NOTE

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 Imaging colon cancer development in mice: IL-6 deficiency prevents adenoma in azoxymethane-treated Smad3 knockouts

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Abstract
The development of colorectal cancer in the azoxymethane-induced mouse model can be observed by using a miniaturized optical coherence tomography (OCT) imaging system. This system is uniquely capable of tracking disease development over time, allowing for the monitoring of morphological changes in the distal colon due to tumor development and the presence of lymphoid aggregates. By using genetically engineered mouse models deficient in Interleukin 6 (IL-6) and Smad family member 3 (Smad3), the role of inflammation on tumor development and the immune system can be elucidated. Smad3 knockout mice develop inflammatory response, wasting, and colitis associated cancer while deficiency of proinflammatory cytokine IL-6 confers resistance to tumorigenesis. We present pilot data showing that the Smad3 knockout group had the highest tumor burden, highest spleen weight, and lowest thymus weight. The IL-6 deficiency in Smad3 knockout mice prevented tumor development, splenomegaly, and thymic atrophy. This finding suggests that agents that inhibit IL-6 (e.g. anti-IL-6 antibody, non-steroidal anti-inflammatory drugs [NSAIDs], etc.) could be used as novel therapeutic agents to prevent disease progression and increase the efficacy of anti-cancer agents. OCT can also be useful for initiating early therapy and assessing the benefit of combination therapy targeting inflammation.

Note

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(Some figures may appear in colour only in the online journal)

Abbreviations

AOM azoxymethane
CRC colorectal cancer
IL-6 interleukin 6
OCT optical coherence tomography

Introduction

The causal relationship between the inflammatory response in the body and the development of cancer is becoming a widely accepted paradigm (Coussens and Werb 2002, Landskron et al 2014). Chronic inflammation is strongly associated with and is proposed to be at the root of approximately 1/5th of all human cancers (Westbrook et al 2010). Evidence suggests that an altered microenvironment caused by inflammation is an essential component of all tumors (Grivennikov et al 2010). A fuller examination of immune cells and pathways throughout the time course of development of cancer will provide fuller validation of the relationship between inflammation and tumor development. Colorectal cancer (CRC) is a representative example of the dual interplay between the immune system and cancer (Bergomas et al 2011). The Smad3−/− (129/Sv background) mouse treated with azoxymethane (AOM), a potent carcinogen used to induce colon cancer, provides a developmental model allowing for examination of the early stages of disease development (Neufert et al 2007, Tian et al 2011).

Our approach for investigating CRC development uses OCT imaging in a double knockout mouse model. The relationship between inflammation and cancer can specifically be studied in Smad3−/− mice, which model ulcerative colitis (UC). UC is an inflammatory bowel disease known to put patients at high risk for CRC. The Smad3 gene encodes for a transcription factor that mediates TGFB receptor signaling, which is known to be a tumor suppressor. The inactivation of the Smad3 gene can lead to chronic intestinal inflammation, which causes accelerated adenoma development. This progression suggests a synergistic effect of inflammation and tumor suppressor deficiency in aggressive growth of adenomas in Smad3−/− ApcMin/+ mice (129/Sv strain) (Sodir et al 2006). Inhibition of the pro-inflammatory cytokine IL-6 has previously been found to suppress the growth of colon cancer (Lu et al 2006, Ataie-Kachoie et al 2014, Waldner and Neurath 2014, Yao et al 2014). This study tests whether OCT imaging accurately detects early lesions and lymphoid aggregates in the distal colon and whether blocking inflammatory cytokine IL-6 prevents adenoma development and lymphoid aggregates in Smad3−/− mice.

Along with assessing tumor development, immune dysregulation in the mouse model can be analyzed to determine the tumor induced immunosuppression by measuring the extent of thymic atrophy, stress-induced shrinkage of the thymus caused by disease, and splenomegaly, an enlargement of the spleen due to chronic inflammation (Coletta et al 2004, Nakanishi et al...
The colonic immune response can also be assessed through the number and size of lymphoid aggregates. Lymphoid aggregates are clusters of immune cells that play an essential role in the immune response to perturbations of the mucosal environment. It has been shown that the size of the aggregates increase with the severity of UC rather than the quantity (Yeung et al. 2000). The use of OCT to detect lymphoid aggregates in the distal colon is a novel technique and could prove to be a valuable tool in the analysis of CRC disease development. Despite the well-known role of inflammation in de novo tumor development, the biological function of lymphoid tissue in a cancerous environment has been inadequately investigated (Nascimbeni et al. 2004, Kim et al. 2006, Bergomas et al. 2011, Hahn et al. 2011).

