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Alterations of the Expression of Signal-Transducing CD3 ζ and Costimulatory CD28 Molecule in Peripheral Blood T Lymphocytes in Patients with Cervical Cancer

Zaburzenia ekspresji łańcucha CD3 ζ i cząsteczki kostymulującej CD28 limfocytów T krwi obwodowej u chorych na raka szyjki macicy

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Abstract

Background. Mechanisms of abnormal immune function in patients with cervical cancer are object of intense investigations. It has been recently reported that peripheral blood T cells in cancer patients express reduced levels of the T cell receptor signal-transducing CD3 ζ chain and costimulatory CD28 molecule.

Objectives. Estimation of the proportion of CD3 $^{+}/\zeta^{+}$ and CD3 $^{+}/$ CD28 $^{+}$ cells in peripheral blood in patients with cervical cancer and normal subjects before and after anti-CD3 monoclonal antibody *ex vivo* stimulation.

Material and Methods. Studies were performed in previously untreated 20 patients with cervical cancer and 16 age and sex matched healthy subjects. Peripheral blood mononuclear cells were stimulated *ex vivo* with anti-CD3 monoclonal antibody for 72 hours. The proportions of CD3 $^{+}/\zeta^{+}$ and CD3 $^{+}/$ CD28 $^{+}$ were estimated before and after stimulation.

Results. The authors showed significantly lower proportion of CD3 $^{+}/\zeta^{+}$ cells in patients with cervical cancer compared with controls before stimulation ($p = 0.001$) and after 72 h of stimulation ($p = 0.01$). Stimulation with anti-CD3 did not significantly change the percentage double-stained cells in normal subjects. Anti-CD3 stimulation in cervical cancer patients increased ($p < 0.001$), but did not normalized the proportion of CD3 $^{+}/\zeta^{+}$ cells. The mean proportion of CD3 $^{+}/$ CD28 $^{+}$ cells were significantly lower before ($p = 0.001$) and after stimulation ($p = 0.001$) in patients with cervical cancer compared with controls. In all studied subjects stimulation with anti-CD3 did not increased the expression of CD28 molecule.

Conclusion. The down regulation of CD3 ζ chain and CD28 molecule on peripheral blood T cells may be one of the mechanisms leading to immune deficiency in cervical cancer patients (*Adv Clin Exp Med* 2004, 13, 4, 589–594).

Key words: CD3 ζ , CD28, stimulation, cervical cancer.

Streszczenie

Wprowadzenie. Mechanizmy zaburzeń odporności u chorych na raka szyjki macicy są przedmiotem intensywnych badań. W ostatnich latach u chorych na nowotwory wykazano obniżoną ekspresję białka sygnałowego CD3 ζ i molekuly kostymulującej CD28 limfocytów T krwi obwodowej.

Cel pracy. Ocena ekspresji odsetka komórek CD3 $^{+}/\zeta^{+}$ i CD3 $^{+}/$ CD28 $^{+}$ krwi obwodowej u chorych na raka szyjki macicy i u osób zdrowych przed i po stymulacji przeciwciałem monoklonalnym anty-CD3.

Materiał i metody. Badanie przeprowadzono u 20 chorych na raka szyjki macicy przed leczeniem i u 16 osób zdrowych odpowiadających wiekiem i płcią badanym chorym. Komórki jednojądrzaste krwi obwodowej stymulowano przeciwciałem monoklonalnym anty-CD3 przez 72 godziny. Odsetek komórek CD3 $^{+}/\zeta^{+}$ i CD3 $^{+}/$ CD28 $^{+}$ oceniano przed i po stymulacji.

Wyniki. U chorych na raka szyjki macicy wykazano statystycznie istotnie niższy odsetek komórek CD3 $^{+}/\zeta^{+}$ w porównaniu z grupą kontrolną przed stymulacją ($p = 0,001$) i po 72-godzinnej stymulacji przeciwciałem monoklonalnym anty-CD3 ($p = 0,01$). Stymulacja anty-CD3 nie zmieniała istotnie odsetka podwójnie znakowanych limfocytów

tów u osób zdrowych. U chorych na raka szyjki macicy stymulacja znamienne zwiększała ($p < 0,001$), ale nie normalizowała odsetka komórek $CD3^+/ζ^+$. Średni odsetek limfocytów $CD3^+/CD28^+$ był istotnie niższy przed ($p = 0,001$) i po stymulacji ($p = 0,001$) u chorych na raka szyjki macicy w porównaniu z grupą kontrolną. U wszystkich badanych osób stymulacja przeciwciałem monoklonalnym anti-CD3 nie zwiększała ekspresji antygenu CD28.

