PHOTOIMMUNE SUPPRESSION AND PHOTOCARCINOGENESIS

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1. ABSTRACT

The primary cause of non-melanoma skin cancer, the most prevalent form of human neoplasia, is the ultraviolet (UV) radiation found in sunlight. Exposing mice to UV radiation induces skin cancers that are highly antigenic. Upon transfer of an UV-induced skin cancer to a normal syngeneic mouse, the tumor cells are recognized and rapidly destroyed by the immune system of the recipient. This raises the question of how these cancers avoided immune destruction during their development in the UV-irradiated host. This question was answered when it was discovered that in addition to being carcinogenic, UV radiation was also immunosuppressive. Studies with immune suppressed transplantation recipients, and biopsy proven skin cancer patients have confirmed that UV-induced immune suppression is a risk factor for skin cancer development in humans. It is of great importance, therefore, to understand the mechanisms underlying UV-induced immune suppression. The focus of this manuscript will be to use some examples from the more recent scientific literature to review the mechanisms by which UV radiation suppresses the immune response and allows for the progressive outgrowth of antigenic skin tumors.

2. INTRODUCTION

The carcinogenic effects of UV radiation are well known (1). It is estimated that in the year 2002, between 1 to 2 million new cases of skin cancer will be diagnosed in the United States alone (www.cancer.org/statistics). Fortunately, because of their highly visible location, skin cancers are more rapidly diagnosed and more easily treated than other types of cancer. Regardless, approximately 10,000 Americans a year die from skin cancer. Similar statistics are reported throughout the industrialized world. The morbidity and mortality associated with skin cancer is a major public health concern.

The first step in UV-induced carcinogenesis is DNA damage. This leads to a cascade of events orchestrated by the tumor suppressor gene *p53*. UV exposure induces transcription of the *p53* gene, resulting in production of p53 protein that causes cell cycle arrest and affords the cell an opportunity to repair its damaged DNA. If the damage is so extensive and repair is not possible, the cell undergoes apoptosis. Unfortunately, UV exposure mutates the *p53* gene at a high frequency so this critical function is lost, cells with mutations survive, and skin cancers develop [reviewed by Ouhtit et al. (2)].

UV-induced mutations in *p53* and the associated effects on apoptosis help to explain the carcinogenic effect of UV exposure. However, UV radiation has another biological effect that potentates its carcinogenic potential. In addition to being a complete carcinogen, UV radiation is also immunosuppressive. Margaret Kripke first recognized the immune suppressive effect of UV radiation in a series

of experiments in which UV-induced skin cancers were transplanted into normal age and sex-matched syngeneic recipient mice. None of the tumors grew when transplanted into normal mice. Tumor growth was only apparent if the recipient mice were immune compromised. This observation indicated that the tumors were highly antigenic. They were recognized by the immune system of the recipient mice and rejected. Only when the immune system of the recipient was compromised, did the UV-induced skin cancer grow progressively. These experimental results, however, did not explain how the antigenic tumors could develop and grow in the UV-irradiated host, who presumably had an intact immune system. The only way to explain these findings was to propose that UV exposure was somehow compromising the immune system of the irradiated animal. Experimental proof of this hypothesis was provided by observing progressive growth of the transplanted tumors in mice that were exposed to a sub-carcinogenic dose of UV radiation (3). These initial experiments gave rise to a new discipline merging elements of photobiology, cancer research, dermatology and immunology, known as photoimmunology. Epidemiological studies with immune suppressed transplant patients (4) and studies with skin cancer patients (5), support the hypothesis that the immune suppression induced by UV exposure is a major risk factor for skin cancer induction. In addition, photoimmunologists over the years have provided evidence that UV exposure can suppress a wide variety of immune reactions, including the immune response to viral, bacterial and fungal antigens (6). This type of immune suppression can occur after a single exposure to doses of UV radiation that are easily obtained during normal occupational and recreational exposure (7, 8). Because the immune suppression induced by UV exposure has the potential to adversely affect human health and well being, this manuscript will focus on reviewing the mechanisms involved.

3. MECHANISMS UNDERLYING UV-INDUCED IMMUNE SUPPRESSION

3.1. Molecular targets in the skin

In order for electromagnetic energy (i.e., UV radiation) to be converted into a biological signal, it must interact with a chromophore in the target tissue, the skin. To date three molecular targets have been identified that absorb UV energy and convert it into a biological signal, DNA, urocanic acid and peroxidation of membrane lipids.

3.1.1. DNA

UV radiation is a well-known DNA-damaging agent. The most abundant UV-induced DNA lesions are pyrimidine dimers and 6-4 photoproducts at dypyrimidine sites (UV signature mutations), although recent data has indicated that the pyrimidine dimers, and not 6-4 photoproducts are responsible for the majority of mutations induced by UV radiation (9). As mentioned above, UV-induced *p53* mutation is the initiating event in skin cancer formation (10). There is also considerable evidence that the initiating event in UV-induced immune suppression is DNA damage, and particularly the formation of pyrimidine dimers. Evidence supporting this conclusion was generated in a series of experiments in which repairing UV-induced DNA damage blocked the induction of immune suppression.

The first set of experiments took advantage of the endogenous light-activated DNA repair mechanism found in *Monodelphis domestica*. This marsupial possess a repair enzyme that complexes to UV-induced pyrimidine dimers, and upon absorbing energy provided by visible light, splits the dimer and restores the DNA to its un-damaged configuration. This photoreactivating enzyme is highly specific in its ability to repair pyrimidine dimers and studies by Ley and colleagues demonstrated that shining visible light on the marsupials after, but not before UV-irradiation, reversed pyrimidine dimer formation and reduced the incidence of skin tumors (11). Kripke and her colleagues used *M. domestica* to test the hypothesis that DNA was the molecular target for immune suppression. In these experiments, the marsupials were first exposed to UV and then treated with a contact allergen. As expected, and as shown previously with mice and humans, UV exposure suppressed the generation of contact hypersensitivity in UV-treated marsupials. The marsupials were then treated with visible light immediately following UV exposure. Treating the UV-irradiated marsupials with photoreactivating light had two effects. First, the number of pyrimidine dimers in the skin was reduced dramatically. Second, no immune suppression was noted in animals exposed to UV and then exposed to visible light. Because repairing pyrimidine dimer formation *in vivo* blocks the induction of immune suppression, these findings provided the first evidence that DNA is the target in the skin that absorbs UV and initiates immune suppression (12).

Because there are fundamental differences in the nature of the immune response generated in marsupials, and placental mammals, concerns were raised as to whether the data generated with marsupials could be due to some unknown artifact. To address these concerns, a second set of experiments was performed using mice. Because mice do not possess an endogenous set of dimer repair enzymes, a method was designed to introduce the enzyme into the target cells. To accomplish this goal, liposomes containing the bacteriophage excision repair enzyme, T4N5 were applied to the skin of mice. These multilameller lipid vessels penetrate the cell membranes of epidermal cells, where they are destabilized by the acidic pH, thus delivering their contents intracellularly. Electron microscopic examination of murine skin treated with these liposomes documented delivery of the enzyme into the cytoplasm and nucleus of keratinocytes and Langerhans cells (13). When T4N5-containing liposomes were applied to the skin of UV-irradiated mice the number of pyrimidine dimers in epidermal DNA was reduced, and the induction of immune suppression was blocked. Control liposomes containing a heat-inactivated enzyme preparation were inert, and did not reverse the immune suppressive effect. The reversal of immune suppression by T4N5-containing liposomes was noted regardless of whether systemic suppression (i.e., UV irradiation at one site, antigen administration at another non-irradiated site) of contact hypersensitivity (CHS) or systemic suppression of delayed-

type hypersensitivity (DTH) was used as immunological endpoints. In addition, applying T4N5-containing liposomes the skin of UV-irradiated mice blocked the induction of immune-regulatory suppressor T cells (see section 3.2). These findings confirm the data generated in marsupials, and provide further support for the hypothesis that UV-induced pyrimidine dimer formation is the initiating step in UV-induced immune suppression (14).