Here we utilize a miniaturized endoscopic optical coherence tomography (OCT) imaging system to nondestructively examine morphological changes in the distal colon of the AOM-induced mouse model of colon carcinogenesis. OCT has previously been proved successful in the detection of early cancerous lesions in the distal colon of the murine model (Hariri et al. 2007, Winkler et al. 2010). However the use of OCT to detect lymphoid aggregates in the mucosal layer of the colon is a novel technique, which could provide additional information on immune exhaustion and level of disease. We monitored in vivo morphological changes in the distal colon over a three-month period. The size and number for both the tumors and lymphoid aggregates were measured at each time point for every mouse. In addition, histological analysis of each colon along with spleen and thymus size was used to further support the relationship between inflammation and disease.

**Materials and methods**

**Animal model**

Twenty-four 129/Sv strain mice generated by breeding Smad3IL6<sup>+/−</sup> mice to have either knockout, heterozygous, or wild-type gene coding for IL-6 and Smad3 were used in the study. All 24 mice were injected i.p. with azoxymethane (AOM, Sigma, ST. Louis, MO) 10mg kg<sup>−1</sup> weekly for 4 weeks starting at 6 weeks of age.

**Endoscopic optical coherence tomography system**

The endoscopic ultra-high resolution OCT system has previously been described in detail (Tumlinson et al. 2004, Hariri et al. 2007, Winkler et al. 2010). OCT measures near-infrared light backreflected from tissue to generate cross-sectional images of tissue microstructure. The OCT image resolution was 8 μm lateral and 4 μm axial. In adherence to protocol previously approved by the University of Arizona Institutional Animal Care and Use Committee, OCT images were collected from the 24 mice at 3-week intervals over a 12-week period. Preceding imaging, the mice were provided Pedialyte solution in place of chow for 24h. Prior to imaging they were anesthetized by administering a mix of 100mg kg<sup>−1</sup> ketamine plus 10mg kg<sup>−1</sup> xylazine intraperitoneally. The distal colon was then rinsed with 6ml of warm saline to remove any remaining fecal matter. Water-based lubricant was applied to the 2mm diameter OCT endoscope, which was then inserted 30mm inside the distal colon. Within the stationary endoscope outer glass envelope, the imaging optics translated and rotated to obtain 30mm longitudinal, 1.4mm deep images at 24 evenly spaced rotations (15 degrees apart) around the circumference of the colon. Imaging time was approximately 15min.
The adenomas observed in the OCT images were identified according to criteria previously described by Hariri et al. (2007), including mucosal thickness twice the local average, attenuation of signal, and faint boundaries between the mucosa and submucosa tissue boundary. Maximum adenoma depth and lateral extent was measured from the OCT images and adenoma volume was computed assuming a spherical shape. Tumor burden at each time point was computed as the sum of the calculated volume of all of the adenomas. Lymphoid aggregates were identified as a break in the mucosal or submucosal regions with an area of homogenous intensity inside of the break. Lymphoid aggregate maximal lateral extent was measured from the OCT images. The diagnostic criteria for both adenoma and lymphoid aggregates can be found in table 1.

### Table 1. Diagnostic criteria used to identify lymphoid aggregates and adenoma observed in the OCT images. Adenoma criteria previously defined by Hariri [21].

<table>
<thead>
<tr>
<th>Healthy</th>
<th>Adenoma</th>
<th>Lymphoid Aggregate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lateral dimension</td>
<td>N/A</td>
<td>&gt;1 mm</td>
</tr>
<tr>
<td>Mucosal thickness</td>
<td>Constant</td>
<td>Moderate protrusion</td>
</tr>
<tr>
<td>Tissue boundaries</td>
<td>Visible</td>
<td>Faint to obscured</td>
</tr>
<tr>
<td>Signal attenuation</td>
<td>Constant</td>
<td>Moderate attenuation</td>
</tr>
</tbody>
</table>

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### Ex vivo imaging and tissue processing

After the final imaging time point the animals were sacrificed, and the distal 30 mm of the colon, spleen, and thymus were excised. The colon was sliced longitudinally and flattened to expose the lumen. The spleen and thymus were weighed. Gross images were taken of all excised organs. Ex vivo analysis of the colon was used as a gold standard in order to verify the number and location of lymphoid aggregates and tumors found in OCT at the final time point. The colon tissue was first fixed for 24 h in a 10% formalin solution. The tissue was then transferred to a 70% EtOH solution until embedding. The colon tissue was embedded in paraffin wax positioned to obtain en face sections. Eight micrometer thick sections were obtained and stained for hematoxylin and eosin.

