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Apoptosis of immune cells in the tumor microenvironment and peripheral circulation of patients with cancer: implications for immunotherapy

Theresa L. Whiteside*

University of Pittsburgh Cancer Institute, 200 Lothrop Street, BST 211, W1041 Pittsburgh, PA 15213-2582, USA

Abstract

Rapid turnover of lymphocytes observed in patients with cancer appears to be driven by increased apoptosis of T lymphocytes or insufficient thymic output of recent thymic emigrants (RTE). Using multicolor flow cytometry and apoptosis assays, we found that $CD8^+CD95^+Annexin^+$ T cells are dying at a rate that is significantly higher in patients with cancer than in normal controls (NC). $CD8^+$ effector subsets of T cells were particularly vulnerable to apoptosis. Thymic excision circle (TREC) analysis of peripheral blood lymphocytes showed a decreased number of RTE in these patients. Together, the data suggest that a high rate of T-cell turnover might contribute to immunologic imbalance in patients with cancer and have unfavorable effects on immunotherapy, including therapeutic antitumor vaccines.

Keywords: Recent thymic emigrants; Apoptosis assays; TREC; $CD8^+$ T lymphocytes

1. Introduction

For many years, interactions between tumors and the host immune system have been a subject of much interest and controversy. Work in my own laboratory has long been directed toward demonstrating that tumors exert a deleterious effect on immune cells and that tumor progression is invariably linked to selective and pervasive impairment of immune cells. Mechanisms responsible for this impairment may vary depending on the nature of the tumor milieu, and newer data suggest that immunosuppressive effects of the tumor extend to the periphery, far beyond its microenvironment [1,2]. Studies of the mechanisms responsible for dysfunction of immune cells in cancer-bearing hosts are essential for the development of strategies to prevent or reverse tumor-induced effects and to protect immune cells in this hostile microenvironment.

This review focuses on one of the many mechanisms of tumor-mediated interference with the host immune system, namely, on apoptosis of T lymphocytes in the circulation of patients with cancer. Lymphocytes re-circulate between tissues and blood (Fig. 1), and their homeostasis is regulated via the thymic output of naïve lymphocytes and by death in the periphery of lymphocytes that have completed their functions or are no longer useful. It now appears that apoptosis of lymphocytes in the tumor microenvironment disturbs normal homeostasis, leads to rapid and perhaps selective lymphocyte turnover and to a loss of effector cells, which die and thus fail to control tumor growth.

2. Apoptosis of T cells at the tumor site

We have observed that tumor-infiltrating lymphocytes (TIL) in human solid tumors contain variable proportions of T cells with fragmented DNA (TUNEL+) [3]. Also, TIL obtained from human solid tumors are frequently dysfunctional, as measured in standard in vitro functional assays [4]. Signaling defects in the TCR as well as NFκB activation pathways in TIL have been described, which appear to be responsible for a loss of function in these cells [5–7]. In contrast, T cells infiltrating inflammatory, non-cancerous sites are largely able to respond normally to exogenous stimulants, and TUNEL+ T cells are uncommon in inflammatory infiltrates [3]. The presence of T cells with low or no expression of the ζ chain, a major signal-transducing molecule of the TCR complex, in the tumor was identified as a significant prognostic and survival biomarker in patients with oral carcinoma [8]. Patients with tumors infiltrated by TIL expressing normal levels of ζ were found to have a significantly better 5-year survival than those with presumably dysfunctional TIL devoid of ζ [8]. This finding emphasizes the importance of TIL function in the control of tumor growth. A loss of ζ and of TIL function may both be manifestations of apoptosis, as described earlier [9]. It is, therefore,

* Tel.: +1-412-624-0096; fax: +1-412-624-0264.
E-mail address: whitesidetl@msx.upmc.edu (T.L. Whiteside).