Wnioski. Obniżenie ekspresji łańcucha CD3ζ i antygenu CD28 limfocytów T krwi obwodowej może być jednym z mechanizmów prowadzących do zaburzeń odporności u chorych na raka szyjki macicy (*Adv Clin Exp Med* 2004, 13, 4, 589–594).

Słowa kluczowe: CD3ζ, CD28, stymulacja, rak szyjki macicy.

The role of immune mechanisms in the control of cancer has been suggested by several studies [1, 2], but the mechanisms whereby tumors escape immunosurveillance still remain poorly understood.

Several possible mechanisms have been proposed to explain the failure to control tumor growth: lack of recognition of tumor associated cell antigens, induction of T-cell anergy, absence of secondary stimuli necessary for T-cell activation, decrease or lack of expression of major histocompatibility complex (MHC) class I molecules, decrease of cytokine production. Recently, a decrease in lymphocyte signal-transduction molecules, described in cancer patients, has been proposed as a possible mechanism leading to impairment of the immune system [3–5].

The T cell receptor (TCR) associated complex of CD3 transmembrane proteins is involved in the transduction of activation signals following engagement of the TCR. The CD3 complex comprises at least six CD3 protein subunits: gamma, delta, epsilon and plus the ζ-ζ homodimer also referred as TCRζ. The CD3ε subunit amplifies the activation signal initiated by antigen binding and is responsible for signal transduction function in addition to providing the key signal to direct transport of assembled TCR/CD3 complex from the Golgi apparatus to the cell surface. The consequences of reduced ζ chain levels are likely to be functionally important [3] because the phosphorylation of the immunoreceptor tyrosine-based activation motifs in the intracytoplasmic component of the ζ chain by protein kinases is an important early event in T-cell activation. Reduced levels of ζ chains result in a relative lack of tyrosine residues for phosphorylation and a reduced recruitment and phosphorylation of downstream signal-transducing molecules, such as ZAP-70, leading to a failure of T-cell activation, proliferation and cytokine production [6]. Consistent with this mutant mice lacking CD3ζ chain are unable to respond to antigen, exhibit greatly reduced proliferative response and the expression level of TCR on these cells is 5-fold lower than those on wild type cells [3, 7].

In addition to the expression of the ζ chain, expression of CD28 is of importance for the proper function of T lymphocytes. Induction of T-cell effec-

tor functions after TCR recognition of antigen bound to MCH molecules on antigen presenting cells requires costimulatory signals [8–10]. The interaction of CD28 on T cells with B7 family molecules on antigen presenting cells is considered to be of critical importance for T cell activation. [8–10]. CD28 is a primary T cell costimulatory molecule, which is expressed constitutively on almost all T cells. Upon interaction with ligands CD28 transduces the signal which enhances T cell proliferation, cytokine secretion and sustains T cell response [11]. Triggering of TCR/CD3 alone in the absence of a costimulatory signal not only fails to induce immune response, but may also lead to a state of hyporesponsiveness or anergy [12, 13]. CD28 negative transgenic mice exhibit profound defects in mitogenic responses and germinal centers are not formed in response to immunization [14, 15].

In the present study the authors investigated the expression of CD3ζ chain and CD28 molecule on unstimulated and anti-CD3 monoclonal antibody (MoAb) stimulated peripheral blood T lymphocytes. According to authors' knowledge, no such studies have been reported so far.

Materials and Methods

Patients

The study was performed in 20 patients with cervical cancer in III and IV stage of disease, aged 22–73 (median 50) and 16 age and sex matched healthy subjects. The stage of the disease, defined according to the FIGO classification, was assessed using standard procedures. All cases of cervical cancer were histological defined as squamous cell cancer. Studies have been carried out with ethical committee approval.

Isolation of Cells

Peripheral blood mononuclear cells (PBMC) were separated by buoyant density gradient centrifugation on Gradisol L (Kutno, Poland) from freshly drawn peripheral venous blood, washed three times with 0.9% saline.

