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Humoral immune response to abnormal MUC1 in subjects with colorectal adenoma and cancer

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Abstract

Colorectal cancer (CRC) expresses a hypoglycosylated (abnormal) form of MUC1 different than MUC1 expressed in normal colon, which elicits antibodies in patients with CRC. This form of MUC1 is expressed in other abnormal but non-malignant lesions in the colon, such as adenomatous polyps, precursors to CRC. Estimates of the prevalence of anti-MUC1 antibodies in subjects with these lesions are lacking. We evaluated IgM and IgG anti-MUC1 antibodies in 148 subjects with non-advanced adenomas (NAA), advanced adenomas (AA), colorectal cancer, hyperplastic polyps (HPP), and normal controls. We hypothesized that the prevalence of anti-MUC1 antibodies would increase along the adenoma-carcinoma sequence as more dysplastic tissues express more abnormal MUC1. Anti-MUC1 IgM was found in 5/47 (10.6%) of normals, 5/45 (11.1%) of NAA, 7/47 (14.9%) of AA, and 4/20 (20.0%) of CRC ($p=0.70$). The prevalence of anti-MUC1 IgG was 8/47 (17.0%) of normals, 14/45 (31.1%) of NAA, 14/47 (29.8%) of AA, and 6/20 (30.0%) of CRC ($p=0.36$). We found no significant differences in the prevalence of anti-MUC1 antibodies between subjects along the adenoma-carcinoma sequence. However, in an exploratory analysis, the median normalized anti-MUC1 IgG OD level of the combined abnormal groups (NAA, AA, CRC) was significantly higher than the normals (0.045 vs. 0.030 OD; $p=0.017$). Our data support further studies into the potential role of anti-MUC1 immunity in preventing progression of premalignant colon lesions to colon cancer.

Keywords

MUC1; mucin; tumor immunity; colon adenomas; colorectal cancer

INTRODUCTION

MUC1 is a mucin that is expressed on epithelial cells of the respiratory, genitourinary, and gastrointestinal tracts (Vlad et al., 2004). Abnormal expression of MUC1, characterized by

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overexpression and hypoglycosylation (Vlad et al., 2004), is seen on the vast majority (75–100%) of colorectal tumors (Ajioka et al., 1996; Cao et al., 1997; Ho et al., 1996; Limburg et al., 2000; Percinel et al., 2007; Zotter et al., 1987). Of subjects with colorectal cancer (CRC), 20–48% have anti-MUC1 antibodies (Kotera et al., 1994; Nakamura et al., 1998). The presence of anti-MUC1 IgG in the serum has been associated with prolonged survival in pancreatic cancer (Hamanaka et al., 2003) and early stage breast cancer (von Mensdorff-Pouilly et al., 2000). Furthermore, factors other than the presence of cancer contribute to the development of MUC1 immunity and some normal individuals also have anti-MUC1 antibodies. Certain events that can induce inflammation in tissues that express MUC1 can lead to its overexpression and hypoglycosylation. Examples such as lactation and/or mastitis, pelvic surgery, mumps virus, and current smoking have been associated with the presence of anti-MUC1 antibodies in healthy women (Cramer et al., 2005; Vlad et al., 2004) with reduced lifetime risk of ovarian cancer.

Adenomatous polyps are precursors to colon cancer in the model known as the adenoma-carcinoma sequence (Vogelstein et al., 1988). Many adenomas (19–76%) show aberrant expression of MUC1 (Cao et al., 1997; Ho et al., 1996; Limburg et al., 2000; Percinel et al., 2007) and increased MUC1 expression has been associated with increasing dysplasia along the adenoma-carcinoma sequence (Cao et al., 1997; Ho et al., 1996; Percinel et al., 2007). Data on the antibody response to MUC1 in subjects with colonic adenoma are limited. Using Western blot, Nakamura *et al* reported anti-MUC1 IgG antibodies in 8/23 (35%) of Japanese subjects with colon adenomas, compared with 5/31 (16%) healthy subjects (Nakamura et al., 1998). However, the Nakamura study did not address differences between non-advanced and advanced adenomas. Furthermore, it is unclear how the results of this small study generalize to an American population, given the differences in the epidemiology of gastrointestinal cancers in Japan and the United States (Dunn, 1975; Flood et al., 2000).

The aim of this study was to estimate the prevalence of anti-MUC1 antibodies among subjects with colon polyps and colorectal cancer by examining serum from a cross-section of subjects along the adenoma-carcinoma sequence. We hypothesized that the prevalence of anti-MUC1 antibodies would increase with increasing degree of dysplasia, due to the effect of increased exposure to the tumor form of MUC1. We were especially interested in IgG antibodies as indicators that class-switching has occurred, which requires activation of MUC1-specific helper T-cells.

