Decreased ζ Chain Expression and Apoptosis in CD3+ Peripheral Blood T Lymphocytes of Patients with Melanoma

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Abstract

Expression of T-cell receptor- or Fcγ receptor III-associated signal-transducing ζ chain is important for the functional integrity of immune cells. We found that significantly higher proportions of circulating CD3+ T cells as well as natural killer cells had low or absent expression of the ζ chain in patients with advanced melanoma than in normal donors (P < 0.0005). Decreased ζ expression was always observed in a small subset of circulating CD3+ T cells that were in the process of apoptosis, i.e., bound Annexin V or were terminal deoxynucleotidyl transferase-mediated nick end labeling positive. Up to 80% of T cells in the peripheral blood of patients with melanoma were Fas+, with the mean percentage of Fas+CD3+ cells significantly higher in patients (P < 0.004) than normal controls. These Fas+CD3+ T cells were found to preferentially undergo apoptosis. Annexin V binding, the loss of Fas expression from the cell surface as well as ζ down-regulation, which are associated with early apoptosis, were detected in a proportion of circulating Fas+CD3+. In Jurkat cells incubated with agonistic anti-Fas antibody (CH-11), a rapid loss of Fas expression from the cell surface coincided with Annexin V binding and preceded the loss of ζ chain during early apoptosis. In a subset of Jurkat cells coinfected with human melanoma cells, Annexin V binding and ζ degradation as well as DNA fragmentation were observed, indicating that the tumor-induced T-cell death. Triggering of death receptors expressed on activated T lymphocytes was accompanied by the loss of ζ expression. On the other hand, soluble factors secreted by melanoma cells induced down-regulation but no apoptosis in activated normal T cells. In the circulation of patients with melanoma, apoptosis of immune effector cells may be related to the state of chronic activation, resulting in the up-regulation of death receptors and increased susceptibility to apoptosis.

Introduction

The presence of signaling defects in the TCR3 pathway of peripheral blood T cells in patients with melanoma has been reported by several investigators (1–3). These defects, which included decreased expression of the ζ chain and the downstream proteins, p56lck or ZAP-70, were reported to be associated with poor survival in patients with melanoma (1). In contrast, ex vivo incubation of T cells in the presence of IL-2 appeared to correct the signaling defects and to restore functions in these T cells (2). Also, the ζ chain expression was found to normalize in T cells of patients who were responders to therapy with IL-2 (2).

Experiments performed in situ, using biopsies of melanoma lesions (2) and biopsies of other solid tumors, e.g., oral carcinomas (4) or ovarian carcinomas (5), indicated that T lymphocytes infiltrating tumors often had low or undetectable expression of the TCR-associated ζ chain. Moreover, decreased expression of this signaling molecule was found to be biologically significant, because patients with advanced oral carcinoma (stages III and IV) whose tumor-infiltrating lymphocytes were defective in expression of ζ had significantly shorter 5-year survival than those with normal expression of ζ in tumor-infiltrating lymphocytes (4).

More recent observations have indicated that a substantial proportion of CD3+ T cells in the peripheral circulation of patients with melanoma undergo spontaneous apoptosis and become TUNEL+ upon a short period of ex vivo incubation in medium at 37°C (6). Because these T cells express activation markers, it has been assumed that activation-induced cell death is responsible for their demise. Because the two phenomena, spontaneous apoptosis and four-chain down-regulation, were also observed in the peripheral blood T cells of patients with melanoma, a question arose as to whether they were related. In other words, the possibility was considered that the process of the ζ chain down-regulation was a result of the apoptotic pathway induced in activated T cells by the tumor or tumor-related soluble factors not related to TCR triggering, such as membrane-bound or soluble FasL. We have shown earlier that the ζ chain contains motifs recognized by caspase-3 and caspase-7 and thus can serve as a substrate for these caspases (7). The presence on the surface of human tumor cells of apoptosis-

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3 The abbreviations used are: TCR, T-cell receptor; IL, interleukin; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; FasL, Fas ligand; TNF, tumor necrosis factor; NK, natural killer; Ab, antibody; PBMC, peripheral blood mononuclear cell; DPBS, Dulbecco's PBS; PE, phycoerythrin; FSC/SSC, forward angle scatter/side angle scatter; PBL, peripheral blood lymphocyte; MFI, mean fluorescence intensity.
inducing factors, such as membrane-associated FasL, TNF-related apoptosis-inducing ligand (TRAIL), or TNF-α, which are able to induce death in activated T lymphocytes, has been described by many investigators (8–10). On the other hand, the expression of FasL, or of mRNA for FasL in human melanomas has been a controversial issue (11–13). The controversy arose because of the paucity of FasL protein or mRNA expression in experiments with melanoma cell lines in some laboratories (12, 13).

