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Hematopoietic Derived Cell Infiltration of the Intestinal Tumor Microenvironment in *Apc^{Min/+}* Mice

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Abstract

Tumors consist of a heterogeneous population of neoplastic cells infiltrated by an equally heterogeneous collection of nonneoplastic cells that comprise the tumor microenvironment. Tumor growth, invasion, and metastasis depend on multiple interactions between these cells. To assess their potential as therapeutic targets or vehicles for tumor specific delivery of therapeutic agents, we examined the contribution of bone marrow derived cells (BMDCs) to the intestinal tumor microenvironment. Hematopoietic stem cells expressing the enhanced green fluorescent protein (eGFP) were transplanted into lethally irradiated *Apc^{Min/+}* mice, and their engraftment was analyzed by confocal microscopy. The results showed abundant infiltration of eGFP cells into the small intestine, colon, and spleen compared to heart, muscle, liver, lung, and kidney. Within the intestine, there was a pronounced gradient of engraftment along the anterior to posterior axis, with enhanced infiltration into adenomas. Immunofluorescence analysis showed that osteopontin was expressed in tumor stromal cells but not in nontumor stromal populations, suggesting that gene expression in these cells is distinct. Tumor vasculature in *Apc^{Min/+}* mice was chaotic compared to normal intestinal regions. Our data suggest that BMDCs can be harnessed for tumor-targeted therapies to enhance antitumor efficacy.

Keywords

tumor microenvironment; hematopoietic stem cells; bone marrow derived cells; colon cancer; *Apc^{Min/+}* mice

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Supplementary Figure 1

Supplementary Figure 1, which shows the infiltration of intestinal tumors by leukocytes in nontransplanted *Apc^{Min/+}* mice, can be found online. Please visit journals.cambridge.org/jid_MAM.

Supplementary Figure 2

Supplementary Figure 2, which shows the expression of SDF1 in intestinal tumors in nontransplanted *Apc^{Min/+}* mice, can be found online. Please visit journals.cambridge.org/jid_MAM.

Introduction

Adult stem cells hold a tremendous potential for use in the therapeutic treatment of human diseases, including cancer. Hematopoietic stem cells (HSCs) were the first to be used for stem cell therapy in allogeneic hematopoietic stem cell transplants (Jenq & van den Brink, 2010). This allowed the treatment of individuals with inherited anaemias or immune deficiencies. In cancer therapy, it allowed the administration of high doses of radiation or chemotherapy that were myeloablative, but could increase the killing of tumor cells, by rescuing the resulting loss of the patient's bone marrow system (Jenq & van den Brink, 2010). In recent years, accumulating data have shown that hematopoietic derived cells play a critical role in tumor growth, progression, and response to therapy (Coussens & Werb, 2001, 2002). They are recruited to tumor sites, where together with endothelial cells, fibroblasts, pericytes, and the extracellular matrix, they comprise the tumor stroma or microenvironment. Collectively, these cells actively contribute to tumor growth and invasion by secreting a diverse combination of growth factors, hormones, angiogenic factors, cytokines, and proteolytic enzymes that promote carcinogenesis and aid in the evasion of the immune system by cancer cells (Chantrain et al., 2008; Hofmeister et al., 2008; Hanna et al., 2009). Increasing lines of evidence suggest that effective anticancer therapies should target not only the tumor cells but also the tumor-associated stromal cells that provide a permissive environment that sustains tumor survival and potentially promotes tumor recurrence (Schwarz et al., 1999; Pietras et al., 2002; Weaver et al., 2002; Hall et al., 2004; Wang et al., 2004, 2005).

Colon cancer is the third most common cancer and the second leading cause of cancer deaths in the United States, with an estimated 146,290 new cases diagnosed and 56,000 deaths occurring each year (American Cancer Society, 2009). Treatment often consists of surgery, radiation, or chemotherapy (Hanna et al., 2009). Although these methods have been modestly effective in the treatment of some patients, the risk of recurrence has remained significantly high. Over the years, substantial effort has been invested in developing new therapies that target various signaling pathways in the transformation of normal cells to cancer cells, as well as enhancing existing therapies by combining those that target multiple pathways. However, these have remained problematic due to acquired resistance and hematopoietic and gastrointestinal toxicities resulting from their lack of specificity, and have limited the doses that can be administered to a patient (Berger et al., 1988; Nicoletta et al., 2003; Chau & Cunningham, 2006). There remains a great need to develop therapeutic strategies to reduce toxicity while simultaneously enhancing efficacy.

