Killing of Skin-derived Tumor Cells by Mouse Dendritic Epidermal T-Cells

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ABSTRACT

Dendritic epidermal T-cells (DETC) are a unique population of T-cells that reside normally in mouse epidermis and express a y6 T-cell receptor. We have reported previously that DETC acquire in culture the capacity to lyse the YAC-1 lymphoma, a conventional target for natural killer cells. The aim of the present study was to characterize this cytotoxic potential, using a spectrum of skin-derived mouse tumors. Cytotoxicity was measured by a 51Cr release assay and by the visual assessment of target cell lysis. Long-term DETC lines, established from CBA, AKR, and BALB/c mice by mitogenic stimulation and repeated feeding with interleukin 2 (5 units/ml), were used as effectors. Skin-derived tumor targets included 5 melanoma lines and the transformed keratinocyte line Pam 212. Each DETC line lysed skin-derived tumors as well as YAC-1 targets effectively in the 18-h 51Cr release assay, and target lysis occurred in a non-major histocompatibility complex-restricted manner. By contrast, freshly isolated spleen cells lysed YAC-1 but not skin tumor targets. Moreover, confluent monolayers of melanoma or Pam 212 targets were disrupted completely by added DETC lines but not by spleen cells. The cytolytic activity of DETC appeared to be specific for tumor cells, since normal mouse keratinocyte monolayers remained intact under the same conditions. Finally, DETC freshly isolated from skin failed to exhibit significant cytotoxicity but acquired this capacity 10–14 days after mitogenic stimulation and feeding with interleukin 2 (5 units/ml). We conclude that DETC possess the potential to recognize, bind, and lyse tumor cells that originate in skin.

INTRODUCTION

Skin cancer is the most frequently observed primary human malignancy in the United States (1); this finding should not be surprising, because the skin is constantly exposed to environmental carcinogens, including chemicals, viruses, and UV radiation. Moreover, it can be argued that this incidence would be even higher were it not for normal immunosurveillance, as exemplified by the increased incidence of skin tumors in patients who are immunosuppressed (2, 3). It is, therefore, important to understand mechanisms by which transformed skin cells are ordinarily recognized and eliminated by immune cells during the preclinical stage of cutaneous carcinogenesis.

Two dendritic leukocyte populations reside in normal mouse epidermis, both of which are thought to play important roles in immunosurveillance against skin tumors. (a) Langerhans cells are the major antigen presenting cell in epidermis (reviewed in Ref. 4); these cells have been shown to present tumor-associated antigens to CD4+ T-cells (5, 6). (b) DETC are a unique subset within the recently defined keratinocyte-presenting cell in epidermis (reviewed in Refs. 7 and 8), that reside normally in mouse epidermis and express a y6 T-cell receptor. An activated by co-culturing with transformed keratinocytes. A widely accepted concept then is that malignant transformation or other "stressful" events occurring in neighboring cells activate DETC to become killer leukocytes, which, in turn, eliminate otherwise harmful cells via cytotoxicity (12, 15). Thus, the present study was conducted to determine whether DETC are capable of recognizing and killing tumor cells that are derived from skin.

MATERIALS AND METHODS

Mice. Female CBA and BALB/c mice were purchased from Harlan (Indianapolis, IN) and used when 6–8 weeks old. Mice were housed in the University Animal Resource Center where they were provided food and water ad libitum according to NIH guidelines.

