of calcineurin in circulating lymphocytes prevents nuclear localization of the transcription factor nuclear factor of activated T-cells (NFAT), and blocks expression of important genes, such as cytokine genes, involved in T cell activation. The result is profound systemic suppression of the immune system.

The systemic immune suppression produced by these drugs is followed by a dramatic rise in the incidence of skin cancer beginning about 4 y after transplantation (reviewed in Berg and Otley, 2002). These skin cancers are causally related to sunlight because they arise predominantly on sun-exposed skin and they contain mutations in the tumor suppressor gene p53 that are characteristic of those produced by UV radiation (McGregor et al., 1997). The skin cancers are also directly related to the drugs because when drug treatment ceases tumor formation subsides (Otley et al., 2001). Lymphocytes isolated from CsA-treated transplant patients are deficient in DNA repair synthesis following UV exposure (Vamvakas et al., 1996; Weinstein et al., 2000; Herman et al., 2001). Therefore, the systemic inhibition of lymphocytes is considered to cause both the immune suppression and the skin cancer.

Topical use of calcineurin inhibitors is considered to avoid these problems because topical administration avoids systemic exposure, does not produce systemic immune suppression, and therefore does not have the same risk of skin cancer. Here, we show that calcineurin inhibitors CsA and ascomycin inhibit DNA repair of UV damage and reduce UV-induced apoptosis in human keratinocytes, the target cell for many skin cancers.

Results

CsA inhibits cyclobutane pyrimidine dimer repair in normal human epidermal keratinocytes (NHEK) cells
We investigated the effect of the calcineurin inhibitor CsA on DNA repair in UVB-irradiated NHEK cells, using the dot-blot method. Cyclobutane pyrimidine dimers (CPD) formed in DNA immediately after irradiation and then declined in intensity during 24 h incubation because of DNA excision repair (Fig 1, top, row B vs row C). CsA added at 1 μg per mL to the cell culture media for 1 h (row D) or 24 h (row E) prior to irradiation reduced CPD repair during the subsequent 24 h. The intensities of the dots were quantified by image analysis (Fig 1, bottom). Whereas NHEK removed about 66% of the CPD (p < 0.001), the cells treated before irradiation with CsA for either 1 or 24 h removed no more than 35% of the CPD, significantly less than untreated cells (p = 0.023, 0.009, respectively). We observed similar findings in HaCaT-immortalized keratinocytes (data not shown).

CsA and ascomycin inhibit DNA repair in HaCaT Ascomycin is a calcineurin inhibitor structurally unrelated to CsA but that also binds an immunophilin (FKBP12) to
calcineurin. We found that it too inhibited DNA repair in HaCaT cells measured by dot-blots (Fig 2). In the first set of experiments, UVB-irradiated HaCaT cells repaired more than 80% of the CPD from their DNA during 24 h, but pre-treatment for 24 h with CsA at either 0.1 μg per mL or 1.0 μg per mL significantly inhibited this repair (p<0.05 for either). In a second set of experiments (closed bars), ultraviolet B (UVB)-irradiated HaCaT cells at a passage number higher than that of the CsA experiment were less efficient than in the first experiment in repair during the 24 h following irradiation. Nevertheless, ascomycin at either 0.1 or 1.0 μg per mL significantly inhibited repair of CPD (p<0.05 for either), and there was no significant difference between these two doses. As a technical note, over the years, we have observed that the growth rate and repair efficiency of HaCaT cells decline with passage number.