Colorectal cancers in humans and mice are highly infiltrated with hematopoietic derived inflammatory cells. Among these, mast cells have been shown to play a role in adenoma development (Gounaris et al., 2007). Regulatory T cells were shown to exert a protective role against cancer by controlling inflammation in an IL-10 dependent manner (Erdman et al., 2003, 2005); however, molecular signals within the tumor microenvironment emanating from the tumor are believed to promote their expansion in an IL-17-dependent manner within adenomatous polyps to promote inflammation and tumor growth (Gounaris et al., 2009). Using *in situ* immunohistochemical analyses and gene expression profiling of human colorectal cancers, Galon and colleagues (2006) proposed that the type and location of tumor infiltrating inflammatory cells in these and potentially other cancers may be good prognostic indicators of the clinical outcome of therapeutic treatment. Collectively, these findings indicate that a thorough characterization of the types, distribution, and function of hematopoietic derived cells in the tumor stroma must be undertaken to develop effective therapies targeting both tumor and stroma for total tumor killing.

In the present study, we established a mouse model to analyze the intestinal tumor microenvironment and identify the cellular participants in tumor progression. The goal is to understand the interactions between hematopoietic derived cells and tumor cells in the intestinal compartment. We utilized the *Apc^{Min/+}* mouse as a genetic model of gastrointestinal tumorigenesis. This mouse carries the *Min* allele of the adenomatous polyposis coli (*Apc*) gene that contains a truncation mutation of this tumor suppressor gene (Moser et al., 1990; Shoemaker et al., 1997). Mice that are heterozygous for this allele spontaneously develop multiple adenomatous polyps throughout the gastrointestinal tract through the somatic inactivation of the wild-type *Apc* allele in undifferentiated stem cells found at the base of intestinal crypts (Moser et al., 1992; Shoemaker et al., 1997). This phenotype is similar to that found in humans with familial adenomatous polyposis (FAP), an inherited form of colon cancer caused by a germline mutation in the *Apc* gene (Shoemaker et al., 1997). The same gene is mutated in a majority of sporadic human colorectal cancers (Kinzler & Vogelstein, 1996). Recent studies have implicated hematopoietic derived cells in polyp formation and adenoma progression (Gounaris et al., 2007, 2009). We examined the distribution of hematopoietic-derived cells within the intestinal tumor microenvironment of *Apc^{Min/+}* mice by confocal microscopy and examined their activated state in tumor compared to normal tissue microenvironment. Our studies indicate that hematopoietic derived cells are a major component of the intestinal tumor microenvironment and support the notion that these cells can be harnessed as targets to enhance tumor specific efficacy of existing therapies.

Materials and Methods

Mice

C57BL/6-Tg(ACTB-EGFP)10sb/J mice expressing enhanced green fluorescent protein (eGFP) under the control of the chicken beta actin promoter were used as donors for bone marrow transplantation (BMT). C57BL/6J-*Apc^{Min}*/J mice (*Apc^{Min/+}*) were used as BMT recipients. Both strains were purchased from Jackson Laboratories (Bar Harbor, ME, USA) but were bred and maintained at the Mouse Core Facility of the Center for Colon Cancer Research at the University of South Carolina (USC), Columbia, SC. All aspects of the animal experiments have been conducted in accordance with the guidelines and approval of the USC Institutional Animal Care and Use Committee.

Bone Marrow Isolation and Transplantation

Donor mice were anesthetized by inhalation of 2% isoflurane in oxygen prior to sacrifice by cervical dislocation. Bone marrow (BM) cells were flushed from the femur and tibia using a 21-gauge needle into phosphate buffered saline (PBS) containing 2% fetal calf serum. BM cells were washed with PBS and counted using a hemacytometer. Four-week-old recipient *Apc^{Min/+}* mice were lethally irradiated (950 rads administered at 200 rads/min) using a Varian Clinac linear accelerator. $3-5 \times 10^6$ mono-nucleated cells were transplanted into the lethally irradiated recipient mice by tail vein injection. Transplanted mice were administered sterile water containing 0.018% Baytril antibiotic (Bayer Animal Health Division, Shawnee, KS, USA) for two weeks post-transplantation to prevent infection.