The second aim of this study was to estimate the prevalence of anti-MUC1 antibodies in subjects with hyperplastic polyps (HPP). In contrast with adenomatous polyps, hyperplastic polyps are not considered pre-cancerous lesions. Although hyperplastic polyps do not display increased expression of MUC1 compared with normal colonic mucosa (Biemer-Huttmann et al., 1999; Ho et al., 1996; Percinel et al., 2007), aberrant expression of other mucins has been documented (Biemer-Huttmann et al., 1999; Percinel et al., 2007). The third aim was to investigate whether demographic and clinical covariables were associated with anti-MUC1 antibody levels.

MATERIALS AND METHODS

Subject population

Serum samples (n=184) and clinical data were obtained from the Digestive Diseases Tissue Resource at the University of Pittsburgh Medical Center. All patients signed an IRB-approved consent form to donate blood for research purposes between 2003 and 2007 at the time of colonoscopy or colon surgery. Clotted blood was spun and serum collected, divided into aliquots, and frozen the same day the blood was drawn. Subjects were classified based on their most advanced histology and put into clinical groups as normal (n=47), non-advanced adenoma

(NAA; n=45), advanced adenoma (AA; n=47), CRC (n=20), or hyperplastic polyp (HPP; n=21). Four subjects were excluded from analysis: two due to insufficient information, and two due to a history of Crohn's disease.

ELISA for anti-MUC1 antibodies

Sera were tested twice for anti-MUC1 antibodies by Enzyme Linked Immunosorbent Assay (ELISA) in a blinded fashion. A synthetic peptide of 100 amino acids was made in the University of Pittsburgh School of Medicine Peptide Synthesis Facility. It contains five MUC1 tandem repeats of the sequence PDTRPAPGSTAPPAHGVTS and represents the abnormal (non-glycosylated) form of the molecule. Ninety-six well Immulon plates (Thermo Scientific) were coated overnight with 20ug/ml MUC1 100-mer peptide or 2.5% bovine serum albumin (negative control). Diluted serum (1:20, 1:40) was added in duplicate wells for 1 hour, and plates were then washed 5 times. Goat anti-human IgG or IgM (Sigma Aldrich) conjugated to alkaline phosphatase was added to the wells for 1 hour and then washed. The substrate p-nitrophenyl phosphate (Sigma Aldrich) was added to all wells and incubated for 30 minutes before stopping the reaction with NaOH. Absorbance was read on a spectrophotometer (Thermo Labsystems MRX Revelation) at 405 nm. A positive control (serum from a subject vaccinated with the same MUC1 peptide with known titers of anti-MUC1 IgM and IgG) was included on each plate.

Statistical analysis

The optical density (OD) value reported is the average of the duplicate MUC1 wells minus the average of the duplicate background control wells, divided by the positive control on that plate to account for inter-assay variability. The second trial was normalized to the first trial using a smooth-curve normalization procedure (Cleveland, 1979). Repeat trials of the ELISA were highly correlated, even at very low OD levels; thus, the assay was reliable (Supplemental Figure).

Because there is no anti-MUC1-positive reference serum from unimmunized controls available, the following procedure was used to select an appropriate cutoff for "positive" in a blinded fashion: a) the median of the pooled averaged data for all five groups was determined (the median rather than the mean was used because data are highly skewed); b) the median was subtracted from the data to yield the quantity K; c) large values of K (obvious outliers) were eliminated; d) the median value of K was determined for positive K only; e) the cutoff was set to 3 times this value. This procedure was then applied to the normal group to determine the value of the cutoff. If the data were normally distributed, this would be equivalent to a cutoff of approximately 2 standard deviations above the mean. We determined the cut-off value for being negative versus positive for IgM to be OD 0.615 (not shown) and OD 0.085 for IgG (Figure 1).

After OD levels were dichotomized into positive and negative as above, the Fisher's exact test was used to compare the prevalence of anti-MUC1 antibodies in subjects along the adenoma-carcinoma sequence (normals, NAA, AA and CRC). Because of the small sample size in each group and the small effect sizes we encountered, the NAA, AA, and CRC were collapsed into one group known as the abnormal group for an exploratory analysis using Fisher's exact test and logistic regression. OD levels were also compared on an ordinal scale using Wilcoxon rank sum test.

To address the second aim of the study, the prevalence of the anti-MUC1 antibodies in subjects with HPP was estimated using the same procedure as above, and compared to normals using Fisher's exact test.

