Assessment of Treg Cells CD4⁺CD25⁺ in Chronic Cirrhotic Liver Disease and Hepatocellular Carcinoma Egyptian Patients

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**Background and Aims:** Dysfunction of the host immune system in cancer patients can be due to a number of factors, including secretion of tumor-derived immunosuppressive factors or induction of immune tolerance against tumor-specific antigens. Several studies suggest that suppression of tumor-associated antigen reactivates lymphocytes by CD4⁺CD25⁺ regulatory T (Treg) cells. This study was designed to evaluate whether CD4⁺CD25⁺ Treg cells in chronic liver disease and hepatocellular carcinoma (HCC) patients exhibit an expanded Treg pool and to correlate it with liver tumor markers and grading.

**Methods:** Blood samples were collected from 20 patients with cirrhotic liver disease (CLD), 15 HCC patients and 10 healthy control subjects. Alpha fetoprotein (AFP), HBV and HCV antibodies were detected by EIA. HCV was confirmed by immunoblotting and RT-PCR. To evaluate HCC grading, abdominal ultrasound guided liver biopsy was done. Patients were categorized into moderately differentiated (grade II) and poorly differentiated (grade III) groups. Cytometric analysis of CD4⁺CD25⁺ Treg cells in PBMCs was performed using anti-CD3, anti-CD4, anti-CD25, anti-CD45RA, and IgG-isotype control (FITC and PE).

**Results:** Both CLD and HCC groups were 80% positive for HCV while only 20% of CLD and 11% of HCC patients were positive for HBV. The mean percentage of CD4⁺CD25⁻ T cell population demonstrated a highly significant increase in comparing HCV to HCC patients [2.47±0.66 vs. 8.96±1.38 (P<0.001)] and when comparing both group to controls [1.15±0.5 (P<0.01)]. Nine HCC patients were in grade II while 6/15 were in grade III. Their mean CD4⁺CD25⁻ T cells percentage was 9.12±1.52 and 8.73±1.33, respectively. A negative correlation was found between mean CD4⁺CD25⁻ T cells percentage and AFP serum level in HCC patients (r=-0.923) while Treg cells with patients tumor grades (II and III) (r=-0.474 and 0.582, respectively). CLD showed a significant correlation with AFP level (r=0.962).

**Conclusions:** Tumor specific Treg cells may limit the efficacy of anti-tumor response. Treg cells correlate properly with the unique marker AFP and with tumor grades. Better understanding of the underlying mechanism of Treg regulation or of the strategy for controlling Treg cells may lead to effective HCV immunotherapy and enhancing immunity against cancer.

**Keywords:** CD4⁺CD25⁺ Treg Cell, HCV, Hepatocellular Carcinoma, Liver Cirrhosis

Introduction

The emergence of a tumor results from the disruption of cell growth regulation as well as from failure of the host to provoke a sufficient immunological anti-tumor response (1). Indeed, most cancer patients do not develop a satisfactory immunological antitumor response, implicating the existence of tumor-specific immune evasion strategies (2, 3). Dysfunction of the host immune system in cancer patients can be due to a number of factors.
factors, including the secretion of tumor-derived immunosuppressive factors, such as IL-10 and TGF-β (4) or the induction of immune tolerance against tumor-specific (5).

In vitro studies demonstrate that CD4+CD25+ T cell population appears to be a homogeneous population of suppressors that do not contain memory or activated T cells (6). CD4+CD25+ T cell population present in normal mice is a potent inhibitor of polyclonal T cell activation. Suppression is mediated by a cytokine-independent, cell contact-dependent mechanism that requires activation of the CD4+CD25+ cells via the TCR (7). It is well known that TILs and, to a lesser extent, PBLs from patients with advanced-stage cancer have a poor immune response (8). This tumor-induced immunosuppression includes diminished responses to recall antigens, decreased proliferative T-cell responses and loss of cytokine production, and defective signal transduction in T cells and natural killer cells (9). There is also evidence for increased apoptosis among CD8+ T cells in PBLs from cancer patients and mice with experimental tumors.

Several studies suggest that suppression of tumor-associated antigen reactivates lymphocytes by CD4+CD25+ regulatory T (Treg) cells (10) in cancer patients and that elevation of Treg may down-regulate tumor-specific immunity. In healthy humans, this population accounts for 5-10% of peripheral CD4+ T cells. Treg, characterized by coexpression of CD4 and CD25 markers, are thought to be a functionally unique population of T cells and function to maintain immune homeostasis (1). Of note, Treg can inhibit the immune response mediated by CD4+CD25− and CD8+ T cells because it has been reported that Treg play an important role in preventing allograft rejection, graft-versus-host disease, and autoimmunity. The observation that Treg-depleted mice develop a broad range of autoimmune diseases suggests that this T cell subset plays a crucial role for the control of T cell-mediated immunity (11).