Isolation and Transplantation of Purified Hematopoietic Stem Cells

Hematopoietic stem cells were purified according to Masuya et al. (2003). Briefly, BM cells were isolated from donors as described above. Samples were made into single cell suspensions by filtering through a 40- μ m nylon mesh. BM cells with densities from 1.063–1.077 g/mL were collected by gradient separation using Nycodenz (Accurate Chemical and Scientific, Westbury, NY, USA). Lineage positive cells were removed by incubating with anti-Cd11b, anti-Gr-1, anti B-220, anti-CD4, anti-CD8, anti-Ter-119 followed by

immunomagnetic beads (Dynabeads M-450) coupled to sheep anti-rat IgG (Dynabeads, Great Neck, NY, USA). The resulting Lin⁻ cells were stained with PE-conjugated anti-Sca-1, APC-conjugated anti-c-kit, and biotin conjugated anti-CD34, followed by streptavidin-conjugated PharRed (Pharmingen, San Diego, CA, USA). Propidium iodide (1 µg/mL) was added, and cells were resuspended in PBS containing 1% bovine serum albumin for cell sorting. Five color cell analysis and cell sorting were carried out using a FACSVantage Cell Sorter (Becton Dickinson, San Jose, CA, USA) with appropriate isotype-matched controls. Single Lin⁻, Sca-1⁺, c-kit⁺, and CD34⁻ cells were sorted into 96-well plates using CloneCyt system. The single clonal populations were cultured for 7 days as described (Masuya et al., 2003), and viable clusters of cells derived from single cells were transplanted into lethally irradiated recipient mice by tail vein injection. Radioprotective Lin⁻, Sca-1⁻, c-kit⁻, CD34⁻ BM cells from recipient mice were also transplanted to prevent post-transplantation death. Mice were kept in water containing Baytril for two weeks post-transplantation to prevent infection.

Immunohistochemistry and Confocal Microscopy

To determine the contribution of engrafted eGFP-positive bone marrow derived cells (BMDCs) to the tumor microenvironment, tissues from transplanted mice were analyzed by confocal microscopy. The entire intestine, kidneys, heart, lung, liver, spleen, and soleus muscle were excised from three control C57BL/6 mice, three *Apc*^{Min/+} mice at 8 weeks post-transplant, and three *Apc*^{Min/+} mice at 12 weeks post-transplant, and fixed in freshly prepared 4% paraformaldehyde in PBS, pH 7.2. Following fixation, the tissues were rinsed with PBS and vibratome sections were cut at a thickness of 100 µm. Samples were stained with phalloidin conjugated to either Alexa 568 or Alexa 633 (Invitrogen, Carlsbad, CA, USA) at a dilution of 1:100. Phalloidin specifically binds to f-actin and provides information on the overall tissue architecture. Nuclei were stained with a 1:20,000 dilution of 4',6-diamidino-2-phenylindole (DAPI, Invitrogen). Fluorochromes for confocal microscopy were obtained from Molecular Probes (Eugene, OR, USA). Samples were imaged with a Zeiss LSM510 META confocal scanning laser microscope.

To determine the distribution of eGFP-labeled cells throughout the different regions of the intestine, the entire gastrointestinal (GI) tract was dissected out and cut into approximately 5 cm segments. A vibratome section (100 µm thick) was then cut from the cross section of the proximal end of each segment and stained and imaged as described above. To obtain data for the entire cross section of the intestine, images were collected across the section at a magnification of 20× and montaged using Adobe Photoshop software (Adobe Systems, Inc., San Jose, CA, USA). To quantitate the extent of migration of eGFP positive cells to each cross-sectioned region of the intestine, images were analyzed using the Image Pro Plus image analysis software (Media Cybernetics, Inc., Silver Spring, MD, USA). The settings were set at a threshold where pixels in the green channel below a value of 30 were assigned to black and pixel values 30 and above were assigned to white. The number of pixels in each category was then converted to tissue area by multiplying the total number of pixels in each region of interest (ROI) by the area of each pixel (at 10× magnification each pixel = 3.2 µm²) used in the collection of the confocal images. For each cross section of the intestine, the ROI included areas that contained villi and excluded the lumen and muscle layers of the intestine. All images within a dataset were collected and analyzed with identical instrument settings.

