Depletion of CD8+ or CD4+ Lymphocytes Enhances Susceptibility to Transplantable Ultraviolet Radiation-induced Skin Tumours

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Abstract. Background: Immunosuppressed patients are extremely susceptible to cutaneous squamous cell carcinoma, suggesting that immunosurveillance by T lymphocytes protects against this ultraviolet radiation-induced tumour. Materials and Methods: To determine the relative contribution of CD8+ and CD4+ lymphocytes to immunosurveillance, we tested the effects of CD8+ or CD4+ T lymphocyte depletion on the susceptibility of C3H/HeN mice to a syngeneic UVR-induced skin tumour cell line. Results: Both anti-CD8 and anti-CD4 treatment significantly enhanced the growth of transplanted tumours. In CD8-depleted animals, tumours grew rapidly in all animals. Tumour growth in CD4-depleted animals was slower, and 50% of these mice eventually rejected their tumours. In CD4-depleted mice that did not reject their tumours, an early period of tumour growth was followed by partial regression of the tumour; a second phase of rapid tumour growth then supervened. Conclusion: Our findings suggest that both CD8+ and CD4+ lymphocytes contribute to immunosurveillance against skin cancer.

Nonmelanoma skin cancer (NMSC) is the most common post-transplant malignancy (1), with the risk of NMSC in transplant recipients reaching 250-fold the risk in the general population (2-4). Most of the skin tumours that develop in transplant patients are squamous cell carcinomas (SCC); the ratio of SCC to basal cell carcinomas in transplant patients is approximately 4:1, the inverse of the ratio in the general population (4). In addition, transplant recipients tend to develop multiple tumours, which are more locally aggressive and have a greater tendency to metastasize than skin tumours in the general population (2, 4). In one study, skin cancer was reported to account for 27% of deaths in heart recipients in Australia who survived more than 4 years after transplantation (5). Given the ever-increasing incidence of NMSC worldwide and the expanding population and prolonged survival of chronically immunosuppressed transplant patients, the potentially life threatening nature of the skin tumours these patients develop has become an issue of increasing public health concern (2, 4).

The presence of signature p53 mutations in skin tumours arising in transplant patients indicates that ultraviolet radiation (UVR) is an important etiologic factor for NMSC in immunocompromised individuals, as well as in the general population (6). However, NMSC risk in transplant patients is also highly dependent upon the type and duration of immunosuppressive therapy being administered (2-4). Immunosuppression in transplant patients appears to impair the protective immune response against UVR-induced NMSC (2, 3, 7-11). There has been limited investigation of the specific immune defects that underlie increased NMSC susceptibility in transplant recipients. Renal transplant patients with NMSC have significantly reduced numbers of CD4+ T lymphocytes compared to patients without NMSC,
while the numbers of total lymphocytes, CD8+ lymphocytes and CD19+ cells do not differ between the groups (12). The fact that regressing NMSC contain many more CD4+ T lymphocytes than non-regressing tumours suggests that these cells are essential components of the immune defense against UVR-induced NMSC in man (13-16); however, this hypothesis has not been tested directly.

Early studies in mice verified a causal role for treatment-related immunosuppression in fostering the development of UVR-induced NMSC. These studies demonstrated that treating mice with immunosuppressive agents such as cyclophosphamide, methotrexate, cortisone, anti-lymphocyte globulin, azathioprine, or prednisolone reduced the numbers of Langerhans cells and T lymphocytes in the skin, shortened the latent period for the emergence of UVR-induced skin tumours, and increased the tumour yield per mouse (17-19). In addition, a study by Norbury et al. (20) indicated an important role for T cells in protection against UVR-induced tumours, as T cell-depleted animals developed tumours more rapidly than immunocompetent animals. Girardi et al. (21) recently demonstrated that mice deficient in either γδ or ω T cells were more susceptible to transplantable, chemically-induced skin tumours than wild-type animals. Moreover, they also demonstrated that both CD8+ and CD4+ cells producing interferon-γ (IFN-γ) contributed to the protective immune response against the tumours.

