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Proteomic Analysis Reveals Innate Immune Activity in Intestinal Transplant Dysfunction

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Abstract

Background—Many patients with intestinal failure require intestinal transplantation (ITx) to survive. Acute cellular rejection poses a challenge in ITx because its biologic components are incompletely understood. New methodologies for its integrative and longitudinal analysis are needed.

Methods—In this study, we characterized episodes of acute cellular rejection in ITx recipients using a noninvasive proteomic analysis. Ostomy effluent was obtained from all patients undergoing ITx at University of California, Los Angeles from July 2008 to September 2009 during surveillance endoscopies in the first 8 weeks post-ITx. Effluent was analyzed using 17-plex Luminex technology and matrix-assisted laser desorption/ionization proteomics.

Results—Of 56 ostomy effluent samples from 17 ITx recipients, 14% developed biopsy-proven rejection at a median of 25 days post-ITx. Six had mild rejection, two were indeterminate for rejection, and no graft loss was seen in the first 3 months posttransplantation. Effluent levels of five innate immune cytokines were elevated in the posttransplantation phase: granulocyte colony-stimulating factor, interleukin-8, tissue necrosis factor- α , interleukin-1 β , and interferon- γ . Proteomic analysis revealed 17 protein features differentially seen in rejection, two identified as

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human neutrophil peptide 1 and 2. This was confirmed by the presence of human neutrophil peptide-positive lamina propria neutrophils in biopsy tissue samples.

Conclusions—Proteomic and cytokine analysis of ostomy effluents suggests an early and unappreciated role of innate immune activation during rejection.

Keywords

Intestinal transplantation; Rejection; Proteomics; Innate immunity

Intestinal transplantation (ITx) offers a potential way of survival for patients with intestinal failure who have developed life-threatening complications (1). Overall, patient and graft survival rates have improved significantly over the past several years (2, 3). Nonetheless, acute cellular rejection (ACR) continues to pose major challenges in these patients, occurring in up to 50% of recipients and accounting for the majority of grafts loss. Currently, endoscopic biopsy is the only way for clinical assessment of ACR (4). Limitations of endoscopy include that it is invasive and associated with risks of bleeding, infection, or perforation. In addition, ACR is also often patchy (missed by ~30% of endoscopies) (5), and confounded by its presentation, which may be similar to infection. This creates challenges to both clinical management and biologic research (6). New noninvasive techniques to analyze ACR are thus needed to further advance the ITx field.

The intestine is the largest immune organ in the body, with 80% of the total immune cells (7). Study of ITx recipients is further complicated by the presence of an immunogenic chimeric donor cell population (8, 9), and the underlying biology of ACR remains unclear. Recently, an underappreciated role of the innate immune system has been suggested in solid organ transplantation with evidence from both mouse models and human studies (10). Upregulation of proinflammatory mediators has been shown to occur independently of adaptive immunity, even before the T-cell response (11–14). Moreover, the profile of this early cytokine upregulation suggests it might be due to the epithelial stress response to tissue injury (12, 15).

High-throughput proteomics allows simultaneous detection of a panel of proteins, which are differentially expressed in healthy and disease states. This technology has been successfully applied to profile proteins from different forms of clinical specimens (16), and protein combinations provide potentially innovative biomarkers which are useful in diagnosis or disease monitoring. In the search of biomarkers for ACR, various metabolites and proteins have been suggested as potential biomarkers for allograft monitoring in both human and mouse study (6, 17, 18).

The purpose of this study was to evaluate the feasibility of using protein profiles of ostomy effluents as a strategy to monitor ACR, in particular those related to innate immune activation, which may represent a precondition of the graft for rejection. We used both high-throughput proteomic analysis and candidate immunoassay protein detection of ostomy effluents to search in a noninvasive manner for molecular profiles of epithelial stress and innate immunity during ACR. Our analyses uncovered several proteins that appeared to differ among patients with early ACR, notably the elevation of antimicrobial peptides and cytokines characteristic of early innate immune activation.

RESULTS

Patient Demographics and Sample Collection

During the study interval, 16 patients received 17 ITx. One patient had to receive a second transplant, which was counted as an independent ITx case. Recipient demographics included

the following: 16 children (<21 years old) and 1 adult; 12 combined liver and intestinal grafts, and five isolated intestinal grafts. Four children developed ACR, which was diagnosed at a median of 25 days (6 – 47 days) after ITx. Within the rejection patients, one had four episodes, one patient had two episodes, and two patients had one episode each, and no graft loss was seen in the first 3 months post-transplantation. Of the eight cases, six were diagnosed as mild, and two had indeterminate rejection. Complete resolution of rejection was achieved by treating with a steroid pulse in all cases except one. One patient with recurrent mild rejection despite steroids received plasmapheresis.