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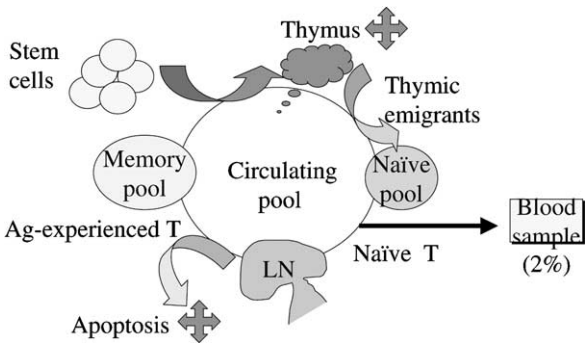

Fig. 1. A schematic for lymphocyte re-circulation, indicating the two sites (thymus and periphery) at which apoptosis of lymphocytes occurs. A blood sample obtained from the peripheral circulation contains at best 2% of all re-circulating lymphocytes.

not surprising that apoptosis of lymphocytes in the tumor is considered to be a poor prognostic factor [10]. In many recent studies, the presence and proportions of apoptotic TIL in the tumor was shown to correlate with FasL expression on the tumor cells in situ (reviewed in [11]). Considerable evidence has accumulated indicating that FasL expression on the tumor contributes to apoptosis of $CD95^+$ TIL [11].

3. Concomitant apoptosis of TIL and circulating T cells in patients with cancer

More recently, we have reported that increased proportions of TUNEL+$CD3^+$ T cells are detectable in the peripheral blood of patients with cancer relative to normal controls (NC) [1,12]. These newer data reporting signaling dysfunction and spontaneous apoptosis in circulating T cells of patients with cancer strongly suggest that immunosuppressive effects of the tumor extend beyond its microenvironment. If this hypothesis is correct, then a direct association should exist between dysfunction and apoptosis seen in TIL and in autologous PBL. This hypothesis was recently tested by us, combining in situ analysis of TIL and tumor cells with studies of paired PBL-T in 28 patients with oral carcinoma [13]. This study provided two new observations: (1) concomitant dysfunction of T cells in both TIL and autologous PBL-T was demonstrated in proliferation assays using anti-CD3 Ab; (2) low ζ chain expression correlated with increased proportions of apoptotic T cells in paired TIL and PBL (Table 1). In addition, we confirmed that low ζ expression, depressed immune function and apoptosis of T cells correlated with high levels of FasL expression on the tumor [13]. These immune defects occurred concomitantly in a subset of patients but not in all with advanced disease. It is possible, therefore, that the presence of a constellation of these particular defects may allow the identification of a subset of patients with particularly poor prognosis. Overall, the results of this study suggest that human carcinomas exert systemic immunosuppression which is, in part, mediated via the Fas/FasL pathway.

Table 1
Associations between apoptosis and ζ expression in PBL and TIL of patients with oral carcinoma

	Normal	Reduced	Total
ζ expression in PBL			
PBL apoptosis			
<5%	14	3	17
>5%	2	9	11
Total	16	12	28
ζ expression in TIL			
TIL apoptosis			
None	13	1	14
Present	5	8	13
Total	18	9	27

The data are numbers of patients. The ζ expression in PBL or TIL was measured by immunocytochemical staining and apoptosis by TUNEL staining. Significant associations were observed between reduced ζ expression and apoptosis in PBL ($P = 0.0015$) and in TIL ($P < 0.0019$). Reproduced with permission of the American Association for Cancer Research [13].

4. Apoptosis of T cells in the circulation of patients with cancer

The presence of spontaneous apoptosis of T cells in the peripheral circulation of patients with cancer has been so far described for melanoma, breast carcinoma, and head and neck cancer (HNC), including oral carcinoma [1,14,15]. In the circulation of patients with metastatic melanoma, T cells which undergo apoptosis are $CD3^+CD95^+$, and the proportion of such cells significantly ($P < 0.004$) exceeds that in the circulation of NC [1]. These $CD95^+$ T cells are sensitive to apoptosis, preferentially bind Annexin and show elevated levels of caspase-3 activity as well as decreased expression of the TCR-associated ζ chain [1,12,16]. In some patients with cancer, as many as 95% of circulating T cells are $CD95^+$. We evaluated a cohort of 37 patients with HNC as well as 35 age-matched NC for expression of apoptosis markers (Annexin V-binding, caspase-3 activation), TCR-associated ζ chain and the death receptor CD95 (Fas) in circulating $CD3^+$ T cells by multicolor flow cytometry [17]. The results indicated that in patients with HNC, 74% ± 15% (mean ± S.D.) of $CD3^+$ T cells were Fas^+ cells compared to 52% ± 13% in NC ($P < 0.0001$; Fig. 2). Thus, Fas^+ T cells represent the major population of circulating lymphocytes in these patients. Furthermore, 29% ± 16% of the Fas^+CD3^+ T cells bound Annexin V in patients and only 14% ± 7% in NC ($P < 0.0001$). In patients with HNC, $CD95^+CD3^+$ cells preferentially apoptosed and showed a loss of ζ chain expression. The most important finding, however, documented that proportions of $CD8^+Anx^+$ T cells were significantly elevated in patients with cancer relative to controls and that largely $CD8^+$, and not $CD4^+$, T cells bound