### Data analysis

Data from in vivo OCT images was utilized to create a 2D tumor and lymphoid aggregate map of the virtually flattened colon at each time point. The map x and y axes represent distance from the anus and rotation, respectively. Maps were created by condensing the twenty-four, 30 mm longitudinal scans into 1 mm lateral segments, creating a $24 \times 30$ element matrix. For each segment the presence of adenoma or lymphoid aggregate was noted.

Histology slides of each colon were analyzed using a 2x, 4x, and 10x objective on an upright microscope equipped with a color camera. The sensitivity and specificity of OCT to the presence of adenoma and lymphoid aggregates was determined by comparing the results obtained from the OCT images to the gold standard histology images. The largest diameter of each tumor was measured from histology and compared to OCT measurements. Lymphoid aggregate clustering around a tumor was determined using histology by counting all lymphoid aggregates within a 2 mm distance of the tumor.
Results and discussion

No animals were lost as a result of the imaging procedure. However, 9 mice died or were euthanized as a result of unresolvable health issues due to the systemic effects of gene mutation. If mice completed 3 of the 4 imaging time points and histology was obtained than the mouse data was utilized. Six of the 15 mice that completed the study developed tumors.

At the final time point, with gold standard histology available, OCT showed perfect (100%) sensitivity and specificity to the 9 histologically-confirmed adenoma. Therefore, OCT measures of adenoma at earlier time points can be accepted with high confidence. Using OCT, we calculated a final tumor burden ranging from 0.333 mm$^3$ to 31.862 mm$^3$. Using histology, we calculated a final tumor burden ranging from 0.519 mm$^3$ to 16.92 mm$^3$. The results were highly correlated with a Pearson correlation of 0.993. The discrepancy between histology and OCT in the highest tumor burden measured is a result of sampling artifact in histology, with sections possibly not taken at the largest diameter of the tumor, as well as the effects of tissue shrinkage during histology processing.

OCT imaging detects lymphoid aggregates in the colon

OCT was successful in detecting larger lymphoid aggregates, with a sensitivity of 87.8% and a specificity of 100% to the 61 histologically-confirmed aggregates of size 0.5 mm diameter or greater. Sensitivity to the 89 histologically-confirmed aggregates of 0.3 mm diameter or greater dropped to 70.7%, however the specificity remained at 100%. Overall sensitivity of OCT to all size lymphoid aggregates (168 total, which ranged in size from 0.1 to 2.5 mm diameter) was 49.0%. An example OCT image, showing an adenoma and lymphoid aggregates, is shown in figure 1. A comparison of a histological section, showing 4 larger (>0.5 mm) lymphoid aggregates and multiple small aggregates, with an OCT-generated map (figure 2) shows the ability of OCT to accurately identify large but not small lymphoid aggregates (there were no adenoma in this mouse). An improvement in the contrast mechanism of the OCT imaging system may be required to visualize small lymphoid aggregates in vivo.

No correlation between number of lymphoid aggregates and number of tumors was found. However, the median diameter of the lymphoid aggregates in the Smad3$^{-/-}$ group (the group with the largest tumor burden) was higher than that of the Smad3$^{+/+}$ group (full data in table 2 of Supplementary Data) (stacks.iop.org/PMB/61/N60/mmedia). This finding suggests that the size of the aggregates rather than their number increases with disease severity, in congruence with earlier findings (Yeung et al 2000).

Figure 1. OCT image (30 mm in length) of the distal colon of a wild-type mouse, showing the development of an adenoma (boxed area) on the proximal (left) side over three different time points. The arrow in the figure is pointing to a mucosal lymphoid aggregate.
Examination of histology of the mouse with the largest tumor burden revealed lymphoid aggregates clustering around the largest adenoma (figure 3). Less lymphoid aggregate clustering was observed in the other mice with lower tumor burden (supplementary data, table 4) (stacks.iop.org/PMB/61/N60/mmedia). Although this is a preliminary finding, it may support the idea of inflammatory immune cell recruitment by the cytokines and chemokines produced by the neoplastic epithelial cells and stromal cells during disease development.

**Figure 2.** The location of lymphoid aggregates is highly correlated between the *in vivo* OCT cross sectional image 30 mm in length (top), analyzed OCT map (middle), and *ex vivo* intensified image of an *en face* histology section (bottom) of the mouse colon. The correlating lymphoid aggregates are paired through shapes. The location of the cross sectional OCT image on the OCT map is emphasized by the darkened row. Multiple very small (<0.2 mm) lymphoid aggregates are seen only in the histology section, and are circled with a dashed line.