A third set of experiments, using a different measurement of immune suppression also provided evidence supporting DNA damage as the first step leading to UV-induced immune suppression. In these experiments the ability of UV-damaged Langerhans cells to mediate UV-induced local immune suppression was measured. Local immune suppression is the term photoimmunologists use when referring to the situation in which antigens, in most cases small molecular weight contact allergens, are placed directly onto the UV-irradiated skin. When the recipient is challenged 5 to 7 days later with the same contact allergen, his/her immune response is suppressed (15, 16), and in the case of mice, antigen-specific suppressor T cells are found in their lymphoid organs (17). Formal proof that the epidermal Langerhans cells were the target of UV-radiation was presented by Cruz and colleagues, who found that injecting UV-irradiated, antigen-pulsed Langerhans cells into normal mice resulted in the induction of immune suppression and the induction of long lasting tolerance in the recipient mice (18). Kripke and co-workers asked whether UV-induced DNA damage transforms the Langerhans cells from a potent antigen-presenting cell into a tolerance-inducing cell. In these experiments dendritic cells from hapten-painted mice were used to immunize normal recipients. The antigen-presenting cells were isolated from mice that were painted with the fluorescent hapten, fluorescein isothiocyanate (FITC). Previous experiments indicated that Ia-positive, FITC-positive cells isolated from the draining lymph nodes of normal mice, presumably antigen positive migrating Langerhans cells, were very effective antigen presenting cells. When these cells were injected into normal recipient mice (i.e., a dendritic cell vaccine), a vigorous immune reaction was noted in the recipient mice. On the other hand, when the FITC-positive cells were isolated from mice that were first exposed to UV and then painted with hapten at the site of UV exposure, no immune response was observed in the recipient mice (19). Does DNA damage, specifically the induction of pyrimidine dimers, alter cutaneous antigen presenting cell function? Here again, the question was addressed by applying T4N5-containing liposomes to the mice immediately after UV exposure and prior to FITC application. The draining lymph nodes were removed and the dendritic cells enriched by metrizamide gradient centrifugation. Dendritic cells isolated from UV-irradiated mice, or isolated from mice exposed to UV and treated with liposomes containing heat-inactivated T4N5, failed to induce an immune response. When, however, the dendritic cells were isolated from mice exposed to UV and treated with T4N5-containing liposomes, little to no immune suppression was noted. In addition, the number of pyrimidine dimers found in dendritic cells isolated from UV-irradiated T4N5-treated mice was significantly reduced (20). These findings suggest that reversal of UV-induced pyrimidine dimer formation in epidermal Langerhans cells reverses the induction of immune suppression, thus providing further evidence supporting the hypothesis that DNA damage is the initiating event.

The data presented above suggest, but do not conclusively prove that UV-induced DNA damage in Langerhans cells is the initiating event in suppressed cutaneous antigen-presenting cell function. This is because DNA damage also activates keratinocytes to secrete immune regulatory cytokines, such as interluekin (IL)-10 (21). One of the primary immunological functions of IL-10 is modulation of antigen presenting cell function (22). Therefore, it is not clear if applying T4N5-containing liposomes to the skin of UV-irradiated mice has a direct effect on Langerhans cells or if it works *via* an indirect mechanism by modulating keratinocyte-derived cytokine production. To directly address this question FITC-positive, dimer positive, Ia-positive dendritic cells were isolated from the draining lymph nodes of UV-irradiated mice. The purified dendritic cells were then cultured, *in vitro*, in the dark with liposomes containing photolyase, a pyrimidine dimer-specific photoreactivating enzyme isolated from *Anacystis nidulans*. After a period of time, the cells were washed and exposed to photoreactivating light. The dendritic cells were then injected into normal recipient mice, and their ability to vaccinate the recipient mice was used as the immunological endpoint. Photoreactivation reduced the numbers of pyrimidine dimers present in the dendritic cells and totally restored antigen-presenting cell function. Treating the dendritic cells with the photolyase-containing liposomes only, or with only photoreactivating light, failed to restore antigen-presenting cell function. Similarly, if the sequence of events was reversed, and the photoreactivating light was applied prior to liposome application, no reversal of immune suppression was noted. Because the photolyase was applied *in vitro*, to cultures devoid of keratinocytes, these findings indicate that the UV-induced DNA damage to epidermal Langerhans cells is the initiating event in UV-induced local suppression of CHS (23).

Similar data, implicating UV-induced pyrimidine dimer formation as the initiating event in UV-induced immune suppression in humans have been generated. Wolf and colleagues (24) exposed patients with a prior history of skin cancer to a UV source that closely mimicked sunlight (5.5% UVB, 94.5% UVA). Immediately following UV exposure, T4N5-containing liposomes were applied to the irradiated site. Transmission electron microscopy clearly demonstrated that the enzyme penetrated through the skin and could be found in the cytoplasm of Langerhans cells and keratinocytes. In addition, a slight, although significant reduction in the numbers of pyrimidine dimers in the T4N5-treated skin was noted. Next the effect that T4N5-treatment had on UV-induced cytokine production was measured. As mentioned above, animal studies indicated that repairing UV-induced pyrimidine dimer formation *in vivo* blocked the production of immune regulatory cytokines such as IL-10 and tumor necrosis factor-alpha (TNF-alpha) (25, 26). Identical results were found in

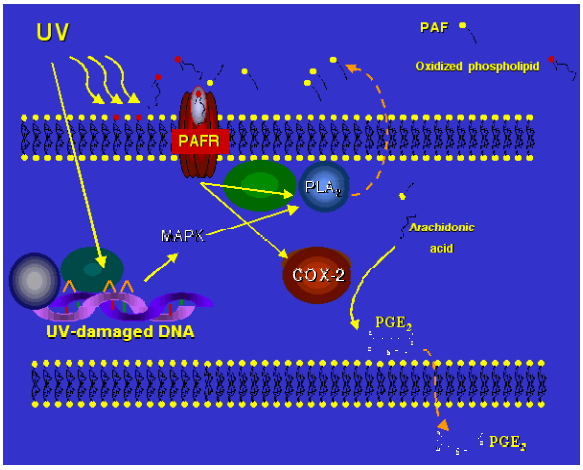

Figure 1. Suggested pathway for UV-induced production of PAF and the initiation of the immune suppressive cytokine cascade. Upon UV-irradiation two initial events occur. Free-radical activation causes the oxidation of phosphatidylcholine and production of PAF-like molecules at the membrane. UV-induced pyrimidine dimer formation activates p38 MAP kinase that initiates G2/M checkpoint delay and activates PLA_2. Activated PLA_2 stimulates the production of *bona fide* PAF and catalyzes the release of arachidonic acid from the cell membrane. PAF up-regulates the transcription of COX-2, which stimulates the production of PGE_2. PAF and PAF-like molecules are secreted and bind to PAF receptors on adjacent keratinocytes, amplifying the suppressive signal.

human patients exposed to UV radiation. Both IL-10 and TNF-alpha were up regulated (mRNA and protein) in UV-irradiated skin, and the application of T4N5-containing liposomes prevented the up-regulation of these immune regulatory cytokines.

Stege et al used photolyase-containing liposomes to repair UV-induced DNA damage and arrived at a similar conclusion (27). In this case two different immunological endpoints were examined. Interferon-gamma (IFN-gamma)-treated keratinocytes up-regulate the expression of intracellular adhesion molecule-1 (ICAM-1), a molecule required for immune and inflammatory reactions in the skin. Exposure to UV radiation suppresses the up-regulation of ICAM-1, and previous studies have suggested a correlation between ICAM-1 suppression and susceptibility to skin cancer induction (28). Treating UV-irradiated skin with photolyase-containing liposomes blocked the UV-induced suppression of ICAM-1 up-regulation. The second immunological assay examined was suppression of CHS. Nickel sensitive patients generate a vigorous hypersensitivity when nickel sulfate is applied to their skin. UV exposure suppresses this reaction. Stege et al (27) found, in close agreement with data generated using mice (14), that application of photolyase-containing liposomes reversed UV-induced suppression of CHS.

These findings, using a variety of immunological endpoints, and multiple methods of repairing pyrimidine dimer formation, all support the hypothesis that UV-induced DNA damage is the initial event in UV-induced immune suppression. Moreover, a recent study by Yarosh et al reminds us of the essential role that UV-induced pyrimidine dimer formation plays in skin cancer induction (29). In this study T4N5-containing liposomes were applied to the skin of Xeroderma pigmentosum patients. Xeroderma pigmentosum patients are uniquely susceptible to the carcinogenic effects of sunlight because of a genetic defect that renders them unable to repair UV-induced pyrimidine dimer formation. Using actinic keratoses as a surrogate measure of skin cancer induction, Yarosh et al, found a significant reduction in the rate of new cancers in patients treated with T4N5-containing liposomes (68% reduction $p< 0.004$) compared to placebo-treated controls. Here again, repair of UV-induced pyrimidine dimer formation in humans yields results that are similar to those previously generated in experimental animals (11, 30), reduction in skin cancer incidence and inhibition of immune suppression.

