Dendritic Epidermal T Cells in Ultraviolet-irradiated Skin Enhance Skin Tumor Growth by Inhibiting CD4+ T-Cell-mediated Immunity

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ABSTRACT

Chronic UV irradiation of the skin not only causes skin cancer in humans but modifies immune responses generated within the epidermis, resulting in impaired immunity against a variety of infectious as well as noninfectious agents. In mice, tumors induced by chronic UV irradiation grow faster when transplanted into mice that are immunosuppressed by UV irradiation. To investigate epidermal cells (EC) in UV-irradiated skin that induce the induction of immunity against tumors, the murine UV-induced LK2 regresser tumor was used. This tumor grows initially in vivo and then spontaneously regresses. In vivo T-cell depletion was used to determine that regression of LK2 tumors in unirradiated mice was mediated mainly by CD8+ T lymphocytes, with minor involvement of CD4+ T cells. Immunization of mice with tumor antigen-pulsed EC prepared from unirradiated mice enhanced immunity against subsequent inoculation of LK2 tumors, augmenting regression of the LK2 tumor due to increased activation of both CD4+ and CD8+ T-cell subsets against the tumor. By contrast, immunization with EC prepared from UV-irradiated skin inhibited the induction of antitumor immunity, enhancing LK2 tumor growth. This was caused by the dendritic epidermal T cells that remained within this UV-irradiated EC preparation inhibiting activation of CD4+ T cells, without affecting CD8+ T cell function. Hence, during the development of murine UV-irradiated skin tumors, dendritic epidermal T cell inhibition of CD4+ T cell activation may enable this skin tumor to escape immune-mediated destruction.

INTRODUCTION

Chronic exposure to UV irradiation causes skin cancer in humans and mice (1, 2). In addition to the induction of tumors, UV irradiation modifies a variety of immunological responses (3, 4). For example, immunity against cutaneous tumors and infectious agents such as leishmania and herpes simplex virus is impaired (reviewed in Ref. 5). Studies into the UV-induced inhibition of CHS have demonstrated that UV irradiation impairs antigen presentation such that tolerance results in UV-exposed mice instead of effector immunity (6, 7). Systemic immunosuppression is also induced in UV-exposed mice, as when antigen is applied to skin distal to the irradiated site, transferable lymphocytes with suppressor function are activated (6, 8).

The cellular changes induced by UV irradiation responsible for permitting tumor growth remain unknown. Murine epidermis contains two types of resident bone marrow-derived cells that have been implicated in immune responses against skin tumors: LC, MHC class II+ immature dendritic cells that function as potent APC (9); and DETC, which comprise 1–5% of epidermal cells, depending on mouse strain (10). DETC express Thy-1, CD3, and the yb T-cell receptor for antigen on the cell surface (11, 12). DETC have been shown to have direct activity against tumors, demonstrated by cytotoxicity against the murine melanoma cell lines K1735 and CM3205 (13). DETC have also been proposed to have suppressive rather than effecter function (14, 15), because when haptenated and injected into mice, these cells suppress the induction of antigen-specific immunity, probably due to inhibition of activated T-cell proliferation (16). LC have also been implicated in the induction of antitumor immunity, because LC pulsed with soluble tumor fragments from a chemically induced tumor induced protective immunity when used to immunize naive mice (17).

Both of these cell types are depleted from the epidermis by exposure to UV radiation, and whereas LC repopulate the epidermis over time, the DETC remain depleted (18–20). Those LC that remain in the UV-irradiated epidermis induce anergy in CD4+ lymphocytes of the Th1 subset and thus have altered function (21, 22). In a study of UV-irradiated and unirradiated Thy-1+ EC, DETC function was unaffected by in vitro UV irradiation, since unresponsiveness resulted from injection of both haptenated, irradiated, and unirradiated cells (23). UV-irradiated epidermis is transiently infiltrated with MHC class II+ macrophages identified by CD11b in both humans and mice (24, 25). The induction of tolerance to the hapten DNFB after UV exposure has been shown to be dependent on these cells because epidermal cells isolated from UV-irradiated mice, depleted of CD11b+ cells, fail to induce tolerance upon inoculation into syngeneic mice (26).

Whereas some UV-induced changes in cells of the skin immune system have been shown to inhibit the induction of CHS, it is unknown whether these cellular changes influence the growth of UV-induced skin tumors. To investigate this, we used the UV-induced regressor tumor LK2, which upon transplantation into syngeneic mice is immunologically rejected. This tumor, therefore, models a developing tumor that activates protective immunity. We then investigated the ability of tumor antigen-pulsed EC from UV-irradiated mice to inhibit the development of this protective immunity.

MATERIALS AND METHODS

Mice. Inbred male and female C3H/HeNCr mice were between 8 and 12 weeks of age at the beginning of experiments. Mice were used in accordance with the University of Sydney Animal Experimental Ethics Committee guidelines.