For immunohistochemical staining, vibratome sections were incubated with 3% goat serum at room temperature for 1 h to block nonspecific binding of mouse immunoglobulins. Sections were incubated with various primary antibodies at room temperature for 1 h. The primary antibodies used were the following: mouse monoclonal antibody against CD45.2 (leukocyte common antigen) was from BD Biosciences, Pharmingen (San Jose, CA, USA),

while rabbit polyclonal antibodies against stromal cell-derived factor-1 (SDF-1) and osteopontin were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). After three 5-min washes with PBS, the sections were stained with the appropriate secondary antibody conjugated to Rhodamine red, followed by three 5-min washes with PBS. Immunostained slides were then examined under a Zeiss confocal laser-scanning microscope. All images were captured using a digital imaging system.

Analysis of Bone Marrow Engraftment by Flow Cytometry

To assess engraftment of BMDCs, peripheral blood was obtained from the retro-orbital sinus of recipient mice at 4, 8, and 12 weeks post-transplantation. Red blood cells were lysed with ammonium chloride lysis buffer (150 μ M NH₄Cl, 10 mM Na₂CO₃; 0.1 μ M EDTA, pH 7.4). Donor-derived T-cells, B-cells, granulocytes, and monocyte/macrophage lineages were analyzed by staining with biotin-conjugated anti-Thy-1.2(30-H12), anti-CD45R/B220(RA3-6B2), or a combination of anti-Gr-1(RB6-8C5) and anti-Cd11b antibodies, respectively, followed by incubation with PE-Cy5 conjugated streptavidin. All antibodies were purchased from BD Biosciences Pharmingen (San Diego, CA, USA). Labeled cells were analyzed in a Beckman Coulter Epics-XL Flow Cytometer and CXP Analysis software.

Assessment of Tumor Vasculature by Latex Perfusion

Latex perfusion was performed as described previously (Fuseler et al., 2010) with modifications. Twelve-week old *Apc^{Min/+}* mice were anesthetized with xylazine (5–10 mg/kg) and ketamine (50–100 mg/kg) in PBS. The heart was exposed by sternotomy and the right atrium clipped to prevent backflow. Visceral blood was flushed completely by perfusion with 5 mL warm heparin-saline (10 U/mL) solution. The animals were then injected with 3 mL of a curable yellow silicone latex rubber injection compound Microfil MV-22 (Flow Tech, Inc., Carver, MA, USA) to perfuse the entire vasculature. The atrium and arterial cannula were then clamped, and the animal was placed under refrigeration at 4°C overnight to allow polymerization. The following day, the entire intestinal tract was dissected out and placed in a solution of 50% glycerin and water. To allow microscopic examination of the three-dimensional vascular bed, the tissue was cleared by incubation for successive 24-h intervals in solutions containing 75, 85, and 100% glycerin. Images were taken under an Olympus dissecting microscope using a DP71 digital camera (Olympus, Tokyo, Japan). Image contrast was adjusted using Adobe Photoshop software. Tumor vascularization was assessed by counting the number of blood vessels per field view. The numbers reported are the average of five field views.

Results

Distribution of Bone Marrow Derived Cells along the Intestinal Tract of *Apc^{Min/+}* Mice

To track of the fate of BMDCs in the intestinal tract, we transplanted whole bone marrow or purified hematopoietic stem cells from transgenic donor mice expressing eGFP into lethally irradiated *Apc^{Min/+}* and wild-type C57Bl/6 recipients. Analysis of peripheral blood from recipient mice by flow cytometry indicated that transplanted marrow successfully engrafted at four weeks with 86% to 98% of leukocyte cells expressing eGFP and 31% to 53% stained with anti-B220 antibodies (B cells) (Fig. 1A–C). The same percentages were observed in leukocytes stained with antibodies against Gr-1⁺ (granulocytes), and CD3⁺ (T cells), and in blood samples analyzed at 8 and 12 weeks post-transplant (data not shown).

