Decreased Expression of Signal-transducing ζ Chain in Peripheral T Cells and Natural Killer Cells in Patients with Cervical Cancer

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ABSTRACT

An impaired immune response is frequently observed in patients and experimental animals with advanced cancer. We and others have shown alterations in CD3-associated signal-transducing ζ molecules in tumor-infiltrating T cells and peripheral blood lymphocytes (PBLs) of patients with advanced cancer. By using flow cytometric analysis of permeabilized cells with a monoclonal antibody (TIA-2) that reacts with the cytoplasmic domain of the ζ chain, here we demonstrate a marked decrease (P < 0.01) in the expression of the signal-transducing CD3 ζ chain of PBLs in patients with cervical cancer (n = 22) as compared to PBLs from healthy donors (n = 21). In addition, PBLs isolated from patients (n = 23) with cervical intraepithelial neoplasia (CIN), to a lesser but significant (P < 0.01) extent, expressed reduced CD3 ζ levels as compared to those from healthy donors. This decreased expression of ζ chains was also observed on CD16+ natural killer cells in PBLs from patients with cervical cancer. Surface expression of CD3 ζ on PBLs was also decreased in cervical cancer patients as compared to healthy donors, but not on PBLs from patients with CIN. CD3 ζ chain expression significantly (r = 0.53, P < 0.01) correlated with the ability of the PBLs to produce tumor necrosis factor in response to anti-CD3 stimulation. These findings suggest that alterations of signal-transducing ζ molecules commonly occur in patients with cervical cancer and to a lesser extent with CIN, and that they are associated with reduced cellular functions such as production of tumor necrosis factor.

INTRODUCTION

Tumor-infiltrating and to a lesser extent peripheral lymphocytes from cancer patients are known to have a poor immune response which could be normalized upon culture in recombinant interleukin 2 (1). A multitude of different mechanisms could account for this, including decreased production of growth-promoting cytokines, the presence of immunosuppressive cytokines (2), and macrophages with immunosuppressive activity (3, 4).

The cytoplasmic domain of the CD3 ζ subunit in the TCR complex is involved in signal transduction and subsequently activation of T cells (5). Recently, Mizoguchi et al. (6) have reported a decrease in CD3 ζ chain levels in T cells from tumor-bearing mice. We have observed that lymphocytes from colon cancer patients had decreased expression of the CD3 ζ, CD3 ε, and CD16 ζ molecules (7, 8). Others also have shown alteration of CD3 ζ in lymphocytes from patients with renal cell carcinoma (9, 10), melanoma (11), ovarian cancer (12), and upper gastrointestinal cancer (13). Decreased expression of CD3 ζ has been correlated with reduced proliferative responses following antigenic challenge (9) and with reduced cytokine production (10–12). In addition, a correlation has been found between tumor progression and decreased levels of CD3 ζ expression (8). The decrease of CD3 ζ expression was found to be more pronounced in TILs as compared to PBLs in patients with colorectal and renal carcinomas (7–9). Moreover, Zea et al. (11) recently demonstrated that the overall survival rate of melanoma patients with low TCR ζ levels was significantly lower than that of patients with normal TCR ζ levels. Recently, we have shown that one of the possible mechanisms behind these structural changes in the TCR complex is related to hydrogen peroxide secreted from tumor-associated macrophages (14).

Cxca is the second most common cancer in women worldwide (15) and is known to be related to HPV (16). Herein, for the first time, we report that PBLs from Cxca patients express less CD3 ζ and CD16 ζ as compared to PBLs from normal controls. These deficiencies in expression of signal-transducing molecules were associated with reduced production of TNF when PBLs were stimulated with anti-CD3 mAb.