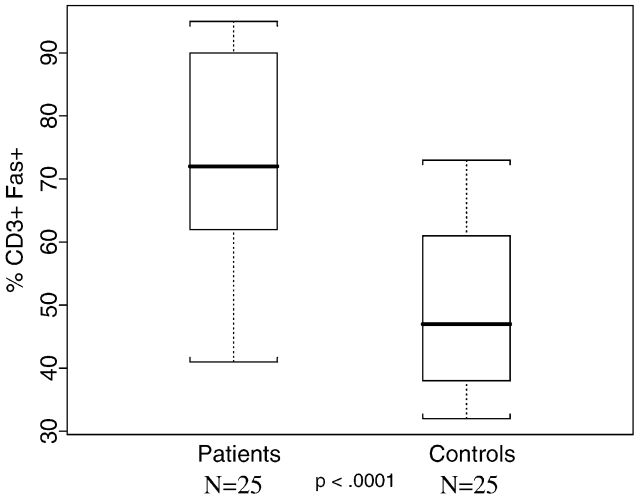

Fig. 2. A box plot comparing the proportions of $CD3^+Fas^+$ cells in the peripheral circulation of patients with HNC ($n = 37$) and normal controls ($n = 37$). The black bar indicates the median; the box shows the interquartile (25–75%) range; the whiskers extend to 1.5 times the interquartile range.

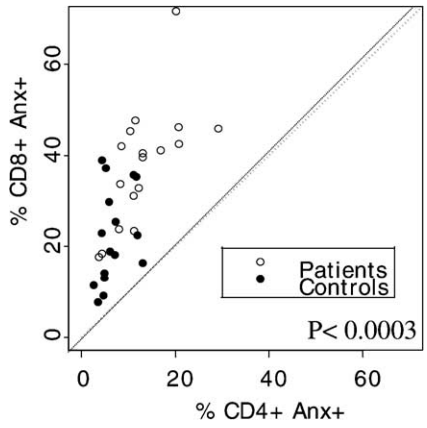

Fig. 3. $CD8^+$ T cells preferentially bind Annexin V in both patients with HNC ($n = 17$) and normal controls ($n = 17$). Note that the proportions of Annexin V^+CD8^+ T cells are significantly higher in patients than controls ($P < 0.0003$).

Annexin V ($P < 0.0003$) in patients or NC (Fig. 3). Thus, $CD8^+$ T cells were especially sensitive to apoptosis. The highest proportions of $Fas^+Annexin^+$ T lymphocytes were seen in a subset of patients with active disease (AD). These results indicated that Fas^+, activated $CD8^+$ T cells, which are enriched in the circulation of patients with HNC, are primed to die, leading to a rapid turnover of T lymphocytes and possibly contributing to a loss of antitumor effector cells.

5. Clinical significance of $CD8^+$ T-cell apoptosis in patients with cancer

The phenomenon of preferential demise of $CD8^+$ T cells in the circulation of cancer patients was studied further in another cohort of patients with HNC. The goal of this study was to establish associations between apoptosis of $CD8^+$ T cells and disease, its activity, stage and other clinicopathologic categories as well as behavioral characteristics of the patients. Because patients with HNC are generally older, and age could exert considerable influence on their immune system, we first evaluated effects of age on apoptosis of circulating $CD8^+$ T cells in 41 patients and 40 age-matched NC [18]. Both Annexin-binding and CD95 expression on $CD8^+$ T cells were found to be age dependent, increasing linearly with age in NC and in patients with AD. However, the percent of $CD8^+Anx^+CD95^+$ T cells was age independent in both NC and patients, and a trend toward lower proportions of these cells during aging was observed in patients with NED. Overall, HNC patients had significantly elevated percentages of $CD8^+Anx^+$ T cells relative to NC ($P < 0.0002$), although this characteristic did not discriminate between patients with AD versus NED: in 58% of AD versus 57% of NED patients, the percent of $CD8^+Anx^+$ T cells was >30. Significantly more $CD8^+$ than $CD4^+$ T cells bound Annexin, consistent with the hypothesis that $CD8^+$ T cells are particularly susceptible to apoptosis and are turning over more rapidly in patients with HNC than in NC.

