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REVIEW

Theresa L. Whiteside

Signaling defects in T lymphocytes of patients with malignancy

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Abstract In patients with cancer, alterations in the expression of T-cell receptor-associated molecules in tumor-infiltrating lymphocytes (TIL) as well as in circulating lymphocytes have been reported. By quantitative flow cytometry analysis, decreased or absent expression of the ζ chain in $CD4^+$ or $CD8^+$ T cells as well as in natural killer (NK) cells was demonstrated in patients with malignancies. Changes in the expression of ζ are biologically significant, because the absence or low expression of this signaling molecule in TIL of patients with stage III or IV head and neck cancer predicts a significantly shorter 5-year survival than that of patients with normal ζ expression in TIL. Preliminary evidence indicates that expression of ζ in TIL may not only influence survival but also predicts a favorable response to biologic therapies. Patients with cancer also show significantly greater spontaneous ex vivo apoptosis in peripheral blood mononuclear cells (PBMC) compared to normal controls, as measured by a terminal deoxynucleotide transferase-mediated dUTP nick end labeling (TUNEL) assay. While no correlation could be established between the proportions of cells with low ζ chain expression and those that spontaneously apoptose ex vivo, the ζ chain has been shown to be cleaved by caspases in T cells coincubated with tumor cells or with T cells exposed to CH-11 antibody, which induces apoptosis upon crosslinking Fas on the cell surface. The results suggest that low/absent ζ chain expression and lymphocyte apoptosis may be manifestations of negative effects of the tumor on the host immune system.

Key words Tumor-infiltrating lymphocytes · ζ chain expression · Malignancy · Lymphocyte apoptosis

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T.L. Whiteside
Department of Pathology and Otolaryngology,
University of Pittsburgh School of Medicine,
Pittsburgh, PA 15213, USA

T.L. Whiteside (✉)
University of Pittsburgh Cancer Institute,
211 Lothrop Street, W1041 BST,
Pittsburgh, PA 15213, USA
e-mail: whitesidetl@msx.upmc.edu
Tel.: +1-412-6240096; Fax: +1-412-6240264

Introduction

Human solid tumors are often infiltrated by substantial numbers of mononuclear cells, including T lymphocytes [1, 2]. For many years now, "tumor-infiltrating lymphocytes" (TIL) have been the subject of great interest because of early reports of a possible association between the presence of TIL and a favorable prognosis [3, 4]. While it has not been possible to fully confirm the association in more recent studies [5], it has been clearly demonstrated that TIL are a source of tumor-specific T lymphocytes which, upon culture in the presence of IL-2, demonstrate antitumor activity [1, 6, 7]. Furthermore, T cell lines derived from TIL obtained from patients with melanoma and expanded in the presence of IL-2 have been successfully used by Rosenberg and colleagues to identify and characterize several melanoma-associated antigens [8, 9].

At the same time, evidence from a number of laboratories indicates that *fresh* human TIL isolated from melanoma or other human tumors are functionally impaired and do not respond fully in proliferation or cytotoxicity assays [10, 11]. When tested for phenotypic characteristics by flow cytometry, fresh TIL are mixtures of $CD8^+$ and $CD4^+$ T cells, which express activation markers and are mostly $CD45RO^+$ [1, 12]. Yet, unlike normal T lymphocytes, these memory T cells are not responsive or only partly responsive to mitogens or antigens in ex vivo assays and are not capable of mediating cytotoxicity against autologous tumor targets [1, 11, 13].

Fresh TIL as well as tumor-associated lymphocytes (TAL) obtained from, e.g. ascitic fluids of patients with

ovarian carcinoma, have been shown to express the type 2 cytokine profile and to lack expression or express low levels of IL-2 or IFN-γ [14, 15]. At the same time, T cells separated from inflammatory lesions of patients without cancer and from the peripheral blood of healthy individuals consistently show normal functional responses to mitogens or antigens [14, 16]. These observations suggest that the tumor microenvironment has negative effects on immune cells. The failure of the host to eliminate tumor may, therefore, be a consequence of the impaired functional state of T cells among TIL or TAL, including the inability of these cells to signal normally upon T cell receptor (TcR) engagement [17].