Tumor Cell Line. The LK2 cell line was derived from an UV irradiation-induced squamous cell carcinoma that arose in a C3H/HeNCr mouse exposed daily to solar-simulated UV irradiation over a period of approximately 36 weeks. The solar-simulated UV irradiation was provided by a bank of six Cosmolux RA, UVA-emanating tubes flanking a single FS72 UBV-emaniting tube, with a total irradiance of 3.79 × 10−3 W/cm2 UVA (320–400 nm) and 3.3 × 10−4 W/cm2 UBV (280–320 nm), as measured using an International Light IL1350 radiometer with SED038 UVA and SED240 UBV detectors, respectively. The total dose of UV for the induction of the tumor was approximately 1.3 kJ/cm2 UVA and 65 J/cm2 UBV. The LK2 tumor line was established and maintained in tissue culture at 37°C and 5% CO2 in DMEM (Trace Biosciences, Castle Hill, NSW, Australia) containing 10% NCS (CSL, Parkville, Victoria, Australia), 20 mM HEPES buffer, and 8 mM l-glutamine.

Preparation of TE. LK2 tumors were grown in mice that had been immunosuppressed by prior exposure to UV irradiation over 5 days, giving a total dose of 6.82 J/cm2 UVA and 0.59 J/cm2 UBV. The tumors were excised...
when approximately 10–15 mm in diameter, placed into sterile PBS, disaggregated mechanically, and then sonicated to disrupt cell membranes. The supernatant collected after centrifugation at 1500 × g for 30 min at 4°C was dialyzed against PBS (12–14 kDa cut-off; SpectraPor membrane tubing; SpectruM Medical Industries, Inc., Houston, TX) and used as the source of tumor antigen. Protein concentration was determined by Lowry assay.

**UV Irradiation of Mice for EC Preparation.** The dorsal trunk skin of mice was close-shaved to remove hair 1 day prior to UV irradiation. The mice were exposed to UV irradiation daily for 5 consecutive days to give a cumulative UV dose of 6.82 J/cm² UVA and 0.59 J/cm² UVB, which induces immunosuppression. The UV irradiation source was the same as that used for the induction of the LK2 tumor. Mice were unstrained in plastic boxes without lids placed 40 cm below the light source during the exposure. Exposed skin was excised from mice for the preparation of EC, either within 30 min or 3 days after the final irradiation.

**Preparation of EC.** EC were prepared according to a modification of a method described previously (10). Briefly, mice were sacrificed; then the dorsal trunk was shaved and chemically depilated (Veet, Rockkitt, and Coleman, Sydney, Australia). The excised skin was cut into small pieces, placed into HepesBSS containing 0.3% trypsin (Boehringer-Mannheim, Mannheim, Germany) and 300 units/ml DNase (Amersham, Takara, Japan), and incubated for 16 h at 37°C. The epidermis was mechanically separated from the underlying dermis and reinfused in a fresh 0.3% trypsin solution containing 300 units/ml DNase for 20 min at 37°C, after which an equal volume of DMEM containing 10% FCS was added. EC were dislodged by gentle agitation, filtered through nylon mesh (250 μm; Swiss Screens, Seven Hills, NSW, Australia), and washed. Following preparation, EC were cultured for 2 h in DMEM containing 10% FCS, 20 mM L-glutamine, and 0.05 mM 2-mercaptopethanol (complete medium) supplemented with 10 units/ml murine recombinant GM-CSF (Genzyme, Cambridge, MA) prior to use.

**In Vitro Depletion of T-Cell Subsets.** MHC class II⁺ EC were removed prior to the 2-h culture by incubation in monoclonal anti-Ia antibody (TIB 93; ATCC, Rockville, MD) as hybridoma supernatant diluted 1:4 in complete medium for 30 min at 4°C. Thy-I⁺ EC were removed by incubation in purified monoclonal anti-Thy-I.2 antibody (T-24—31.7 Ref. 27) diluted 1:500 in corn CSF (Genzyme, Cambridge, MA) as hybridoma supernatant diluted 1:4 in complete medium. Thy-1⁺ EC were removed by incubation in monoclonal anti-Thy-I.2 antibody (T-24—31.7 Ref. 27) diluted 1:500 in complete medium. EC were then washed and incubated at 37°C for 35 min in low-toxicity rabbit complement (Cedarlane Laboratories, Homby, Ontario, Canada) diluted 1:30 in PBS containing 5% FCS. Dead cells were removed by treatment with 0.05% trypsin and 300 units/ml DNase in HepesBSS for 10 min at 37°C and then washed. Depletion of class II⁺ or Thy-1⁺ cells was confirmed by immunofluorescence.

**Antigen Pulsing of EC and Immunization Protocol.** EC were washed twice with HepesBSS containing 10% FCS and once with HepesBSS prior to exposure to TE. EC were incubated with 1000 μg protein/ml TE diluted in HepesBSS for 30 min at 37°C. EC were washed as before to remove any remaining TE and resuspended for immunization in HepesBSS. Mice were immunized with 10⁶ viable EC by s.c. injection into the dorsal trunk.