UVB dose–response of repair with CsA at 6 h We investigated the effect in NHEK cells of UVB dose on CPD repair in the presence of calcineurin inhibitors. In this experiment the results of the dot-blot assay were converted to CPD per 10^6 DNA bases using λ-DNA with a known number of CPD/10^6 bases as calibration standards. In Fig 3, we compared the frequency of CPD remaining in DNA at 6 h post-UVB in untreated cells with those pre-treated for either 24 or 48 h with 1 μg per mL of CsA. After a UVB dose of 250 J per m^2 UVB, CPD remained in the DNA at 6 h, and there was no difference among cells with or without CsA. After 500 J per m^2, the cells treated with CsA showed slightly more CPD/10^6 base, but with 750 J per m^2 there was a clear and significant difference after only 6 h between cells untreated and pre-treated with CsA (p<0.05). Pre-treatment for 48 h with CsA produced only a small difference from pre-treatment for 24 h. The difference between CsA treated and untreated cells remained between 15 and 30 CPD/10^6 bases after 24 h (data not shown). Assuming an excess of 15 CPD/10^6 bases and a human genome of 2 x 10^9 bases, the effect of the calcineurin inhibition was to allow about 30,000 of these pre-mutagenic lesions to persist in each irradiated cell.

Cell survival and apoptosis after UVB and CsA We expected that the persistence of DNA damage would reduce...
survival of calcineurin-inhibited cells. On the contrary, we found that the survival of NHEK cells treated with 1 μg per mL CsA cells was similar to untreated cells at 250 J per m² UVB, and at 500 J per m² UVB the pre-treated cells showed at least a 40-fold increased colony-forming ability that was statistically significant (Table I). Similar results were observed for HaCaT cells (Table I) and JURKAT cells, an immortalized T cell line (data not shown). Pre-treatment of HaCaT cells with 1 μg per mL ascomycin also increased colony-forming ability after 500 J per m² of UVB by more than 300-fold compared with untreated cells (p = 0.006, Table I).

The drug-induced increase in colony-forming ability after UVB suggested that apoptosis was inhibited. Apoptosis in UVB-irradiated NHEK cells was measured by the induction of caspase-3 activity per 10⁵ cells at 24 h after UVB (Fig 4).

Low levels of caspase-3 activity were found in untreated cells and UVB doses of up to 250 J per m² produced little change (data not shown), but 500 J per m² UVB increased caspase-3 activity 2- to 8-fold after 24 h (Fig 4). Pre-treatment for 24 h with either CsA or ascomycin at 1 μg per mL significantly reduced UVB-induced caspase-3 (Fig 4). We found variability from experiment to experiment in the amount of caspase-3 at baseline and after UVB, and this made combining data from different experiments problematic. In Fig 4, we present one experiment for CsA and one for ascomycin, and we observed similar results in a total of six experiments. Taken together, the results suggest that these calcineurin inhibitors reduced UVB-induced apoptosis relative to controls even in the face of increased persistent DNA damage.

CsA and ascomycin inhibit UV-induced nuclear translocation of NFAT The best-understood mode of action for calcineurin inhibitors is blocking the nuclear localization of NFAT, a transcription factor for genes activated in T cells. Huang et al (2000) reported that very high doses of UVB (4000 J per m²) induced activation of NFAT in mouse epidermal cells and skin, and this was blocked by high doses of CsA (12 μg per mL). We found that NFAT was localized to the nucleus of NHEK cells at doses between 100 and 500 J per m² UVB (Fig 5A) in a concentration-dependent manner. The localization began within 5 min after 500 J per m² UVB and increased up to 4 h (Fig 5B). We found that localization declined by 24 h (data not shown), whereas Huang et al (2000) reported that the maximum level of activation in mouse cells was 18–24 h after UVB. NFAT localization to the nucleus is dependent on calcium mobilization (Huang et al 2000), and we found that in NHEK cells, CsA and ascomycin at 1 μg per mL reduced nuclear localization following UVB.