The purpose of this brief review is to summarize current evidence for the possibility that tumor induces abnormalities in the signaling pathways mediated via TcR in TIL and TAL as well as peripheral blood T cells (PBL-T) of patients with cancer. In addition, the mechanism(s) that might be responsible for TcR-associated signaling defects in these T cells are discussed. The overall objective is to emphasize the likely possibility that human tumors can and do induce immune cell dysfunction, and that the understanding of the mechanisms involved in subversion of antitumor responses is important for the design of future clinical trials with biologic agents, which are expected to upregulate host antitumor responses.

Decreased expression of the TcR-associated ζ chain in T cells of patients with cancer

The TcR-associated ζ chain is responsible for transduction of signals delivered via the receptors and, therefore, its expression is important for activation of T cells [18]. Recent studies have demonstrated that abnormalities in expression of ζ and other signal transduction molecules associated with TcR are present in TIL or TAL obtained from patients with cancer [17, 19–23]. Initially, these abnormalities were reported in mice bearing established tumors [24, 25]. In addition to depressed Ca^{++} mobilization and other functional defects, splenocytes of these mice demonstrated low expression levels of the ζ chain as well as $p56^{lck}$ and $p59^{fyn}$ [25]. Subsequently, using antibodies to signaling molecules in Western blots, the same abnormalities were found in TIL and TAL of patients with colon, renal, prostate, cervical or ovarian carcinomas [19, 21, 22, 23, 26], as well as melanoma [27, 28] and head and neck cancer (HNC) [17]. Expression of these proteins is either decreased or absent in T cells isolated from tumor or from the peripheral blood of patients with cancer in comparison to those obtained from healthy donors. In addition to decreased expression of ζ chain, $p56^{lck}$ or $p59^{fyn}$, various other functional defects have been observed in TIL [14, 17]. Western blots are neither sensitive nor quantitative, however, and the variability in expression of signaling molecules, ranging broadly from their absence in T cells of some patients to normal levels of expression in T cells of others [23], creates concerns about the consistency of these results. A survey of tumor-bearing mouse strains has failed to detect decreases in expression of the ζ chain in Western blots [29].

The possibility has been suggested that the signaling defects observed in TIL or PBL of patients with cancer are artifacts induced by granulocyte- or monocyte-derived proteases released during tissue or blood processing [30]. However, the absence or low expression of ζ in T lymphocytes infiltrating tumors observed by immunocytochemistry in cryosections of human tumor biopsies of many different tumor types indicates that these abnormalities exist in situ, and thus cannot result from tissue processing [14, 17, 28]. In individual patients, absent or low ζ or $p56^{lck}$ expression detected in TIL or TAL often does not correlate with normal or near-normal levels of ζ expression in PBL-T, further confusing the interpretation of the results. In aggregate, low ζ expression seems to be consistently detectable in lymphocytes derived from the tumor microenvironment but is less frequently observed in patients' PBL-T. These findings could be interpreted as evidence that ζ abnormalities are induced in T cells by the tumor and thus are less pronounced in patients' PBL-T than at the tumor site.

Quantitative flow cytometry applied to the TcR and ζ chain analysis in T cells of patients with cancer has greatly facilitated the interpretation of these somewhat perplexing findings. It soon became apparent that by scoring the mean fluorescent intensity (MFI) of T cells stained for expression of the TcR-associated ζ or ε chains using specific antibodies, significant differences could be detected between T cells of patients with cancer and those of normal controls. As shown in Table 1, expression of both chains is decreased in T cells obtained from cancer patients relative to PBL-T of normal donors. We have performed this type of analysis with TIL, TAL and PBL-T obtained from patients with metastatic melanoma, ovarian carcinoma, and HNC and have consistently observed lower ζ and ε expression in T cells of patients with cancer than normal controls. In our experience, ζ expression is usually lower than that of