MATERIALS AND METHODS

Patients and Controls. All samples were coded by W. M. Kast and shipped to Stockholm, Sweden. The results of the assay were sent back prior to decoding. Blood samples from

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1 The abbreviations used are: TCR, T-cell receptor; Cxca, cervical carcinoma; CIN, cervical intraepithelial neoplasia; HPV, human papillomavirus; NK, natural killer; PBL, peripheral blood lymphocyte; TNF, tumor necrosis factor; TIL, tumor-infiltrating lymphocyte; mAb, monoclonal antibody.
healthy donors were obtained from the Blood Bank, University Hospital (Leiden, the Netherlands). Blood samples of patients were taken in the Departments of Gynecology, University Hospital, Loyola University Cancer Center (Maywood, IL), and Reinier de Graaf Hospital (Delft, the Netherlands). Informed consent from blood donations was obtained from all individuals. Cxca and CIN status of the patients were diagnosed at the Department of Pathology, University Hospital, Loyola University Cancer Center, and at the Department of Gynecology, Reinier de Graaf Hospital. Blood samples from the Cxca patients were either taken before (n = 12) or after (n = 9) the operation.

PBLs of healthy donors and patients were purified by centrifugation on a Ficoll (Pharmacia, Uppsala, Sweden) gradient and routinely stored in liquid nitrogen in 10% DMSO (Sigma Chemical Co., St. Louis, MO) + 45% FCS.

mAbs and Fluorescence-activated Cell Sorting Analysis. The expression of CD3 ζ and CD16 ζ was investigated using flow cytometric analysis of permeabilized cells as described previously (7, 8). Briefly, PBLs were fixed with 0.5% formaldehyde (Sigma) in PBS for 20 min on ice and permeabilized with digitonin (10 μg/ml; Sigma) for 5 min on ice. Then cells were incubated with a saturating concentration of anti-CD16 mAb (TIA-2, IgG1) specific for the intracellular part of the ζ chain (17) or IgG1 isotype control antibody, followed by staining with a rabbit anti-mouse-FITC antibody as described previously (18). After blocking with normal mouse serum, cells were double stained with phycoerythrin-conjugated mAbs to CD3 or CD16 (Dakopatts, Glostrup, Denmark) and then subjected to flow cytometric analysis using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). CD3+ or CD16+ cells were gated and measured for mean fluorescence intensity of ζ molecules. For analysis of cell surface molecules, FITC-conjugated mAbs to CD3 or CD4 and phycoerythrin-conjugated mAb to CD8 (Becton Dickinson) were used without fixation or permeabilization.

Cytokine Production Assay. To induce cytokine production, PBLs (1.5 × 10^6/ml) were incubated for 48 h in a 24-well plate (Costar, Cambridge, MA) precoated with mAbs to CD3 (10 ng/ml: final concentration, OKT-3). Then supernatants were collected and stored at −70°C. TNF contents were determined by testing their cytotoxic effect on WEHI 164 clone 13 cells in a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide colorimetric assay (19). IFN-γ contents were determined using the sandwich ELISA in accordance with the instructions of the manufacturers (Medgenix Diagnostics SA, Fleurs, Belgium).

Statistics. Differences between groups were compared using Student’s t test and were considered statistically significant when the P values were <0.05. The relationship between CD3 ζ level and TNF production was tested using the Spearman rank correlation coefficient.

RESULTS

Decreased Expression of CD3 ζ and CD16 ζ in PBLs of Patients with Cxca. We quantitatively assessed the CD3 ζ levels of PBLs in patients with Cxca (n = 22; 51.5 ± 13.1 years old), with CIN (n = 23; 42.8 ± 7.5 years old), and in healthy donors (n = 21; 49.6 ± 8.2 years old) by using flow cytometric analysis of the permeabilized cells. PBLs from Cxca patients expressed significantly less CD3 ζ level than those from healthy donors (P < 0.01), as shown in Fig. 1 and Table 1. A considerable variation in the expression of CD3 ζ among different patients was observed, ranging from a marginal decrease to an almost absent expression. Some of the Cxca patients with severely advanced stages showed almost total disappearance of the CD3 ζ chain in PBLs (data not shown). There was no significant difference in the CD3 ζ values derived from PBLs of Cxca patients taken before (n = 12) as compared to after (n = 9) the operation. In addition, and of considerable interest, PBLs from patients with CIN, to a lesser extent, had reduced levels of the CD3 ζ molecule as compared to healthy donors (P < 0.01, Fig. 1 and Table 1).