All experiments on the fresh and cultured cells were carried out by double labelling with anti-CD3 ζ , anti-CD28 (Serotec, UK) and anti-CD3 monoclonal antibody (MoAb) (Serotec, UK) as previously described [16]. The cells were fixed and permeabilized according to the method described by Anderson et al. [17].

Culture Conditions

The PBMC were resuspended to 1×10^6 PBMC/ml in RPMI 1640 medium (Gibco, Paisley, UK), supplemented with 10% fetal calf serum (Flow Labs, UK), 100 μ g/ml streptomycin, 2 mM L-glutamine, 100 j/ml penicillin, 0.1 mM glucose and were culture with 10 ng/ml anti-CD3 MoAb (Becton-Dickinson, San Jose, CA, USA). Control cultures without stimulants were included in each experiment. The cultures were incubated at 37°C in humidified atmosphere, containing 5% CO₂, for 72 hours.

Permeabilization of Cells and Flow Cytometry Analysis

Briefly, the cells were incubated for 30 min at 4°C with 2 ml of a mixture of 1 ml 4% paraformaldehyde in phosphate-buffered saline (PBS) and 1 ml of 1:10 dilution of FACS lysing solution (Becton Dickinson) in distilled water. Cells were washed with PBS containing 0.5% Tween-20 and incubated for for 20 min with AB serum and anti- ζ chain MoAb. Cells were then washed twice using PBS containing 0.5% Tween-20 and incubated with goat-anti-mouse-FITC, washed twice with PBS/Tween-20 and resuspended for 5 min in mouse serum, diluted 1 : 2000. Cells were then incubated for 20 min with phycoerythrin-conjugated MoAb to CD3, washed twice in PBS/Tween-20, resuspended in PBS, and analyzed by flow cytometry using FACScalibur flow cytometer (Becton, Dickinson). Negative controls were always used by omitting the MoAb as well as incubating the cells

with mouse Ig of the same isotype as MoAbs conjugated with fluoresceine or phycoerythrin.

The results were expressed as the proportion of the peripheral blood CD3⁺ lymphocytes subpopulations co-expressing the ζ chain or CD28 molecule. At least 10 000 events per sample were analyzed in double staining analysis.

Statistical Analysis

Statistical analysis was performed using non-parametric Mann-Whitney *U* test.

Results

The mean proportion of CD3⁺/ ζ ⁺ cells was significantly lower in patients with cervical cancer compared with controls before stimulation ($38.0\% \pm 25.4\%$ vs. $69.1\% \pm 17.3\%$; $p = 0.001$) and after 72 h of stimulation with anti-CD3 ($65.6\% \pm 27.0\%$ vs. $78.7\% \pm 13.3\%$; $p = 0.01$) (Tab. 1). Stimulation with anti-CD3 did not significantly change the percentage of double-stained cells in normal subjects. Anti-CD3 stimulation in cervical cancer patients significantly increased ($p < 0.001$), but did not normalize the proportion of CD3⁺/ ζ ⁺ cells (Tab. 1).

The mean proportions of CD3⁺/CD28⁺ cells were significantly lower before ($48.2\% \pm 15.0\%$ vs. $80.0\% \pm 20\%$; $p = 0.001$) and after stimulation ($52.0\% \pm 18.0\%$ vs. $82.0\% \pm 18\%$; $p = 0.001$) in patients with cervical cancer compared with controls (Tab. 2). In all studied subjects stimulation with anti-CD3 did not increase the expression of CD28 (Tab. 2).

Discussion

There are only few reports regarding the expression of ζ chain in peripheral blood T cells in cervical cancer patients. Nieland et al. [18] and

Table 1. The mean proportion CD3⁺/ ζ ⁺ cells in group of cervical cancer patients and healthy subjects before and after anti-CD3 monoclonal antibody stimulation

Tabela 1. Średni odsetek komórek CD3⁺/ ζ ⁺ w grupie chorych na raka szyjki macicy i grupie kontrolnej przed i po stymulacji przeciwciałem monoklonalnym anti-CD3

	The group of patients with cervical cancer (Grupa chorych na raka szyjki macicy) n = 20; x% \pm SD%	Healthy subjects (Grupa kontrolna) n = 16; x% \pm SD%	p
Before stimulation (Przed stymulacją)	38.0 \pm 25.1	69.1 \pm 17.3	p = 0.001
After stimulation (Po stymulacji)	65.6 \pm 27.0	78.7 \pm 13.3	p = 0.01
p	p < 0.001	ns	