In the present report, we demonstrate that specific depletion of either CD8+ or CD4+ T lymphocytes significantly enhances the susceptibility of mice to a transplantable UVR-induced skin tumour, confirming that both cell types play important roles in immunosurveillance against UVR-induced skin NMSC.

**Materials and Methods**

The UV2237 cell line, derived from a UVR-induced skin tumour by Dr. M. Kripke (22), was the kind gift of Dr. S. Ullrich (M.D. Anderson, Houston, TX, USA). Cells were grown in 10% CO2 as monolayer cultures, as previously described (23). Cells were harvested for injection using 0.05% trypsin in 0.53 mM EDTA (Atlanta Biologicals, Norcross, GA, USA). Trypsin was neutralized by the addition of medium containing 10% FBS. Suspended cells were washed 3 times in PBS before subcutaneous injection into the flanks of young adult C3H/HeN mice (Charles River Laboratories, Wilmington, MA, USA). Growth of the injected tumours in C3H/HeN mice was monitored periodically using digital vernier calipers for up to 42 days after tumour injection. Tumour size was calculated by multiplying length by width by height for each tumour. The studies were conducted in accordance with all relevant local, state and national animal welfare guidance in facilities accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care.

For depletion of CD3+CD4+ lymphocytes, mice were injected intraperitoneally with 0.5 mg depleting anti-CD4 monoclonal...
antibody (GK1.5) on days –1 and +1 relative to tumour cell injection. CD3+CD8+ lymphocyte depletion was accomplished by intraperitoneal injection of 0.1 mg depleting anti-CD8 monoclonal antibody (YTS 169, kind gift of Dr. Robert Fairchild, Cleveland Clinic Foundation, Cleveland, OH, USA) on days -3, -2, and day 0 relative to tumour injection, and weekly thereafter.

Differences between treatment groups were evaluated using Student’s t-test for the continuous variables CD4+:CD8+ T lymphocyte ratio and tumour volume. P values less than 0.05 were considered statistically significant.

Results

Initial experiments determined the optimal dose for testing the effect of CD4+ T lymphocyte depletion on growth of UV2237 cells. As shown in Figure 1, tumour growth was dose-dependent. Two million tumour cells per injection resulted in the rapid appearance of large tumours in all injected mice. Lower numbers of cells (1 x 10⁶ or 0.5 x 10⁶) produced smaller tumours over a somewhat longer time-course. Based on these findings, we selected 0.5 x 10⁶ cells as an inoculum that was expected to be large enough to establish tumours in all mice, but small enough to permit us to detect the effects of CD4+ T lymphocyte depletion on tumour emergence and rejection.

C3H mice treated with GK1.5 had 90-100% depletion of CD4+ T lymphocytes 16 days after treatment; CD8+ lymphocyte numbers were unaffected. Depletion of CD4+ T lymphocytes was long lasting, with little CD4+ T lymphocyte repletion at any time-point examined up to 42 days after treatment (Figure 2). C3H mice treated with YTS169 had greater than 95% depletion of CD8+ cells; this depletion was evident for up to 21 days (data not shown), when the animals were sacrificed due to large tumour size.

We injected 0.5 x 10⁶ UV2237 cells subcutaneously into 8 untreated, 4 CD8+ T lymphocyte-depleted, and 4 CD4+ T lymphocyte-depleted C3H/HeN mice and measured tumour growth (Figure 3). All of the immunocompetent control mice rejected UV2237 cells by 36 days after injection. In contrast, all CD8+ T lymphocyte-depleted mice rapidly grew very large tumours that required the animals to be sacrificed at 21 days post-injection. These data confirm the key role of CD8+ T lymphocytes in protecting against NMSC. CD4+ lymphocyte-depleted mice developed tumours that were significantly larger than tumours in immunocompetent mice. Some CD4+ T lymphocyte-depleted mice were able to reject the tumours, although regression occurred more slowly than in untreated animals. In half of the CD4+ T lymphocyte-depleted animals, tumours continued to grow for up to 42 days after injection. UV2237 tumour growth was biphasic in CD4+ lymphocyte-depleted animals: the average tumour size reached a peak at 9 days post-injection; by 21 days post-injection, tumours were no longer palpable; at 29 days post-injection, tumours in some animals began to regrow and continued to enlarge until 42 days post-injection, when the study was terminated. These studies indicate that CD4+ T lymphocytes also contribute to effective immunosurveillance against NMSC.