### Table I. Colony-forming ability of UVB-irradiated NHEK and HaCaT cells

<table>
<thead>
<tr>
<th>Cell</th>
<th>UVB (J/m²)</th>
<th>Colonies per plate ± SEM</th>
<th>Ratio</th>
<th>p value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>−CsA</td>
<td>+CsA</td>
<td>+CsA/−CsA</td>
</tr>
<tr>
<td>NHEK</td>
<td>0</td>
<td>516 ±14</td>
<td>561 ±19</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>99 ±16</td>
<td>103 ±13</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>0</td>
<td>12 ±1</td>
<td>&gt;40.0</td>
</tr>
<tr>
<td>HaCaT</td>
<td>0</td>
<td>61 ±4</td>
<td>100 ±3</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>18 ±3</td>
<td>8 ±1</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>1 ±0</td>
<td>15 ±2</td>
<td>15.0</td>
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<table>
<thead>
<tr>
<th>Cell</th>
<th>UVB (J/m²)</th>
<th>Colonies per plate ± SEM</th>
<th>Ratio</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>−Asc</td>
<td>+Asc</td>
<td>+Asc/−Asc</td>
</tr>
<tr>
<td>HaCaT</td>
<td>0</td>
<td>566 ±22</td>
<td>716 ±6</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>330 ±45</td>
<td>326 ±73</td>
<td>1.0</td>
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<td>250</td>
<td>80 ±4</td>
<td>132 ±9</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>0</td>
<td>104 ±8</td>
<td>&gt;315</td>
</tr>
</tbody>
</table>

UVB, ultraviolet B; NHEK, normal human epidermal keratinocytes; CsA, cyclosporine A; Asc, ascomycin.
The number of colonies formed (average of three replicates) by UVB-irradiated cells pre-treated with 1 μg/mL CsA or ascomycin for 24 h compared with untreated cells.

*NS, not significant. Two-tailed t test except differences from zero by the one-sample t test.

Figure 4
Cyclosporine A (CsA) and ascomycin (Asc) inhibit ultraviolet B (UVB)-induced caspase-3 activation in normal human epidermal keratinocytes (NHEK). NHEK were pre-treated as indicated with 1 μg per mL CsA or ascomycin, irradiated with 500 J per m² UVB, incubated for 24 h, and caspase-3 activity measured. The plot shows a mean of two replicates.
Examples of NHEK cells stained for NFAT are shown below the graphs in Fig 5. The nuclear localization after 5 min or 4 h is apparent relative to unirradiated cells, whereas CsA- or ascomycin-treated cells fail to show this intensity of nuclear fluorescence.

**Discussion**

Systemic calcineurin inhibitors have had great success in improving organ transplantation, but they have the undesirable side effect of increasing the risk of cancer in sun-exposed skin. Topical calcineurin inhibitors have also been successful for diseases such as atopic dermatitis because their steroid-sparring effect minimizes dermal atrophy, striae, and telangiectasia, and they were considered as preventing the skin cancer risk. But, evidence is accumulating that systemic immune suppression is not the only cause of the increased risk of skin cancer (André et al., 2004), and at least in the case of renal transplant patients, their DNA repair capacity is impaired (Vamvakas et al., 1996; Herman et al., 2001).

We report here that the calcineurin inhibitors CsA and ascomycin each inhibited repair of UV-induced DNA damage in human keratinocytes, the target cell for most skin cancers. The inhibition is achieved with as low as 0.1 μg per mL drug, and as quickly as within 1 h after exposure, whereas the preferred experimental conditions are 1 μg per mL and 24 h pre-incubation. The concentration of CsA in the epidermis of a patient treated for psoriasis for 3 d is 2.8 μg per mL (Fisher et al., 1988), and the ascomycin-related drug tacrolimus is topically applied at 1000 μg per mL (0.1% wt/vol), whereas pimecrolimus is topically applied at 10,000 μg per mL (1% wt/vol). The magnitude of the effect of CsA and ascomycin on repair of CPD are dependent on the dose of UVB, but is observed with a dose of 500 J per m² UVB producing less than 100 CPD/10⁶ DNA bases, which is well with the range of minimal erythemal doses for skin types I–III (Hönigsmann et al., 1987; Hemminki et al., 2002).