To address the third aim of the study, subject demographic and clinical variables were compared to anti-MUC1 OD levels using Kruskal-Wallis chi-squared tests and Spearman's rank correlations in exploratory analyses. The following variables were examined: age (continuous), race (categorical), gender (binary), history of ever smoking (binary), aspirin use (ordinal), presence of one or more first degree relatives with a history of cancer (binary), and if applicable, location of lesion (right/left where right colon included cecum, ascending colon, hepatic flexure, transverse colon, and splenic flexure, and all others were categorized as left colon). Logistic regression was used on the dichotomized data to control for clinical variables related either to the anti-MUC1 OD levels or to clinical groups.

All statistical tests were two-sided, and considered significant at level 0.05; no correction for multiple comparisons was applied.

RESULTS

Using the highly reproducible ELISA (Supplemental Figure) and the objectively determined OD cut-off value for categorizing each sample as antibody-positive or antibody-negative (Figure 1), we determined the prevalence of anti-MUC1 IgM to be 5/47 (10.6%) of normals, 5/45 (11.1%) of NAA, 7/47 (14.9%) of AA, and 4/20 (20.0%) of CRC (Figure 2a). While IgM prevalence showed a steady increase with more dysplastic samples, anti-MUC1 IgG prevalence was similar in NAA, AA, and CRC. The anti-MUC1 IgG level qualified as positive in 8/47 (17.0%) of normals, 14/45 (31.1%) of NAA, 14/47 (29.8%) of AA, and 6/20 (30.0%) of CRC (Figure 2b). These data revealed a trend towards increased prevalence in abnormal categories but the differences were not statistically significant ($p=0.70$ for IgM and $p=0.36$ for IgG).

We then collapsed the three abnormal groups (NAA, AA and CRC) to increase the number of subjects in that group and thus the power of the analysis. The prevalence of anti-MUC1 antibodies in the abnormal groups did not significantly differ from the normals for IgM or IgG ($p=0.62$ and 0.11 , respectively). However, when results were adjusted for age and gender using logistic regression, the analysis revealed that the prevalence of anti-MUC1 IgG at the 1:20 serum dilution was statistically higher in the abnormal group compared to the normal controls ($p=0.049$). We did not see a difference for IgG at 1:40 dilution likely due to very low OD values. Similar results were obtained when the above OD values were analyzed on an ordinal scale using the Wilcoxon rank sum test. There was no significant difference between the median OD level of the normal and the abnormal groups for IgM at 1:20 ($p=0.46$; Figure 3a), but the median OD level for anti-MUC1 IgG was significantly higher in the abnormal versus the normal group (0.045 vs. 0.030; $p=0.017$; Figure 3b).

We investigated the prevalence of anti-MUC1 antibodies in HPP as a separate category due to lack of evidence that MUC is abnormally expressed in HPP. The prevalence of anti-MUC1 IgM was 6/21 (28.6%), and the prevalence of IgG was 3/21 (14.3%). The prevalence was slightly higher in HPP than in normal samples for both IgM and IgG, but it was not significantly different for either isotype ($p=0.70$ and 0.34 , respectively).

Analysis of demographic data revealed that subjects in the sample were 45.0% female, 84.4% Caucasian, 11.1% African American, and had a mean age of 60.6 years. Race and sex were similar across the clinical groups, but there was a significant association between age and group ($p=0.006$); the CRC group had the highest mean age. In an exploratory analysis, we compared anti-MUC1 antibody levels with the clinical variables to determine if any of them were associated with anti-MUC1 antibody levels. There was a significant association between anti-MUC1 IgG level and racial group at the 1:20 dilution ($p=0.010$). For African Americans, the median OD was 0.082 (interquartile range: 0.039 – 0.512), and for Caucasians, the median OD was 0.042 (interquartile range: 0.022 – 0.082). There were very few subjects in the American

Indian/Alaskan Native, Asian/Pacific Islander, and Hispanic categories. Females had slightly higher median OD levels than males for both IgM and IgG at the 1:40 dilution ($p=0.068$ and 0.079 , respectively). We found no significant associations between anti-MUC1 antibody level and age, smoking history, aspirin use, family history or location of lesion.