SMAD3 deficiency increases tumor incidence and burden

Results of tumor burden at the final time point, grouped according to genotype, are shown in figure 4. Since all mice received the carcinogen, a mouse with zero tumor burden is an important result as it enables differentiation of how susceptible the different genotypes were to tumor development. There are six groups based off the combinations of IL-6 wild type, heterozygous, and knock out with Smad3 wild type and knock out. The two mice in the study with IL-6 wild type and Smad3 wild type and knock out. The two mice in the study with IL-6 wild type and Smad3 heterozygous were moved into the IL-6/Smad3 wild type group, because previous studies have shown that Smad3 wild type (Smad3$^{+/+}$) and Smad3 heterozygous (Smad3$^{+/−}$) mice to display the same systemic effects (Seamons *et al* 2013).
Consistent with the resistance of 129/Sv strain to AOM-induced adenoma development, wild-type mice exhibited a low incidence of adenoma development. However, knockout of Smad3 increased the incidence and the tumor burden, whereas haploinsufficiency of IL-6 was protective of tumor development in Smad3−/− mice.

Spleen and thymus weight also followed the same trends, with the highest spleen weight in the Smad3−/− Il6+/+ group (258 mg average) while the weight in wild type (89.2 mg average)
and in the Smad3^{+/−} Il6^{-/−} group (77.5 mg average) are within the normal size range. The thymus weight was lowest in the Smad3^{-/-} Il6^{+/+} group (50 mg average) suggesting tumor induced systemic inflammatory effect on the host immune system while the weight in wild type (54.6 mg average) and in the Smad3^{+/+} Il6^{-/-} (58.5 mg average) mice is almost similar. Although trends were clear, these results are not statistically significant due to variability and low number of mice in this pilot study.

These pilot results suggest that the SMAD3 signaling acts as a tumor suppressor in the distal colon, as we observe that the Smad3^{-/-} group had the greatest tumor burden (figure 4). The results also demonstrate that the IL-6 heterozygous group had no adenoma (figure 4), no splenomegaly and normal thymus size, suggesting IL-6 as a potential target for therapeutic development in CRC patients with mutations in TGFβ signaling.

Conclusions

Using the OCT results to calculate tumor burden per mouse, we were able to make a correlation between the disease development in the colon and gene inhibition. Although a study with a larger number of mice is needed for confirmation, our results suggest that SMAD3 deficiency promotes tumor development in an IL-6 dependent manner in a strain that is resistant for carcinogen-induced tumor development. These findings support previous studies which have shown that the knockout of both alleles of IL-6 inhibits angiogenesis and tumor growth (Nagasaki et al. 2014, Waldner et al. 2014). However, this is the first report to suggest that haploinsufficiency of IL-6 is sufficient to prevent carcinogen induced tumorigenesis in Smad3^{-/-} mice. Studies from cancer patients reveal that plasma IL-6 levels are correlated with tumor stage and size, as well as patient survival rates. This correlation makes IL-6 a good prognostic marker, as well as a possible therapeutic target (Hong et al. 2007).

Our results also show that OCT imaging accurately detects early lesions in the distal colon of mice with high confidence. OCT may be useful for early detection of CRC in humans, and may also visualize the effects of treatment in both humans and mouse models. The study also introduces OCT as a novel imaging technique to detect lymphoid aggregates in the mucosal and submucosal layers of the colon. The detection of these lymph structures will provide important additional analysis of the tumor microenvironment required for CRC disease development. Further study of lymphoid aggregates is necessary to prove the possible relationship between size of lymphoid aggregates and level of disease based off our initial results. In this limited study, sensitivity and specificity of OCT detection of adenoma and larger lymphoid aggregates is shown to be high compared to histology, and OCT has the advantage of being a non-destructive method. In comparison to the clinical standard colonoscopy, which can only visualize the surface of the colon, OCT provides cross-sectional image information, and thus information on subsurface lymphoid aggregates and the extent of adenoma into the mucosa, which can be important for full understanding of colon disease (Jenkins et al. 1997).

Currently, cancer is usually treated as an already advanced and often intractable disease (Khansari et al. 2009). Prevention and early detection are better, more economical ways to save lives. These methods keep the disease from damaging tissue and also prevent immune exhaustion, a condition which may have life-long consequences for the patient. The use of OCT to detect early lesions and inflammatory markers (lymphoid aggregates) in the colon can provide additional information for chemoprevention and chemotherapeutic studies, as well as monitor patient health, helping to make colorectal cancer a curable disease.
Acknowledgments

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