In this paper, we report that down-regulation of ζ expression observed in Fas+ T or NK cells in the circulation of patients with advanced melanoma appears to be a result of Fas-mediated apoptosis. The process of ζ chain down-regulation could be induced during the direct tumor cell-T cell interactions, after cross-linking of the Fas receptor on activated T cells either by an agonistic Ab or by the tumor expressing FasL.

Materials and Methods

Patients and Controls. Twenty-two patients with melanoma, who were seen in the University of Pittsburgh Cancer Institute (UPCI) outpatient clinic between February 1998 and September 1999, were included in this study. The protocol for collection of patient samples for research studies was approved by the Institutional Review Board. The patients were randomly selected by their physicians, based on availability and willingness to participate in this study. All patients signed an informed consent. The patients included 9 females (ages 32–75 years.) and 13 males (ages 39–73 years). At the time they were entered in the study, all patients had stage III or IV active disease, except for 5 patients, who had no evidence of disease. All were being evaluated for entry in one of the UPCI protocols but were not treated at the time of blood donation for this study. Previous therapies that these patients received included various biological therapies (peptide-based vaccines, n = 9; cytokine or gene therapy, n = 4), chemotherapy, and/or radiotherapy (n = 7). Only two patients had not received any previous treatment.

Normal volunteers (n = 11) were initially recruited from among the laboratory personnel and included 5 males and 6 females. These controls were substantially younger than patients, and their median age was 33 years compared with 66 years for patients. All normal volunteers were in excellent health at the time of the study. In addition, we included a smaller group of 6 normal volunteers, who were age-matched with the patients (median age, 66 years) and consisted of 5 males and 1 female. In all experiments, patient and control specimens were processed and handled in the same way, and patient samples were always tested together with at least one laboratory control.

PBMCs. Venous blood was obtained from patients and controls (20 ml) in the morning and collected in heparinized tubes. Blood samples were hand-carried to the laboratory and immediately processed by Ficoll-Hypaque gradient centrifugation. PBMCs were recovered from the gradient interface, washed in DPBS, counted in a trypan blue dye, and promptly used for experiments.

To determine the proportion of PBMCs with evidence of apoptosis, TUNEL positivity for DNA fragmentation and Annexin V binding for surface membrane changes were evaluated on aliquots (from 1 × 10⁶ to 2 × 10⁶ cells/assay) of the patients’ or control cells immediately after their isolation. These assays were performed as described previously (6). In some cases, spontaneous apoptosis was determined after 24 h incubation of PBMCs in medium as described by us earlier (6). Measurements of ζ expression were performed by flow cytometry on freshly harvested lymphocytes (14).

Staining for Flow Cytometry. PBMCs (3.5 × 10⁶) were washed in DPBS, divided into 5 × 10⁵ cell aliquots, and individually incubated in the presence of the following PE-labeled monoclonal Abs: anti-CD3, anti-CD4, anti-CD8, anti-CD14, anti-CD16, anti-CD56, anti-CD19, IgG, or IgG2b isotype controls (all from Becton Dickinson, San Jose, CA) for 45 min on ice. All antibodies were pretreated on normal PBMCs to determine their optimal dilutions. After incubation, the PBMCs were washed twice in DPBS containing 0.1% BSA and 0.1% NaN₃ and fixed with 2% (w/v) paraformaldehyde in DPBS for 30 min at room temperature prior to flow cytometry.