DISCUSSION

This study provides estimates of the prevalence of serum anti-MUC1 antibodies in U.S. subjects with benign and neoplastic colonic polyps and cancer. Furthermore, the assessment of antibody prevalence uses a novel, unbiased method for determining the cutoff values for positive. Our estimates are consistent with the previous report that concluded that anti-MUC1 antibodies were more prevalent in subjects with CRC and adenomas than in healthy controls (Nakamura et al., 1998). Although our study did not demonstrate statistically significant differences in the prevalence of anti-MUC1 antibodies in subjects along the adenoma-carcinoma sequence, the trend was consistent with our hypothesis. The prevalence of anti-MUC1 IgM increased along the adenoma-carcinoma sequence. We interpret this to reflect increasing exposure to abnormal MUC1 with increasing dysplasia. Antibodies of the IgG isotype are markers of a coordinated immune response because the process of class-switching from IgM to IgG requires the help of activated T-cells. Given that IgG antibodies against tumor antigens, including MUC1, have been correlated with improved prognoses in cancer (Albanopoulos et al., 2000; Hamanaka et al., 2003; von Mensdorff-Pouilly et al., 2000), an early immune response against a premalignant lesion, by extension, may be protective against malignant transformation. We saw increased prevalence of anti-MUC1 IgG in the abnormal groups over the normals but there was no increase from less to more dysplastic groups. Differences in the timing of the development of the immune response may account for this pattern. Individuals who mount a robust IgG response against MUC1 in the early stages of the adenoma-carcinoma sequence may avoid progressing to later stages. This would tend to have a leveling effect on the prevalence of the antibody in the abnormal groups.

Our finding that IgG prevalence and median OD value were higher in the combined abnormal groups compared to normal controls suggests that abnormal colon lesions are associated with a specific response to abnormal MUC1. The difference is small but may be clinically significant, given that the antibody level needed to confer protection against the development of tumors is unknown. Furthermore, if IgG is taken as surrogate marker of increased function of MUC1-specific T cells, slight differences in IgG levels may indicate a much greater and thus biologically significant differences in T cell function. T cell responses will be evaluated in a prospective study that will collect live peripheral blood mononuclear cells in addition to serum samples.

We observed no difference in anti-MUC1 antibodies in HPP as compared to normals. This makes sense because HPP do not express increased levels of MUC1 (Biemer-Huttmann et al., 1999; Ho et al., 1996; Percinel et al., 2007), and it is in keeping with the adenoma-carcinoma sequence model, which does not include HPP in the sequence.

In our exploratory analysis of demographic and clinical variables, we found that race was significantly associated with anti-MUC1 antibody level. The median OD level for anti-MUC1 IgG at the 1:20 serum dilution for African Americans was nearly twice that of Caucasians. This is an interesting finding given that African Americans have a higher incidence of certain autoimmune and inflammatory diseases, often with a more severe course (Barnato et al., 2008; Molokhia and McKeigue, 2006; Peters et al., 2007; Verma et al., 2007). None of the other variables was significantly associated with anti-MUC1 antibody level. Further studies are needed to determine if clinical variables predict a specific immune response to MUC1.

This investigation has limitations. Due to the cross-sectional design of the study, we cannot conclude that the colon lesions primed this antibody response. A longitudinal cohort study with serial blood draws is required to answer that question. Based on new evidence that antibodies to abnormal MUC1 can be elicited through several known inflammatory conditions (Cramer et al., 2005), two scenarios are likely: dysplastic lesions in the colon can either elicit a new anti-MUC1 response or trigger a memory response elicited by a prior event.

This study was underpowered to detect small differences between the clinical groups that nevertheless might be biologically significant. Due to the exploratory nature of this work, no adjustments for multiple testing were made, so the associations we found may be due to chance. However, we consider that highly unlikely because our results are consistent with what could be expected based on previous studies (Kotera et al., 1994; Nakamura et al., 1998).

Further studies are warranted to explore the temporal relationship between the anti-MUC1 response and colon lesion formation, and to determine if the anti-MUC1 response is associated with longer survival in colorectal cancer, prevention of recurrent adenomatous colon polyps, or protection against progression of adenomas to advanced adenoma or carcinomas.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Distribution of normalized OD for anti-MUC1 IgG for each clinical group in the adenoma-carcinoma sequence and for subjects with hyperplastic polyps (1:20 serum dilution). Triangles indicate the median normalized OD for each group. The horizontal line indicates the cutoff for positive expression in the serum (OD 0.085).

Figure 2.
Percent of subjects who were positive for anti-MUC1 IgM (a) and IgG (b), by group (1:20 serum dilution). Bars are 95% confidence intervals.

Figure 3. Boxplot of log OD values showing median and interquartile ranges for anti-MUC1 IgM (a) and IgG (b) at the 1:20 serum dilution. The difference between the combined abnormal groups (NAA, AA, and CRC groups) and subjects with normal colons was significant for IgG ($p=0.017$), but not for IgM.