The studies reviewed above provide compelling evidence for UV-induced pyrimidine dimer formation as the initiating lesion in both cancer induction and photoimmune suppression. Although it is easier to conceptualize how UV-signature mutations in the DNA binding sites of the tumor suppressor gene *p53* can contribute to skin cancer induction, the association between UV-induced DNA damage and immune suppression is not as readily apparent. We suggest the following (**Figure 1**). Following genomic stress, a cell must progress through a series of checkpoints that determine whether that cell lives or dies. Ultraviolet exposure promotes arrest at the G2/M checkpoint to allow for DNA repair (31). If DNA repair is successful, progression through the cycle continues, if not, apoptosis and cell death results. As recently shown by Fornace and colleagues, MAP kinase activation plays a critical role in the initiation of G2/M delay following UV irradiation (32). Ultraviolet-induced DNA damage activates MAP kinase p38, which subsequently initiates a cascade of events that promotes G2/M cell cycle arrest. MAP kinase activation is also involved in the activation of phospholipase-A_2, the first enzymatic step in the synthesis of prostaglandin E_2 (PGE_2) (33) and platelet activating factor. Previously, we showed that UV-induced PGE_2 starts a cascade of events that ultimately leads to systemic immune suppression (34). Very recently we discovered that UV-induced platelet activating factor is upstream of PGE_2 production and appears to be one of the earliest events in the cytokine cascade that results in systemic immune suppression (35). We propose that a side effect of the process used by cells to repair UV-induced DNA damage and maintain genomic integrity *in vivo* is the induction of immune suppression. The MAP kinase pathway controls G2/M check point delay and at the same time increases the enzymatic activity of phospholipase-A_2. This results in the biosynthesis of platelet-activating factor, thus driving systemic immune suppression (see section 3.3.2). There are a number of other observations in the literature supporting the suggestion that the maintenance of genomic integrity and the induction of UV-induced immune suppression are closely linked. First, reactive oxygen species, which are well known for their ability to induce DNA damage (36),

and induce immune suppression (37), induce platelet activating factor synthesis (38). Second, no induction of immune suppression was found in UV-irradiated apoptosis-deficient Fas and/or Fas-ligand-deficient mice (39, 40). Third, in the past we have been able to induce cytokine production and systemic immune suppression by inducing non-specific DNA damage with HindIII-containing liposomes (41). We propose that the immune suppression induced by HindIII containing-liposomes works *via* a MAP kinase, platelet activating factor dependent mechanism, and studies are in progress to directly test this hypothesis.

3.1.2. Urocanic Acid

Urocanic acid, a deamination product of histidine is abundant in the stratum corneum, the outermost layer of the skin. Urocanic acid is normally found in the trans-configuration, and upon UV exposure it is isomerized to the cis-form. In a landmark paper in photoimmunology, De Fabo and Noonan reported that the action spectra for UV-induced immune suppression, UV-induced DNA damage, and isomerization of urocanic acid were remarkably similar. Based on their inability to induce immune suppression after tape stripping, which removes the stratum corneum, De Fabo and Noonan concluded that the photoreceptor is located superficially in the upperlayer of the skin, suggesting a role for urocanic acid (42). This initial observation prompted many others to investigate the role of cis-urocanic acid in UV-induced immune suppression, and this topic has been the subject of a number of reviews (43, 44). Cis-urocanic acid is found in the serum of UV-irradiated mice (45). Moreover, cis-urocanic acid treatment will mimic many of the effects of total body UV radiation. For example, injecting cis-urocanic acid into mice suppressed DTH to herpes simplex virus and induced antigen-specific suppressor T cells (46, 47). Intravenous injection of cis-urocanic acid caused systemic impairment of antigen presenting cell function, similar to what is observed following UV exposure (48). Topical treatment with cis-urocanic acid or the intracutaneous administration of cis-urocanic acid altered the morphology and function of epidermal Langerhans cells (49, 50). Treating splenic T cells with cis-urocanic acid activates them to secrete the immune regulatory cytokine IL-10 (51), a cytokine that is known to be essential for UV-induced immune suppression (52, 53). In addition, isomerization of urocanic acid has also been shown to play a role in UV-induced photoaging (54).

One criticism of the approach used in many of the above mentioned studies is that the cis-urocanic acid applied was used in amounts that generally exceeded the endogenous level found in UV-irradiated skin. An important advance in the field was the development of a monoclonal antibody specific for cis-urocanic acid (45). For the first time, it was possible to neutralize the activity of cis-urocanic acid *in vivo* and ask what effect this has on photoimmune suppression. Some very interesting results were obtained from these types of studies. They suggested that cis-urocanic acid was involved in suppressing some but not all immunological endpoints commonly used to measure UV-induced immune suppression. Mice were exposed to UV radiation, injected with the monoclonal antibody and then two different endpoints of immune function were examined, systemic suppression of DTH and CHS. Two different groups found that administration of anti-cis-urocanic acid antibody blocked UV-induced suppression of DTH, but had no effect on UV-induced suppression of CHS (55, 56). In a third study, injecting UV-irradiated mice with anti-cis-urocanic acid antibody yielded only partial restoration (50 to 60%) of CHS (57). Contrast this to the situation seen when T4N5-containing liposomes or photolyase-containing liposomes were used to reverse immune suppression, regardless of the assay used, total and complete restoration of immune function was noted. This suggests that the photoisomerization and production of cis-urocanic acid can account for some but not all of the suppressive effects of UV radiation on the immune system.

Of course the critical question is; do the immune regulatory mechanisms involved in UV-induced suppression of DTH and/or CHS reflect what is really happening during skin carcinogenesis? Does it really matter that anti-cis-urocanic acid monoclonal antibody only partially reverses CHS? What about the role of cis-urocanic acid in carcinogenesis? Although there is one report in the literature suggesting that applying cis-urocanic acid to the skin can contribute to photocarcinogenesis, the conclusions derived must be considered with caution due to the fact that the urocanic acid was applied topically in supra-physiological doses (58). Data in a very recent paper, however, using the anti-cis-urocanic acid antibody to neutralize endogenous cis-urocanic acid suggest that this pathway is involved in photocarcinogenesis (59). In this paper the authors tested the hypothesis that recombinant IL-12 will overcome the immune suppressive effects of cis-urocanic acid. Endpoints included DTH, CHS and tumor antigen presentation. In all cases tested cis-urocanic acid induced immune suppression and IL-12 reversed the effects, mimicking what is known about the effects of IL-12 on UV-induced immune suppression (60-62). The most relevant experiment from the point of view of this review, was the last figure in the paper. In this experiment mice were injected with monoclonal anti-cis-urocanic acid antibody prior to UV exposure. Another set of mice received isotype-matched IgG prior to exposure, and the third group of mice was simply exposed to UV radiation. Although the appearance of the first tumor in the UV + anti-cis-urocanic acid group and the UV only group was the same, the monoclonal anti-cis-urocanic acid antibody significantly delayed the rate of tumor induction. After 200 days of UV exposure 100% of the UV only-treated mice developed a tumor, whereas only 50% of the UV + anti-cis-urocanic acid antibody-treated mice developed tumors. After 300 days of irradiation 80% of the mice treated with UV + anti-cis-urocanic acid antibody developed skin tumors. Because this antibody neutralizes the function of cis-urocanic acid *in vivo*, these data indicate that this pathway of immune suppression is involved in photocarcinogenesis.

The glaring gap in our understanding of cis-urocanic acid-induced immune suppression is the complete lack of data concerning the mechanisms involved. For

example, 17 years after the initial report, the receptor for cis-urocanic in lymphoid cells remains elusive. Histamine (57) and the GABA (63) receptors have been suggested as possible candidates, but hard biochemical data to support these hypothesis remain to be produced. Similarly, very little is known about the molecular mechanisms by which cis-urocanic modulated cellular function and induces immune suppression. Palaszynksi et al reported that treating cultured fibroblast cell lines with histamine increases production of cyclic AMP. When cis-urocanic acid was added to the cells cAMP production was suppressed (64). The authors suggest that modulation of this important second messenger by urocanic acid may modulate cytokine production, which could alter immune function. Unfortunately, more recent studies have indicated that cis-urocanic acid does not alter cytokine production by keratinocytes (65) suggesting cAMP modulation is not the mechanism underlying immune suppression. Much more work is needed to understand how cis-urocanic acid modulates cellular function, at the molecular level, to suppress the immune response.

3.1.3. Membrane Lipid Peroxidation and Free Radical Formation

Irradiating cells with UV perturbs the function of a variety of molecules. UV-induced DNA damage and isomerization of urocanic acid are two prime examples. UV radiation is also well known for its ability to alter cellular redox equilibrium leading to free radical formation and membrane lipid peroxidation. This molecular response to stress may also contribute to UV-induced immune suppression. Anti-oxidant treatment blocks UV-induced impairment in antigen presentation (66), abrogates the UV-induced suppression of CHS (37), and interferes with the induction of tolerance (67).