Engraftment of donor-derived eGFP-expressing cells was analyzed along the GI tract of *Apc^{Min/+}* mice to determine their anterior-posterior distribution. Tissue sections were taken at 8 and 12 weeks post-transplant from representative regions throughout the length of the

small intestine and the colon as shown in Figure 1D (inset). Sections were stained with phalloidin conjugated to Alexa 568 and examined for both red and green fluorescence. The extent of marrow-derived cell infiltration was quantified in each section by determining the percentage of eGFP-labeled pixels in different regions of the images. In all animals examined, we observed a pronounced gradient of hematopoietic-derived cell engraftment along the anterior-to-posterior axis in morphologically normal regions of the gastrointestinal tract (Fig. 1D). The left panel represents quantitation of engraftment in the intestine at 8 weeks post-transplant, while the right panel represents intestinal engraftment at 12 weeks post-transplant. Proximal regions of the intestine exhibited a more extensive infiltration by eGFP-expressing cells into the lamina propria compared to the distal regions, with very little engraftment observed in the colon (Fig. 1D,E). The same gradient was observed in the GI tract of transplanted wild-type C57Bl/6 recipients (data not shown), indicating that the positional “homing” of transplanted BMDCs was not dependent on the *Apc^{Min/+}* allele. Importantly, there was a striking increase in GFP-labeled cells within the tumors (Fig. 1E, arrows), as compared to adjacent tumor-free regions. This frequently caused a spike in the number of eGFP-labeled cells quantified in specific regions of the intestine such as those seen in sections 1, 5, 6, 7, and 9 of the transplanted mice at 12 weeks post-transplant shown in Figure 1E (tumors are indicated by the arrows).

To determine if the pattern of intestinal BMDC infiltration in transplanted mice was similar to that in untransplanted mice, we examined representative intestinal sections in 12-week-old *Apc^{Min/+}* mice that were age-matched to the transplanted mice at 8 weeks post-transplant. These sections were stained with antibodies against the CD45.2 leukocyte common antigen. A representative result is shown in Supplementary Figure 1.

Although the staining is less intense due to the cell surface localization of CD45 as compared to the cytoplasmic and high level of eGFP expression in the transplanted mice, the data showed that the villus and lamina propria of the small intestine in *Apc^{Min/+}* mice were infiltrated by BMDCs, with increased infiltration in tumor-bearing regions (Supplementary Fig. 1, arrows). The results showed that at 8 and 12 weeks post-transplant, the pattern of eGFP-expressing BMDC infiltration of the gastrointestinal tract in transplanted mice reflected that which is observed in unmanipulated, untransplanted *Apc^{Min/+}* mice and was not due to intestinal damage resulting from pretreatment with irradiation prior to transplantation. Collectively, our data indicated that as early as 4 weeks post-transplant and throughout the lifetime of the transplanted *Apc^{Min/+}* mice, donor-derived eGFP expressing cells infiltrate the intestinal stroma at a sustained level with enhanced infiltration into tumor-bearing sections.

Enhanced Infiltration of Intestinal Tumors by BMDCs

Preferential infiltration of tumors by eGFP-expressing cells was more pronounced in colon tissues examined at higher magnification (Fig. 2). Although a relatively lower number of eGFP-labeled cells infiltrated this more distal region of the GI tract, the infiltrating cells exhibited a more distinct association with stromal regions of tumors compared to adjacent normal areas of the colon (Fig. 2C–D). eGFP-labeled cells were found in the stromal compartment of the tumor and in the lamina propria of normal colonic tissue. Quantification of marrow-derived cell infiltration in different regions of the images taken from the colon showed that 75–80% of regions containing a tumor were eGFP-labeled compared to 50% of the normal colon tissue immediately surrounding the tumors, and only 10% in colon tissue distal to the tumor, similar to that found in wild-type mice transplanted with eGFP positive cells (Fig. 2B).

Examination of the small intestines at higher magnification revealed similar extensive engraftment of eGFP positive cells that were detected in the crypt, villi, and the lamina

propria and throughout the stromal compartment of adenomas (Fig. 3A–C). Although eGFP-expressing cells were mostly found in the interstitial tissues of the intestine, occasional eGFP positive cells were found associated with the epithelial lining of the gut in the regions that included tumors (arrow in Fig. 3C). Enhanced infiltration was also observed in intestinal lymph nodes proximal to regions containing adenomas (Pena and Acharya, personal communication). Furthermore, preliminary immunohistochemical analyses showed that infiltrating eGFP positive cells included macrophages, leukocytes, T-cells, fibroblasts, and endothelial cells (data not shown). In summary, these data indicate that tumors in both the small intestine and colon are highly infiltrated with a variety of hematopoietic derived cells.

