Granzyme B as a prognostic marker of cervical intraepithelial neoplasia

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Summary

Granzyme B (GrB) is a serine protease synthesized in T lympocytes (CTL), released after T-cell activation resulting from exogenous stimulation. With perforin, GrB discharges apoptotic signals to a target cell and therefore constitutes a marker to identify activated CTL. We aimed to quantify GrB expression by immunohistochemistry staining in 12 tissue fragments of cervical carcinoma, 33 cervical intraepithelial neoplasias treated by LLTEZ and nine cervical pieces without disease. Activated cytotoxic lymphocyte mean values (20 HPF-400x) in both epithelial and stromal pars were 7.11 cells in tissue without neoplasia, 33.45 cells in cervical intraepithelial neoplasia and 139.75 cells in carcinoma samples, with a statistical difference between them. Comparative analysis in the CIN group showed an expressive difference between cases with disease recurrence (19.28 cells) and without recurrence (37.26 cells). Thus, the relation between number of activated CTLs found at the moment of treatment and clinical evolution determined in this study, suggest GrB use as a prognostic marker.

Key words: Granzyme B; Cervical intraepithelial neoplasia; Prognostic marker; Immunologic marker.

Introduction

Cell-mediated immune responses (CMI) are important for disease control. Cervical cancer is the second genital cause of female death and has a strong impact, specially in developing countries.

Persistent infection with oncogenic human papillomavírus (HPV) types is associated with development of cervical neoplasia, which encompasses a spectrum of cervical epithelial cell abnormalities, ranging from preinvasive cervical intraepithelial neoplasia (CIN) to cervical cancer.

DNA from HPV can be found in more than 94% of cervical carcinomas worldwide; this association suggests that it might be possible to develop either prophylaxis or therapies for cervical neoplasia based on the manipulation of human immune response to HPV [1], even though, HPV infection and cancer development is not an absolute association. A great number of infected women present regression and do not progress to cancer [2].

Cell-mediated immune responses (CMI) are important for disease control [3]. Human cytotoxic T-lymphocytes (CTL) and natural killer cells together comprise the means by which the immune system detects and rids higher organisms of virus-infected or transformed cells. De Gruijl *et al.* [4] have shown T-cell infiltrates surrounding CIN lesions.

Granzyme B (GrB) is a serine protease specifically expressed by activated CTL, and is a primary molecular mediator of apoptosis by CTL and natural killer cells. After T-cell receptor activation, GrB is released from CTL cytoplasmic granules by exocytosis, enters the target cells and in the presence of perforin, initiates caspase processing [5]. It promotes DNA fragmentation and is directly involved in cell death [6].

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Objective

In the present study we attempted to predict CIN outcome after treatment by measuring the specific CMI number of T cells determined by GrB expression at the infection site, the cervix, and to compare this number with CIN evolution after treatment, analyzing recurrence of disease.

Methods and Patients

Thirty-three patients aged between 20-50 years, not pregnant and not immunosuppressed, from the Lower Genital Tract Pathology Sector of the Department of Gynecology, UNIFESP (Universidade Federal de São Paulo) participated in this study after informed consent.

CIN diagnosis was assessed by cytology, colposcopy and histopathologic examinations. All patients received outpatient treatment with large-loop excision of the transformation zone (LLETZ). Complete lesion excision could be histologically assessed. Strict histopathologic parameters were absence of deep thermal effects and free surgical margins. Follow-up after treatment ranged from 24 to 48 months.

Tissue

Formalin-fixed paraffin-embedded tissues were cut into 3-um thick sections and mounted on 3-amino-propyl-triethoxy-silane coated slides (Apes, Sigma, MO, USA) for hematoxylin and eosin and immunohistochemical staining.

Immunohistochemistry

Tissue sections were deparaffinized using xylene; endogenous peroxidase was blocked and antigen retrieval by microwave treatment was performed for staining with GrB antibody. Sections were subsequently washed and preincubated with normal serum and incubated with the primary antibody (Mouse anti-Granzyme B N-19, Santa Cruz, CA, USA).

Sections were incubated with DAB (3,3-diaminobenzidine tetrahydrochloride, Sigma Chemical Co. 02,D5637, USA) and hydrogen peroxide followed by counterstaining with hematoxylin, and then dehydrated and mounted. Tonsilar fragments were used as a positive control.