How does UV-induced free radical formation activate immune suppression? Some have suggested that UV-induced cytokine production is involved. Within minutes of UV exposure, the Src tyrosine kinase, which is normally found at the inner surface of the plasma membrane, is activated. This leads to a cascade of events that results in the activation of downstream mediators including H-Ras, Raf-1, c-jun amino-terminal protein kinase (JNK) and ultimately phosphorylation of positive regulatory sites in the activation domain of c-jun leading to the activation of AP-1 and NF-kb. Activation of these transcription factors is a critical step in the formation of many of the immune regulatory cytokines secreted by UV-irradiated keratinocytes (68), one of the first steps leading to immune suppression (52, 69). Addition of free radical scavengers (N-acetylcysteine) blocks intracellular signaling and interferes with the activation of AP-1 (70, 71). Because UV-induced signal transduction was blocked when cells were treated with compounds such as vanadate and low concentrations of Triton X-100, which are known to interfere with membrane organization, it was assumed that one target of UV was the cell membrane. Data to support the cell membrane as a target of UV came from a series of experiments performed by Devery and colleagues (72). To rule out a role for UV-induced DNA damage in this system, HeLa cells were enucleated and then exposed to UV radiation. Both AP-1 and NF-kb were activated in UV-irradiated enucleated HeLa cells. Osmotic shock, which presumably causes membrane effects without DNA damage, caused the same effects (73).

Unfortunately the radiation and cell lines used in the above-mentioned studies raises some concerns. All the previous work used UVC (200 to 280nm) radiation and for the most part HeLa cells. The problem in using UVC radiation is that it is not environmentally relevant, all of the UVC in sunlight is filtered out by the atmospheric ozone layer and does not reach the biosphere. Further, the target of UV radiation is the skin, so the exclusive use of HeLa cells in the previous work raises the question as to whether the same mechanisms are involved in UV-induced keratinocyte-derived cytokine secretion. Using cell-free cytostolic keratinocyte extracts, Simon et al confirmed that the target of UV radiation that induced NF-kb activation was a cell membrane component (74). A subsequent paper from the same group provided further support for the hypothesis. Murine fibrosarcoma cells were genetically engineered to over-express heat shock protein 70. Heat shock proteins are normally used by cells to increase their resistance to environmental insults, particularly UV radiation. Over expression of heat shock protein 70 significantly blocked cell killing by UVB and UVA radiation and by agents that induce oxidative stress, such as hydrogen peroxide. In addition, the production of immune regulatory cytokines by cells exposed to UV radiation was significantly suppressed by over expressing heat shock protein 70 (75). These findings suggest that UV-induced alteration of membrane redox potential can activate transcription factors in epidermal cells and drive cytokine production, ultimately leading to UV-induced immune suppression. Moreover, these data indicate that the activation of AP-1, NF-kb and cytokine transcription can occur independently of UV-induced DNA damage and occurs after UV-induced oxidative stress and membrane lipid peroxidation.

3.2. Transmission of the suppressive signal from the skin to the immune system.

Once the initial event occurs, be it DNA damage, isomerization of urocanic acid, oxidative stress, or a combination of all three, the suppressive signal must be transmitted to the immune system. Two prominent mechanisms appear to be involved, migration of UV-damaged Langerhans cells to the draining lymph nodes, and the release of immune regulatory biological response modifiers and cytokines.

3.2.1. Migration of UV-damaged Langerhans cells

The skin is the first line of immunological defense against external environmental pathogens. Staining epidermal sheets with monoclonal anti-Ia antibody reveals a dendritic cell network. The function of the Langerhans cells that form this network is to capture the invading microorganism, ingest it, process its antigens, and then migrate to the draining lymph node where it presents the antigen to T cells and initiates a protective immune response (76). Exposure to UV alters antigen presentation by epidermal Langerhans, leading to suppression of the

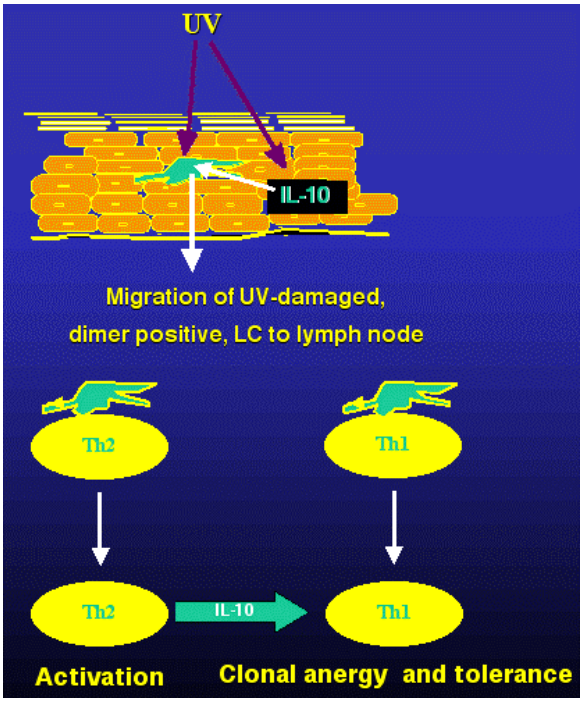

Figure 2. Local immune suppression by UV radiation. Exposing the skin to UV radiation induces DNA damage to Langerhans cells and the surrounding keratinocytes. Keratinocyte DNA damage causes the release of immune modulatory cytokines such as IL-10 that alter Langerhans cell function. UV-damaged Langerhans cells carry hapten to the draining lymph nodes where Th1 cells are tolerized and Th2 cell activation occurs normally.

immune response to contact allergens applied directly to the UV-irradiated skin. Photoimmunologists often refer to this form of immune suppression as "local immune suppression" (**Figure 2**). One consequence of applying contact allergens directly to UV-irradiated skin, however, is systemic immune tolerance.

Toews and colleagues (15) were the first to report that UV exposure affects Langerhans cell function. Exposing mice to low dose UV radiation alters both the morphology and function of Langerhans cells. Not only was the dendritic network of Ia-positive, ATPase-positive dendritic cells destroyed by UV radiation, but mice sensitized through the UV-irradiated skin failed to generate a contact hypersensitivity response. Perhaps the most interesting result presented in this study was the observation that re-immunization of these mice, at a distant non-irradiated site, also failed to induce a response. This suggests immune tolerance is activated when contact allergens are applied to UV-irradiated skin. The subsequent discovery of antigen-specific suppressor T lymphocytes in the spleens of UV-irradiated mice supported this hypothesis (17). Evidence that Langerhans cells were the target cells that transmit the suppressive signal from the skin to the immune system came from the studies of Cruz et al (18). Here Langerhans cells were isolated from the skin by fluorescent activated cell sorting, exposed to UV radiation and then conjugated with hapten. These cells were then injected into normal recipient mice and their ability to induce a CHS reaction in the recipient mouse was used to measure their immune function. Compared to the positive response found when normal Langerhans cells were injected into recipients, administration of UV-irradiated Langerhans cells resulted in immune suppression and tolerance induction.

Multiple mechanisms appear to be involved in the generation of immune tolerance by UV-irradiated Langerhans cells. One, as mentioned above is the generation of suppressor T cells. Another is the induction of clonal anergy. This was discovered in experiments in which UV-irradiated Langerhans cells were used to present antigen to two different classes of T cell clones. T cells, both CD4+ and CD8+, can be segregated into different subtypes based on the immune reactions they participate in and the type of cytokines they secrete. Type 1 (Th1) T cells secrete IFN-gamma but not IL-4, and are generally involved in helping immune reactions associated with the production of complement fixing antibodies and inflammation. Type 2 (Th2) T cells secrete IL-4 and not IFN-gamma, provide help for the production of non-complement fixing antibodies and are generally involved in the immune reactions associated with allergy. Normal Langerhans cells present antigen equally well to Type 1 and Type 2 T cells. UV-irradiated Langerhans cells, on the other hand efficiently present antigen to Type 2 cells, but do not stimulate Type 1 T cells very well (77). In addition, antigen presentation by UV-irradiated Langerhans cells rendered the Type 1 T cells tolerant. Adding recombinant IL-2 to the cultures broke tolerance, indicating that that UV-irradiated Langerhans cells induced a state of clonal anergy (78).