We also examined the proportions of $CD8^+Anx^+$ T cells in HNC patients stratified according to disease stage, nodal involvement and tumor site. The only interesting association that emerged concerned a significant decrease of $CD8^+Anx^+$ T cells in N2 disease relative to N0 or N1. Although counterintuitive, this observation suggests that patients with poorer prognosis had fewer $CD8^+Anx^+$ T cells, perhaps because of rapid apoptosis of these cells in more advanced disease [18]. There was no association detected between smoking or alcohol abuse and proportions of $CD8^+Anx^+$ or $CD8^+Anx^+CD95^+$ T cells in the circulation of patients with HNC.

6. RTE and apoptosis in patients with cancer

Cumulatively, our results suggest that a high rate of $CD8^+$ T-cell apoptosis in the peripheral blood of patients with cancer may be associated with disease progression and poor prognosis. To compensate for the loss of peripheral T cells, their replacement via a thymus-dependent (i.e. output of T cells by the thymus) or thymus-independent pathway (i.e. peripheral expansion of pre-existing memory T cells) could occur. To quantify thymic output in patients with cancer, we used the thymic excision circle (TREC) PCR-based technology [19]. We studied TREC levels and the frequency naïve T-cell subsets in the circulation of 30 patients with HNC and 30 age-matched NC. The presence of recent thymic emigrants (RTE) in the periphery was first related to age, because of a well-known principle that thymic function deteriorates as individuals age. We found that HNC patients had fewer TRECs than NC ($P = 0.004$) at any age, an indication that either the patients were producing fewer RTE than NC or

that RTE were turning over more rapidly in patients than in NC. Using multicolor flow cytometry, we then determined the proportions of naïve $CD8^+CD45RO^-CD27^+$ and naïve $CD4^+CD45RO^-CD27^+$ T-cell subsets in patients and NC. The proportions of these naïve T cells were also significantly lower in patients with cancer relative to NC, and they decreased with age at a similar rate in both patients and NC. This again suggested that naïve T cells were either not generated as rapidly in cancer patients as in NC or that they were being more rapidly removed from the peripheral circulation. In any event, there was no difference in the number of TRECs in PBMC of HNC patients with AD versus those with NED. When we examined the data for associations between TRECs and T or N stage of the patients' tumors, a significant association emerged for the N stage and TRECs at $P < 0.04$. The patients who had involved lymph nodes and thus worse prognosis also had fewer TRECs in PBMC than patients without nodal involvement. In addition, patients with a carcinoma of the larynx and better prognosis had higher percentages of naïve $CD8^+$ T cells than those with oral carcinoma, which has worse prognosis. These significant associations between the TREC number and the stage and site of the tumor in a small cohort of patients with HNC provide evidence that immunologic function of the host, in this case, thymic output or lymphocyte turnover, is an important component of antitumor defense.

7. Selective apoptosis of antitumor effector cells

Next, we considered the possibility that the observed apoptosis of $CD8^+$ T cells in patients with cancer was not a global event but was directed at the subsets of T cells responsible for antitumor functions. Using multicolor flow cytometry, we evaluated two subsets of circulating T cells known to play an important role in antitumor defense: $CD8^+CD45RO^-CD27^-$ and $CD8^+CD28^-$ effector cells in groups of patients with HNC and in NC. We found that the frequency of $CD8^+CD45RO^-CD27^-$ was significantly increased in the circulation of all HNC patients regardless of the disease status ($P < 0.0003$). However, expression of the ζ chain in these cells was dramatically decreased, and their ability to respond to exogenous stimuli by IFN-γ expression was compromised [20]. Annexin V was found to bind to a higher proportion of this effector cell subset than to the other subsets within the naïve T-cell compartment. Thus, the expanded effector $CD8^+CD45RO^-CD27^-$ T cells appear to be dysfunctional and destined for apoptosis in the circulation of patients with HNC.