In addition, patients and experimental models with cancer showed that Treg down-regulates the activity of effector function against tumors, resulting in T-cell dysfunction in cancer-bearing hosts. These observations have led to the hypothesis that tumor-bearing hosts with advanced cancers have an increased population of Treg, which might inhibit the tumor-specific T-cell response. In fact, an increased population of Treg has been reported in patients with ovarian cancer, lung cancer, and breast cancer (12, 13). Regulatory T cells that recognize organ-specific antigens are attracted to the involved organ, are re-stimulated by their target antigen, and mediate suppression. Suppression may be mediated by the production of suppressor cytokines in the target organ with bystander suppression of the effector cells or suppression may be mediated by direct cell-cell contact of the suppressors with the effectors (14).

Notably, removal of regulatory T cells can also evoke effective antitumor immunity in mice injected with syngeneic cancer cells. Therefore, depletion of Treg may enhance the anti-tumor immunity of host, but the pathogenic and mechanistic relationship between cancer and Treg is still unclear (15). The aim of this study was to evaluate whether CD4+CD25+ Treg cells in HCV chronic liver and HCC patients exhibit an expanded Treg pool and to correlate it with liver diagnostic investigations and tumor grading.

Materials and Methods

Blood samples were collected from 20 patients with chronic hepatitis C (mean age: 50±9.9 years) and 15 patients with HCC (mean age: 60±8.3 years). Alpha-feto protein and HCV positive antibodies were detected by EIA and immunoblotting techniques confirmed by RT-PCR. The HCC group grades were categorized into grade I, II, III. We consecutively recruited all asymptomatic patients who showed an increase of 1.5 times or higher in aminotransferases levels, as recorded at least twice during 6 or more months, and those who had no major contraindication to liver biopsy. The histologic grading was classified according to the criteria of the METAVIR Cooperative Study Group they subclassified into 3 grades I, II, III. Ten blood samples from normal control subjects (mean age: 37±7.4 years), who were negative for anti-HCV antibodies were also included in this study.

Laboratory investigation

All studied groups were subjected to: 1) liver function tests: ALT, AST, GGT, total protein, alkaline phosphatase, direct and indirect bilirubin (CX5 auto-analysers); 2) alpha-feto protein tumor marker: Enzyme Immune Assay (EIA) (Can-Ag kit); and 3) evaluation of Treg in peripheral blood mononuclear cells (PBMCs) from an adequate number of HCC patients and correlating them with tumor burden.

Flow cytometric analysis

To determine the Treg cell phenotype percentage, three- and four-color flow cytometry of whole blood
or isolated CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells was performed using the following antibodies: anti-CD4; anti-CD11a; anti-CD25; anti-CD45RA; and IgG1-isotype control (either FITC-, PE-, peridinin chlorophyll protein-; all purchased from BD PharMingen). For whole blood staining, 50 µl of whole blood was incubated with appropriate amounts of fluorochrome-labeled antibodies in the dark at room temperature for 30 min, washed once, and analyzed. To compare the phenotype of recently in vitro activated T cells with the CD4⁺CD25⁺ population, 1 ml of whole blood was stimulated with 10 ng/ml PMA (Sigma) and 0.5 µg/ml Ionomycin (Sigma) for 48 h before FACS analysis. Flow cytometry was performed on a Becton Dickinson FACS Calibur and Cell Quest software was used for analysis.

**Statistical analysis**

Data was statistically analyzed using SPSS (Statistical Package for Social Science) program version 11.0. Chi-square test was done for qualitative variable analysis and P<0.05 was considered significant. Fischer’s exact test for 2x2 tables was used when expected cell count of more than 25% of cases was less than 5 and P<0.05 was considered significant.

Student’s t-test was done for normally distributed quantitative variables to measure mean and standard deviation and P<0.05 was considered significant. ANOVA test was done to compare three variables: one quantitative variable and the other two were quantitative variables of normally distributed variables and P<0.05 was considered significant. Further, Mann-Whitney test was done for quantitative variables which were not distributed normally and P<0.05 was considered significant.

**Results**

Both CLD and HCC groups were 80% positive for HCV while only 20% of CLD and 11% of HCC patients were positive for HBV (Table 1). The mean percentage of CD4⁺CD25⁺ T cell population demonstrated a highly significant increase in comparing HCV to HCC patients [2.47±0.66 vs. 8.96±1.38 (P<0.001)] and when comparing both group to controls [1.15±0.5 (P<0.01)]. Nine HCC patients were in grade II while 6 were in grade III. Their mean CD4⁺CD25⁺ T cells percentage was 9.12±1.52 and 8.73±1.33, respectively. A negative correlation were found between mean CD4⁺CD25⁺ T cells percentage and AFP serum level in HCC patients group (r=-0.923) while T regs with patients tumor grades (II and III) (r=0.474 & 0.582, respectively). CLD patients showed a significant correlation with AFP level (r= 0.962).