Another mechanism underlying the induction of immune tolerance after contact allergens are applied to the skin of UV-irradiated mice involves UV-induced immunoregulatory cytokines. The two important cytokines appear to be TNF-alpha and IL-10, and each plays a unique role in the induction of immune suppression and tolerance. This was delineated in a series of experiments performed by Streilein and colleagues. Previous studies from this group suggested that UV radiation induces tolerance and impairs induction of CHS by different mechanisms. This conclusion was based on the finding that antibodies to TNF-alpha reversed the suppression of CHS (79) whereas treating Langerhans cells with recombinant IL-10 induces tolerance (80, 81). What was not clear was whether UV-induced TNF-alpha and/or IL-10 contributed equally to immune suppression and tolerance induction. Niizeki and Streilein set out to determine the role of each in local suppression of CHS and the induction of immune tolerance. To do this, recombinant cytokines or cytokine-specific monoclonal antibodies were injected into mice and the effects on immune suppression or immune tolerance were measured. In the first set of experiments, recombinant IL-10 was injected into the epidermis, and then a hapten was applied to the epidermis directly above the site of cytokine injection. When CHS was measured in these mice, no difference from the positive control was noted. However, if the mice were rested for 14 days and then re-sensitized with

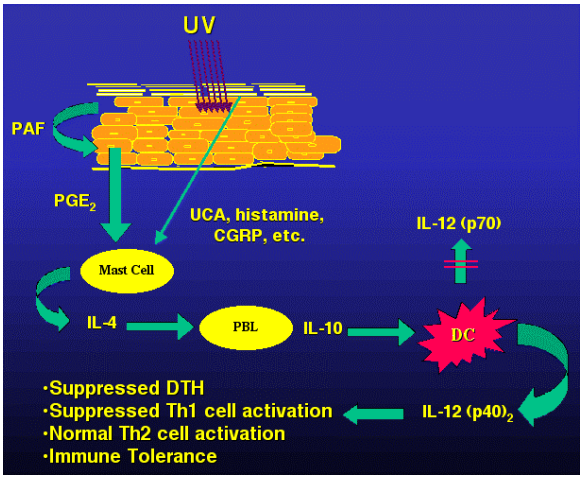

Figure 3. Systemic immune by UV radiation. Exposing the skin to UV radiation induces a cytokine cascade that ultimately alters the ability of distant dendritic cells to secrete IL-12p70. Critical steps include the production of soluble mediators of immune suppression by keratinocytes. We suggest that products produced in the skin, such as PAF, PGE_2, cis-urocanic acid and CGRP, target dermal mast cells to secrete cytokines such as IL-4, that ultimately induce IL-10 secretion by peripheral blood lymphocytes. IL-10 suppresses IL-12 secretion by distant dendritic cells, which blocks efficient antigen presentation to Th1 cells and suppresses DTH and tumor rejection.

the same hapten, they failed to respond suggesting that IL-10 injection did not suppress CHS but did induce tolerance. This conclusion was confirmed in experiments where UV-irradiated mice were injected with anti-IL-10, which blocked immune tolerance but had no effect on immune suppression. Conversely, injecting UV-irradiated mice with anti-TNF-alpha blocked immune suppression but did not reverse tolerance induction. These data show that UV-induced TNF-alpha suppressed CHS, whereas UV-induced IL-10 induced tolerance (82). Subsequent experiments showing that dendritic cells isolated from mice exposed to UV and treated locally with hapten cannot secret the immune adjuvant IL-12 (83), a cytokine required to drive inflammatory Th1-driven immune reactions, is consistent with the known effects of tolerance-inducing IL-10 on cytokine production (22).

Identifying the source of the UV-induced TNF-alpha and IL-10 was next. These cytokines are produced by UV-irradiated keratinocytes and previous studies by a number of laboratories showed that injecting supernatants from these cells could induce immune suppression [reviewed in (84)]. So it was reasonable to assume that the source of the immune modulatory cyokines in UV-irradiated skin was the keratinocyte. However, these cytokines are also produced by dermal mast cells, and both the induction of immune suppression and immune tolerance was absence in UV-irradiated mast cell deficient mice (85, 86). Similar findings, using the systemic model of immune suppression, were reported by Hart and colleagues (see section 3.3.2) supporting the emerging view that dermal mast cells play an essential role in providing the cytokines that drive immune suppression after UV exposure.

3.3.2. UV-induced biological response modifiers and cytokines

Biological response modifiers and cytokines play an essential role in UV-induced immune suppression. Many of the experiments documenting a role for cytokines in UV-induced immune suppression use a model generally referred to as "systemic immune suppression" (**Figure 3**). In this system, the UV radiation is applied at one site, and the hapten or antigen is applied at a distant unirradiated site. When the mice (87) or human volunteers (88), are subsequently challenged, their immune response is suppressed. One of the principal mechanisms by which UV exposure induced systemic immune suppression is via the release of immune modulatory cytokines and biological response modifiers. We believe that these immune modulatory factors target dendritic cells in distant lymph nodes, and the net result is similar to the immune deviation observed when UV-irradiated Langerhans cells are used to present antigen to T cell clones (77). When antigen-presenting cells were isolated from the spleens of UV-irradiated mice, the activation of Th1 cells was suppressed whereas Th2 cell activation occured normally. The role of cytokines in the impairment of systemic antigen presenting cell function was confirmed in experiments in which the immune deviation was reversed with monoclonal anti-IL-10 (53).

An important question regarding UV-induced systemic immune suppression concerns the mechanism by which the immune suppressive signal is transmitted from the skin to the immune system. Considerable evidence exists supporting a role for UV-induced biological response modifiers in activating systemic immune suppression, including PGE_2, cis-urocanic acid, histamine, IL-10, IL-4, and TNF-alpha (89). Although the interplay between these various UV-induced cytokines is complex and not completely understood, it does appear that a cytokine cascade is activated that ultimately induces immune suppression (PAF $\rightarrow$ PGE_2 $\rightarrow$ IL-4 $\rightarrow$ IL-10 $\rightarrow$ suppress distant dendritic cell IL-12 production). As pointed out above, the initial signal that drives cytokine production is UV-induced DNA damage (21, 24, 26, 27, 41). Previous studies from our laboratory suggest that an early step in this cytokine cascade is UV-induced PGE_2 production, which then causes down stream effects, including the secretion of IL-4 and IL-10 into the serum (34). The ultimate target of the these immunoregulatory cytokines is the dendritic cell, as one consequence of total body UV-irradiation on dendritic cell function is to suppress the secretion of IL-12p70 while at the same time promoting IL-12p40 homodimer production (90). Suppressed IL-12p70 secretion coupled with production of the IL-12p40 homodimer, a natural antagonist of biologically active IL-12, may explain why antigen presenting cells isolated from the lymphoid organs of UV-irradiated mice fail to present antigen to Th1 clones (53). Additional evidence to support the idea that the cyokines produced following UV-irradiation target dendritic cell IL-12 production comes from the finding that injecting recombinant IL-12 into mice

will overcome UV-induced immune suppression, the induction of suppressor T cells and the induction of tolerance (60, 91). The mechanism by which IL-12 reverses UV-induced immune suppression is independent of its effect on IFN-gamma production, but rather involves suppression of cytokine production by IL-12. The suppression of cytokine production by IL-12, in part by interfering with cytokine gene transcription, is a novel function for IL-12 (62).

Although our previous studies indicate an essential role for UV-induced keratinocyte-derived PGE_2 in systemic immune suppression, the earliest molecular events that occur immediately following UV exposure are not well defined. Some have suggested that UV exposure can directly activate PGE_2 synthesis in keratinocytes (92), whereas others have suggested that PGE_2 secretion results only after a synergistic interaction between UV and endogenous mediators, such as histamine (93). This raises the possibility that the molecular targets of UV radiation in keratinocytes are upstream of cyclooxygenase (COX)-2 activation.

One potential candidate is platelet activating factor (PAF). Although PAF is not expressed in normal skin, keratinocytes and corneal stromal cells secrete PAF in response to UV exposure (94-96). Of particular interest are the observations that keratinocytes express PAF receptors on their surface (97) and that PAF upregulates COX-2 gene expression and PGE_2 secretion by keratinocytes (98). Moreover, PAF receptor antagonists block UV-induced apoptosis (94). These studies suggest that UV-induced PAF may be upstream of PGE_2 in the cascade of events that lead to UV-induced immune suppression.

This appears to be the case. We found that treating keratinocytes with PAF activates the transcription of COX-2 and IL-10 reporter gene constructs. In addition, PAF suppressed DTH *in vivo*. More importantly, injecting UV-irradiated mice with PAF-receptor antagonists totally blocked UV-induced systemic immune suppression. In addition, injecting mice with PAF-like molecules (i.e., oxidized phosphatidylcholine) induced systemic immune suppression (35). We propose that upon UV-irradiation of the skin the following occurs. First, is the induction of pyrimidine dimers in the DNA of irradiated keratinocytes. UV-induced DNA damage activates MAP kinase p38, which activates phospholipase A_2 (PLA_2). The enzymatic removal of *sn-2* side chains from phosphatidylcholine by PLA_2, followed by acetylation of the free hydroxyl moiety results in the synthesis of PAF (99). PAF is then released by the UV-irradiated cells (95, 96), binds to the PAF-receptors on adjacent keratinocytes and induces them to secrete PAF and up-regulate production of PGE_2 and other immune modulatory biological response modifiers (98). In addition, UV-induced free radical formation can contribute to this pathway by oxidizing phosphatidylcholine, causing the formation of PAF-like lipids that bind to the PAF receptor and activate cytokine synthesis (100). Activating the pathway with PAF-receptor agonists will induce immune suppression, or short circuiting this pathway in UV-irradiated mice by flooding the system with PAF-receptor antagonists, will block immune suppression (35).