BMDC Engraftment in Other Organs

We examined the engraftment of BMDCs in extra-intestinal organs. The highest concentration of eGFP-labeled cells was found in the spleen, a hematopoietic tissue (Fig. 4A), although a significant number was also observed in the lung (Fig. 4F). Only sporadic eGFP-labeled cells were found in tissues such as the heart, the soleus muscle, liver, and kidney (Fig. 4B–E). Interestingly, the level of infiltration of normal colonic regions distal to colon tumors was similar to that found in the heart or liver (Figs. 2B, 3), underscoring the enhanced recruitment of these cells to the stromal regions of colonic tumors. The extent and pattern of engraftment of donor-derived eGFP-expressing cells into the intestines and other organs was similar in mice that were transplanted with whole bone marrow or with donor marrow that was enriched for hematopoietic stem cells, i.e., Lin[−] Sca-1⁺ c-kit⁺ CD34[−] [100 cell prep, (Masuya et al., 2003)], suggesting that the engrafted cells were most likely derived from transplanted hematopoietic stem cells.

Differential Expression of Osteopontin in BMDCs

Recent studies have demonstrated that stromal and tumor cells co-evolve during tumorigenesis (Littlepage et al., 2005). Molecular signals secreted by the tumor are thought to modulate gene expression in stromal cells within tumors such that the gene expression signatures of tumor-associated stromal cells are distinct from that of stromal cells associated with normal tissues. To begin to determine if BMDCs associated with intestinal tumor cells exhibit differential expression of certain genes as compared to BMDCs associated with normal regions, we examined the expression of osteopontin (OPN), an acidic cytokine that functions in cellular remodeling associated with pathological insults and inflammation (Denhardt & Noda, 1998; Denhardt et al., 2001). This protein is highly upregulated in adenomatous polyps of *Apc^{Min/+}* mice relative to morphologically normal mucosa (Chen et al., 2004), is produced by a number of hematopoietic derived cells, and is induced by inflammatory stimuli (Denhardt & Noda, 1998; Denhardt et al., 2001). We stained tumor-containing and normal intestinal sections of *Apc^{Min/+}* mice that have been transplanted with eGFP-expressing marrow with an antibody against OPN. The results showed that while both normal and tumor regions of the small intestine are abundantly infiltrated by hematopoietic derived cells, OPN expression was up-regulated only in BMDCs specifically associated with tumors (Fig. 5A,B) but not in those associated with normal regions of the intestine (Fig. 5C,D). We examined OPN expression in BMDCs associated with the liver and spleen and show that while both tissues are infiltrated with these cells to different extents, OPN protein is undetectable in stromal cells within these organs (Fig. 5E,F). These results provide evidence that BMDCs in tumor stromal compartments do express different genes from those in normal stromal compartments of intestinal or extra-intestinal tissues.

We further examined tumor and nontumor tissues for expression of stromal cell derived factor 1α (SDF-1), a chemoattractant cytokine. This cytokine is expressed by stromal cells such as fibroblasts and endothelial cells (Muller et al., 2001; Salvucci et al., 2004) and specifically interacts with the transmembrane G-coupled receptor, CXCR4 (Federspiel et

al., 1993). A number of BMDCs have been shown to express high levels of CXCR4 receptor and can be mobilized from the bone marrow to sites producing the SDF-1 ligand (Jin et al., 2006; Yang et al., 2008). We stained tumor-bearing and nontumor regions of the small intestine of transplanted mice with antibodies against SDF-1. The results showed that SDF1 protein levels were elevated in tumor-bearing regions compared to nontumor regions where the protein was undetectable (Fig. 5G,H, respectively). Correspondingly, the eGFP-expressing BMDCs homed in specifically to the regions with elevated levels of SDF1. We stained liver and spleen tissues with antibodies against SDF1 and showed that in these tissues where we found very different numbers of infiltrating BMDCs, SDF1 protein was undetectable (Fig. 5I,J, respectively). These results indicate that expression of SDF1 correlated with enhanced and specific homing of BMDCs to tumor-bearing regions in the intestine. We further stained intestinal sections from unmanipulated, untransplanted *Apc^{Min/+}* mice and found that tumor-bearing regions have elevated levels of SDF1 compared to nontumor regions (Supplementary Fig. 2, arrows).