Interpretation of immunohistochemical staining

Infiltrate analysis of the CIN lesions was performed in 20 high power fields (HPF, 400x), consisting of 50% epithelial and 50% stromal cells, selected from highly positive concentration areas using an interactive video-overlay-based measuring system (Eclipse E400, Nikon, Japan).

Infiltrating cells positive for GrB were counted by two independent observers.

Statistical methods

The expression of GrB marker in the different dysplasia groups (with and without recurrence) was compared using the Mann-Whitney two-sample test.

Results

GrB activity was detected in all cases of CIN. The number of cells ranged from 7 to 113 (mean 33.45). Preponderant distribution was in the stroma.

The mean number of GrB positive cells was significantly higher in the group without recurrence (37 cells) than that of GrB positive cells (19) in the group with recurrence (Table 1).

Table 1. — *Distribution of granzyme B expressing cells in 33 cases of grade 3 cervical intraepithelial neoplasia submitted to LLETZ with and without recurrence of dysplasia and a reliable interval.*

Group	N	Interval	GrB
With recurrence	7	11-35	19.28
Without recurrence	26	7-113	37.26

N = number of cases; GrB = positive granzyme B lymphocytes; Mann-Whitney Test; Z calc = –1.696; *p < 0.05.

There were 21.9% (n = 7) recurrent cases: 57.1% (n = 4) CIN III cases; 28.57% (n = 2) CIN I cases and one invasive carcinoma (14%). Interval between treatment and recurrence diagnosis ranged from 12 to 41 months (mean = 24 months). There was no case of persistence of disease in the first year of follow-up.

Stromal distribution was predominant in the group with recurrence and there were no differences between the groups regarding epithelial distribution. Age and parity were compared and there were no differences between the two groups.

Discussion

After treatment, in the premalignant stage, disease eradication with a minimal possibility of recurrence is expected. Avoiding invasion is very important; a woman affected by cervical invasive cancer has last nine years of her life's years estimative. The mortality rate for all stages of invasive cervical cancer is about 30%.

In this study the issue was to assess whether there is an effective role of cytotxic T cells in restraining premalignant lesions of the cervix.

A high percentage of activated CTLs were found. This result confirms that in CIN many infiltrating lymphocytes with cytotoxic potential are activated. Most cases with a high number of activated CTLs did not recur, showing a significantly higher number of lymphocytes than in the group with recurrence.

Nevertheless, this activation was not effective in all cases and culminated with relapse of the disease because viruses have evolved a variety of strategies that may delay apoptosis in order to potentially allow them more time to replicate within a host cell.

Incorporation fragments of the host genome allow codification of proteins that resemble Bcl-2, functionally inactivate p53, block Fas-mediated apoptosis or act directly as serine proteinase inhibitors (serpins), like PI-9 [7, 8].

Thus, in the initial phase of the precursor lesion there is a possible inhibitory mechanism of lymphocyte attraction. Therefore a lower number of activated lymphocytes would be noticed, as it happened in our group with recurrence.

Even this lower density of lymphocytes would be sufficient to induce lesion regression because one lymphocyte only is able to destroy many target cells.

Consequently DNA fragmentation is dependent on the state of target cell activation and recruitment to the mitotic cycle. Quiescent cells are refractory to DNA fragmentation, but not to membrane lysis and G0 appears relatively resistant to CTL-induced apoptosis [9]. Cell kinetics is also faster than CTL activation. Probably in cases of recurrence, perforin-GrB conjugation is not enough to permanently stop cell processes of premalignant lesions.

These results indicate that local factors, such as cytokines and immunoselection of premalignant cells, are possibly responsible for immunologic response escape, associated with slight lymphocyte activation.

Our results show that there was granzyme B expression in all cases of CIN with higher expression in non-recurrent CIN cases than in the recurrent ones. It is fundamental that women with early diagnosis and treatment do no suffer future disease recurrence, often through an invasive pathway.

Recurrence prevention demands development of strategies. Identifying clinical risk factors (age over 40, glandular involvement, satellite lesions and remaining disease) and analyzing the immunological response seem to be the most important approaches.

Conclusions

The relation between number of activated CTLs found at the moment of treatment and clinical evolution determined in this study suggest GrB use as a prognostic marker. The patient should be under a strict and long follow-up period if the GrB values are low with the intention of early detection of recurrence.

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