Mast cells play an important role in UV-induced immune suppression. Both UV-induced local (86) and systemic immune suppression (101) are absent in mast cell deficient mice. Moreover, patients with a history of basal cell carcinoma have a significantly higher number of dermal mast cells than age-and sex matched controls, and chronic sun exposure increases the numbers of mast cells at the sunlight exposed site (102). It is interesting to note that mast cells are activated by PAF (103). Perhaps UV-induced PAF activates dermal mast cells to secrete IL-4, IL-10 and other soluble mediators, thus contributing to immune suppression. This would reconcile our data showing inhibition of UV-induced immune suppression with PAF-receptor antagonists in wild type mice, and Hart's and/or Streilein's findings demonstrating no UV-induced immune suppression in mast cell deficient mice. Alternatively, two of the soluble mediators of UV-induced immune suppression including calcitonin gene-related peptide (104-107), and cis-urocanic acid, induce mast cell degranulation (108, 109). It is entirely possible that UV radiation, or the soluble factors produced in response to UV exposure, induce dermal mast cells to secrete cytokines thereby serving as a critical player in the immune suppressive pathway.

Another cell that may be playing an important role in producing the cytokines involved in transmitting the signal from the skin to the immune system is the infiltrating macrophage. Cooper and colleagues observed that a non-Langerhans cell macrophage-like cell (CD1a-, CD11b+) migrated into human epidermis 72 hours post UV exposure (110, 111). The infiltrating macrophages were found to present antigen to CD4+ T cells (suppressor-inducer), T cells that help in the activation of CD8+ immune regulatory suppressor T cells (112, 113). Kang et al found that these inflammatory macrophages secreted large amounts of IL-10. According to Kang et al the major source of IL-10 in the UV-irradiated epidermis is the CD11b+ macrophage (114). In addition to secreting IL-10, UV-induced CD11b+ cells fail to secrete IL-12 upon activation. In all probability, increased secretion of IL-10, with concomitant failure to secrete IL-12, contributes to UV-induced immune suppression and tolerance induction (115). Similarly, the expression of co-stimulatory molecules on macrophages from UV-irradiated skin is low, suggesting that antigen-presentation by UV-induced, IL-10 secreting, B7-low, CD11b+, macrophages may contribute to tolerance induction (116).

To further dissect the mechanisms involved, Cooper and colleagues initiated a series of animal experiments. In mice and humans the same sequence of events occurs; immediately after UV irradiation one sees a depletion of epidermal Langerhans cells and 72 hours later, an influx of inflammatory macrophages, monocytes and neutrophils. Applying hapten to the skin of the UV-irradiated mice immediately after UV exposure resulted in the suppression of CHS, but not immune tolerance. Immune tolerance was only observed if the hapten was

applied 72 hours after UV exposure, indicating a temporal association between macrophage infiltration and tolerance induction (117). Confirmation of the importance of infiltrating macrophages in tolerance induction came from subsequent experiments in which haptenated epidermal cells from UV-irradiated mice were injected into normal syngeneic controls. When monoclonal anti-CD11b was used to remove these cells from the tolerizing epidermal cell suspension, tolerance induction was lost (118, 119). In addition, injecting UV-irradiated mice with anti-CD11b monoclonal antibody partially blocked UV-induced suppression of CHS (120). Hammerberg and colleagues also noted that epidermal structure in UV-irradiated, anti-CD11b-injected mice was better preserved and less keratinocyte damage was noted in these mice versus UV-irradiated isotype-matched-injected controls. These findings suggest that in addition to inducing immune tolerance, UV-activated CD11b macrophages may also contribute to UV-induced immune suppression by serving to amplify UV-induced keratinocyte damage.

An intriguing and unique mechanism that further contributes to UV-induced immune suppression was recently described by Hammerberg and colleagues (121). UV exposure activates the complement cascade and the third component of complement (C3b) is found in UV-irradiated skin. Hammerberg and co-workers wanted to determine if C3b played any role in UV-induced immune suppression. They acquired C3-deficient mice and exposed them to UV radiation. These mice were resistant to the effects of UV, and generated a normal CHS reaction. Further, blockade of C3 cleavage to C3b, and accelerated degradation of C3b by soluble complement receptor 1 in wild type mice blocked UV-induced immune suppression. Blocking complement activation also suppressed UV-induced tolerance induction, and UV-induced immune tolerance was absent in C3-deficient mice. Treatment with soluble complement receptor 1 reduced the infiltration of CD11b+ leukocytes into the epidermis and dermis of UV-irradiated skin but did not reverse the UV-induced depletion of epidermal Langerhans cells. These data suggest a novel mechanism in which ligation of CD11b, by UV-activated C3b molecules, modifies cutaneous CD11b+ cells so that these antigen-presenting cells are unable to sensitize in a primary immune response, but actively induce antigenic tolerance. Although complement is not generally considered a biological response modifier or an immune regulatory cytokine, here is a further example of a soluble mediator secreted by epidermal cells in response to UV radiation playing a critical role in the induction of immune suppression and tolerance.

Another potential source of cytokines may be cells undergoing apoptosis. As mentioned above, there is a close association between UV-induced immune suppression and the maintenance of genomic integrity. Strains of mice that are genetically deficient in Fas (lpr) and/or Fas-ligand (gld) are resistant to UV-induced immune suppression (39, 40). Cells undergoing apoptosis can and do secrete some of the cytokines that are essential for UV-induced immune suppression. Interleukin 4 and 10 are two examples (52, 122). Gao et al report that apoptotic T cells secrete IL-10 and promote immune deviation (i.e., suppressed Th1 immune reactions with normal Th2 immune reactions) (123). This observation was recently confirmed and extended by Tomimori et al (124), who used UV exposure to induce apoptotic T cell death and found that the apoptotic T cells released IL-10. Similar findings have been reported after T cell apoptosis in response to the oral administration of antigen (oral tolerance). In this case IL-4, IL-10 and immune suppressive transforming growth factor-beta were secreted by cells undergoing apoptosis (125).

3.3. Cells that mediate UV-induced immune suppression.

3.3.1. Antigen presenting cells.

As mentioned above, direct irradiation of the skin alters the antigen presenting cell function of epidermal Langerhans cells. Because transfer of these cells into normal mice will transfer UV-induced immune suppression, and UV-induced tolerance induction, it is clear that UV-damaged Langerhans cells can mediate UV-induced immune suppression (18, 19). Furthermore, the immune deviation (i.e., shift towards a type 2 phenotype) that occurs during systemic immune suppression, as well as tolerance induction, can be attributed to the effect of immune modulatory cytokines on IL-12 secretion by lymphoid dendritic cells (83, 90). Clearly, antigen-presenting cells from UV-irradiated mice can mediate UV-induced immune suppression.

3.3.2. UV-induced suppressor T cells

3.3.2.1. IL-4 secreting Natural Killer T-cells

The induction of antigen-specific suppressor T cells following UV exposure is a well known and often reproduced phenomena. As mentioned above, Kripke's pioneering work with murine UV-induced skin cancers indicated that subcarcinogenic UV exposure will suppress the immune response of mice and allow for the progressive growth of highly antigenic UV-induced skin cancers (3). Subsequent studies by Kripke and colleagues and Daynes and co-workers demonstrated that UV-induced immune suppression could be transferred to normal age-matched syngeneic mice with antigen-specific, CD4+, CD8- T cells (87, 126-128). Moreover the suppressor T cells found in the lymphoid organs of UV-irradiated mice play a vital role in the induction of skin cancer in the UV-irradiated primary host (129). In these experiments two sets of mice were lethally x-irradiated. One set was reconstituted with normal spleen and lymph node cells, and the other set was reconstituted with spleen and lymph node cells from mice exposed to a subcarcinogenic, but immune suppressive dose of UV radiation. Skin grafts from mice exposed to UV radiation were then placed onto the two sets of recipients. This experimental design allowed the investigators to separate the carcinogenic effect of UV from its immunosuppressive effect. Because the grafted skin received that same carcinogenic insult, any differences in tumor incidence would reflect the contribution of the suppressor T cells to the development of primary tumors. Because Fisher and Kripke observed a higher probability of cancer development in mice that were reconstituted with the suppressor T cells, these findings support the

hypothesis that suppressor T cells control the development of primary skin cancers in UV-irradiated mice.

Suppressor T cells are also induced by a single acute UV exposure. Rather than tumor rejection, after a single exposure to UV radiation immunological endpoints such as CHS and DTH are used to monitor immune function. In most cases tested, regardless of what antigen was used to immunize the UV-irradiated mice, suppressor T cells can be found in their spleens (17, 130-133). Although many of these reports are over 20 years old, little was done in the intervening years to determine the mechanisms by which UV-induced suppressor T cells mediate their effects. Failure to clone suppressor T cells, failure to clone the putative restriction element (i.e., I-J), and complicated pathways to describe their mode of action, pathways whose complexity bordered on the ridiculous, contributed to the general demise of interest in suppressor T cells. Indeed, the concept of suppressor cells as a separate class of T cells was abandoned. On the other hand, many of the *in vivo* phenomena that originally stimulated research into mechanisms underlying immune suppression are still regarded as solid, and UV-induced immune suppression, as a result of immune modulation by suppressor T cells, remains a prime example.