Table 2. The mean proportion of CD3⁺/CD28⁺ in cervical cancer patients and healthy subjects before and after anti-CD3 monoclonal antibody stimulation**Tabela 2.** Średni odsetek komórek CD3⁺/CD28⁺ w grupie chorych na rak szyjki macicy i grupie kontrolnej przed i po stymulacji przeciwciałem monoklonalnym anty-CD3

	The group of cervical cancer patients (Grupa chorych na raka szyjki macicy) n = 20; x% ± SD%	The group of healthy subjects (Grupa kontrolna) n = 16 x% ± SD%	p
Before stimulation (Przed stymulacją)	48.2 ± 15.0	80.0 ± 20.0	p = 0.001
After stimulation (Po stymulacji)	52.0 ± 18.0	82.0 ± 18.0	p = 0.001
p	ns	ns	

Kono et al. [19] similarly to our study have reported low ζ chain expression in peripheral blood T lymphocytes. De Gruijl [20] also found the decreased expression of ζ chain in tumor-infiltrating T cells. The authors limited their studies to assessment of ζ chain expression on non-stimulated lymphocytes. In this study the authors also evaluated the effect of anti-CD3 stimulation on the ζ chain expression. This type of stimulation mimics events occurring after antigen binding by stimulating T cells with monoclonal antibody specific for T cell receptor complex. Monocytes present in PBMC served in the model as accessory signal necessary for T-cell stimulation. In the study the authors report significantly lower expression of CD3 ζ chain in peripheral blood lymphocytes in patients with cervical cancer compared with normal subjects, which increased but did not normalize after anti-CD3 *ex vivo* stimulation.

There is no clear understanding of the mechanisms by which tumor affect the expression of T cell signal transduction molecules. It is postulated that the tumor microenvironment is the source of factors causing depression of the ζ chain. This may include macrophage-mediated products such as NO [21], H₂O₂ [22], IL-10 [23] and TGF- β [24], which could be responsible for apoptosis of T cells. It has been also shown that exposure of T lymphocytes on IL-4 *in vitro* during T cell activation or expansion could suppress antitumor activity and lead to a depression of CD3 ζ expression [25]. Recently Taylor et al. [26] identified a circulating factor isolated from ovarian cancer patients which can mediate suppression of CD3 ζ chain. Investigations into molecular mechanisms leading to the reduced ζ expression in advanced ovarian cancer have indicated that it appeared to result rather from increased degradation and not from decreased synthesis [27].

In the past years there have been significant advances in the understanding of the role of CD28/B7 pathway in regulating the T cell response. However, a few studies have investigated

expression of costimulatory CD28 on peripheral blood T cells in cancer patients [28–30]. To the best of authors knowledge there is only one paper on the expression of CD28 on peripheral blood T lymphocytes in cervical cancer patients [31]. These authors showed that the level of CD28⁺ T lymphocytes is lower in patients group compared with normal controls. They also found that the peripheral blood CD8⁺/CD28⁺ count may be used as predictive factor of clinical response to chemotherapy in patients with advanced cervical cancer [31]. Similarly, the authors observed significantly lower proportion of CD3⁺/CD28⁺ cells in advanced stage of cervical cancer patients compared with controls. The lack of the effect of anti-CD3 stimulation on CD28 molecule expression in studied patients indicates profound abnormalities in costimulatory pathways in cervical cancer.

The mechanisms underlying the abnormalities in CD28 expression in cancer patients are not fully elucidated and require further studies. It has been reported that markedly reduced expression of surface CD28 may result from mutation in gene encoding PI-3-kinase, a regulator of cellular traffic of CD28 [32]. It has been also suggested that the loss of CD28 molecule on T lymphocytes may be related to prolonged *in vivo* activation of these cells [33] and/or the release of immunosuppressive factors from tumor cells [34]. Presented observations suggest that TCR/CD3/CD28 activation pathway in cervical cancer patients are severely impaired. Significantly lower CD3 ζ and CD28 antigen expression observed in studied patients before and after stimulation may lead to the state of hyporesponsiveness or anergy and may be one of the mechanisms leading to impaired immune response in cervical cancer. A better understanding of molecular changes in T lymphocytes in cancer would help to develop a strategy for repairing defects in the immune system, and monitor the course of therapy in these patients.

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