Staining for ζ in CD3+ T cells and either for Annexin V binding or Fas (CD95) in preparation for three-color flow cytometry was performed as follows. Two hundred-μl aliquots of cells suspended in PBS (2.5 × 10⁶/ml) were placed in tubes and incubated for 30 min with 10 μl of CD3-PerCP (Becton Dickinson) and 5 μl of CD95-FITC/PE (clone DX2; PharMingen) at 4°C. After the incubation period, the cells were washed twice with 1 ml of PBS and then once with Annexin-buffer (PharMingen). The cells were resuspended in 100 μl of Annexin-buffer, and 1 μl of Annexin-FITC (PharMingen) was added. After an incubation period of 20 min at room temperature in the dark, a 300-μl aliquot of Annexin-buffer was added. The cells were then fixed with 1% of paraformaldehyde for 10 min on ice. The cells were then washed twice with PBS and once with cold saponin (Sigma) 0.1% in PBS + 0.1% BSA solution. The cells were then permeabilized for 30 min on ice in 100 μl of the saponin solution. A 10-μl aliquot of TCR-ζ-PE (clone 2H2D9; Coulter) or Isotype-IgGl (Becton Dickinson) was added at the same time. After the incubation period, the cells were washed twice with saponin solution and then once with PBS. Flow cytometry analysis was performed immediately on a FACSscan.

Flow Cytometry. Three-color flow cytometry analysis was performed on a FACScan (Becton Dickinson) equipped with a single 488-nm argon ion laser. To reliably detect small subpopulations of cells, at least 20,000 events were acquired for each sample. The amplification and compensation were set according to the standard procedure, using negative controls and tested cells stained in a single color or a combination of colors (FL-1, FITC-Annexin V; FL-2, PE-CD95, or PE-4; and FL-3, CD3-PerCP). Control cells were PBMCs obtained from normal healthy donors. The percentages of apoptotic cells were calculated by scoring Annexin V binding cells after backgating on CD3+ cells in the third color. All of the gated mononuclear cell subpopulations were visualized on FSC/SSC dot-plots. To include all apoptotic cells and avoid debris with a high SSC signal, the gate was set to include a wide boundary of mononuclear cells ("open gate"), because apoptotic cells accumulated mainly in the lower FSC/SSC channels (15). The strategy of three-color staining combined with "open gating" provided the means for eliminating a majority of debris and to study apoptotic cells in different subpopulations of T lymphocytes. Separately, apoptotic cells were determined within Fas+ or ζ chain-positive or
kat cells (1 x 10^6 cells/ml) were cultured in RPMI 1640 harvested in the log phase of growth, washed, and checked for viability using a trypan blue dye and incubated with CH-11 Ab to their addition to the assay. Samples of the cells were removed at hourly intervals for evaluation by flow cytometry. The cells were washed, stained as described above, and studied by three-color flow cytometry for expression of Fas, ζ chain, and Annexin V binding. The Jurkat cell line resistant to apoptosis was obtained from Dr. H. Rabinowich (16) and used as control in the above-described experiments.

**Coincubation of Jurkat with Fas Antibody.** Jurkat cells (1 x 10^6 cells/ml) were cultured in RPMI 1640 supplemented with 1 mM L-glutamine, 10% (v/v) fetal bovine serum, 100 μl/ml penicillin, and 100 μg/ml streptomycin, all from Life Technologies, Inc. (Grand Island, NY). Cells were harvested in the log phase of growth, washed, and checked for viability using a trypan blue dye and incubated with CH-11 Ab (500 ng/ml; Upstate Biotechnology, Lake Placid, NY) or isotype control IgM (Upstate Biotechnology) at 37°C in the atmosphere of 5% CO2 in air for various periods of time, up to 8 h. The Ab samples were dialyzed to remove sodium azide (1 x 10^6) prior to their addition to the assay. Samples of the cells were removed at hourly intervals for evaluation by flow cytometry. The cells were washed, stained as described above, and studied by three-color flow cytometry for expression of Fas, ζ chain, and Annexin V binding. The Jurkat cell line resistant to apoptosis was obtained from Dr. H. Rabinowich (16) and used as control in the above-described experiments.

**Coincubation of Jurkat with Melanoma Cells.** Jurkat cells and FEM-X, a melanoma cell line, were obtained from American Type Culture Collection (Manassas, VA). The cells were cultured as described above. Jurkat cells were harvested in the log phase of growth, and melanoma cells were cultured until confluence. After trypsinization, melanoma cells were washed, checked for viability in a trypan blue dye, and mixed with Jurkat cells at the ratio of 5 tumor cells:1 lymphocyte. The cells, which formed a loose pellet, were cocultured for various periods of time (12-24 h) at 37°C in the atmosphere of CO2 in air. Control cocultures included normal human fibroblasts [courtesy of Dr. Elaine Elder, Immunologic Monitoring and Cell Products Laboratory (IMCPL)] incubated with Jurkat cells or Jurkat cells cultured in medium alone. After cocultures were terminated, the cells were stained for flow cytometry and examined for expression of the ζ chain, Annexin V binding, or Fas expression on CD3+ Jurkat cells. At least 20,000 events from the broad CD3+ live gate were acquired for data analysis. Tumor cells were excluded by backgating on CD3+ Jurkat cells and/or by using FSC/SSC.