Some recent advances have shed new light onto this old problem. A important advancement was the discovery that UV-induced CD3+, CD4+, CD8- suppressor T cells mediate their suppressive effects by releasing the well known and *bona fide* immune regulatory factors IL-4 and IL-10 (122). Only two types of CD4+ are known to release IL-4, Th2 cells and a unique subset of T cells known as Natural Killer T cells. Natural Killer T (NKT) cells, unlike conventional T cells, co-express surface antigens found on T cells (T cell receptor, CD3, CD4) and Natural Killer cells (DX5, NK1.1, Fc-gamma receptor, Ly 49a), hence the name NKT cell. Also, although NKT cells express the alpha/beta T cell receptor for antigen on their surface, the level of expression is generally one-third to one-half that found on conventional T cells. Intermediate T cell receptor expression is another hallmark of NKT cells. Natural Killer T cells also secrete high concentrations of immune regulatory cytokines, especially IL-4 within hours of stimulation, *in vivo* (134) and *in vitro* (135). Unlike conventional T cells, NKT cells exhibit an activated phenotype ($CD44^{high}$, $CD62L^{low}$) and are present at very low frequencies in peripheral lymphoid organs, where they account for 2-3% of the total T cell population (136). They are positively selected and restricted by CD1 (137, 138) and exhibit a very restricted T cell receptor usage (139).

We discovered in preliminary experiments that we could remove all suppressive activity by depleting, with antibody and complement, Fc-gamma positive T cells from CD4+ T cells isolated from UV-irradiated mice (Moodycliffe and Ullrich, unpublished observations). At first we thought that the suppressor T cells were a subpopulation of Fc-gamma receptor positive Th2 cells. However, when we isolated CD4+, Fc-gamma receptor positive T cells and stained then for T cell receptor expression, an intermediate pattern of T cell receptor staining was noted. Moreover, when we stimulated the cells with anti-CD3 *in vitro*, we noted early and substantial secretion of IL-4. We repeated these studies by isolating CD4+ T cells from the spleens of UV-irradiated mice and positively selecting the NKT cells using two other markers, DX5 and NK1.1. Here also we isolated a population of cells that expressed intermediate levels of T cell receptor alpha/beta expression and secreted IL-4, early and at high concentrations (140). This prompted us to test the hypothesis that NKT cells are mediating UV-induced immune suppression. CD3+, CD4+, DX5+ T cells were isolated from the spleens of UV-irradiated mice and injected into age-matched syngeneic recipient mice. We found we could transfer UV-induced immune suppression when recipient mice were injected with as few as 1 million CD4+, DX5+ cells. No immune suppression was noted when equal numbers of CD4+, DX5- cells were injected into recipient mice. The UV-induced suppressor T cells were CD1-restricted in that UV-irradiation failed to induced immune suppression in CD1-deficient mice, nor could UV-induced suppressor T cells, generated in wild-type mice, suppress when transferred into CD1-deficient animals. The CD4+, DX5+ NKT cells also suppressed tumor rejection. Here also, transferring as few as 1 million CD4+, DX5+ T cells from mice exposed to subcarcinogenic doses of UV radiation into syngeneic recipient mice suppressed the rejection of highly antigenic UV-induced tumor skin cancer cells. These cells did not suppress the rejection of a chemically induced regressor tumor cell line, MCA-113, indicating that the suppressor cells were antigen-specific. These findings indicate that CD3+, CD4+, T cell receptor intermediate expressing, IL-4 secreting, CD1-restricted NKT cells, isolated from the spleens of UV-irradiated mice transfer suppression of tumor rejection and DTH (140).

3.3.2.2. IL-10-secreting T regulatory type 1 cells

In addition to NKT cells, another UV-induced suppressor T cell has been recently identified. Schwarz and colleagues were studying UV-induced tolerance that results after hapten sensitization through UV-irradiated skin. As mentioned above, immune tolerance, as measured by the inability to generate CHS when the mice are sensitized two to three weeks later on distant non-irradiated skin, results, and tolerance can be transferred by hapten-specific suppressor T cells. Based on the well known fact that CTLA-4 (CD 152) is a marker expressed on activated T cells and one that has been shown to negatively regulate T cell function, Schwarz and colleagues asked if CTLA-4 is involved in UV-induced tolerance. T cells were isolated from UV-irradiated mice and CTLA-4 positive cells were depleted or enriched. When CTLA-4-positive T cells were deleted, transfer of immune suppression was abrogated. When CTLA-4-positive T cells were enriched, transfer of as few as 5×10^5 T cells could suppress CHS. Further, injecting anti-CTLA-4 antibody into the recipient mice, blocked the transfer of immune suppression. Next, the mechanism by which CTLA-4 positive cells mediated immune suppression was examined. Because cytokines have been implicated in UV-induced immune tolerance induction, the secretion pattern of the CTLA-4 positive cells was measured. Upon activation of these cells *in vitro*

they released significant amounts of IFN-gamma, IL-10 and TGF-beta, some IL-2 and no IL-4. Treating the cells with anti-CTLA-4 monoclonal antibody blocked the secretion of IL-10, but not IL-2 or IFN-gamma. This suggested that the CTLA-4-positive cells released IL-10. To determine the role IL-10 in the transfer of immune suppression, CTLA-4-positive cells were injected into the recipient mice, which then received anti-IL-10 antibody. Neutralizing anti-IL-10 antibody totally reversed the transfer of tolerance, indicating that IL-10-secreting CTLA-4-positive cells transfer UV-induced immune suppression. Because the cells secrete IL-10, but not IL-4 they are not Th2 cells but appear to belong to a class of regulatory T cells (Tr1) originally described by Groux and colleagues (141).

4. CRITICAL WAVELENGTHS INVOLVED IN UV-INDUCED SYSTEMIC IMMUNE SUPPRESSION

Ambient UV radiation is divided into two major regions, UVB (290-320 nm) which comprises less than 5% of the UV that reaches the biosphere and UVA (320-400), which comprises at least 95% of the remaining UV radiation. Although the role of UVB in inducing skin cancer and immune suppression is well known (see above), the contribution of UVA to the deleterious effects of sunlight are not as well defined. This is due in part to the fact that over the years most photoimmunologist have relied heavily on FS40 sunlamps, or other equivalent fluorescent bulbs to supply their UV radiation. Although fluorescent sunlamps are excellent sources of UVB radiation, they are poor substitutes for sunlight because their emission, particularly in the UVA region of the solar spectrum, differ significantly from sunlight (142). Because UVA comprises 95% of the UV radiation in sunlight is it important to determine exactly what role it plays in activating immune suppression.

Determining the relative role of UVA in immune suppression may have broad implications besides being of interest to photo-immunologists. Oncologists and dermatologists have been promoting a campaign of "safe sun exposure" to combat the dramatic rise in skin cancer incidence. Using sunscreens is an essential part of this campaign. Until very recently, most sunscreens available in the United States absorbed wavelengths in the UVB region of the solar spectrum, with little to no absorption in the UVA region. This appears to be sufficient to protect against sunlight induced *p53* mutations and non-melanoma skin cancer induction (10, 143). However, the action spectrum for melanoma induction in fish (144), and data suggesting that UVA induces immune suppression raise concerns about the ability of most sunscreens to provide adequate UVA protection.

Unfortunately, the scientific literature concerning the role of UVA in UV-induced immune suppression is contradictory. Examples of UVA suppressing the induction of immunity (145-149) are as numerous as examples where UVA fails to have an effect (150-154). Moreover, recent reports from Reeve and colleagues suggest that prior exposure to UVA radiation can protect against the immunosuppressive effects of UVB (154, 155). These findings question whether it is even desirable to add UVA filters to sunscreens. It is extremely important therefore, to clarify the role of solar UVA in immune suppression.

Two recent papers point out the essential role UVA radiation plays in systemic immune suppression. In the first, experimental animals were used (8). In these experiments, mice were first immunized with the fungal pathogen, *Candida albicans* and then at periods of time post immunization (10 to 30 days) the mice were exposed to UV radiation. The UV radiation was provided by a 1000 watt Xenon light source equipped a Schott WG-320 to mimic the UV radiation present in sunlight. Alternatively, the Xenon light was equipped with a Schott WG-335 filter to cut off all wavelengths below 320 nm, or a Schott WG 360 to cut off all wavelengths below 340. This allowed us to irradiate the mice with UVA + UVB radiation (295 to 400 nm) or UVA I + UVA II (320 to 400 nm) or UVA I only (340 to 400 nm). Exposing mice to UVA + UVB radiation post immunization suppressed the elicitation of DTH to *C. albicans*, in a dose-dependent fashion. Somewhat surprising was the observation that UVA radiation, devoid of UVB was as effective as UVA + UVB radiation in suppressing the elicitation of DTH. In fact, the dose response curves for immune suppression by UVA + UVB radiation and UVA only were identical. Further, the mice were exposed to UVA I radiation and no immune suppression was noted. This suggests that the immunosuppressive wavelengths of UVA reside in the UVA II (320 to 340 nm) region of the solar spectrum. These observations were confirmed by the use of two different sunscreen formulations. Although both provided the same sun protection factor (SPF), one was formulated to absorb only UVB whereas the second was formulated to absorb both UVB and UVA. Only the sunscreen that absorbed UVA afforded immune protection. Indeed no immune suppression was observed when the UVA + UVB absorbing sunscreen was applied to the skin of the mice prior to UV exposure. On the other hand, applying the UVB-only absorbing sunscreen afforded no immune protection. The degree of immune suppression observed in mice exposed to solar simulated UV radiation was the same in mice treated with a UVB only absorbing sunscreen or in mice exposed to UV and treated with the vehicle.