We suspect that the cause of the reduced DNA repair is because of inhibition of calcineurin, as two drugs with different structures and different methods of calcineurin inhibition both inhibited DNA repair. CsA inhibits calcineurin by binding it with cyclophilin A, whereas ascomycin inhibits it by binding it with FKBP12. We further speculate that transcription factor NFAT participates in DNA repair, and that the inhibition of calcineurin prevents dephosphorylation of NFAT1 and its nuclear localization. Consistent with this hypothesis, UVB induces nuclear localization of NFAT1, as part of a “UV response” (Fig 5, Huang et al., 2000). Transcription factors are the first proteins to encounter DNA damage during transcription, and several have been identified in DNA repair complexes (Svejstrup, 2003).

The persistence of DNA damage is itself a risk factor for skin cancer. Diminished DNA repair capacity causes an increased risk for skin cancer (Wei et al., 1993), and polymorphisms in DNA repair genes that may reduce DNA repair are correlated with cancer risk, including skin cancer (Goode et al., 2002). In the extreme case of the disease xeroderma pigmentosum, genetic mutations inactivate DNA repair genes and increase the skin cancer risk by 1000-fold (Kraemer et al., 1987).

These calcineurin inhibitors also inhibited UV-induced apoptosis in addition to DNA repair. CsA has previously been reported to inhibit apoptosis by two mechanisms: (1) by binding of cyclophilin A to calcineurin, it blocks...
dephosphorylation of the apoptosis-inducer Bcl 2-antagonist of cell death (BAD), and (2) by binding of cyclophilin D to mitochondrial permeability transition pore protein, it prevents release of the apoptosis-inducer cytochrome C (Waldmeier et al, 2002). As ascomycin also reduces caspase-3 induction (Fig 4) but does not bind cyclophilin D, we suspect that the first mechanism, blocking BAD dephosphorylation, is important in keratinocytes as it is in other cells (Yang et al, 2004).

Inhibition of apoptosis is recognized as a tumor-promoting event in skin cancer (Bernstein et al, 2002, Guzman et al, 2003). For example, mutational inactivation of the p53 DNA repair gene leads to a reduction of “sunburn cells” or apoptotic keratinocytes, and this is an early event in skin cancer (Ziegler et al, 1994). The combination of persistent DNA damage in cells and the inhibition of apoptotic removal of such cells, both caused by calcineurin inhibitors, creates a potential for increased risk of skin cancer.

This combination of reduced DNA repair and reduced apoptosis leading to skin cancer has been observed in mice, whether the calcineurin inhibitor is administered systemically or topically. Systemic administration of CsA in mice reduced DNA repair synthesis in the epidermis following UVB exposure, and also suppressed the appearance of apoptotic sunburn cells (Sugie et al, 2002). It was disclosed at the time of approval of both tacrolimus and pimecrolimus that topical treatment of mice with the drugs during exposure to solar-simulating light shortened the mean time to skin tumor formation, but both studies were flawed by the greatly increased photocarcinogenesis produced by the vehicle alone. Topical treatment of mice with tacrolimus dramatically increased the induction of skin tumors after exposure to the carcinogen 7,12-dimethyl benz α-anthracene (DMBA) (Niwa et al, 2003).

The published long-term safety studies in humans of topical calcineurin inhibitors have not been designed to detect an increased risk of skin cancer. They have been of only 1 year duration and were underpowered to measure significant changes in skin cancer rates (Kang et al, 2001; Luger et al, 2004). As a point of reference, organ transplantation became widespread in 1965, and the first report of an association with skin cancer appeared from Australia 7 years later (Marshall 1972).