The second subset of effector cells we studied consisted of $CD8^+CD28^-$ T cells [21]. This subset was also significantly expanded in the circulation of patients with HNC and, surprisingly, it contained the highest proportion of $Annexin^+$ cells among the naïve or memory $CD8^+$ T-cell subsets. This implies that these dying effector cells were being rapidly replaced, i.e. had a rapid turnover rate in patients with HNC. The proportion of $CD8^+CD28^-$ effector cells normalized after surgery, with a decrease in their proportion and an increase in the proportion of naïve $CD8^+CD45RO^-CD28^+$ T cells. These data suggest that the presence of tumor has a profound effect on homeostasis of various lymphocyte subsets and that tumor removal might restore normal homeostatic regulation.

Taken together, our results indicate that the effector subpopulations of $CD8^+$ T cells are targeted for apoptosis in patients with cancer. These findings call attention to the possibility that a loss of effector function through such targeted apoptosis might severely compromise antitumor functions of the host immune system and contribute to tumor progression.

8. Tumor-specific effector cells and apoptosis

The next obvious question concerns the fate of tumor-specific effector cells in patients with cancer. To begin to answer this question, we studied Annexin-binding to Vβ-restricted and expanded clones of T cells in PBMC of patients with HNC. In addition, attempts are being made to use peptide-specific tetramers in combination with Annexin to determine whether tetramer-positive T cells show greater levels of apoptosis than tetramer-negative T cells. Our preliminary results suggest that not all $CD8^+$ T cells are equal targets for apoptosis. $CD8^+$ T cells with restricted specificity appear to be most vulnerable. For example, Fig. 4 shows that Annexin preferentially binds to Vβ13.2-restricted T cells and less so to Vβ13.2-negative T cells in the circulation of a patient with HNC. Similar results are emerging from the experiments, using wt. $p53_{149–156}$ tetramer for detection of Annexin-binding peptide-specific $CD8^+$ T cells. Preliminary results are consistent with the hypothesis that tumor antigen-specific $CD8^+$ T cells are at the greatest danger of apoptosis in the tumor microenvironment.

9. Implications of $CD8^+$ T-cell apoptosis for cancer immunotherapy

Our studies have identified a distinct mechanism of lymphocyte death and rapid turnover as yet another item on the growing list of immune deviations present in patients with cancer. These patients experience increased turnover of immune cells driven either by excessive apoptosis of circulating T cells or by a limited thymic output of naïve $CD8^+$ T cell or both. Newer technologies of multicolor flow cytometry, TREC and apoptosis assays facilitated the discovery and confirmed the importance of this phenomenon in cancer biology. The recognition of rapid lymphocyte turnover in cancer patients is important for the following reasons: (1) selective apoptosis of circulating $CD8^+$ T cells is likely to deplete the effector pool, including antitumor effector cells in these patients; (2) persistent and chronic efforts to replace