Either FEM-X cells or normal human fibroblasts (1 x 10^6/well) were seeded in Falcon multiwell plates separated into two compartments by a positron emission tomography membrane (0.2 µm pore size) in medium (1.5 ml in the upper chamber and 2.5 ml in the lower). After 24 h, 1 ml of medium was removed from the lower chamber, and 1 x 10^6 of normal PBL or Jurkat cells were added in 1 ml of fresh medium. For this experiment, PBMCs were either resting or preactivated with phorbol myristate acetate/ionomycin for 4 h (17).

**Statistical Analyses.** All comparisons between melanoma patients and normal controls were tested with the exact two-tailed Wilcoxon test. Spearman rank correlation coefficients were determined, and tests were conducted to assess association among end points for patients and controls both separately and combined.

**Results**

**Expression of ζ in T and NK Cells in Patients and Controls.** Using PBLs obtained from patients with melanoma or from normal donors, expression of the ζ chain in T cells or T-cell subsets as well as NK cells was studied in 21 patients with melanoma and in 17 normal donors. The data presented in Fig. 1 indicate that the MFI for ζ was significantly lower (P < 0.0005) in CD3+, CD4+, CD8+, and CD56+ lymphocytes of the patients than those of the normal controls. A considerable variability was observed in expression of this signaling molecule in patients as well as in T or NK cells of normal donors. These quantitative flow cytometry data conform earlier reports, which suggested that in many (although not all) patients with melanoma, ζ expression was significantly decreased relative to that in normal donor T or NK cells (1-3). Our data also indicated that expression of ζ in NK cells of normal donors was considerably higher than that in CD3+ T cells (median MFI,
Signaling Defects and Apoptosis in T Cells

The MFI values for ζ reflect expression of this signaling molecule in the populations of T or NK cells. It is possible that ζ expression is decreased or lost in some cells but not in others within the population. For this reason, the percentage of T or NK cells with low ζ expression was also determined by taking the mean of all MFIs established for normal controls minus 2 SDs as the cutoff point. The percentage of cells below this cutoff point for calculating cell frequencies. The correlations for both T cells and NK cells were negative (r = 0.92; P < 0.0001), and patients had significantly higher percentages of T and NK cells with low ζ than CD8+ T cells, based on higher MFI. Interestingly, ζ expression was most strongly depressed in NK cells of patients with melanoma, an indication that these effector cells were highly susceptible to the mechanism(s) responsible for down-regulation of ζ. Overall, these observations indicated that ζ associated with TCR or Fcy receptor III was being down-regulated in circulating lymphocytes of patients with advanced melanoma.

The MFI values for ζ versus MFI for ζ (upper plot) and between the percentage of CD3+ CD56+ NK cells expressing low ζ versus MFI for ζ (lower plot). The percentage of T or NK cells with low ζ expression in the circulation of patients with melanoma or normal controls was obtained by taking the mean of MFI established for all normal controls minus 2 SDs as the cutoff point for calculating cell frequencies. The correlations for both T cells and NK cells were negative (r = 0.92; P < 0.0001), and patients had significantly higher percentages of T and NK cells with low ζ than normal controls.

Overall, only 4 of 14 patients had elevated percentages of NK cells with low ζ is significantly elevated in most patients with melanoma relative to normal controls; and (b) the MFI for ζ is an accurate measure of the presence of cells with low or absent ζ in a proportion of T or NK cells.

DNA Fragmentation and Low ζ Expression in T Cells of Patients with Melanoma. We have reported previously that signaling defects in the TCR pathway, specifically low ζ expression in T cells, and ex vivo spontaneous apoptosis occurred in a subset of T cells in the peripheral circulation of patients with melanoma (6). More recent data indicated that a small proportion of TUNEL+ cells detectable in the peripheral circulation of some patients with melanoma all expressed low ζ. The data obtained using PBMCs of a representative patient with melanoma are shown in Fig. 3. On the basis of these data, we reasoned that low ζ in freshly harvested T cells could be a marker for an already initiated process of apoptosis. Therefore, we expected that the T cells with low ζ would preferentially undergo ex vivo spontaneous apoptosis upon their incubation in medium for ~ 12 hours. Therefore, we explored the relationship between MFI of in freshly harvested CD3+ T cells and the percentage of CD3+ TUNEL+ cells (after incubation of the cells in medium to induce spontaneous apoptosis) in the subset of patients with melanoma and in normal controls. As shown in Fig. 4, there was no significant correlation observed between the proportion of TUNEL+ T cells assessed after 24 h incubation of PBMCs in medium and the level of ζ expression in freshly harvested T cells. The normal controls all had high ζ and few spontaneously apoptotic T cells (Fig. 4).