Topical calcineurin inhibitors are approved for use in adults and in children as young as 2 y old. Of special note is their use in vitiligo, in which the drug may precede, follow, or be combined with UVB to stimulate pigmentation (Passeron et al, 2004; Silverberg et al, 2004). An additional indication is the approved use of 0.05% (500 μg per mL) CsA eyedrops for treatment of dry eye. UVB damages the corneal epithelium and endothelium, and DNA repair is an important part of the cellular defense (Brenner and Grabner, 1981). Xeroderma pigmentosum patients are deficient in DNA repair and their rate of neoplasms of the anterior eye, including corneal epithelioma, is elevated about 2000-fold for patients 20 y and younger (Kraemer et al, 1987). They also suffer from corneal clouding and vascularization at an early age.

In summary, the data presented here suggest that the increases in rates of skin cancer observed in organ transplant patients may be as a result of not only systemic immune suppression of circulating T cells but also the inhibition of DNA repair and apoptosis in skin by calcineurin inhibitors. These findings may have implications for the use of topical calcineurin inhibitors in sun-exposed skin and eyes. The FDA has recently issued alerts concerning topical calcineurin inhibitors.3,4

Materials and Methods

NHEK were purchased from Cascade Biologics (Portland, OR) and cultured in their Epilife medium containing the human keratinocyte growth supplement kit. Each experiment was performed with cells from a single donor. HaCaT cells were cultured in the Invitrogen (Carlsbad, CA) Dulbecco minimal essential medium low-glucose medium with 1% pen/strep, 1% l-glutamine, 1% non-essential amino acids, and with 10% heat-inactivated fetal bovine serum (Sigma, St Louis, MO). All cells were incubated in a 37°C incubator with a 5% humidified CO2 atmosphere.

CsA was pharmaceutical grade (identity and purity confirmed by infrared and high-performance liquid chromatography), but equivalent research grade is available from Sigma, St Louis, MO. Ascomycin was from Alexis, San Diego, CA. The drugs were diluted in ethanol, added to the media, and incubated for 1–48 h prior to irradiation. The media were collected and replaced with phosphate-buffered saline (PBS). The cells were irradiated with two FS40 UVB bulbs (Phillips, Twinsburg, OH) filtered with Kodacel paper (Kodak, Rochester, New York) that absorb UVC. Sixty-five percent of the UVB energy was in the 290–320 nm range with a peak emission at 313 nm. The UVB radiation was measured using a Model IL1400A Radiometer/Photometer and a UVB#21303/W#9560 probe (International Light Inc., Newburyport, MA). After irradiation, the PBS was replaced with fresh media or the media containing CsA or ascomycin.

CPD were measured in UV-irradiated DNA by the Southwestern dot-blot method using antibodies against CPD (Yarosh et al, 2002), with the exception that the filters were not baked. Dot blots were loaded with equal OD260 units of DNA extracted from cells, and loading was measured by staining equally loaded neutral agarose gels with ethidium bromide. Dots were visualized using a Kodak IS440CF imaging station (Kodak, Rochester, NY) and quantified by image analysis TotalLab (Phoretix Intl., Durham, NC) and normalized for loading. The blots in Fig 3 were calibrated using standards of λ-DNA irradiated with a range of UV doses. The number of CPD per 106 bases in these standards was determined by the alkaline agarose gel method using λ-HindIII fragments for calibration (Yarosh et al, 2002). The blots were developed by phosphorfluorescence, and the intensity of the images was analyzed by TotalLab.

For colony formation, cells were plated into Corning (Corning, NY) T-75 flasks, and after drug treatment, cells were counted using a Coulter Z1 Particle Counter (Beckman Coulter, Hialeah, FL). One thousand cells were plated into 100 mm cell culture dishes (Corning) and after incubation for 5–6 h the plates in triplicate were irradiated with UVB and fresh media with drug were again added. The cells were incubated for 7 d at 37°C, fixed with methanol, stained with 0.25% methylene blue in PBS, and the colonies were counted.

Caspase-3 was measured using a Rediplate96 EnzCheck-Caspase-3 Assay Kit (Moleular Probes, Eugene, OR). Briefly,1
calcineurin inhibitors decrease DNA repair