Effector Cells. Three long-term cultured DETC lines were used in most experiments: 7-17, established from FACS-purified Thy-1+ EC in AKR mice and 3-28 and 12-12, established from EC in CBA mice. The phenotypic and functional characteristics of these long-term DETC lines have been described elsewhere (11, 12, 14, 16). To test the cytotoxic potential of freshly isolated DETC, EC suspensions were prepared from frozen skin of CBA mice by trypsinization as reported previously (9, 17, 18). Isolated EC were then enriched for DETC by centrifugation through Histopaque (1.083), with cells recovered at the medium interface routinely 5–20% Thy-1+ (DETC). In some experiments, these "interface" EC were purified by FACS for Thy-1+ cells (9). Short-term DETC lines were established by stimulating interface EC with 2 μg/ml concanavalin A, immobilized anti-CD3 mAb (500.A2, kindly provided by Dr. J. Allison, University of California, Berkeley, CA), or with the combination of ionomycin (1 μM) and phorbol 12-myristate 13-acetate (3.3 ng/ml); cells were fed thereafter with fresh medium containing 5 units/ml recombinant IL-2 (R & D Systems, Minneapolis, MN) every 3–4 days.

Targets. Thirteen tumor cell lines were used as targets; their characteristics are summarized in Table 1. When primary cultures of mouse keratinocytes were used, disaggregated EC from BALB/c mice were cultured for 4 days in Dulbecco's modified Eagle's medium supplemented with 20% fetal calf serum, 10 ng/ml epidermal growth factor (Sigma, St. Louis, MO), and 0.4 μg/ml hydrocortisone in 24-well plates (Falcon, Oxnard, CA) (2 × 106 cells/well) (19).

51Cr Release Assay. Assays were carried out as described previously (9, 17). Briefly, 1 × 106 target cells in suspension were incubated for 90 min at 37°C with 100 μCi of 51Cr (specific activity, 2.5 mCi/ml) (ICN Radiochemicals, Irvine, CA). After three rinsings, cells were suspended in complete RPMI. Effector cells were also suspended in complete RPMI, at several different concentrations. Target cells (100 μl/well) and effector cells (100 μl/well) were added to round-bottomed 96-well plates (Falcon) and incubated for 4 or 18 h at 37°C. Plates were then centrifuged for 10 min at 1000 rpm, and 100 μl of supernatant were recovered for radioactivity, with specific lysis determined as:

% of specific lysis = 

Experimental - spontaneous release

Maximal - spontaneous release × 100

Received 2/08/93; accepted 6/21/93.

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Visual Determination of Cytotoxicity Using Tumor Cell Monolayers. Target cells (4 × 10^5 cells/well) were plated onto 24-well plates and cultured for 6 h to form confluent monolayers. Effector cells (2 × 10^6 DETC or 10 × 10^6 spleen cells) suspended in 1 ml of complete RPMI were then added. After incubation at 37°C for up to 18 h, target cells that had been lysed or damaged and nonadherent effector cells were removed by gentle but extensive washing with Dulbecco’s phosphate-buffered saline (140 mM NaCl-3 mM KCl-4 mM Na_2HPO_4-2 mM KH_2PO_4, 1 mM MgCl_2-1 mM CaCl_2, pH 7.4). Subsequently, specimens were fixed and stained for 20 min with 0.1% crystal violet in methanol, followed by rinsing with distilled water (20). Cytotoxicity was assessed macroscopically and microscopically (×200).

RESULTS

Cytotoxicity of DETC Lines against Skin Tumor Cells. Initial experiments were carried out with the B16-F0 melanoma to serve as a prototype. In the 4-h ^51Cr release assay, which is commonly used to measure NK cytotoxicity, DETC line 12-12 caused low but significant lysis of B16-F0 (and YAC-1) targets (Fig. 1). By contrast, freshly isolated spleen cells killed YAC-1 cells, but not the B16-F0 melanoma. Although optimal killing of YAC-1 by 12-12 DETC was observed after 4 h, killing of B16-F0 targets continued to increase up to 18 h. Two other long-term DETC lines (7-17 and 3-28) produced similar effects against the same B16-F0 targets (data not shown).

Four additional melanoma cell lines (B16-F1, B16-F10, Queen’s B16-F10 subline, and Cloudman melanoma) and the transformed keratinocyte line Pam 212 were then tested in parallel. As seen in Fig. 2, 12-12 DETC caused significant lysis of each of these skin-derived tumor targets; 15-20% of melanomas and 20-25% of Pam 212 cells were killed by DETC at an E:T ratio of 100:1. Again, two other DETC lines also killed each of these skin-derived targets (see also Fig. 5). Spleen cells exhibited minimal, if any, killing of these skin-derived targets, even at higher E:T ratios (Fig. 2). By contrast, some of the non-skin-derived targets, including YAC-1, were lysed effectively not only by DETC but also by spleen cells (Fig. 3).