On the basis of these results, the possibility was considered that the decrease in ζ expression could be an early event in the process of apoptosis, whereas DNA fragmentation was its final

Fig. 2 Correlations between the percentage of CD3+ T cells expressing low ζ versus MFI for ζ (upper plot) and between the percentage of CD3+ CD56+ NK cells expressing low ζ versus MFI for ζ (lower plot). The percentage of T or NK cells with low ζ expression in the circulation of patients with melanoma or normal controls was obtained by taking the mean of MFI established for all normal controls minus 2 SDs as the cutoff point for calculating cell frequencies. The correlations for both T cells and NK cells were negative (r = 0.92; P < 0.0001), and patients had significantly higher percentages of T and NK cells with low ζ than normal controls.

Fig. 3 A representative flow cytometry histogram of PBMCs obtained from a patient with melanoma. The cells were simultaneously stained for ζ chain expression and for TUNEL. The gate was set on CD3+ T cells. The great majority of these cells were TUNEL+ and had normal (high) ζ expression. The small minority were TUNEL+ and ζ−.
stage. Therefore, to better define the subset of circulating T cells with low \( \zeta \), Annexin V binding and \( \zeta \) expression were next evaluated by flow cytometry in freshly harvested PBL-T of patients with melanoma and controls. Annexin V binding to the surface of T lymphocytes identifies cells with early apoptotic alterations (phosphatidyl serine "flip") in the cell membrane (18). The representative histogram obtained with cells from a patient with melanoma and shown in Fig. 5 indicates that low \( \zeta \) was only observed in Annexin V+ T cells but not in Annexin V- circulating T cells. Our experiments consistently showed that \( \zeta \) down-regulation was primarily observed in circulating CD3+ T cells that were Annexin V+, i.e., showed membrane changes consistent with early apoptosis.

**Expression of Fas on the Surface of T Cells in Patients with Melanoma.** Preliminary experiments indicated that Fas+ T cells are more numerous in the peripheral blood of patients with melanoma than in normal donors (6). Fas expression on the surface of CD3+ T cells was, therefore, assessed in 22 patients with melanoma included in this study. As shown in Fig. 6, up to 80% of T cells were Fas+ compared with the upper limit of 45% in normal controls. The difference in Fas expression on circulating T cells between the patients and normal donors was significant at \( P < 0.004 \).

We have reported previously that circulating Fas+ T cells were highly sensitive to spontaneous apoptosis in patients with melanoma (6). Current results confirm this observation and indicate that the proportion of Fas+ CD3+ T cells that bound Annexin V (i.e., were in the early stages of apoptosis) was considerably higher than that of Fas+ T cells in patients with melanoma (data not shown). These results are consistent with the finding that Fas+ CD3+ T cells in the circulation of patients with melanoma preferentially undergo apoptosis (6).

Therefore, our next goal was to determine whether \( \zeta \) expression was selectively decreased in Fas+ circulating T cells in patients with melanoma. As shown in Fig. 7, Fas+ T cells with decreased \( \zeta \) expression represented a distinct subset of CD3+ T cells. The important caveat of this type of experiment is that surface expression of Fas on T cells rapidly decreases during early apoptosis (Fig. 7, arrow), and thus, it is necessary to look for T cells that are Fas+ and \( \zeta \)low. Indeed, a subset of such T cells was detected in the circulation of patients with melanoma, as indicated by the data from a representative experiment shown in Fig. 7. This finding suggests that although most of circulating Fas+CD3+ T cells have normal ("high") expression (MFI > 519 in Fig. 7), those that down-regulate Fas also show reduced ("low") expression (MFI, 18). Likewise, Fas+ CD3+ T cells have high \( \zeta \) expression. Therefore, when Fas+, activated T cells present in the circulation of patients with melanoma begin to lose expression of Fas, they also down-regulate \( \zeta \) expression, and this process is detectable only in a subset of Fas+CD3+ cells.

