IDO in Human Gut Graft-versus-Host Disease

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Although rodent graft-versus-host disease (GVHD) models have suggested that indoleamine 2,3-dioxygenase (IDO) is a critical regulator of gastrointestinal GVHD, parallel human studies on IDO expression have not been reported. IDO expression was assessed in 20 patients who underwent duodenal biopsy. IDO was upregulated in epithelial cells. In situ analyses reveal that macrophages and dendritic cells stain positive for IDO, but that most of the IDO^+ cells were a novel population of $CD3^+CD4^+IDO^+$ cells. The proportion of $CD4^+IDO^+$ T cells was significantly higher in patients with moderate GVHD. In situ regulatory T cell and Th17 numbers correlated with overall severity. Although needing confirmatory results from larger sample sets, these data are consistent with the hypothesis that IDO is involved in regulating gastrointestinal GVHD.

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INTRODUCTION

Wider application of allogeneic hematopoietic stem cell transplantation is restricted because of graft-versus-host disease (GVHD). The environmental effects that influence GVHD injury and the natural regulatory mechanisms by which GVHD is controlled at the tissue level are incompletely understood [1].

Indoleamine 2,3-dioxygenase (IDO) catalyzes the first and rate-limiting step of tryptophan catabolism. Decreased tryptophan and/or increased metabolite concentrations elicit T cell anergy or apoptosis (reviewed in [2]). Several aspects of IDO biology make it an intriguing target for transplantation and tolerance (reviewed in [3]).

Previous experimental GVHD models in rodents by us [4,5] and others [6-8] dissected the role of IDO in GVHD. We previously reported [4,5] that: (1) IDO is a critical regulator of GVHD, most strikingly in the colon; (2) IDO can act at the site of colon expression to decrease T cell proliferation and survival, diminishing inflammation and reduce disease severity; and (3) host IDO expression in both epithelial cells and antigen-presenting cells affected GVHD severity. In humans, only 1 in vitro study reported IDO expression by reverse transcription-polymerase chain reaction of monocytes obtained from acute GVHD (aGVHD) patients [9].

Herein we report the first study that assayed IDO protein expression in patients with gastrointestinal (GI) GVHD.

PATIENTS AND METHODS

Patients

Biopsies were performed as diagnostic procedures for digestive symptoms [10]. The Hospital Saint-Louis ethical review board approved the design of this study. A cohort of allografted patients (total n = 20) was studied after myeloablative conditioning. Their median age was 34 years. Samples were obtained from: 8 patients (7 males, 1 female; median age 30 years) without pathologic GI GVHD (grade 0-1); 5 patients (2 males, 3 females; median age 47 years) with pathologic GI GVHD grade 2, and 7 patients (5 males, 2 females; median age 34 years) with pathologic GI GVHD grade 3 to 4. All patients underwent biopsy before being treated with steroids and thus only received cyclosporine as GVHD prophylaxis. Five nontransplanted patients, with active *Helicobacter pylori* infection, were used as controls.

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Methods

Duodenal biopsies during fiber-optic examination were performed, as described [10,11]. All biopsies were performed before any steroid treatment. The histologic grading of aGVHD was according to Sale [12] and Epstein [13]. None had evidence of digestive or systemic viral, bacterial, or fungal infections.

Immunostainings were performed with Ventana Discovery reagents (Ventana, Phoenix, AZ). For IDO staining, polyclonal rabbit antibody (gift from D.M.; dilution 1:500) was used and detected using a DabMAP kit. Specificity of IDO staining was confirmed by incubation of sections with neutralized antibody (IDO antibody incubated with blocking peptide for 2 hours at 4°C before incubation with sections).

Double immunofluorescent staining was performed on frozen sections for IDO/CD4, IDO/CD68, IDO/CD3, IDO/CD123, Foxp3/CD4, and IL-17/CD4. Regulatory T cell (Treg) and Th17 double stainings were performed as described [14]. Additional staining was performed using anti-CD68 (clone KP-1, DAKO, Carpenteria, CA), anti-CD3 (Biocare, Chicago, IL), and anti-CD123 (clone 6H6, Biolegend, San Diego, CA) antibodies. Endogenous peroxidase inhibition and nonspecific binding sites blocking were systematically performed. Controls with irrelevant isotypic antibodies, and absence of primary antibodies were systematically performed.