The variations seen in susceptibility to cytotoxicity among the selected targets most likely reflect combinations of differences in effector-target recognition and target sensitivities to the lethal hit process. To separate these events, we sought to determine whether killing of skin tumors, which were less susceptible than YAC-1 targets, could be enhanced by promoting effector-target contact with added lectins. As shown in Fig. 4, the DETC-resistant target, P815, became susceptible in the presence of 1 μg/ml PHA, as has been reported previously (17). Similarly, the addition of PHA enhanced killing of both the B16-F0 melanoma and Pam 212 keratinocytes, suggesting that skin tumors can be lysed by DETC even more effectively when effector-target contact is promoted by exogenous factors.
PH A (1 μg/ml), or by using transformed Pam 212 keratinocytes as targets. On the other hand, significant cytotoxicity was detected consistently with DETC harvested from 10–14-day EC cultures that had been stimulated with concanavalin A, immobilized anti-CD3, or the combination of phorbol 12-myristate 13-acetate and ionomycin and then expanded in the presence of 5 units/ml recombinant IL-2 (Fig. 5). These short-term EC cultures routinely contained 60–80% DETC as judged by immunofluorescence staining with mAb against the Vγ3 determinant of the TCR (data not shown). Each of our long-term (>3 months) DETC lines (3-28, 12-12, 7-17, 11-20, and 11-28) also showed significant killing of the B16-F0 melanoma, for which 3-28 was the most and 7-17 the least potent. Although the mechanism(s) of DETC activation remains to be addressed, it is clear that cells freshly isolated from skin possess no detectable cytotoxicity against skin tumor targets and that this capacity is inducible by our in vitro culture conditions.

Morphological Assay for Measuring Killing of Skin Tumors by DETC. The standard 51Cr release assay, which has been used widely for testing cytotoxicity of NK cells and cytotoxic T-cells, is a sensitive and reproducible method to examine nonadherent, lymphoid targets. This assay, however, was not ideal for adherent targets. Melanoma cells and keratinocytes, for instance, would adhere to the round-bottomed 96 wells within 1–3 h, blocking access by the cytotoxic effectors. As an alternative, we developed a method that measured the capacity of effector cells to damage tumor cells directly. For this assay, target cells were incubated for 6 h in 24-well plates to form confluent monolayers. After addition of effectors, cell destruction was assessed by macroscopic and microscopic observations of the residual cells, after staining with crystal violet.

With this assay, DETC line 3-28 was observed to cause marked destruction of Pam 212 keratinocytes and B16-F10 (Queen’s) and B16-FO melanoma (Fig. 6). This phenomenon required the DETC, and it correlated with the number of added cells; 1–2 × 106 DETC caused almost complete disappearance of 4 × 105 Pam 212 keratinocyte targets, whereas 0.5 × 106 DETC resulted in only partial destruction. Two other DETC lines (7-17 and 12-12) produced similar effects but required 2–4-fold higher E/T ratios (data not shown). Importantly, spleen cells failed to affect these tumor cell monolayers, even in higher numbers (10 × 106 cells), which is consistent with results from the conventional 51Cr release assays.

Microscopically, the destruction of cell monolayers occurred in the following sequence: (a) adhesion of DETC to tumor cells (within 15 min); (b) formation of discrete foci of cell destruction in which tumor cells were replaced by DETC that then became adherent to the substrate (about 4 h); (c) gradual enlargement of the discrete foci; and (d) complete disappearance of target cell monolayers, leaving many DETC bound to the substrates (by 18 h).