**Assessment of Protective Antitumor Immunity.** Antitumor immunity was assessed by both incidence and growth of subsequently inoculated tumors. Immunized mice received a s.c. inoculation of 2 × 10⁶ LK2 live tumor cells in 50 μl PBS into each flank 10 days after immunization. The development of tumors was assessed by manual palpation 7 days after inoculation of tumors and then every 3–7 days until the end of each experiment (35–40 days). In those mice that developed tumors, tumor diameter was measured using Vernier calipers. In all experiments, a naive group of unimmunized mice was included that received tumor inoculation at the same time as immunized mice.

**In Vivo Depletion of T-Cell Subsets.** Mice were depleted of T-cell subsets according to the protocol described by Cobbold et al. (28). Briefly, mice received i.p. injections of the optimal concentration of purified Ab, previously determined to result in maximal depletion of T-cell subsets, on 3 consecutive days, then at weekly intervals throughout the duration of the experiment. Ab used were anti-CD4 (GKI.5; ATCC) and anti-CD8 (YTS 169.4: 28), used at 1.0 and 4.0 mg protein/injection, respectively. Anti-CD4 Ab removed approximately 80% of splenic CD4⁺ T cells, and anti-CD8 Ab removed approximately 75% of CD8⁺ T cells. The control used, anti-mycobacteria Ab (L22; 1.0 mg protein/injection; Ref. 29), did not alter either CD8 or CD4 T-cell subpopulations.

**Assessment of CHS.** UV-irradiated or control mice were sensitized by application of 50 μg TNBC (Tokyo Kasei, Tokyo, Japan) dissolved in 30 μl acetone on the shaved abdomen. To elicit the CHS, sensitized mice were challenged on both surfaces of one ear with a total of 100 μg TNCB dissolved in 10 μl acetone 5 days after sensitization. Ear thicknesses of both ears were measured using an engineer’s micrometer 24 h later. CHS was determined as the ear swelling response, which was the difference between the challenged and unchallenged ears for each mouse.

**Assessment of Tolerance.** Mice which had been sensitized with TNCB 12 days previously were resensitized with 50 μg TNCB dissolved in 50 μl acetone on the shaved abdomen to assess the secondary immune response. To determine whether mice remained unresponsive, resensitized mice were challenged on both surfaces of the previously unchallenged ear with 100 μg TNCB dissolved in 10 μl acetone 5 days after resensitization. Ear thickness of the challenged ear was measured 24 h later, and the difference in ear thickness of this ear before and after challenge was the ear swelling response.

**Preparation of Splenic Leukocytes and MECLR.** Splenic leukocytes were prepared from BALB/c (H-2b) mice for use as responder cells in the MECLR as described previously by Dunlop et al. (30). EC from control or UV-irradiated C3H/HeN (H-2b) mice were cultured for 3 days in medium containing 10 units/ml GM-CSF. EC (10⁵) were cultured with 2 × 10⁶ splenic leukocytes as described previously (30). Cultures were performed in replicates of six, with data presented as the mean cpm ± SE.

**Immunofluorescent Staining of EC and Flow Cytometry.** For each experiment, EC prepared from three control or UV-irradiated mice were pooled and fixed with 2% paraformaldehyde. EC were washed and resuspended for staining in PBS containing 0.2% sodium azide (PBS-azide). EC were stained using routine methods for indirect immunofluorescence. Ab used for staining were: rabbit anti-S-100 (Sigma Chemical Co., St. Louis, MO); hamster anti-CD3 ε-subunit (2C11; Ref. 31); rat anti-CD11b (Mac-1 α-subunit, M1/70; ATCC); or rat anti-HSA (M1/69; ATCC). EC were incubated in Ab prepared as neat hybridoma supernatants, except anti-S-100, which was used at 1:600 in PBS-BSA for 60 min at 20°C. Secondary Ab included: sheep antirabbit Ig F(ab)₂; sheep antirat at IgG (Silenus Laboratories, Victoria, Australia); or goat antihamster IgG γ+L chain (Caltag, San Francisco, CA).

**Statistical Evaluation.** Tumor growth measurements were taken every 3–7 days after tumor inoculation until the end of the experiment, and the average size of tumors per mouse at each time point over the entire length of the experiment was used for statistical analysis. Tumor growth was analyzed by repeated measures of ANOVA, taking into account all individual tumor measurements throughout the duration of the experiment. The results presented are the means ± SD for 50,000 events were collected for each EC preparation and stored for analysis.
naive mice. Ten days after immunization, these mice and unimmunized control mice were inoculated with $2 \times 10^6$ LK2 cells. The growth of these tumors was monitored over the next 28 days. The LK2 tumor, which was induced by prolonged exposure to solar-simulated UV irradiation, grows for the first 14 days after s.c. injection into immunocompetent mice and then commences to regress; hence, this is a regressor tumor (Fig. 1). The growth of the LK2 tumor was inhibited, although not significantly, by immunization with TE-pulsed EC from normal mice. Immunization with EC from UV-irradiated mice significantly prevented tumor regression. Enhanced tumor growth and incidence was observed in this group compared to the unimmunized group. Hence, immunization with normal EC can enhance the immune response against a normally regressing tumor, whereas UV irradiation of the skin alters the function of EC, resulting in enhanced tumor growth.