Table 1 Expression of cytoplasmic CD3-ε and CD3-ζ in lymphocytes obtained from patients with squamous cell carcinoma of the head and neck. Flow cytometry was performed using permeabilized lymphocytes from lymph nodes or blood of patients or blood of healthy controls and stained with anti-CD3 or anti-ζ monoclonal antibodies. The values are mean fluorescence intensity (MFI) ± SD

Source of lymphocytes	CD3-ε	CD3-ζ
Tumor-involved lymph nodes ($n = 12$)	1289 ± 58	133 ± 13
Patients' peripheral blood ($n = 6$)	908 ± 71	341 ± 28
Normal peripheral blood ($n = 9$)	2014 ± 157*	513 ± 61*

*$P < 0.01$ vs patients' samples

CD-3ε. The MFI for ζ is always lower in TIL or lymph node lymphocytes from tumor-involved lymph nodes than in the paired PBL-T. Our recent studies indicate that even in patients with HNC who have no evident disease (NED) as a result of previous surgery, PBL-T have significantly lower ζ expression than control T cells [31]. Taken together, these findings confirm that the ζ chain responsible for TcR signaling is underexpressed or absent in T cells obtained from cancer patients. As the message for ζ is detectable and appears to have a normal level of expression in these T cells [17], we conclude that post-translational modification(s) are responsible for the deficiency in ζ protein expression observed in patients with malignancy.

Evidence for tumor-induced degradation of the ζ chain

Our studies have indicated that decreased expression of the ζ chain in T cells obtained from patients with cancer is associated with signaling defects, as manifested by reduced or absent mobilization of Ca^{++} and by decreased tyrosine kinase activity upon TcR crosslinking by anti-CD3 antibodies in TIL compared with the same functions in normal PBL-T [17]. In addition, low ζ expression in TAL of patients with ovarian carcinoma is associated with an altered cytokine profile, reflecting a significant decrease in IL-2 or IFN-γ at the mRNA and protein levels in the T cells [14]. These findings as well as similar observations reported by others [32, 33] suggest that underexpression of ζ in tumor-associated T cells translates into signaling abnormalities.

To directly test the hypothesis that tumor induces signaling aberrations in T cells, we coincubated freshly isolated tumor cells (ovarian carcinoma) with normal allogeneic or autologous PBL-T for various periods of time. The aim was to determine whether defects in lymphocyte signaling or other lymphocyte functions are induced as a result of contact with tumor cells. We initially observed reduced expression of the ζ chain in T cells by Western blots and flow cytometry after 24 h of coculture [34]. Furthermore, in vitro-activated T cells were found to be especially susceptible to tumor-induced downregulation of ζ expression. Also, preincubation of T cells with a peptide aldehyde, *N*-acetyl-leu-leu-norleucinal, which is known to inhibit both lysosomal and proteasomal peptidase activity, has been shown to prevent degradation of the ζ chain in these lymphocytes (H. Rabinowich, unpublished data).

These results suggest that tumor cells induce activation of intracellular peptidases in T lymphocytes, and that this tumor-induced enzymatic degradation is responsible for decreased or absent expression of signal-transducing molecules, including the ζ chain in activated T cells. This interpretation fits well with the presence of normal levels of mRNA for the ζ chain observed in TIL and TAL [17, 34] and with the possibility that post-translational modifications in signal-transducing proteins are present in these cells as well as in normal T cells incubated in the presence of tumor cells [34]. Therefore, we began to suspect that coincubation of lymphocytes with tumor might lead to the initiation of an apoptotic cascade in activated T cells. Indeed, using TUNEL or JAM assays, which measure DNA fragmentation, we have confirmed that DNA breaks are detectable in a substantial proportion of lymphocytes coincubated with tumor cells [17, 34]. As caspase activation appears to be the key event in the initiation and execution of apoptosis, we next proceeded to demonstrate that the synthetic peptides Z-VAD-FMK and Z-DEVD-FMK, which are pan-caspase inhibitors, cause a nearly complete inhibition of apoptosis in T lymphocytes coincubated with human tumor cells [34, 35].