Antibodies were covalently linked to Alexafluor 488 or Alexafluor 594 using APEX Antibody Labeling Kits (Invitrogen, Carlsbad, CA). Sections were incubated in PBS pH 7.4 containing 5% bovine serum albumin for 30 minutes at room temperature. Double immunofluorescent stainings were performed by incubation with antibodies applied to sections for 1 hour at room temperature. Sections were finally mounted in Vectashield medium containing 4′,6-diamidino-2-phenylindole. Cell counts and proportions were expressed as median and interquartile range of the number or proportion of cells per field at 400× magnification.

Samples from patients were identified by anonymous 7-digit codes corresponding to laboratory identification. Biopsies were independently evaluated by 2 examiners (P.R. and A.J.). In all cases of disagreement between examiners, a common reading was organized to achieve a consensus on count. For automated counts of epithelial cells expressing IDO, regions of interest corresponding to epithelium and excluding infiltrated cells were independently defined by 2 pathologists (P.R. and A.J.) using CellSens software (Olympus, Rungis, France). For each patient, the average number of epithelial cells expressing IDO was determined on 5 different fields at 400× magnification. Automated counts of $CD4^+$ lymphocytes expressing IDO were performed using CellSens software (Olympus) on double immunolabeled sections with CD4 and IDO. For each patient, the average number of $CD4^+$ lymphocytes expressing IDO was determined on 5 different fields at 400× magnification.

Statistical Analyses

Reproducibility of counts was assessed through examination of discrepancy levels between the examiners and intraclass correlation estimates with 95% confidence intervals. The median level and range of CD4, CD8, CD68, $CD4^+IDO^+$, $CD68^+IDO^+$, Treg, and Th17 characteristics (cell numbers or their ratios) are presented. For the comparison on cell count or proportion of Treg, Th17, $CD4^+IDO^+$, $CD68^+IDO^+$, $CD4^+$, $CD68^+$, IDO^+ epithelial cells between the groups without GVHD (grade 0-1) and mild GVHD (grade 2), between the groups with mild GVHD and severe GVHD (grade 3-4), or between the groups of GVHD and *Helicobacter pylori*, the Mann-Whitney nonparametric test was used. For the correlation between $CD4^+IDO^+$ proportion and Th17 or Treg proportion, a comparison was performed using *Z*-test.

RESULTS AND DISCUSSION

Figure 1A shows expression of IDO in duodenal intestinal cells with a morphology and location consistent with epithelial cells, which was up-regulated in specimens obtained from patients with GVHD compared with those without GVHD. IDO expression in the epithelial cells of nontransplanted controls with *Helicobacter pylori* infection was also detected. These data are consistent with those in rodents and suggest that IDO is up-regulated during the GVHD process.

In addition to IDO expression in epithelial cells in the duodenum of patients with GVHD, IDO-positive mononuclear cells (MNCs) also were readily detected. The specificity of the staining was proven by a blocking peptide (Figure 1A). Double staining surprisingly revealed that most of these IDO^+ MNCs were double-positive $CD4^+IDO^+$ cells. Few MNCs were macrophages ($CD68^+$IDO that were $CD4^-$ (Figure 2A) or $CD4^+$ plasmacytoid dendritic cells [DCs] [$CD123^+IDO^+$] (Figure 2B)). Instead, $CD4^+IDO^+$ coexpressed CD3. Thus, the dominant source of IDO in MNCs was $CD4^+$ T cells (Figure 2B).

We sought to correlate the number of $CD4^+IDO^+$ T cells with other T cell subsets and with the severity of GVHD pathologic lesions. As described in Figure 3A and B, there was no statistical correlation between $CD4^+IDO^+$ T cell numbers and Treg cell subsets, although there was a statistical correlation between CD4/IL-17 and Treg cells with pathologic grade of aGVHD, as we have previously described [14]. Moreover, we found a positive correlation ($P = .03$) between the proportion of CD4/IL-17 and CD4/IDO lymphocytes. The proportion of $CD4^+IDO^+$ cells