The low-affinity FcR for IgG on human NK cells is also associated with ζ-ζ homodimers and ζ-γ heterodimers (17), known to be important in signal transduction of NK cell functions (20). As shown in Table 1, the levels of CD16 ζ in PBLs from Cxca patients were decreased as compared to healthy donors (P < 0.01).

Decreased Expression of Cell Surface TCR/CD3 on PBLs in Patients with Cxca. Since the CD3 ζ molecules have been shown to play a role in the assembly of the TCR-CD3 complex (21), we next analyzed the expression of surface CD3 ε using flow cytometry. The level of CD3 ε on PBLs was also reduced in Cxca patients, whereas no significant difference in the level of the CD3 ε on PBLs from patients with CIN was observed as compared to PBLs from healthy donors (Table 1).

TFN Production from PBLs Stimulated with anti-CD3 mAb Correlates with CD3 ζ Levels. To investigate the correlation between alteration of signaling molecules and functional consequences for T cells, cytokine production assays in response to anti-CD3 stimulation were performed. There was a

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**Fig. 1** Reduced CD3 ζ expression on PBLs in patients with cervical cancer. The expression of CD3 ζ was investigated using flow cytometric analysis of permeabilized cells with a mAb (TIA-2) specific for the intracellular part of the ζ chain. PBL samples from patients with Cxca (Cancer) and CIN and from healthy donors (Healthy) were analyzed. *, statistically significant (P < 0.01) using Student’s t test.

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<table>
<thead>
<tr>
<th>Cancer (n=22)</th>
<th>CIN (n=23)</th>
<th>Healthy (n=21)</th>
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<tbody>
<tr>
<td>Cancer</td>
<td>CIN</td>
<td>Healthy</td>
</tr>
<tr>
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</tr>
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<td>Mean fluorescence intensity of CD3 ζ</td>
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</table>

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**Statistics.** Differences between groups were compared using Student’s t test and were considered statistically significant when the P values were <0.05. The relationship between CD3 ζ level and TNF production was tested using the Spearman rank correlation coefficient.

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The Spearman rank correlation coefficient. The relationship between the CD3 levels and TNF production was significant. However, the correlation between the CD3 levels and IFN-γ production was not significant. This is consistent with the findings of Tartou et al. (10) with PBLs and TILs from patients with renal cell carcinoma. Others have reported that also IFN-γ and interleukin 2 production in response to anti-CD3 stimulation were reduced in patients with low CD3 expression (11, 12).

Aoe et al. (23) have reported that activated macrophages induce structural abnormalities in the TCR-CD3 complex in tumor-bearing mice. We have shown that hydrogen peroxide (H₂O₂) derived from tumor-associated macrophage or lipopolysaccharide-activated monocytes derived from PBLs can down-modulate the CD3 expression, paralleling the impaired Ca²⁺ mobilization and reduced tumor-specific cytolytic activity of CTL (14). A similar mechanism dependent on macrophage-derived H₂O₂ was also shown to down-regulate CD16 expression (14) and NK cytolytic activity (14, 24), which was also shown to be associated with induction of apoptosis in NK cells (25). It remains to be shown whether these same mechanisms can explain the observed reduction of CD3 expression and the impaired TNF production in patients with cervical cancer.