Since our ex vivo coincubation experiments suggested that tumor is capable of inducing both signaling defects and apoptosis in activated T lymphocytes, the next goal was to search for DNA fragmentation in TIL at the tumor site. TUNEL assays were performed in human tumor biopsies and tumor-involved lymph nodes obtained from patients with ovarian carcinoma or HNC [17, 34]. As expected, considerable numbers of TIL were found to be $TUNEL^+$ (apoptotic) in sections of tumor biopsies [17, 34]. Control normal tissues or tumor-uninvolved tissues obtained from patients with cancer contained no or only a low number of apoptotic lymphocytes. Furthermore, by performing TUNEL assays in conjunction with immunostaining for CD3 in tumor biopsies, we were able to confirm that DNA fragmentation occurs in TIL and not in tumor cells [17, 34].

The question remained, however, about the possible relationship between the signaling defects and apoptosis in TIL. T cells coincubated with tumor cells were examined, using two-color flow cytometry. In this type of experiment, it was possible to confirm that both degradation of ζ and DNA fragmentation occurs in the same T cells upon their coincubation with tumor cells. Similar findings have been obtained by immunostaining of tumor biopsies, in which apoptotic $TUNEL^+$ T cells in situ were seen to have decreased ζ, while ζ^+ T cells were not apoptotic (Fig. 1A). In another series of experiments, we have deliberately induced apoptosis in normal activated PBMC, using anti-Fas antibody (Ab) (CH-11). These PBMC were stained for CD3, ζ and TUNEL and examined by flow cytometry. As shown in Fig. 1B, those PBL-T that were $TUNEL^-$ showed strong expression of intracytoplasmic ζ (blue peak), while $TUNEL^+$ (apoptotic) T cells contained little ζ (red peak). Thus, both in situ and ex vivo experiments suggest that ζ degradation accompanies apoptosis which occurs spontaneously in the tumor or is induced via the Fas pathway, respectively.

More recently, a computer-assisted analysis of the amino acid sequence of the ζ chain has revealed the presence of sites sensitive to cleavage by caspases, and we have recently demonstrated that the ζ chain is a substrate for proteolysis mediated by intracellular caspases [36]. These and other experiments in our labora-