Ostomy effluent was collected from the ostomy pouch for the hour before all endoscopic procedures in the post-transplant phase. The amount of effluent collected ranged from 1 to 30 mL. Those samples obtained immediately post-transplantation often totaled less because patients in general had small outputs at that time. Endoscopy was performed weekly beginning on postoperative day 6 to 14 for the first 3 to 4 weeks, then once every 2 weeks until 8 weeks after ITx. When patients presented with clinical symptoms indicating abnormal allograft function, additional endoscopy was performed as needed. In total, 56 ostomy effluent samples were collected from 17 recipients longitudinally along the 8-week posttransplant time course. Each effluent specimen was matched with the results of the biopsies taken at the same time.

Luminex Analysis of Ostomy Effluents

Seventeen cytokines representing both innate and adaptive immunity were evaluated by Luminex assay. Of the 17, only 5 had detectable levels: interferon (IFN)- γ , interleukin (IL)-1 β , IL-8, tissue necrosis factor (TNF)- α , and granulocyte colony-stimulating factor (G-CSF) (Fig. 1A), all of which are commonly produced by innate immune activation. Of these five, only IFN- γ levels were statistically significantly higher in samples with rejection ($P=0.01$; Fig. 1B). IFN- γ levels did not fluctuate with the number of days posttransplant (Fig. 1C), whereas the other four cytokines were all higher in the immediate posttransplant period and gradually decreased with time. IL-8 was shown as a representative example (Fig. 1D). Consistent with reports that IL-8 can be reduced by steroid treatment, IL-8 also decreased after steroid treatment for rejection (Fig. 1E).

Proteomic Analysis of Ostomy Effluents

To comprehensively analyze ostomy effluent proteins, each sample was evaluated by high-resolution matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometer (MS). Four hundred fourteen protein features were selected for further evaluation. Using mixed-effect model analysis, 17 protein features showed significant differences between the rejection and nonrejection groups with P less than 0.05. Two of the three features with the smallest P values for the rejection effect had masses of 3442 and 3775 Da ($P<0.001$ and $P=0.001$, respectively). These two peaks were identified by immunoprecipitation-MALDI as human neutrophil peptides (HNP) 1 and 2, respectively (Fig. 2A,B). As neutrophil influx and HNP expression could be related to infectious enteritis, we compared HNP levels between groups with infection and without infection. The difference was not significant ($P=0.65$ for HNP 1, $P=0.98$ for HNP 2), supporting the association of HNP elevation in ITx patients with rejection-associated innate immunity.

HNPs are part of the human defensin family, which is a major component of antimicrobial peptides. Another member of the antimicrobial peptide family human α -defensin 5 (HD5), corresponding to a peak with a mass of 3583 Da, was also detected in most samples and confirmed by immunoprecipitation-MALDI. HD5 was not statistically different between the rejection and nonrejection groups (Fig. 2C). Similar to other innate cytokines described

previously, all three defensins were elevated early in the posttransplant stage and gradually decreased in the following weeks (Fig. 3A–C).

Confirmation of Elevated HNPs in ACR

Enzyme-linked immunosorbent assay (ELISA) was used to confirm the existence of HNPs in the ostomy effluent. The ELISA data and MALDI data were strongly correlated (Fig. 4A), with the HNP levels higher in rejection samples using ELISA methodology (Fig. 4B). Immunohistochemistry (IHC) was also performed on 37 biopsy sections from patients with biopsy-proven rejection and nonrejection to identify the HNP-expressing cell type in the intestinal mucosa during the rejection episode (Fig. 5). As expected, samples with biopsy-proven rejection demonstrated prominent HNP-positive lamina propria cells (Fig. 5A,C). These were identified as neutrophils by hematoxylin-eosin staining (H&E) morphology (Fig. 5E insert box), and these cells were unapparent in nonrejection samples (Fig. 5C). By evaluating three sections of 10 crypts per biopsy slide, we calculated the average numbers of HNP-positive crypts per 10 crypts for each biopsy (Fig. 5G). The difference between rejection and nonrejection groups was significant with P less than 0.0001. Based on our calculation, we decided three positive crypts per 10 crypts would be used as a reasonable threshold for HNP-positivity in IHC staining. Using this criteria, ACR specimens were 100% (8/8) HNP positive, whereas nonrejection samples were 17% (5/29) HNP positive ($P < 0.001$).