These results further confirm that our observations in transplanted mice reflect those in unmanipulated mice and are not the result of intestinal damage resulting from irradiation during pretransplant conditioning.

Intestinal Tumors in *Apc^{Min/+}* Mice Have a Highly Disorganized Vasculature

To further assess the infiltration of bone marrow derived eGFP-expressing cells into tumors, we compared blood vessel density of tumors and adjacent normal areas. Mice were perfused with liquid latex Microfil to visualize the architecture of blood vessels. As shown in Figure 6, intestinal tumors that are about 2 mm in diameter were comprised of a dense and disorganized vasculature (Fig. 6C) with an average of 55.6 blood vessels counted per field view compared to 17.3 in normal regions (Fig. 6A). This technique revealed that nascent tumors with a diameter of less than 0.2 mm already exhibited a dense, convoluted, and chaotic vasculature (Fig. 6B) compared to adjacent normal regions of the intestine. These results indicate that both large and small tumors are highly infiltrated with BMDCs potentially through an enhanced vasculature system where they may contribute further to tumor vascularization through enhanced angiogenesis (Rafii et al., 2002).

Discussion

The development of adenomas in *Apc^{Min/+}* mice is initiated by the loss of the APC gene in undifferentiated stem cells at the bottom of intestinal crypts (Moser et al., 1990). This results in the stabilization of β -catenin and subsequent activation of genes involved in tumor proliferation. Loss of APC results in genomic instability that leads to further mutations in critical genes that delineate the multistep progression of colorectal cancer both in the inherited form, FAP, and in a majority of the sporadic incidences of the disease (Kinzler & Vogelstein, 1996; Fodde et al., 2001). However, while the loss of APC is necessary and sufficient to initiate adenoma formation, participation of stromal cells residing in the tumor microenvironment has been increasingly shown to be obligatory for tumor progression (Obermueller et al., 2004). The tumor microenvironment contains a variety of mesenchymal cells such as fibroblasts, myofibroblasts, endothelial cells, pericytes, and a variety of inflammatory cells associated with the immune system (Orimo & Weinberg, 2006). Together with the extracellular matrix, they interact with the tumor and secrete molecules that provide a permissive environment that promotes tumor growth and invasion (Coussens & Werb, 2002). While most therapies have been developed to target actively dividing cells, accumulating evidence supporting the critical role of tumor stromal cells in tumor progression (Lin et al., 2001) and response to therapy (Wang et al., 2004, 2005) suggest that they must also be targeted to effectively eliminate tumors (Hanna et al., 2009).

In this study, we examined the contribution of BMDCs to the composition of the intestinal tumor microenvironment. These cells are derived from hematopoietic stem cells, are mobilized from the bone marrow compartment, and are recruited to tumor sites by molecular signals elicited by the tumors (Chantrain et al., 2008). They are thought to be involved in tumor angiogenesis, metastatic progression, and immune escape (Papaspriidonos & Lyden, 2008) and have been shown to fuse with both normal and transformed intestinal stem cells, a phenomenon that may contribute to intestinal tumorigenesis (Rizvi et al., 2006). Their importance is linked to their potential as therapeutic targets, as well as their genetic character, which can be altered through the transplantation of genetically modified hematopoietic stem cells. They can therefore be exploited as a vehicle to deliver genes that may influence tumor phenotype or therapeutic outcome specifically to the tumor microenvironment to enhance therapeutic efficacy while avoiding toxicity to normal cells (Roorda et al., 2009).