**Discussion**

Since the function of Treg cells in solid tumors has sparked interest, the present study investigated the evidence related to the prevalence of Tregs in HCV and HCC. We showed a significant increase in the population of CD4⁺CD25⁺ T cells in the peripheral blood of patients with HCV and HCC in comparison with healthy individuals. Ichihara et al. (16) reported that there is no clear evidence for the mechanism of induction of Tregs in cancer-bearing hosts. There are several possibilities, including specific expansion of Tregs induced by cancer-derived factors, or physiological defense phenomena against the continuous inflammation induced by cancer. Moreover, Somasundaram et al. (17) added that, so far, little is known about the antigen-specificity of human Treg cells. Ligand-specific activation and cell-to-cell contact are required for Treg cells to exert suppressive activity, suggesting that the presence of tumor-specific Treg cells at tumor sites may have profound effects on the inhibition of T-cell responses against cancer. In a second study, a CD4⁺CD25⁺ Treg-cell line was established from a patient with colorectal carcinoma. This T-cell line was tumor-cell dependent in its growth but did not lyse autologous...
tumor cells and suppressed proliferative responses of allogeneic lymphocytes and autologous CTLs as well as the induction of CTLs from autologous PBMCs. These effects were mediated by TGF-β and did not require cell-to-cell contact, which would be in line with induced regulatory capacity. Clearly, further work is needed to understand how the enrichment of T\textsubscript{reg} cells in cancer patients occurs and if accumulation or preferential induction of clonal, oligoclonal, or polyclonal tumor-specific T\textsubscript{reg} cells plays a role during tumor progression.

Murine models have established that selective elimination of T\textsubscript{reg} cells alone or in combination with other treatment options might induce regression of already established tumors. First pilot studies have been initiated in cancer patients to selectively eliminate T\textsubscript{reg} cells. A promising and specific approach might be targeting of CD25 on the surface of T\textsubscript{reg} cells (16). Danull \textit{et al.} (18) used IL-2 diphtheria toxin conjugate DAB (389) IL-2 (denileukin diftitox) to selectively eliminate CD25-expressing T\textsubscript{reg} cells from the PBMCs of cancer patients without inducing toxicity on other cells that expressed CD25 at only intermediate to low levels. This first clinical study specifically eliminating T\textsubscript{reg} cells has shown promising results that need to be further evaluated. Alisa \textit{et al.} reported that identification and characterization of subsets of CD4\textsuperscript{+} T cells that recognize an epitope within the AFP sequence (AFP\textsubscript{46-55}) and tumor antigens may contain epitopes which activate the expansion of inducible regulatory T cells, leading to evasion of tumor control.

However, in our study T\textsubscript{reg} cells which are a subset of T cells revealed a different behavior. A negative correlation was found between mean CD4/CD25\textsuperscript{-} T cells percentage and AFP serum level in HCC patients group (r=-0.999). While CLD patients group showed a significant correlation with AFP level (r=0.770). These data explain of T\textsubscript{reg} that in CLD patients group might add to the immune inhibitory that exit with the approach of tumor development and carcinogenesis. The present study leads to the observations and provide additional insight into the regulatory mechanisms responsible for immunosuppression in human cancer, which facilitates local tumor growth and metastasis. Metastasis often represents the fatal step during the course of malignancy; T\textsubscript{reg} were correlated significantly with patients' tumor grades (II and III) (r=0.474, 0.582). They were the tumor grades which were enhanced by the suppression of immunosurveillance mechanisms. Moreover, T\textsubscript{reg} may also negatively impact the effectiveness of immunotherapies.

Current attempts at immunotherapy for cancer, including cancer vaccination or adoptive transfer of T cells, remain limited in their effect on the regression of established tumors. Even if the effective CTLs are transferred adoptively to the patients or tumor-specific CTLs are generated by tumor vaccination, there are several mechanisms by which tumor cells can escape from tumor-specific T-cell surveillance in the tumor microenvironment, as described above (19). The presence of factors such as, oxidative metabolites, or immunosuppressive cytokines can be predicted to rapidly shut off the effector functions of CTLs. Here, the increased population of T\textsubscript{reg} could be an additional problem to be resolved in immunotherapy for cancer. A better understanding of the underlying mechanism of T\textsubscript{reg} regulation or of the strategy for controlling T\textsubscript{reg} may lead to more effective immunotherapy for cancer (16).

Wolf \textit{et al.} (15) in their study mention that the depletion of T\textsubscript{reg} may become a successful anticancer strategy. In a mouse model, the efficacy of a therapeutic vaccine against melanoma was substantially improved by depletion of T\textsubscript{reg}. They concluded that manipulation of T\textsubscript{reg} in terms of their frequency and functional activity should be added to the therapeutic armamentarium for enhancing tumor immunity in humans. Taken together, these data have established the concept of increased T\textsubscript{reg} cells in solid tumors. An important aspect of future studies would be to clarify and describe an immune therapy by deleting T\textsubscript{reg} cells as a porter of immune inhibition. However, further studies are needed to validate more specific markers as well as more sophisticated and standardized functional assays.

Conclusions

Tumor specific T\textsubscript{reg} cells may limit the efficacy of anti-tumor response. T\textsubscript{reg} cells correlate properly with the unique marker AFP and with tumor grades. Better understanding of the underlying mechanism of T\textsubscript{reg} regulation or of the strategy for controlling T\textsubscript{reg} may lead to more effective immunotherapy for HCV and for enhancing immunity against cancer.

References

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