**EC Collected 3 Days but not 30 min after UV Irradiation Inhibit Immunological Rejection of the LK2 Skin Tumor.** To further investigate the deleterious effects of UV irradiation on EC induction of antitumor immunity, EC were prepared from UV-irradiated mice at two different times after UV exposure. Mice were exposed to UV irradiation over 5 days, and then EC were prepared from the exposed skin either within 30 mm or 3 days later. EC were also prepared from normal, unirradiated mice. All EC were pulsed with TE prepared from the LK2 tumor, and naive mice were immunized with $10^6$ EC from the three different groups. Ten days after immunization, these mice and a group of unimmunized mice were inoculated s.c. with $2 \times 10^6$ LK2 tumor cells, and the growth of these tumors was observed over the next 35–40 days.

In this experiment, immunization of mice with normal, control EC only marginally inhibited growth of the LK2 tumor compared to growth in unimmunized mice (Fig. 2A). Immunization with EC prepared from skin 30 min after UV exposure caused a small, insignificant enhancement of LK2 tumor growth; however, EC prepared 3 days after the final UV exposure significantly enhanced tumor growth in immunized mice compared to the unimmunized mice. None of the immunization protocols had any effect on tumor incidence in this experiment, although normal EC slightly reduced and UV-irradiated EC slightly enhanced tumor incidence (Fig. 2B). Hence, immunization with EC prepared 3 days but not 30 min after UV irradiation enhanced the growth of the LK2 regressor tumor. The inhibition of tumor growth.

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**Fig. 1.** UV-irradiated EC inhibit immunological rejection of the LK2 skin tumor. Groups of mice were immunized with $10^6$ TE-pulsed EC prepared from normal mice (□) or from skin either 30 min (▲) or 3 days (■) after UV irradiation. These mice and a group of naive, unimmunized mice (○) were inoculated with $2 \times 10^6$ LK2 tumor cells 10 days later. A, the growth of tumors (mean tumor diameter) was measured every 3–4 days after tumor inoculation ($P = 0.37$; least significant difference, 0.93). B, tumor incidence (% mice per group with tumors) calculated at each time point. Tumor growth and incidence for immunized groups was compared to that in naive, unimmunized mice. *, $P < 0.05$ by repeated measures by ANOVA (growth) and Mann-Whitney U test (incidence); $n = 7$ group.

**Fig. 2.** EC collected 3 days but not 30 min after UV irradiation inhibit immunological rejection of the LK2 skin tumor. Groups of mice were immunized with $10^6$ TE-pulsed EC prepared from normal, unirradiated mice (□) or from skin either 30 min (▲) or 3 days (■) after UV irradiation. These mice and a group of naive, unimmunized mice (○) were inoculated with $2 \times 10^6$ LK2 tumor cells 10 days later. A, the growth of tumors (mean tumor diameter) was measured over the next 28 days ($P < 0.0001$; least significant difference, 0.99). B, tumor incidence (% mice with tumors) was calculated at each time point. The growth and incidence of tumors in immunized mice was compared to the growth and incidence in naive, unimmunized mice. *, $P < 0.05$ by repeated measures by ANOVA (growth) and Mann-Whitney U test (incidence); $n = 6$ group.
consecutive days and then were sensitized with 50 μg TNCB on the unexposed abdomen and either 30 min or 3 days later. Primary CHS was elicited and measured after 24 h as the difference between the challenged and unchallenged ears. Mice were resensitized 12 days after the initial sensitization, and the ear was challenged 5 days later to detect induction of tolerance. The previously unchallenged left ear was challenged, and ear swelling was measured. Data represent the mean ear swelling response ± SEM. *, P < 0.05 compared to unirradiated control mice; n = 6/group.

regression (as observed in experiment 1) was not as reproducible as the enhancement of tumor growth. The limited ability of EC prepared 30 min after UV exposure to enhance tumor growth was also observed in two repeat experiments.

Systemic Immunosuppression and Tolerance Is Induced by Application of TNCB 30 min or 3 Days after UV Irradiation. The enhancement of tumor growth was much more evident when using EC prepared 3 days compared to 30 min after UV irradiation. Hence, it was determined whether similar immunosuppression to a contact sensitizer would occur. Mice were sensitized with the hapten TNCB either 30 min or 3 days after UV exposure. Sensitization of unirradiated mice led to a large CHS response (Fig. 3). However, application of sensitizer to unexposed abdominal skin of UV-irradiated mice led to a significant reduction in CHS, irrespective of the time after UV irradiation at which sensitization occurred. Hence, mice were systemically immunosuppressed at both 30 min and 3 days after UV irradiation. To detect induction of tolerance, the same mice were resensitized at the same site 12 days after the initial sensitization. The previously unchallenged ear was challenged 5 days later to elicit CHS. Results demonstrate that both groups of UV-irradiated mice were tolerant to resensitization. Thus, despite the enhancement of tumor growth by EC occurring 3 days but not 30 min after UV irradiation, mice were immunosuppressed at both times.