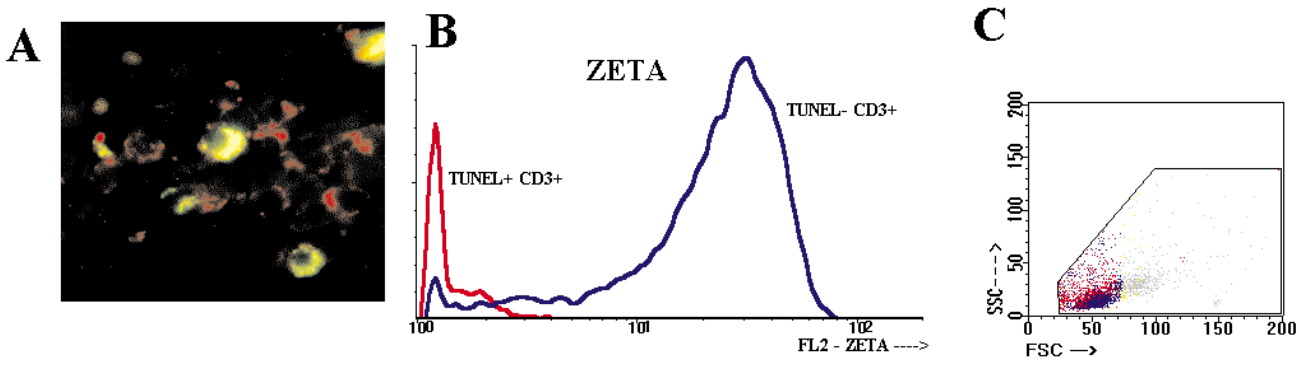

Fig. 1 **A** A section of paraffin-embedded oral carcinoma was immunostained for ζ and then TUNEL reagents were used to detect DNA fragmentation in situ. Among many red (ζ^+) cells, apoptotic (*green*) cells are seen with residual ζ (*red dots*) in the membrane. Apoptotic bodies (*green*/*red*) are also present. **B** Three-color flow cytometry of normal activated PBL induced to apoptose by incubation with anti-Fas (CH-11) Ab. PBL were first activated in the presence of phorbol myristate acetate (2 ng/ml) and ionomycin (1 μ*M*) for 1 day and then incubated with CH-11 Ab for 6 h. Cells were harvested, stained with anti-CD3 Ab conjugated with Per-CP, permeabilized and then stained for flow cytometry with anti-ζ Ab conjugated with PE and with TUNEL-FITC reagents. Multicolor flow cytometry allowed the identification of two distinct $CD3^+$ cell subsets among these PBL: $CD3^+$/$TUNEL^+$ and $CD3^+$/$TUNEL^-$. ζ chain expression was decreased in those T cells which were $TUNEL^+$ (*left peak*). **C** Forward light scatter (FSC) for the stained PBL is shown. Note that apoptotic cells (*red*) have a lower FSC signal than nonapoptotic cells (*blue*)

tory indicate that tumor-induced activation of caspases seems to be responsible for low or absent expression of ζ and perhaps other signal-mediating proteins in T lymphocytes present in the tumor microenvironment or those coincubated with tumor ex vivo.

While coincubation of lymphocytes with tumor can induce both caspase activation and ζ degradation in T cells, and most apoptotic lymphocytes express no or only low levels of ζ, the evidence linking apoptosis with ζ downregulation remains circumstantial. Other mechanisms, including free oxygen radical generation in the tumor microenvironment, have been proposed to account for decreased ζ expression in cancer [37]. Since, however, induction of apoptosis is accompanied by free oxygen radical release, these two mechanisms of ζ degradation are not incompatible. The nature of the signal(s) responsible for the initiation of ζ degradation and its rapid ubiquitination in tumor-associated T cells is not yet known. Furthermore, reports of low ζ expression in chronic infections, including leprosy and AIDS, have appeared [38, 39]. These findings indicate that the phenomenon of rapid ζ degradation is a general one, not restricted to the tumor milieu, or perhaps that T cells are susceptible to a variety of disease-associated stress signals, which can interfere with immune cell functions and even survival. Further studies of signaling defects in TAL are necessary to better define the circumstances and molecular signals that lead to the ζ chain downregulation.

Biologic significance of the ζ chain underexpression in TIL

The finding of decreased ζ expression in T cells present within human tumor biopsies as well as in T lymphocytes coincubated with tumor cells ex vivo indicates that apoptosis induced by tumor might be responsible for degradation of signal-transducing molecules in immune cells [14, 17, 34, 35]. The biological consequences of the decreased ζ chain expression in immune cells could be profound, because of the key role of this protein in TcR signaling. To determine whether degradation of ζ in TIL has biologic significance, we have recently performed a retrospective study of tumor biopsies obtained from 138 patients with oral carcinoma over a period of several years [40]. Semiquantitative analysis of ζ expression in TIL in paraffin-embedded and immunostained section of these tumors combined with the pathologic and survival data available for these patients established that absent or low expression of ζ in TIL was seen in 32% of tumors and was significantly associated with advanced disease (stages III and IV), more nodal involvement and the presence of nodal metastases. Thus, in oral carcinoma patients with advanced disease, normal ζ expression predicts significantly better survival independently of other well-established prognostic parameters [40]. In our study, expression of ζ in TIL was identified as a prognostic factor in oral carcinoma. These findings are the first to suggest a link between ζ expression in T cells accumulating at the tumor site with disease progression and patient survival [40].