DISCUSSION

ITx is the standard of care for patients with life-threatening complications due to intestinal failure. In post-transplant phase patients frequently develop graft dysfunction, manifested by the acute onset of diarrhea or fever. The biologic elements leading to ACR in ITx are incompletely understood, and their delineation may be useful for early diagnosis and new strategies for clinical management. We used ostomy effluents, multiplex cytokine assay, and high-throughput proteomics analysis as a noninvasive biomaterial and biologically robust technology for longitudinal assessment of ITx patients. This was done in response to the unmet need for understanding the mechanism and determining biomarkers of rejection. In this study, we found that increased proinflammatory cytokines and HD5 are detectable in the ostomy effluent early posttransplant and progressively decline over a period of 8 weeks irrespective of the occurrence of rejection. More interestingly, HNP 1 and 2 are increased at the time of biopsy-diagnosed rejection and this is associated with increased neutrophils in the intestinal crypts. These results support the hypothesis that early “innate” tissue injury is an important component of the early graft rejection response.

Transplantation is a complex immunological state in which there is constant interaction between the graft and the recipient's immune system (19). Epithelial cells play an active role in mucosal immune activity by secretion of innate immune cytokines in response to microbial or metabolic stimuli, resulting in the molecular stress response (15, 20). The transplantation event is a major metabolic stress to the intestinal graft, putting the epithelium at risk for a molecular stress response. Consistent with this, four innate immune cytokines (TNF- α , IL-1 β , IL-8, and G-CSF) typical of the epithelial stress response were detectable in ostomy effluents early in the posttransplant period, declining thereafter. IFN- γ remained at high levels throughout the posttransplant phase, perhaps reflecting its probable origin from CD8⁺ T cells and natural killer (NK) cells (21), which are physiologic lamina propria residents and constitutively express IFN- γ (22). More interestingly, IL-8, TNF- α , and IL-1 β (and other epithelial stress response chemokines) are chemotactic for neutrophils and induce both neutrophil and Paneth cell-derived defensins (23, 24).

Paneth cells are another critical cellular component of innate immunity, and make up part of the epithelial barrier at the base of intestinal crypts. Impairment of Paneth cell-derived defensin, notably HD5, is a feature of genetic susceptibility in Crohn's disease (25), a disease with some clinical and pathologic features of intestinal rejection (26). In ITx recipients with ACR, we observed that HD5 levels were higher immediately posttransplant (Fig. 3C), and also after treatment for rejection in two patients (data not shown), perhaps due to steroid induction of HD5 in maturing Paneth cells (27). Along with other animal model studies, our study suggested that restoring HD5 is important for improving mucosal barrier function and protection from deleterious microbial commensals (28–30).

Several innate cell populations have been indicated to play critical roles in animal ACR models, including NK cells (21, 31), dendritic cells (32, 33), and neutrophils (34–36). Neutrophil-derived anti-microbial peptides are known not only to contribute to oxygen-independent microbial cytotoxicity, but also serve as chemotaxins for macrophages, dendritic cells, and T lymphocytes (37, 38). In our study, HNPs were elevated in ITx recipients with rejection as an early graft feature posttransplant (Figs. 2A,B and 3A,B). Fecal calprotectin, another neutrophil-derived protein, has also been reported to be elevated in patients with rejection (6). Neutrophils mediate tissue damage by an array of cytotoxic and pro-inflammatory mechanisms (34). Thus, neutrophils may participate in the balance of transplant integrity both through microbial protection during and after injury (3), and paradoxically by potentially augmenting adaptive immunity and exacerbating rejection. To our knowledge, this is the first human study of the function and distribution of neutrophils in ACR patients.

Although high-throughput MALDI-MS technology allows for the detection of hundreds of proteins simultaneously, there are several limitations that preclude its direct clinical use. It is expensive, time consuming, and requires proficiency in proteomics for sample analysis. In addition, interpretation of MALDI-MS data requires bioinformatics analysis and identification of peptides/proteins. Accordingly, molecules identified by MALDI-MS need development of conventional assays for clinical applications. For this reason, we performed ELISA to validate HNPs in ostomy effluent samples, and evaluated the correlation of ELISA measurements with the proteomic data. The results showed excellent correlation between the two modalities, suggesting that a simple immunoassay (e.g., ELISA) test of ostomy effluent could potentially complement sequential biopsies for ACR diagnosis and monitoring in clinical application.