**Induction of Apoptosis and Low \( \zeta \) by CH-11 Ab in Fas+CD3+ Lymphocytes.** To test the hypothesis that the changes observed in expression of Fas and \( \zeta \) in CD3+ lymphocytes in the peripheral blood of patients with melanoma are related to apoptosis, an ex vivo kinetics experiment was performed as follows. Jurkat cells (Fas+ T cells) were incubated in the presence of agonistic anti-Fas Ab (CH-11 or control IgM at the concentration of 0.5 or 1.0 \( \mu \)g/ml) for 0, 2, 4, and 8 h. At each time point, multiparameter flow cytometry was performed to measure surface expression of Fas and Annexin V binding as well as \( \zeta \) expression in permeabilized Jurkat T cells. As control, a Jurkat cell line resistant to apoptosis (16) was also incubated with CH-11 Ab. In Fig. 8A, it can be seen that Fas expression rapidly decreases on Jurkat cells after the addition of CH-11 Ab, probably as a result of internalization of Fas/Ab complexes, but the percentage of Fas+ cells in the population levels off at 60% by 4 h. At the same time, the total percentage of Annexin V+ cells rises from 12% at time 0 to >40% at 2 h and remains stable afterward. A similar pattern of changes overtime can be seen in Jurkat cells expressing high \( \zeta \), because their proportion decreases from 90% at time 0 to ~70% at 2 h and then stabilizes (Fig. 8B). The percentage of Annexin V+ Jurkat cells in the population reaches the level of 40% at 2 h and remains at this level. Thus, in the population of Fas+ Jurkat cells responding to CH-11 Ab, the proportion of Annexin V+ T cells corresponds to that of T cells with low \( \zeta \). This observation is consistent with the hypothesis that apoptosis may be one of the mechanisms of \( \zeta \) degradation in T cells present in the tumor microenvironment. No changes in expression of Fas or \( \zeta \) or in Annexin V binding occurred in Jurkat cells resistant to Fas-mediated apoptosis that were used as a control in this experiment.

Fig. 8C summarizes the early changes in Annexin V binding as well as Fas and \( \zeta \) expression on Jurkat cells that occur during the first 2 h of incubation with CH-11 Ab. At time 0, close to 90% of these CD3+ cells are Fas+ Annexin V- \( \zeta \)high (Fig. 8C, upper right). At 2 h, the population of cells that are Fas+ Annexin V+ can be identified (Fig. 8C, upper right), representing ~40% of the total cells. This observation indicates that the loss of Fas from the cell surface occurs rapidly and coincides with the gain of Annexin V binding. In the subset of cells that bind Annexin V (~40%), low \( \zeta \) expression is observed in about two-thirds at 2 h (Fig. 8C, upper right, A+ \( \zeta \)low). The loss of Fas appears to precede the loss of the \( \zeta \) chain in Jurkat cells undergoing Fas-mediated apoptosis (data not shown).
Kinetics of changes in Annexin V binding and in expression of Fas and ζ in Jurkat cells undergoing Fas-mediated apoptosis strongly support the hypothesis that both ζ degradation and Fas internalization are early manifestations of the process of apoptosis (Figs. 7 and 8). Because similar changes were seen in circulating CD3+ lymphocytes in patients with melanoma, the Jurkat model used seems to accurately reflect the in vivo events.

Coincubation of Melanoma Cells with T Lymphocytes. The question arises whether apoptosis, decreased ζ expression, and the loss of Fas observed in circulating T cells of patients with melanoma are related to the presence of tumor and whether tumor might be able to induce these changes in activated T cells. To answer this question, we performed a series of coincubation experiments in which a melanoma cell line, FEM-X, or human skin fibroblasts as control, was cultured in the presence of Jurkat cells. Initially, we documented that in the presence of an excess of melanoma cells, Jurkat cells became TUNEL+ (underwent apoptosis) as shown in Fig. 9A. The TUNEL+ T cells were observed only upon coincubation with tumor but not normal tissue cells, such as fibroblasts (data not shown) or in medium alone (Fig. 9C). When expression of ζ was determined by flow cytometry in TUNEL+CD3+ T cells, it was found to be significantly decreased (Fig. 9C). In Jurkat cells coincubated with FEM-X cells, the loss of ζ expression was greatest in a subset of TUNEL+CD3+ cells, but even TUNEL− lymphocytes were found to express less ζ than control Jurkat cells incubated alone or with fibroblasts (data not shown). Similar results were obtained when Annexin V binding and ζ expression were studied by flow cytometry in CD95+ Jurkat cells coincubated with FEM-X melanoma cells in excess (5 tumor cells: 1 lymphocyte), as shown in Fig. 10. In these coincubation experiments, we determined that Jurkat cells, which bound Annexin V after direct interaction with melanoma targets, also expressed low levels of ζ. Thus, coincubation with melanoma cells induced both apoptosis and ζ degradation in activated T lymphocytes.