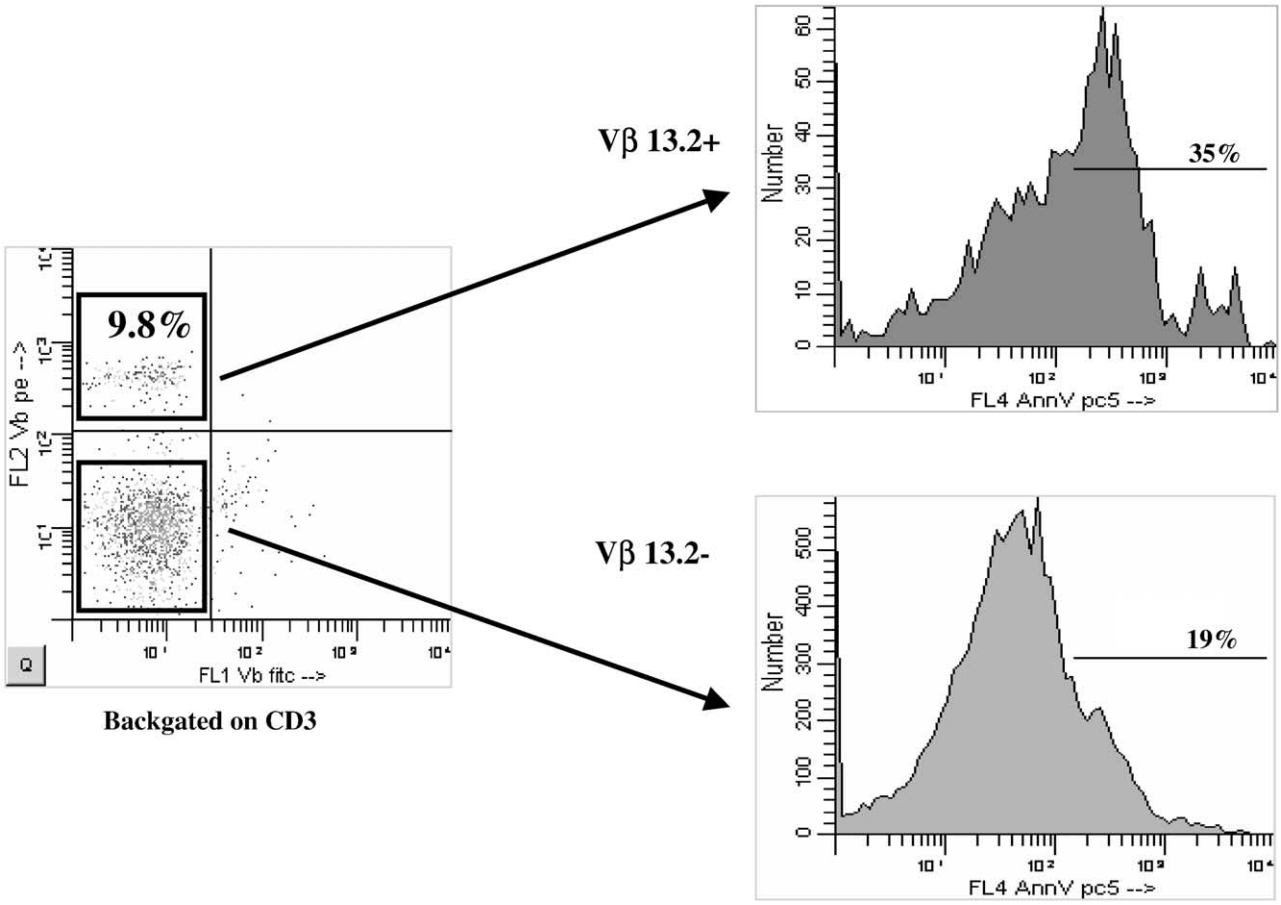

Fig. 4. A subset of Vβ-restricted (13.2$^+$) CD3$^+$ cells in the peripheral circulation of a patient with HNC contains a higher proportion of Annexin V-binding T cells than all the Vβ13.2$^-$ T cells.

the lost CD8$^+$ T cells from the bone-marrow stores increases stress on the aging immune system; (3) chronic depletion of CD8$^+$ T cells may result in the dysregulation of immune responses and a shift toward the Th2 phenotype; (4) a relative paucity of CD8$^+$ T cells, or perhaps distinct subsets of CD8$^+$ T cells introduces immune imbalance, with a possible dominance of CD4$^+$ immunoregulatory T cells [22], and leads to immunosuppression in patients with cancer.

From the immunotherapy perspective, it is now possible to perceive that a rapid loss of effector cells might foil the attempts at strengthening antitumor responses. Cancer vaccines may be especially negatively affected. Because of the ongoing death of CD8$^+$ T cells in cancer, therapeutic anticancer vaccines may not be the best remedy for immune dysfunction associated with cancer. Attempts to increase the frequency of CD8$^+$ tumor-specific effector cells might not be successful in the environment favoring apoptosis and rapid lymphocyte turnover. Future immunotherapeutic strategies have to consider means for restoration of normal lymphocyte turnover or for protection of CD8$^+$ T cells from apoptosis. In this context, cytokines might offer suitable therapeutic opportunities through preventing T-cell demise and sustaining antitumor functions of tumor-specific T cells. However, any attempts at the correction of this process have to be preceded by a better understanding of the mechanisms driving the rapid turnover of lymphocytes in patients with cancer.

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