Allostimulatory Capacity of EC Is Reduced More Intensely at 30 min Than at 3 Days after UV Irradiation. To assess the antigen-presenting function of epidermal APC after UV irradiation, the allostimulatory capacity of EC collected either 30 min or 3 days after UV irradiation was measured. EC collected 30 min after UV irradiation induced a significantly lower MECLR than control EC from unirradiated mice (Fig. 4). The response induced by EC collected 3 days after UV irradiation was significantly greater than that induced by EC collected 30 min after UV but significantly lower than that induced by control EC. Thus, the allostimulatory capacity of EC collected 30 min after UV irradiation was severely reduced and was returning to normal by 3 days. Thus, the effect of UV irradiation on EC enhancement of tumor growth does not parallel the UV-induced inhibition of the allostimulatory capacity of EC.

DETC Are Responsible for the Enhanced Tumor Growth Induced by EC Prepared 3 Days after UV Irradiation. To determine the role of MHC class II+ and Thy-1+ subpopulations of EC in antitumor immunity against the LK2 tumor, these cells were selectively depleted from the EC by Ab-mediated, complement-dependent lysis. EC were prepared from UV-irradiated mice 3 days after exposure. Class II+ and Thy-1+ EC were removed from the EC preparation separately by incubation in monoclonal Ab and complement. Depletion of class II+ cells was 68% effective, and 88% of Thy-1+ cells was removed using this method (determined by immunofluorescence). Although not absolute, using this method we have shown previously that EC depleted of class II+ cells are unable to induce delayed-type hypersensitivity (32).

Mice were immunized with unfractionated EC or with EC from which class II+ or Thy-1+ EC were depleted. All EC were pulsed with TE. Ten days after immunization, these mice and a naive, unimmunized group were inoculated with LK2 tumor cells. EC prepared 3 days after UV irradiation significantly enhanced tumor growth compared to unimmunized mice (Fig. 5A). Removal of the Thy-1+ EC abrogated this growth enhancement induced by unfractionated EC, because LK2 growth in mice immunized with Thy-1-depleted EC was significantly lower than in mice immunized with unfractionated EC. Depletion of class II+ EC did not significantly inhibit tumor growth compared to unfractionated EC. Immunization with neither unfractionated, class II-depleted, nor Thy-1-depleted EC had any effect on the incidence of tumors in this experiment (Fig. 5B). Hence, the growth enhancement induced by immunization with TE-pulsed EC prepared 3 days after UV irradiation of skin appeared to be caused by the Thy-1+ DETC. The class II+ cells did not play a role in this enhancement of tumor growth.

UV Irradiation-Induced Cellular Changes within the Epidermis. To determine the cellular changes within UV-irradiated epidermis, EC were prepared from skin collected from mice UV irradiated either 30 min or 3 days previously. EC were also prepared from unirradiated mice. As detected by S100 staining, LC comprised 3.8% of the epidermis as a whole.
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of unirradiated epidermis (Fig. 6). LC were not significantly reduced 30 min after UV irradiation (4.3%); however, by 3 days after UV exposure, the LC had decreased significantly to 1.7% of total EC. To determine whether exposure of the skin led to hyperproliferation of epidermal keratinocytes, EC were prepared from punch biopsies to enable enumeration of total EC per area of skin. There was no difference in the number of EC prepared from either UV group compared to normal skin (data not shown). Hence, the changes in the percentages of LC were caused by decreased numbers of LC, not increased numbers of keratinocytes. Alternatively to S100, LC were detected by labeling with Ab for HSA. This revealed 5.8% LC in normal epidermis, which is similar to the results obtained with S100 labeling. However, 30 min after UV irradiation, 23% of EC expressed HSA, and even 3 days later, 11% of EC were HSA positive. Microscopic examination revealed that the cells expressing HSA were keratinocytes as well as LC. Hence, UV irradiation induced expression of HSA on keratinocytes.

There were very few CD11b+ (Mac-1+) macrophages present in normal epidermis (<0.5%). However, a significant infiltration was observed 30 min after the 5-day UV-irradiation protocol (2.4%). These cells were reduced to similar levels, as found in unirradiated epidermis, by 3 days after UV exposure. DETC detected by CD3 staining were found to be significantly reduced from 6.6% in unirradiated epidermis to 1.4% 30 min after UV irradiation. The numbers of DETC remained depleted 3 days after UV (1.7%).

EC from UV-irradiated Mice Enhance Tumor Growth by a Failure to Activate CD4+ T Cells. To investigate the role of T lymphocytes in the antitumor immunity against the LK2 tumor, immunized mice were depleted of T-cell subsets by injection with Ab against either the CD4 or CD8 antigen. Unimmunized or EC-immunized mice were injected with Ab against CD4, CD8, or as a control against M. leprae on the same day as they were inoculated with tumors. Injections of Ab continued throughout the duration of the experiment. The incidence and growth of tumors was monitored every 3–4 days.

Injection of the control Ab into unimmunized mice did not affect growth (Fig. 7A) or incidence (Fig. 8A) of the LK2 tumor compared to untreated mice. Neither growth nor incidence of tumors in unimmunized mice was significantly affected by depletion of CD4+ T cells compared to the control Ab-treated mice, although tumor growth was slightly increased. However, injection of anti-CD8 Ab significantly enhanced tumor growth compared to the control Ab-treated mice, although incidence was not significantly affected. Thus, CD8+ T cells primarily control the growth of the LK2 tumor in unimmunized mice, with CD4+ T cells playing a minor role.