In the second study, human volunteers were exposed to solar simulated UV radiation or natural sunlight (156). Delayed type hypersensitivity reactions to recall antigens was used as the immunological endpoint. In the outdoor study, two different groups of subjects were treated with two difference sunscreens, both having a SPF of 25, but having different UVA protection patterns. One sunscreen had an SFP of 25 and a UVA protection factor of 14. The second sunscreen had a SPF of 25 and a UVA protection factor of 6. Both local and systemic immune suppression was measured in the sunlight exposed human volunteers. When unprotected skin was exposed to sunlight, significant immune suppression (local and systemic) was noted. Although both products protected against UV-induced erythema, the degree of immune protection afforded was very different. When the SPF-25,

UVA protection factor 14 sunscreen was applied prior to sun exposure, immune suppression, both local and systemic was blocked. On the other hand, when the SPF 25 UVA protection factor 6 sunscreen was used, the local response was protected, but the systemic response was significantly suppressed. Similar findings were observed when solar simulated light was used. The sunscreen with the higher UVA protection factor yielded better immune protection.

These two studies illustrate a number of important facts concerning UV-induced immune suppression. First, the UVA radiation found in natural sunlight, or that provided by a 1000 watt Xenon solar simulator, suppressed established immune reactions, such as the elicitation of DTH and immunological memory. Because the dose response curves for UVA-induced immune suppression and UVA + UVB (solar simulated)-induced immune suppression are identical, we conclude that the UVA in sunlight is responsible for suppressing immunological memory. Because the UVA radiation in sunlight suppressed the DTH reaction to recall antigens in human volunteers, these findings suggest that sunlight exposure may suppress the immune response to microbial antigens. The results from a study measuring the effects of sunlight on the immune response to *Listeria monocytogenes* indicate that this is the case (7). Second, sunscreens that absorb both UVB and UVA radiation protect against suppression of established immune reactions such as immunologic memory, whereas sunscreens that only absorb UVB do not. Third, these findings clearly show that there is no correlation between the SPF of a sunscreen and its ability to provide immune protection. In both the studies mentioned above, the sunscreens were designed to have equal SPF but different UVA absorbing capabilities. Although they both did a good job in blocking erythema, only the UVA absorbing sunscreen yielded consistent and significant immune protection. These findings indicate that SPF should only be used to describe the ability of a sunscreen to block erythema, and cannot be used to describe the efficacy of a sunscreen when any other endpoint is measured. It is unreasonable, therefore, to expect *a priori* that a sunscreen with a high SPF will provide immune protection or mutation protection, in addition to protection against sunburn. This is especially the case when, as demonstrated in our studies, different wavelengths of UV radiation modulate different biological responses.

6. SUMMARY AND CONCLUSIONS

Photoimmune suppression and photocarcinogenesis are linked. The immune suppression induced by UV radiation is a major risk factor for skin cancer induction. In addition, sunlight exposure will suppress the immune response to infectious organisms, including recall and memory reactions, suggesting that sunlight exposure can depress protection induced by prior vaccination. For these reasons alone, it is critically important to study the mechanisms underlying UV-induced immune suppression. In addition, UV-induced immune suppression represents one of the most widely studied examples of immune suppression by an environmental toxin, and may serve as a model for immune suppression by other immunotoxins in our environment.

The target of UV radiation is the skin, and one of the first steps in the cascade of events that lead to immune suppression is transformation of electromagnetic energy into a biologic signal. Three chromophores have been suggested, DNA, urocanic acid and lipid membranes. It is believed by this author that DNA damage is the most important event that leads to UV radiation induced immune suppression. Indeed, I believe that the immune suppression induced by UV radiation is a side effect of the mechanisms used by cells to maintain genomic integrity. However, equally compelling arguments can be, and have been, made for the primacy of urocanic acid and/or oxidative stress leading to membrane damage as the initial event in UV-induced immune suppression. Now that these chromophores have been identified, the challenge for the future will be to understand how they work, either in an additive, sequential or synergistic fashion to induce immune suppression. Moreover, why did at least three different mechanisms develop during evolution to activate UV-induced immune suppression?

Once the electromagnetic energy is converted into a biologically relevant signal, the immune suppressive signal must be transmitted to the immune system. For the most part it appears that standard immunoregulatory mechanisms are employed. Defects in antigen presentation, immune modulatory cytokines, and the influence of infiltrating immune cells that release immune modulatory cytokines have all been shown to play a role in the induction of immune suppression. Photoimmunologists have contributed to basic immunology in a number of important ways. The immunosuppressive effects of cis-urocanic acid and PAF were first described by photoimmunologists. The key role that altered antigen presenting cell function plays *in vivo* in inducing immune suppression and immune tolerance has long been recognized and manipulated by photoimmunologists. And finally, the critical role that suppressor T cells play in skin cancer induction has keep this generally discredited field of immunology alive. New approaches, identification of cloned chemical mediators of suppression (i.e., cytokines such as IL-4 and IL-10) , and the realization that unique subsets of T cells (i.e., NKT, Tr1 cells) are involved, helped to make the concept of suppressor T cells acceptable again.

How can we prevent UV damage, block immune suppression and reduce the frequency of sunlight induced skin cancer? Some of the approaches that are being used have already been mentioned in this review. Drugs that block PGE_2 production by interfering with the enzymatic activity of COX-2 (157, 158) reduce skin cancer incidence and overcome UV-induced immune suppression (34). Treating mice and humans with liposomes that introduce DNA repair enzymes reduce skin cancer incidence (29, 30) and overcome UV-induced immune suppression (14). Similarly treating UV-irradiated mice with antibodies to cis-urocanic acid reduces skin cancer incidence (59) and overcomes UV-induced immune suppression (55). The

antioxidants in green tea have also been shown to reduce the incidence of skin cancer in UV-irradiated mice (159). It is interesting to note however, that in most of the above-mentioned studies, the treatments used reduced the incidence of skin cancer but did not yield total protection. For the most part 100% of the recipients developed skin cancer regardless of the treatment chosen. What was changed by anti-cis-urocanic acid, the selective COX-2-inhibitor, and by applying T4N5 liposomes, was the time to first appearance of tumors and the numbers of tumors per individual. Generally, the appearance of the first tumor was delayed and the overall numbers of tumors per mouse/individual was reduced. Very few of the treatments significantly reduced the probability of tumor development, at the end of the experiment, all the UV-irradiated mice developed skin cancer. There is one major exception. In a study published by Aanthaswamy et al, sunscreens were applied to the skin of mice prior to irradiation with a UV solar simulator (143). In this study, sunscreen application prevented the appearance of *p53* mutations in the skin of UV-irradiated mice. In addition sunscreen application significantly reduced the probability of tumor development. One hundred percent of mice exposed to solar simulated UV (1000 kJ/m^2 cumulative exposure) radiation, and treated with the vehicle developed skin tumors. Only 2% of mice exposed to UV radiation and treated with a sunscreen prior to each UV exposure developed skin cancer. Ninety-eight percent of the mice treated with sunscreen and exposed to a 1000 kJ/m^2 of UV radiation, remained tumor free. More importantly, the sunscreen and solar simulator exposed mice were irradiated for a prolonged period of time. After cumulative exposure to 1500 kJ/m^2 of UV radiation, 83% of the mice remained tumor free. Some have argued that this suggests that if you prolong the irradiation for a longer period of time, eventually the incidence of tumors in this group will catch up with the controls. This seems doubtful in this case, as some of the mice in the UV + sunscreen-treated group were dying of old age and remained tumor free at the end of the experiment. It is important to note that in preliminary studies, the exact same sunscreens prevented the induction of *p53* mutations in human skin grafted onto the backs of immune-deficient Rag2-/- mice (Ananthaswamy & Ullrich, preliminary observations). Similarly, sunscreen use has been shown to prevent immune suppression in mice (8) and in man (156, 160). Clearly an once or two of prevention, in the form of a sunscreen, will provide a pound of cure.

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