This study is unique in using ostomy effluents (rather than biopsies or serum) as a biospecimen to delineate ACR in human ITx recipients. We emphasize that this study was not designed for transplant rejection biomarker validation. Preliminary results of biomarker discovery have been promising, but the dataset has been small due to many confounding demographic and clinical variables, and there is a dearth of preliminary data to estimate the sample size (19). Also, with only 200 ITx performed per year worldwide, a larger multicenter study and the use of less complex protein quantitation methods is necessary for suitable clinical monitoring. However, this study reveals an early and underappreciated role of innate immunity in ACR, and suggests that monitoring the HNP levels in ostomy effluents has the potential to permit quantitative assessment of ITx in a noninvasive manner.

MATERIALS AND METHODS

General

Institutional review board approval was obtained in compliance with the Health Insurance Portability and Accountability Act guidelines, for this study including all patients undergoing ITx between July 2008 and September 2009 at the Dumont-University of

California, Los Angeles (UCLA) Transplant Center. Demographics, type of transplant, endoscopic procedure, postoperative immunosuppression, feeds, infectious status, and histopathology were recorded. ACR was defined according to the consensus statement that was developed at the 8th International Small Bowel Transplant Symposium in 2003 (4).

Immunosuppression Regimens

Our standard immunosuppression regimen included induction with an IL-2 receptor antagonist such as daclizumab (Zenapax, Roche Laboratories, Nutley, NJ) or rabbit antithymocyte globulin (Thymoglobulin, Genzyme, Cambridge, MA). Maintenance therapy included tacrolimus (Prograf, Fujisawa USA, Deerfield, IL), corticosteroids, and mycophenolate mofetil (CellCept, Roche Laboratories, Nutley, NJ). Target tacrolimus trough levels were 10 to 18 ng/mL. ACR was diagnosed by histopathology and treated with high dose steroids and antithymocyte globulin. In sensitized patients or patients suspected of antibody mediated rejection, a combination of Rituximab 375 mg/m² (2) for one dose, plasmapheresis for 5 days, and immune globulin (Gammagard, Baxter, Westlake Village, CA) 1 g/kg was used both pre- and posttransplant.

Intestinal Surveillance

Protocol endoscopy and biopsy of the transplanted intestine were obtained once weekly beginning posttransplant day 6 to 14 for the first 3 to 4 weeks, then once every 2 weeks until 8 weeks after the operation. Endoscopy was also performed, and biopsies obtained, when patient presented with clinical symptoms, such as diarrhea or fevers, indicating abnormal allograft function. Multiple biopsies from various portions of the transplanted graft were obtained, and paraffin-embedded sections stained with H&E were graded for ACR using standard rejection criteria (4) by UCLA gastrointestinal pathologists. Abnormal results prompted further endoscopic surveillance as indicated.

Ostomy Effluent Collection and Proteomic Evaluation

Ostomy effluents were freshly collected from the ostomy pouch an hour before all endoscopic procedures. The effluent specimens were immediately placed on ice, centrifuged at 2000 rpm for 7 min at 4°C, and the supernatant were stored at -80°C for batch processing at the UCLA High Throughput Clinical Proteomics core laboratory. The detailed procedure for sample processing and proteomic evaluation is illustrated in the supplemental material (see Supplemental Digital Content 1, <http://links.lww.com/TP/A421>). Briefly, each sample was processed identically for protein extraction, fractionation, and MALDI-MS analysis. Proteomic data were analyzed by various statistical methods.

Luminex Evaluation of Cytokines

An aliquot of protein extraction from ostomy effluent was used for Luminex evaluation, with the Bio-Rad human cytokine 17-plex assay (G-CSF, granulocyte-macrophage-CSF, IFN- γ , IL-10, IL-12, IL-13, IL-17, IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, MCP-1, MIP-1 β , and TNF- α) according to the manufacturer's instructions (Bio-Rad, Hercules, CA). All samples were mixed with a matrix of 2 mg/ml bovine serum albumin, 2 mM phenylmethylsulfonyl fluoride, 20 mM ethylenediaminetetraacetic acid and 1% Triton X-100 in equal volume to the sample volume. This same matrix was used to reconstitute one tube of cytokine standard. Data were acquired and analyzed on a Bio-Plex 200 system (Bio-Rad, Hercules, CA), and cytokine levels were normalized to protein concentrations to account for the different protein amount in each sample.

ELISA and IHC of HNPs

The total amount of HNP1 to 3 in protein extractions were quantified in duplicates by an ELISA kit following manufacturer's instructions (Cell Sciences, Canton, MA), using a standard curve constructed for each plate. Protein concentrations were also determined by BCA assay for each sample, and HNP1 to 3 levels were normalized to total protein (pg/mg).