Cytotoxicity of DETC Is Directed against Tumor Cells. Having confirmed the ability of DETC lines to damage skin tumor cells in the visual assay, we then sought to assess target specificity by comparing the susceptibility of Pam 212 keratinocytes (“tumor” cells) with primary cultures of mouse keratinocytes (“normal” cells). The DETC
line 3-28 again caused complete destruction and disappearance of Pam 212 keratinocytes, whereas it failed to damage normal keratinocytes (Fig. 7). As described previously, cell destruction by DETC did not take place diffusely but originated in discrete foci (Fig. 8, C-F). By contrast, normal keratinocytes, which showed a typical cornified appearence in the absence of effector cells (Fig. 8G), remained unaffected by the added DETC (Fig. 8H).

DISCUSSION

In the reported experiments, we have observed that dendritic epidermal T-cells are capable of recognizing and killing tumor cell lines derived from mouse skin. Susceptible targets included five melanoma lines and the transformed keratinocyte line, Pam 212. With respect to the target specificity, DETC were found to lyse not only these skin-derived tumors but also non-skin-derived tumors. Spleen cells, which did kill non-skin-derived tumors, however, failed to lyse the skin tumor targets, suggesting the "effector selectivity" in this killing. Killing was independent of the genetic background of effector and target cells, indicating that recognition occurs in a non-MHC-restricted manner. Finally, in a newly developed visual assay, DETC destroyed skin-derived tumor cells grown in monolayers with high efficiency.

Based on these observations and on earlier studies (9, 10, 17), DETC-mediated cytotoxicity appears to resemble that of conventional αβ T-cells when activated to express non-MHC-restricted killing or lymphokine activated killing (reviewed in Ref. 22). Moreover, γδ T-cells from sources other than skin have been observed to possess similar attributes (23–25). The features of cytotoxicity that these cells
have in common include: 1) killing of NK-susceptible targets, 2) independence from MHC restriction, and 3) a dependence on cytokines for the development of their killing potential. With respect to DETC, they appear to fulfill the first two features of lymphokine activated killing. Less obvious was the absolute dependence of DETC-mediated killing on IL-2, which was provided continuously in our culture system; DETC did not survive in the absence of this cytokine.

We have observed recently that a second cytokine, IL-7, which is produced by keratinocytes (26), also promotes the growth of DETC in vitro. Importantly, IL-7 has been demonstrated to possess a similar, if not even greater capacity than IL-2 to promote the generation of non-MHC-restricted killer αβ T-cells in vitro (27–30). These killer αβ T-cells, although produced in culture, appear to have physiological relevance, since the development of tumor metastasis has been prevented in adoptive transfer studies (31). Likewise, it has been reported that transfection of tumor cells with an IL-7 gene leads to their rapid rejection after inoculation in vivo, indicating that high local concentrations of IL-7 will support the generation of killing activity (32).

Taken together, these findings may suggest that keratinocyte-derived IL-7, rather than IL-2, is the critically required cytokine in the acquisition of cytotoxic potential by DETC.

DETC, when freshly procured from skin, did not express cytotoxicity. Rather, they acquired the capacity to kill skin-derived targets only after 10–14 days in culture, thus duplicating observations made previously with YAC-1 lymphoma targets (9). We interpret this to indicate that DETC in situ also require activation before cytotoxicity can be expressed. In fact, a related scenario appears to apply to γδ T-cells in mouse intestine. Whereas cells from normal mice exhibit significant killing of tumor targets soon after procurement, cells recovered from germ-free animals do not suggest, that cell activation signals (initiated by microorganisms) lead to the maturation of “immature” γδ T-cells into killer leukocytes (23). The major difference would be that DETC appear to be “immature,” whereas intestinal γδ T-cells are “activated” in normal mice.