Mice were immunized with TE-pulsed EC prepared from the skin of normal, unirradiated mice. Immunized mice were then inoculated with LK2 tumors 10 days later and on the same day were injected with the initial dose of Ab for the depletion of T lymphocytes (Figs. 7B and 8B). A naive, unimmunized group of mice was inoculated with LK2 tumors at the same time but did not receive injections of Ab. Mice immunized with the normal EC and treated with the control Ab showed reduced tumor growth compared to naive mice (Fig 7B), and the incidence of tumors in these immunized mice was also significantly reduced compared to naive mice (Fig. 8B). Thus, immunization with normal EC induced enhanced rejection of the LK2 tumor. When CD4+ T cells were depleted with Ab, the growth of tumors was significantly faster than in the control-treated mice, and rejection of tumors was significantly inhibited. When CD8+ T cells were depleted, the tumors grew at a significantly accelerated rate compared to control Ab-treated mice, and none were rejected. Hence, EC from normal, unirradiated mice inhibit tumor growth by enhancing both CD4+ and CD8+ T lymphocyte-mediated immunity, although CD8+ T cells appeared to play the greater role.

Mice were also immunized with EC prepared from the skin of donor mice 3 days after 5 days of UV irradiation. These EC were pulsed in vitro with tumor extract and then used to immunize groups of mice. Ten days after immunization, these mice were inoculated with LK2 tumors and were injected with Ab to induce T cell depletion, as described above. A group of naive, unimmunized mice was inoculated with LK2 tumors at the same time but were not injected with Ab. The growth of tumors in immunized, control Ab-treated mice was significantly faster than in naive mice (Fig. 7C). Depletion of CD8+ T cells enhanced tumor growth compared with that in control Ab-treated mice immunized with UV-irradiated EC. Depletion of CD4+ T cells had no significant effect on tumor growth compared to the same control Ab-treated mice. There were no significant differences in tumor incidence between any groups in this experiment (Fig. 8C). Hence, UV irradiation affects the EC suspension; therefore, CD4+ T lymphocyte-mediated antitumor immunity is not induced, whereas CD8+ T cells remain capable of controlling LK2 tumor growth.
DISCUSSION

We developed a UV-induced regressor tumor model to examine the immunological defects in UV-irradiated skin that inhibit the rejection of skin tumors. The LK2 tumor used in this study is a squamous cell carcinoma, which is a common type of UV-induced epidermal skin tumor in humans. Because this tumor regresses in immunocompetent, syngeneic mice, it models development of a skin tumor that the immune system is able to control. This enabled us to study the mechanism by which UV irradiation effects on cells of the skin immune system enables tumors to develop. Immunization of mice with EC from normal, unirradiated skin enhances rejection of the UV-induced LK2 regressor tumor by activating CD4+ T lymphocytes in conjunction with an enhancement of the CD8+ T lymphocyte activity that controls LK2 growth in unimmunized mice. EC prepared from skin immediately after UV irradiation with a dose of UV shown to be systemically immunosuppressive had only a small influence on the development of an immune response against the tumor. By contrast, EC prepared from skin 3 days after UV irradiation inhibited CD4+ T-cell activation, which led to enhanced growth of the normally regressing tumor. The EC in UV-irradiated skin responsible for the enhanced tumor growth appeared to be the DETC. Thus, UV radiation has been shown to alter the immune system of the skin such that DETC within the epidermis 3 days, and to a lesser extent immediately following UV exposure, inhibit activation of antitumor CD4+ T lymphocytes, thus enhancing tumor growth.

Normal epidermis contains 1–5% Thy-1+ DETC, however, Thy-1 is also expressed weakly on a small number of keratinocytes (33). In our experiments, the Thy-1 antibody used binds to approximately 40% of keratinocytes, although this staining is considerably weaker than DETC staining. Using this Thy-1 antibody and complement to deplete DETC, we achieved an 88% reduction in brightly stained Thy-1+ EC. This depletion did not alter total EC numbers significantly and hence could not have removed the weakly Thy-1+ keratinocytes. Whereas we cannot exclude the possibility that a small number of Thy-1+ keratinocytes in the UV-irradiated epidermis were removed by Thy-1 antibody and complement and that these cells were responsible for inhibiting the antitumor immune response, this is unlikely as keratinocytes remained the major cell type contained within Thy-1-depleted EC. Keratinocytes have been shown not to play any direct role in the induction of immunity, because haptenated keratinocytes irradiated in vitro induce neither immunity nor unresponsiveness when injected into naive mice (23). It is also unlikely that UV-induced infiltration of Thy-1+ inflammatory T cells would have been responsible for inhibition of the induction of antitumor immunity in our experiments. A T-cell infiltrate is mainly evident in the dermis from 24–72 h after UV irradiation (34), and using our UV protocol, no infiltrating T cells were observed in the epidermis either 30 min or 3 days after irradiation of the skin. Hence, it is most probable that DETC were the cell type responsible for the inhibition of antitumor immunity in our model, although they remained in very small numbers. Depletion of MHC class II+ cells did not prevent UV-irradiated EC from increasing the growth and incidence of the LK2 tumor, thus indicating that class II+ EC within the UV-irradiated skin were not responsible for the enhanced tumor growth.