Downregulation of ζ in PBL-T obtained from patients with cancer

Decreased expression of ζ in PBL-T obtained from patients with cancer has also been reported from many laboratories [17, 19, 21, 22, 23, 27, 31]. By flow cytometry, MFI was found to be lower than the normal mean in 11/12 patients with metastatic melanoma in our laboratory, and in 6/12 patients, the percentages of T cells with low ζ expression (defined as less than the

mean MFI−(2 × SD) of normal expression) ranged from 50% to 100% (unpublished data). Thus, aberrations in expression of the ζ protein are not detected in all patients with cancer, and when they are detected, not all circulating T cells are affected. Therefore, when small numbers of patients are studied, these defects might be missed altogether (see, for example, reference 41). Our observations are in agreement with the results reported by Zea and colleagues, who observed a marked decrease in expression of the ζ chain in PBL-T in 19 of 44 patients (43%) with metastatic melanoma [27]. In this study, decreases in several tyrosine kinases were also found in PBL-T of 57% of the patients tested as well as lower IL-2 and IFN-γ production relative to that in healthy individuals or in patients with melanoma whose ζ expression was unimpaired [27]. Importantly, overall survival of melanoma patients with low ζ expression in PBL-T was significantly shorter than that of patients with normal ζ expression [27]. It appears that similar to the findings in TIL described above, aberrant ζ expression in PBL-T has biologic significance.

In patients with HNC, who had NED as a result of previous therapies, the expression of the ζ chain was found to be significantly decreased ($P < 0.0001$) in $CD8^+$ and $CD4^+$ cells as well as in $CD3^-CD56^+CD16^+$ natural killer (NK) cells of the peripheral blood relative to the expression in cells of normal age- or sex-matched controls tested at the same time as the patients [31]. Among these 17 HNC patients, those who had developed new primary tumors or recurrences since surgery for the primary disease or those with more aggressive types of carcinoma had significantly lower ζ expression in PBL-T than patients without recurrences or with a better prognostic profile [31]. These observations suggest that low ζ expression in immune cells in the peripheral blood may be a marker for advanced or aggressive disease.

While the mechanism(s) responsible for the observed downregulation of expression of TcR-associated signaling proteins in PBL-T remains unknown, there are indications that it may be related not only to the presence but also to the aggressiveness of the malignancy. Preliminary results in our laboratory indicate that both in HNC and in metastatic melanoma, the observed ζ chain aberrations are related to apoptosis, which might be tumor-induced, and which leads to DNA fragmentation in lymphocytes at the tumor site [42] as well as in a subset of activated circulating T or NK cells. We find that downregulation of ζ expression in PBL-T obtained from patients with HNC or melanoma is accompanied by spontaneous apoptosis detectable in a substantial proportion of T cells ex vivo, while no such apoptosis is seen in normal PBL-T handled in parallel with the patients' cells (unpublished data).

The preliminary evidence linking low ζ expression in PBL-T to premalignant lesions in women with cervical intraepithelial neoplasia (CIN) was obtained by Kiessling and colleagues [19]. In a study including 22 patients with cervical carcinoma, 23 with CIN and 21 normal controls, they observed significant decreases in the expression of ζ in PBL-T of both patient groups relative to that in controls. Furthermore, CD3ζ chain expression correlated with a significantly decreased ability of the patients' PBLs to produce tumor necrosis factor (TNF) in response to anti-CD3 crosslinking [19]. These findings suggest that both the downregulation of ζ expression and cytokine production by PBL-T become impaired early in the course of cancer progression and, because both aberrations appear to be greater in T cells obtained from patients with carcinoma than in those from patients with CIN, may reflect the extent of disease progression. The implication of these findings is that signaling abnormalities in T cells and the functions of these cells become more compromised as the disease progresses and that ζ might be a biomarker of this progression.