Several different stimuli transduce cell activation signals in DETC. Such signals include: (a) mitogenic lectins (11, 17); (b) coculturing with a transformed keratinocyte line, Pam 212 (14); (c) epicutaneous application of irritant chemicals (33); and (d) epidermal injury caused by injected autoreactive cytotoxic T-cells (34). These cell activation signals, most likely being transduced through the TCR, lead to the production of IL-2, the expression of IL-2 receptor, and vigorous proliferation (17). Our working hypotheses are that the same signals will also lead to the “maturation” of DETC into “killer leukocytes” and that IL-7 produced locally by keratinocytes will promote this maturational process. We do not believe, however, that the TCR/CD3 complex is the sole receptor that mediates the recognition of tumor targets, since neutralizing mAb against Vγ3 TCR, γδ TCR or CD3e failed to block the lysis of skin-derived tumors by DETC (data not shown). Studies are currently in progress in our laboratory to identify the alternative receptor molecule(s) that may mediate the cytotoxic recognition of tumors by DETC.

Our visual assay for cytotoxicity was considerably more sensitive than the standard 51Cr release assay for adherent target cells. With the visual assay, almost complete destruction of Pam 212 keratinocytes was achieved at an E:T ratio of 5:1, whereas specific lysis based on 51Cr release was no more than 25%, even at 100:1. This high sensitivity may be attributed to the high affinity of DETC for target cell monolayers (35) and an effective formation of foci of attack, as was seen microscopically. An additional advantage of this visual assay is its resemblance to in vivo circumstances; effector cells can attach to the mass of target cells, as would be the case for solid tumors in vivo. Similar observations have been made for αβ TCR cytotoxic T-cells (36). These killer cells also cause the detachment of adherent targets from their substrates, and this phenomenon, “loss of adhesion,” has been proposed to represent a unique form of immune damage caused by killer leukocytes, since it is not seen in the complement-mediated lysis of the same targets.

DETC-mediated cytotoxicity appeared to be directed against transformed cells; DETC lines disrupted Pam 212 KC monolayers almost completely, whereas normal KC remained intact under the same conditions (Fig. 7). In this regard, we have observed that nontransformed cell targets, syngeneic lymphoblasts that are also ordinarily resistant to DETC-mediated killing, can be lysed effectively when target recognition is bypassed by adding PHA. Although not yet tested in a skin-derived cell system, it is likely that the process of target recognition serves as a mechanism by which DETC differentiates transformed cells from normal cells.

Finally, compelling circumstantial evidence suggests in vivo relevance for DETC-mediated cytotoxicity. DETC in situ and DETC lines express mRNA for perforin, a molecule required for lethal hit by killer leukocytes (37). The epidermic application of phorbol esters or chemical carcinogens decreases surface densities of DETC in vitro (38, 39). Exposure of skin to UVA radiation (320–400 nm) with a psoralen photosensitizer or to UVB radiation, both of which possess carcinogenic potential, depletes the epidermis of DETC, as well as Langerhans cells (40, 41). It is of interest that DETC are not replaced during the latent period of tumor development, whereas Langerhans cells are, suggesting that the absence of DETC may contribute to the survival of transformed cells. Moreover, Bachelez et al. (42) observed a preferential infiltration of γδ T-cells into cutaneous lesions in patients with malignant melanomas, and they demonstrated that these cells are capable of killing autologous melanoma targets in 51Cr release assays. Although these human γδ T-cells are not precise equivalents of murine DETC, these observations indicate that γδ T-cells, as a class, may play an important role in protective immunity against skin tumors.

Thus, we propose that both populations of dendritic leukocytes play important roles in the surveillance against skin tumors in murine epidermis. Langerhans cells, which have been studied extensively, present tumor associated antigens to conventional αβ TCR-bearing CD4+ T-cells, which, in turn, will eliminate these tumors in vivo (5, 6). The second cell, DETC, may recognize and eliminate tumor cells on the basis of non-MHC-restricted cytotoxicity. Further studies will be required to determine how these resident cells interact and thus to unravel the processes by which cellular transformation is monitored and then deleted in skin.

ACKNOWLEDGMENTS

We wish to thank Dale Edelbaum for his technical assistance and Betty Janes for secretarial assistance.

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