Standard IHC procedures were used on biopsy sections (5 μ m). Each section was incubated with anti-HNP 1 to 3 antibody (rabbit, 1:2000) or preimmune serum (rabbit, 1:2000) as negative control. Anti-Rabbit IgG biotinylated antibody was used as secondary and streptavidin-peroxidase conjugated antibody was used as tertiary (Vector Laboratories, Burlingame, CA), according to the manufacturer's protocol, and the sections were developed by diaminobenzidine tetrahydrochloride substrate (Pierce, Rockford, IL). For quantitation, 10 crypts in 3 different fields on 1 biopsy slide were evaluated for positivity of HNP staining. The number of HNP-positive crypts per 10 crypts was counted and averaged.

Statistics

All data analyses were conducted in R software (<http://www.r-project.org>). To correct the pseudoreplicate effect from repeated sampling over time and the noisy nature of the human study, a mixed-effects model (package nmle) was used to evaluate each protein feature in proteomic or Luminex data. For the initial screening of the protein candidates associated with rejection, we set subject level as the random effect in the mixed-effect model, and the fixed effects included rejection status, posttransplant days, type of transplant, immunosuppressant, feeds, infection, and age. For confirmation of the significance of the HNP in rejection, we only included posttransplant days and rejection status as fixed effects, and subject level as random effect to optimize the mixed-effect model. Kruskal-Wallis test was used for group comparison in ELISA. Fisher's exact test was used to compare HNPs-positivity between rejection groups in IHC. Pearson's correlation coefficient was calculated to assess the correlation between the measurements of HNPs using ELISA and MALDI.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

Cytokine levels in ostomy effluents measured by Luminex 17-plex assay. All cytokine levels were normalized to the total protein level in each sample respectively, and log₁₀ transformed. (A) Five cytokines had detectable levels (above the assay background level indicated in *dashed line*): granulocyte colony-stimulating factor (G-CSF), interferon (IFN)- γ , interleukin (IL)-8, IL-1 β , and tissue necrosis factor (TNF)- α . Data were presented as mean $\pm$ standard error of the mean. *Gray bar*, rejection samples; *white bar*, nonrejection samples. (B) IFN- γ levels compared between rejection and nonrejection groups. Each *filled circle* represents one sample, the *solid line* in each box is the mean for each group. (C, D) Levels of IFN- γ and IL-8 at different times posttransplant. *Open circle*, nonrejection sample; *filled circle*, rejection sample. The trend line was drawn by linear regression; *solid line*, rejection samples; *dashed line*, nonrejection samples. (E) Relationship of IL-8 levels to steroid treatment. *Filled square*, patient C; *open square*, patient G; *filled circle*, patient J; *open circle*, patient L. *Arrows*, episodes of biopsy-proven rejection and steroid treatments. MIP, macrophage inflammatory protein; MCP, monocyte chemoattractant protein.

FIGURE 2.

Defensin levels compared between rejection and nonrejection groups. (A) Human neutrophil peptide (HNP) 1, (B) HNP 2, and (C) human α -defensin 5 (HD5). Each line represents one sample. *Gray shadow*, the distribution of the sample value. *Long bold line*, the mean of each group.

FIGURE 3.

Change of defensin levels in posttransplant stage. (A) Human neutrophil peptide (HNP) 1, (B) HNP 2, and (C) human α -defensin 5 (HD5). *Open triangle*, nonrejection sample; *filled triangle*, rejection sample. The regression lines represent the trends of the samples over time, with the top line representing the rejection samples trend.

FIGURE 4.

Validating human neutrophil peptide (HNP) detection using ELISA. (A) Correlation analysis of HNP level in ELISA and matrix-assisted laser desorption/ionization data. R^2 is calculated by Pearson's correlation analysis. (B) Comparison of ELISA HNP levels in rejection and nonrejection patients. Each *square* represents one sample. Kruskal-Wallis test was used to examine the significance of the difference ($P=0.0587$).

FIGURE 5. Representative tissue sections of (A, C, E) rejection and (B, D, F) nonrejection biopsy specimens for human neutrophil peptide (HNP) immunohistochemistry (IHC; magnification, $\times 100$). Anti-HNP1–3 rabbit serum (1: 2000 dilution) was used to detect HNP (A, B); Non-immune rabbit serum (1: 2000 dilution) was used for negative controls (C, D); H&E staining was used to differentiate different cell types (E, F). The insert in (E) highlighted the lamina propria neutrophils in the rejection sample (Magnification, $\times 1000$). (G) IHC score for HNP positivity compared between rejection and nonrejection groups. Y axis: positive crypts per 10 crypts. Each *open square* represents one sample and the *bold line* represents the mean.