To examine the function of DETC, both freshly prepared Thy-1+ EC and DETC cell lines have been used. Injection either s.c. or i.v. of Thy-1+ EC conjugated to the hapten TNCB has been shown to induce unresponsiveness to subsequent hapten challenge (35). Similar investigations with DETC cell lines resulted in immunological tolerance when injected into mice after FITC conjugation. The tolerance was antigen specific and occurred irrespective of the route of administration (15). Additional studies of lymphocytes from mice sensitized with DETC led to the proposal that tolerance was induced by inhibition of activated T-cell proliferation, as well as direct cytotoxic activity of DETC against these T cells (14, 16). Although UV irradiation of the skin reduces markedly the density of DETC within the epidermis (19), Cruz et al. (23) have shown that both unirradiated and irradiated Thy-1+ EC conjugated to DNFB induce hyporesponsiveness of equal magnitude when injected i.v., thus demonstrating that this function of DETC is unaffected by in vitro UV irradiation. Hence,
LC within tumor antigen-pulsed EC have previously been shown to induce protective immunity against the progressively growing, chemically induced S1509a fibrosarcoma (17). In those experiments, Thy-1+ DETC were removed prior to antigen-pulsing of the EC to demonstrate the protective effects of LC. This suggests that within unfractionated EC suspensions, LC induce, and possibly DETC may inhibit, protective immunity. Because the LK2 tumor is highly immunogenic, inducing protective immunity in the absence of immunization, LC may have been able to enhance this immunity, even in the presence of suppressive DETC.

To investigate this issue further, we studied the cell types present 30 min and 3 days after our UV irradiation protocol. The enhanced tumor immunity induced by unirradiated EC was small and inconsistent; therefore, it was not possible to determine the cell type responsible by

DETC from both unirradiated and UV-irradiated epidermis appear to induce unresponsiveness to contact sensitizers. This is consistent with our results that demonstrated that DETC from UV-irradiated skin inhibited the development of protective immunity against the normally regressing LK2 tumor. However, UV irradiation was necessary for this effect on tumor growth because EC from unirradiated mice enhanced tumor regression. This raises the question as to why DETC were only able to inhibit tumor immunity when present in EC prepared from UV-irradiated, but not unirradiated, skin.

Fig. 7. EC from UV-irradiated mice enhance tumor growth by a failure to activate CD4+ T cells. Tumor growth (mean tumor diameter) was measured in unimmunized mice (A), mice immunized with 10⁶ TE-pulsed EC from normal mice (B) or EC prepared from UV-irradiated skin collected 3 days after exposure (C). Mice were inoculated with LK2 tumors (10 days after immunization in those mice that were immunized) and at the same time injected with Ab to deplete T-cell subsets. A group of naive, unimmunized mice that did not receive Ab treatment was included in each experiment. The growth of the tumors was then observed every 3–4 days after tumor inoculation in these immunized and unimmunized mice that had been treated with anti-CD8 Ab (△), anti-CD4 Ab (●) or the control M. leprae Ab (○), or the naive, untreated mice (▲). Within each experiment, tumor growth was compared to the growth in mice treated with the control Ab (○). *P < 0.05 by repeated measures by ANOVA (A: P < 0.0001; least significant difference, 1.872; B: P < 0.0001; least significant difference, 0.850; C: P < 0.0001; least significant difference, 1.137); n = 7/group.

Fig. 8. Tumor incidence in mice depleted of T-cell subsets. Tumor incidence (percent age of mice per group with tumors) was determined in the same groups of mice as described in Fig. 7, i.e., in unimmunized mice (A), mice immunized with EC prepared from normal mice (B), or EC prepared from UV-irradiated skin collected 3 days after exposure (C). Each experiment contained a group of naive, unimmunized mice (▲). Incidence of the LK2 tumor was calculated at each time point during the experiments, and within each experiment tumor incidence was compared to that in mice treated with the control Ab (○). *P < 0.05 by Mann-Whitney U test; n = 7/group.
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antibody-mediated depletion studies. EC obtained 30 min after UV irradiation only marginally enhanced tumor growth compared to EC isolated 3 days after completion of the UV irradiation protocol; hence, we compared the cell types present at these two times. DETC were reduced at both time points, suggesting that UV effects on DETC alone are unlikely to be responsible for enhanced tumor growth at 3 days but not 30 min. By contrast, LC were not reduced 30 min after UV irradiation but were by 3 days. This raises the possibility that DETC, in the absence but not presence of LC, may have been able to enhance tumor growth. Because UV irradiation reduced DETC to 25% of their normal numbers, those DETC that remained in the UV-irradiated skin were sufficient to inhibit tumor rejection. It is possible that UV irradiation altered DETC function, thus enabling these cells to exert this inhibitory function. This is supported by evidence that EC, from which DETC were depleted and then UV irradiated in vitro, were unable to induce antitumor immunity against the S1509a fibrosarcoma (36).