Restoration of ζ expression and protection of T cell signaling

Not all T lymphocytes isolated from the peripheral blood, body fluids or tumor tissues of patients with cancer are dysfunctional, and restoration of proliferative and cytolytic functions have been reported to occur in the presence of exogenous cytokines, especially IL-2, after the cells are removed from the tumor milieu [28, 43]. The best examples of this phenomenon are instances of selection and culture in the presence of IL-2 of long-term T cell lines in patients with melanoma or renal cell carcinoma [8, 9, 44]. These observations mean that in the population of immune cells derived from the tumor-bearing host, some T cells are not affected and perhaps could be rescued from tumor-induced downregulation of signaling molecules.

The data presented above suggest that the proportion of T cells with impaired signaling vs. those with normal ζ expression may be quite low in patients with a small tumor burden and good prognosis. In contrast, the majority of PBL-T are likely to have low ζ expression in patients with advanced or aggressive cancer. Normalization in the level of ζ expression in melanoma TIL cultured in the presence of 1000 IU of IL-2 for 48 h has been observed in our laboratory [28]. It is difficult to conclude at present that such normalization represents a reversal of tumor-induced proteolytic degradation of signaling molecules. More likely, it reflects selection in culture of those T cells that have not yet entered the apoptotic pathway and thus are able to respond to exogenous IL-2. Preliminary in vitro experiments suggest that preincubation of lymphocytes in the presence of cytokines, including IL-2, IL-12 or IL-7, in part protects these cells from tumor-induced apoptosis during the subsequent coincubation with tumor (unpublished data). Although the mechanism of such protection is currently unknown, it is possible that cytokines induce increased expression of inhibitors of apoptosis such as

the Bcl-2 family members or FLIP and IAPs, which target different caspases [45, 46].

Only limited data are available for the in vivo effects of IL-2 on expression of the ζ chain in T cells obtained from patients with cancer. In a clinical trial performed in patients with metastatic melanoma at our institution, normalization of ζ expression was observed in a complete responder to a high-dose IL-2 therapy but not in several other patients who did not respond clinically [28]. On the other hand, Farace and colleagues, who studied ζ expression in PBL-T obtained from patients with advanced cancers, including renal cell carcinoma, hepatic colorectal metastases, HNC and others, reported no evidence for reversal of low ζ expression after IL-2 therapy [47]. In patients with ovarian carcinoma treated with intraperitoneal IL-2 at our institution several years ago, PBL-T collected prior to therapy were available for assays of ζ expression by flow cytometry. We determined that responders to this therapy had normal pretherapy expression of ζ in T cells, while significantly depressed ζ levels were detected in nonresponders (Kuss and Whiteside, unpublished data). Thus, in this set of patients, normal expression of this signaling protein was predictive of a favorable clinical response to cytokine biotherapy.

Conclusions

Human tumors or tumor cell lines have been shown to induce defects in the expression and function of signaling molecules associated with the TcR-associated signaling pathway in T lymphocytes. The expression of the best studied of these molecules, the ζ chain, has been found to be absent or decreased in T cells of the tumor site as well as of the peripheral circulation from patients with cancer, particularly those with a high tumor burden, but also in T cells from those with NED. In patients with stage III or IV oral carcinoma, the absence or low expression of ζ in TIL independently predicts a significantly shorter 5-year survival than that of patients with normal ζ expression in TIL. In patients with ovarian carcinoma, the presence of normal ζ expression in PBL-T prior to IL-2 therapy is predictive of a clinical response to IL-2. Therefore, normal expression of this signaling molecule is biologically important and may not only influence survival but also predict a favorable response to biologic therapies.

The mechanisms involved in tumor-induced down-regulation of ζ expression in T cells are under intense scrutiny, but preliminary evidence indicates that direct contact with tumor or tumor-derived factors might initiate the apoptotic pathway in T lymphocytes. Intracellular caspases in activated T cells participate in degradation of ζ and perhaps other signaling molecules. In order to prevent signaling defects and apoptosis of T cells in the tumor-bearing hosts, it will be necessary to unravel the mechanism(s) involved in tumor-effector T cell interactions. These studies are of critical importance to future immunotherapy of cancer. Currently, it appears that the protection of antitumor effector cells from tumor-induced dysfunction and death might have to become the primary objective of cancer immunotherapy.

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