Coincubation of T Cells with Supernatants of Melanoma Cells. To answer the question of whether down-modulation of ζ and early apoptosis could be induced by soluble products in tumor cell supernatants, we coincubated Jurkat cells
with activated or resting lymphocytes, normal fibroblasts, or FEM-X cells separated by permeable membranes. After the 24-h
coincubation, ζ chain expression was found to be decreased only
in a subset of Jurkat cells or activated PBLs but not resting
lymphocytes coincubated with FEM-X (Fig. 11). Activated
PBLs or Jurkat cells cocultured with medium alone or normal
fibroblasts did not show or showed low levels of decreased ζ
expression, respectively. The majority of these cells were not
Annexin V⁺. However, this suggests that FEM-X cells secrete
a soluble factor that can induce ζ down-regulation but not
apoptosis in Jurkat cells.

Discussion
The purpose of our studies was to seek an explanation for
and possibly relate to one another the three separate phenomena
observed to occur in T lymphocytes isolated from peripheral
blood of patients with melanoma. These lymphocytes were
found to undergo rapid spontaneous apoptosis upon their incu-
bation in medium, as reported previously (6). A high proportion
of T cells in the peripheral circulation of patients with mel-
ana was found to be Fas⁺. In addition, a substantial proportion
of these T cells showed decreased expression of the ζ chain,
which is an important signaling molecule associated with TCR
(19) as well as Fcy receptor III in NK cells (20). On the basis of
our previous experiments that demonstrated that the ζ chain
contains caspase-sensitive motifs and is a substrate for

caspase-3 and caspase-7 (7), we formulated a hypothesis that
linked Fas expression on patients’ T cells with sensitivity to
Fas-mediated apoptosis and to ζ degradation as a manifestation
of an early apoptotic process. The hypothesis was tested by

Fig. 8. In A, changes in Fas expression and Annexin V binding during incubation of Jurkat cells (CD3⁺Fas⁺) with agonistic CH-11 Ab (μg/ml) or
isotype control IgM. In B, changes in ζ expression and Annexin V binding during incubation of Jurkat cells with CH-11 Ab (μg/ml) or isotype control.
In C, the histogram summarizes early changes in Annexin V binding, Fas, and ζ expression observed during incubation of Jurkat cells with CH-11
Ab. The dot-plots show that after 1 h of incubation with CH-11 Ab, some Jurkat cells were ζ⁺, and that ζ⁺ cells had decreased Fas expression. After
2 h of incubation with CH-11 Ab, more Jurkat cells were ζ⁺, and two-thirds of these cells were ζlow or ζ'. The data are from one of two experiments
performed.
Fig. 9 Coincubation of Jurkat cells with FEM-X melanoma cells. After 5 h coincubation, a substantial proportion of Jurkat cells were TUNEL+ (A). In contrast, Jurkat cells incubated in medium (R) were not TUNEL+. In C, a flow cytometry histogram is shown indicating that among Jurkat cells coincubated with FEM-X, two subpopulations were distinguishable: CD3+ T cells with higher ζ expression, and CD3+ T cells with low ζ expression. Note that even among CD3+ T- cells, a subset of cells had low ζ expression. A representative experiment of three performed is shown.