Discussion

CD8+ lymphocytes are commonly considered to be the chief effector cells in immune clearance of tumours (22, 23). These cells use a combination of soluble cytolytic factors and cell surface molecule-based killing mechanisms to destroy emerging skin tumour cells (reviewed in 24). Our studies, using the UV2237 cell line, demonstrated conclusively that reducing the number of CD8+ cells resulted in very rapid tumour growth in all of the depleted animals. These results are in accordance with the recent results of Girardi and colleagues (21), who showed that repleting αβ T cell receptor-deficient mice with CD8+ cells from mice that had...
reverted chemically-induced skin tumours partially restored the ability of the recipients to reject these same tumours.

Our data demonstrated that depletion of CD4+ T lymphocytes also impaired the protective immune response against an established UVR-induced skin tumour cell line. The biphasic growth of tumour cells in CD4-depleted animals suggests that there may be two distinct phases in which CD4+ cells act in immune surveillance. During the early phase of immunosurveillance (0-10 days post-injection), it is unlikely that CD4+ cells are functioning to promote an antigen-specific response. However, during the latter phase (day 29 onward), CD4+ cells may function as helper cells to activate and expand CD8+ CTL and to promote anti-tumour antibody production. The work of Girardi et al. (21) suggests that the CD4+ lymphocytes important for anti-tumour activity are likely to be TH-1 cells, since mice deficient in IFN-γ show enhanced susceptibility to chemically-induced skin cancer. Depletion of CD4+ Langerhans cells was unlikely to play a role in the observed effects, as Langerhans cells of the murine epidermis are generally CD4- (25) and are not recognized by the GK1.5 antibody we used for CD4+ lymphocyte depletion (26).

There are several CD4+ T lymphocyte populations in mice, including conventional TH-1 and TH-2 T lymphocytes, CD4+CD25+ regulatory T lymphocytes, and NK-T lymphocytes. TH-1 and TH-2 T lymphocytes are characterized on the basis of the cytokines they secrete (24-30), with TH-1 secreting predominantly IFN-γ and TH-2 secreting predominantly interleukin-4 (IL-4) and IL-10. NK-T cells are a unique subset of cells that express both T cell and NK cell markers and frequently have a limited TCR repertoire (reviewed in 31, 32). NK-T cells comprise less than 1% of splenic T cells, and approximately 60% of these cells express CD4. CD4+ NK-T lymphocytes can express both TH-1 and TH-2 cytokines (reviewed in 32). UVR exposure has been reported to induce CD4+ NK-T lymphocytes which, when adoptively transferred to irradiated mice, suppressed tumour immunosurveillance and allowed progressive growth of a transplantable UVR-induced skin tumour cell line (33). CD4+CD25+ regulatory T lymphocytes producing IL-10 and transforming growth factor-β are believed to prevent the development of autoimmunity, suppress transplant rejection, and promote tumour growth (34-40). In our studies, depletion of CD4+ T lymphocytes clearly compromised immune surveillance, allowing more rapid growth of a UVR-induced tumour cell line. Thus, any increase in immune surveillance resulting from depletion of CD4+CD25+ regulatory T lymphocytes or immunosuppressive CD4+ NK-T lymphocytes was outweighed by a decrease in immune surveillance due to removal of conventional CD4+ TH-1 T lymphocytes.

Our studies are the first specifically to test the role of CD4+ and CD8+ immune cells in immunosurveillance against a UV-induced skin tumour. Our findings suggest that immunosurveillance is a complex response, in which both CD4+ and CD8+ immune cells play distinct but critical roles.

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