Regardless of the underlying mechanism, the alterations in signal-transducing molecules observed in patients with cancer have important clinical implications regarding the monitoring of immune status in patients and the evaluation of therapeutic strategies. Measuring CD3 chain expression using flow cytometric

**Table 1**  Down-regulation of ζ chain expression in T cells and NK cells of Cxca patients

<table>
<thead>
<tr>
<th>Mean fluorescence intensity</th>
<th>Cancer patients (n = 22)</th>
<th>CIN (n = 23)</th>
<th>Healthy donors (n = 21)</th>
<th>Cancer patients:CIN</th>
<th>Cancer patients: healthy donors</th>
<th>CIN: healthy donors</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3 ζ</td>
<td>45.3 ± 15.0</td>
<td>57.8 ± 16.5</td>
<td>78.0 ± 10.0</td>
<td>NS*</td>
<td>0.01</td>
<td>0.01</td>
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<tr>
<td>CD16 ζ</td>
<td>36.9 ± 5.0</td>
<td>39.5 ± 9.0</td>
<td>45.1 ± 6.7</td>
<td>NS</td>
<td>0.01</td>
<td>NS</td>
</tr>
<tr>
<td>CD3 ε</td>
<td>36.7 ± 5.0</td>
<td>44.9 ± 6.0</td>
<td>47.9 ± 5.0</td>
<td>NS</td>
<td>0.01</td>
<td>NS</td>
</tr>
<tr>
<td>CD4</td>
<td>38.9 ± 4.7</td>
<td>41.5 ± 6.9</td>
<td>42.0 ± 6.4</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>CD8</td>
<td>42.5 ± 4.9</td>
<td>43.9 ± 6.8</td>
<td>45.9 ± 7.2</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

* The expression of CD3 ζ and CD16 ζ on PBLs was investigated using flow cytometric analysis of permeabilized cells, using a mAb (TIA-2) which reacts with the cytoplasmic domain of the ζ chain. The surface expression of CD3 ε, CD4, and CD8 on PBLs was analyzed without permeabilization. Data are expressed as mean ± SD. The P value corresponds to Student's t test.

**Table 1 continue**

**DISCUSSION**

In the present study, we have found that the expression of the CD3 ζ and CD16 ζ chains was decreased in patients with Cxca. The surface expression of CD3 ε on PBLs was also reduced in Cxca patients as compared to healthy donors. Interestingly, CIN patients, with an early localized lesion and thought to have a normal immune status, also expressed reduced CD3 ε levels on PBLs as compared to healthy donors, although with a smaller decrease than in Cxca and with normal surface CD3 ε expression. Recently, we have reported that the significant correlation between the CD3 ε levels and TNF production (r = 0.53, P = 0.01), as shown in Fig. 2. The correlation between the CD3 ζ levels and IFN-γ production however was not significant (r = 0.32, P = 0.22, results not shown).

Aoe et al. (23) have reported that activated macrophages induce structural abnormalities in the TCR-CD3 complex in tumor-bearing mice. We have shown that hydrogen peroxide (H₂O₂) derived from tumor-associated macrophage or lipopolysaccharide-activated monocytes derived from PBLs can down-modulate the CD3 ζ expression, paralleling the impaired Ca²⁺ mobilization and reduced tumor-specific cytolytic activity of CTL (14). A similar mechanism dependent on macrophage-derived H₂O₂ was also shown to down-regulate CD16 ζ expression (14) and NK cytolytic activity (14, 24), which was also shown to be associated with induction of apoptosis in NK cells (25). It remains to be shown whether these same mechanisms can explain the observed reduction of ζ expression and the impaired TNF production in patients with cervical cancer.

Regardless of the underlying mechanism, the alterations in signal-transducing molecules observed in patients with cancer have important clinical implications regarding the monitoring of immune status in patients and the evaluation of therapeutic strategies. Measuring ζ chain expression using flow cytometric
analysis on a routine basis might provide a reliable and convenient way to investigate whether the restoration of altered signal-transducing molecules within patients correlates with a favorable prognosis during the course of immunotherapy. Alterations in signal-transducing molecules in T cells of Cxca patients will impair the response to therapeutic antitumor vaccines. Therefore, research aimed at developing drugs that counteract suppression of antitumor immunity should provide new and promising avenues for the treatment of cancer, especially if combined with immunotherapy.

REFERENCES
Decreased expression of signal-transducing zeta chain in peripheral T cells and natural killer cells in patients with cervical cancer.

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