Because LC numbers remained normal 30 min after our UV irradiation protocol, we used the MECLR to examine their function. The allostimulatory capacity of EC, a measure of the APC function of the EC, was lower at 30 min than 3 days after our UV radiation protocol, although it remained depressed compared to unirradiated EC at both times. A second type of class II+ APC was present in the epidermis 30 min but not 3 days after UV irradiation. These were macrophages characterized by CD11b that have previously been described to infiltrate the epidermis following exposure to UV irradiation (24, 25). In mice, CD11b+ macrophages are responsible for the induction of tolerance to the contact sensitizer DNFB 72 h after a single UV exposure (26). However, at this same time point, the CD11b+ macrophages were found to be responsible for the early recovery in allostimulatory capacity in the same system, at a time when LC were not present (24). Thus, these CD11b+ cells, although inducing immunosuppression, may contribute to the MECLR. Using our UV irradiation protocol, the 30-min time point at which these CD11b+ macrophages were present was the time when the allostimulatory capacity was more depressed, suggesting that LC function may have been depressed at 30 min after UV irradiation. Hence, it is unlikely that functional LC were preventing DETC from inhibiting tumor rejection 30 min after UV irradiation.

It is intriguing that the EC prepared 30 min after UV irradiation contained two populations of cells expected to inhibit tumor rejection, the CD11b+ macrophages and the DETC, and yet the EC at this time after UV irradiation had little effect on tumor growth. However, by 3 days after UV irradiation, the LC were depleted, and CD11b+ cells were no longer present within the epidermis, leaving only the DETC to inhibit tumor rejection. It is possible that different mechanisms could be involved in the induction of immunity and UV-induced tolerance in other tumor systems. Our UV irradiation contained both UVAs and UVB, as does the solar spectrum, and the mice were given daily suberythemal doses of UV to model sunlight exposure, whereas other irradiation protocols involving only the UBV wavelengths may give different results.

To investigate the effector mechanism that enhances growth of the LK2 tumor in mice immunized with DETC from UV-irradiated skin, the CD4+ and CD8+ T lymphocyte subsets were depleted from immunized mice. The LK2 tumor, a squamous cell carcinoma, regresses upon s.c. injection into naive, syngeneic mice. This regression was found to be primarily mediated by CD8+ T lymphocytes, with a small contribution from CD4+ T cells. Thus, without prior immunization, LK2 activates primarily CD8+ T cells, but CD4+ T cells are also important. The immunological rejection of some UV-induced tumors has previously been ascribed to the function of T lymphocytes (2, 4). Recently, it has been shown by Ward et al. (37) that CD8+ T cells are responsible for regression of these tumors. CD4+ T cells were found to play a larger part in the regression of LK2 tumors in mice immunized with normal EC; however, CD8+ T cells were also active in rejection of tumors in these mice. By contrast, CD8+ but not CD4+ T cells controlled growth of the LK2 tumor in mice immunized with EC from UV-irradiated skin. Hence, DETC collected from UV-irradiated mice 3 days after exposure inhibited activation of the CD4+ T cells, without affecting activation of CD8+ T cells. Because both CD4+ and CD8+ T cells were able to regulate growth of the LK2 tumor, inhibition of CD4+ T-cell activation by EC from UV-irradiated mice led to enhanced tumor growth.

Whereas a role for CD8+ T lymphocytes in immune-mediated destruction of UV-induced tumors is well defined, the role of CD4+ T cells in antitumor immunity is less clear, with evidence for both effector and suppressor functions. Kosugi et al. (38) have shown that depletion of CD4+ T cells in antitumor immunity is less clear, with evidence for both effector and suppressor functions. Kosugi et al. (38) have shown that depletion of CD4+ T cells diminishes the antitumor immune response induced by CD8+ T cells (38), demonstrating a CD4+ T-cell enhancement of immunity consistent with our findings. Immunophenotypic comparisons of regressing and nonregressing human skin tumors demonstrated a major role for CD4+ T cells in regression of human malignant melanoma, basal cell carcinoma, and squamous cell carcinoma (39–41). Thus, although CD8+ T lymphocytes were the major effector cell responsible for rejection of the LK2 tumor in this study, an important role for CD4+ T cells is consistent with previous observations. It is likely that UV-irradiation-induced inhibition of CD4+ T-cell activation could enhance skin tumor growth, even in the presence of tumor-reactive CD8+ T cells.

In conclusion, we used an UV-induced skin tumor that is rejected by the immune system to investigate the mechanism by which UV irradiation affects cells of the epidermis to enable developing skin tumors to escape immune destruction. Our data indicate that DETC within the epidermis 3 days after UV irradiation inhibit the activation of CD4+ T lymphocytes, without affecting activation of CD8+ T cells. This absence of activated CD4+ T cells enabled the tumors to grow at an accelerated rate, despite CD8+ T cells controlling tumor growth. LC or CD11b+ MHC class II+ cells within UV-irradiated epidermis were not involved in UV-induced growth enhancement of this skin tumor.

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REFERENCES


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