Fig. 10 Annexin V binding and ζ chain expression in Jurkat cells coincubated with melanoma (FEM-X) cells for 8 h. As control, Jurkat cells were coincubated with normal human fibroblasts. Expression of ζ was obviously decreased, and the proportion of Annexin V binding cells increased in Jurkat cells coincubated with tumor cells.

examining fresh and permeabilized PBL-T obtained from patients with melanoma and normal controls by three-color flow cytometry for differences in expression of ζ and Fas and in the ability of T cells to bind Annexin V. In addition, Fas-mediated apoptosis was induced in Jurkat cells (used as model T cells) incubated in the presence of CH-11 Ab, tumor cells, or supernatants of tumor cells to follow changes in ζ and Fas expression over time.
The results of these experiments suggest that the downregulation of β observed in Fas⁺/CD3⁺ T cells of patients with melanoma or in Jurkat cells induced ex vivo with CH-11 Ab or tumor cells may be a result of apoptosis occurring in activated lymphocytes. The possibility exists that chronic antigenic stimulation experienced by T cells in the peripheral circulation of patients with melanoma results in activation of these T cells and their "exhaustion" and death. This process of a chronic activation and death could lead to a rapid T-cell turnover in patients with melanoma, similar to increased turnover of T cells that occurs in patients with HIV and other persistent infections (21, 22). At this time, it is unclear whether this turnover of CD3⁺ T cells represents activation-induced cell death, as suggested earlier (23), or is an example of tumor-induced cell death. In fact, the distinction between the two may not be easily made in vivo, in that the presence of tumor and of circulating tumor antigen-antibody complexes in patients with advanced cancer could induce persistent T-cell activation and drive the turnover. It is also unclear whether tumor-specific T cells are preferentially targeted for apoptosis or simply "caught" in the process by virtue of expressing activation markers. We and others are currently experimenting with the tetramer technology (24) to be able to detect and follow the fate of tumor antigen-specific T cells in the circulation of patients with melanoma.

To study the mechanisms responsible for the greater sensitivity of patients' T cells than normal T cells to apoptosis, we induced apoptosis in PBMCs by incubating them in the presence of CH-11 Ab or TNF-α (6). Surprisingly, we observed that the levels of spontaneous apoptosis were almost the same as those of apoptosis induced by either of these agents (6). This meant that cells expressing Fas and/or TNF receptor were primed to undergo spontaneous apoptosis, and that these cells did not increase >40% upon Ab cross-linking, an indication that the mechanism of resistance to apoptosis exists and probably involves intracellular protective elements, such as FLIP, c-IAP, the Bcl2 family members and others (26-28). Recent experiments indicate that one such protective molecule, FLIP short, universally expressed in T lymphocytes, is cleaved in tumor-associated lymphocytes recovered from ascites of patients with ovarian carcinoma. This observation suggests that at least some protective mechanisms might be disarmed in the presence of the tumor and supports the role for the tumor itself in apoptosis of T cells. Expression of functional FasL on human tumor cells, including melanoma, has been described by us and others (8, 10, 11, 29-31). Thus, membrane-associated or soluble FasL could contribute to the demise of T cells in patients with cancer (32).

Programmed cell death of mature T cells, so-called pro-propriocidal death, has been shown to play a critical role in mature T-cell homeostasis and in peripheral tolerance (33, 34). The consequence of pro-propriocidal death by, e.g., Fas-mediated apoptosis, of a large proportion of activated T cells in the circulation of patients with melanoma is the increase in a turnover of T cells. It is apparent that such a turnover may be a factor in the overall immune competence of patients with cancer as well as patients with HIV, whose T cells have been shown to turnover.

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4 Rabinowich, H., submitted for publication.
at a rapid rate (21). One way to prevent this rapid T-cell turnover is to protect lymphocytes from apoptosis. Preliminary experiments indicate that cytokines IL-7, IL-12, IL-15, and perhaps IL-18 may be able to offer such protection. Furthermore, it may be clinically important to determine the extent of the turnover of T cells in patients with cancer. Our data suggest that down-regulation of \( \zeta \), which appears to be associated with early apoptosis, might provide a marker for lymphocyte turnover. Regarding low \( \zeta \) expression in circulating T cells of patients with melanoma, an association with poor survival was reported (1). In studies of other cancers, low \( \zeta \) in T cells was linked to advanced disease status (4, 35–37) or to aggressiveness of the disease (14, 38). Thus, its association with early apoptosis suggests that T-cell turnover may be an important prognostic factor. In the absence of readily clinically applicable methods for evaluation of T-cell turnover, \( \zeta \) chain down-regulation, Annexin V binding, or caspase-3 activation in T cells (6) might prove to be of use as markers of early apoptosis. Additional studies are indicated to further evaluate the clinical utility of these markers in patients with cancer.

References


Decreased ζ Chain Expression and Apoptosis in CD3⁺ Peripheral Blood T Lymphocytes of Patients with Melanoma

Grzegorz Dworacki, Norbert Meidenbauer, Iris Kuss, et al.

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