To harness the potential of hematopoietic derived cells to enhance therapeutic efficacy, it is necessary to determine their distribution and contribution to the tumor stromal compartment. In this study, we utilized the *Apc^{Min/+}* mouse as a model to study the intestinal tumor microenvironment. Our results showed that tumors in these mice are highly vascularized even at the early stages of polyp formation. These results are consistent with previous studies wherein the complexity of the tumor microvasculature in these mice was determined by quantification of the vascular bed in latex perfused intestinal polyps by fractal dimension and image analysis (Fuseler et al., 2010). To examine the hematopoietic component of the intestinal tumor stroma, we transplanted hematopoietic stem cells expressing eGFP into lethally irradiated *Apc^{Min/+}* mice and analyzed intestinal tissues from engrafted mice by confocal microscopy. We determined that BMDCs are distributed unevenly throughout the intestinal tract. Quantification of eGFP fluorescence in sections taken along the length of the intestine showed a gradient of engraftment along the anterior-to-posterior axis, with more extensive infiltration in the proximal regions compared to the distal regions, and a striking accumulation of BMDCs in regions containing a tumor. As early as 4 weeks post-transplantation, eGFP-expressing cells derived from donor mice were detected in the crypt, villus, and lamina propria of the small intestine, and to a lower extent, in the lamina propria of the colon. Where tumors existed, eGFP-labeled cells infiltrated the stromal and interstitial compartment of the tumors. They appeared to accumulate in regions where elevated levels of the chemoattractant cytokine, SDF1, were present suggesting that SDF1 may play a role in their mobilization to tumor sites. It is possible that infiltration of the intestinal compartment might be a result of intestinal damage in response to pretransplant conditioning of the recipient mice with irradiation, or an immune response due to exposure of tissues to bacteria or viruses. However, the gradient of BMDC infiltration across the intestinal tract and their abundance in tumor sites compared to nontumor sites argue that the pathology and inflammation associated with tumor growth may be acting as a stimulus for enhanced BMDC infiltration. This argument is further bolstered by the observation that when intestinal sections from age-matched, nontransplanted *Apc^{Min/+}* mice were immunostained for the CD45 common leukocyte antigen, the results showed that leukocyte distribution in the unmanipulated system was similar to that in transplanted mice. Furthermore, we observed elevated levels of SDF1 within tumor-bearing regions in these tissues compared to nontumor-bearing regions. Thus, transplantation of eGFP expressing donor marrow into irradiated recipients is a valid technique for tracking the fate of genetically modified BMDCs at the time points examined in this study.

Low levels of BMDC infiltration was observed in nontumor regions of the colon, and in extra-intestinal tissues examined with the exception of the spleen, a hematopoietic organ, and the lungs, which can be infiltrated by leukocytes in response to bronchial inflammation. Costaining of eGFP expressing cells with various cell-specific cell surface markers indicated

that among the infiltrating cells were leukocytes, pan T-cells, helper T-cells, fibroblasts, and endothelial cells (data not shown). This is consistent with previous observations that the intestinal compartment in both wild-type and *Apc^{Min/+}* mice is highly infiltrated with hematopoietic-derived cells (Kettunen et al., 2003; Rizvi et al., 2006; Purhonen et al., 2008). Studies by Kettunen et al. showed that intestinal immune responses in wild-type and *Apc^{Min/+}* mutant mice were mostly similar and that intestinal inflammation was not responsible for initiating tumorigenesis in these mice. Thus, the enhanced recruitment of immune cells into the tumors that we observed was most likely in response to signals emanating from the tumor. Gounaris et al. (2007) showed that infiltration of preneoplastic polyps by mast cells is critical for their development. Depletion of mast cells by pharmacological methods or by genetic disruption of mast cell development resulted in drastic remission of existing polyps (Gounaris et al., 2007). Mast cells secrete an array of inflammatory cytokines, angiogenic and vascular permeability factors, and proteases (Galli et al., 2005), all of which may actively contribute to the growth of initiated polyps. Early studies by Erdman et al. (2003, 2005) showed that regulatory T cells (Tregs) exerted a protective role by inducing regression of tumors in *Apc^{Min/+}* mice through the suppression of focal mastocytosis in adenomatous polyps (Gounaris et al., 2009); however, aberrant Tregs in polyp-ridden mice can expand in an IL-17 dependent manner and switch to a proinflammatory, cancer-promoting function (Gounaris et al., 2009). In other studies, tumor stromal cells have been shown to be direct targets of cytotoxic agents. Vincent et al. (2010) showed that 5-fluorouracil, a chemotherapeutic agent that targets the enzyme thymidylate synthase (Danenbergh, 1977; Longley et al., 2003) and has been used in the clinical management of a variety of cancers for decades, selectively kills tumor-associated myeloid-derived suppressor cells, a phenomenon that was suggested to be a necessary component of its antitumor effect. These observations underscore the wide-ranging role of hematopoietic derived cells in tumor progression and therapeutic outcome, and targeting them for therapy might be a viable undertaking.

During tumor progression, both tumor and stromal cells have been shown to co-evolve as they respond to molecular signals that they elicit from each other (Littlepage et al., 2005). This suggests that the gene expression pattern of tumor-associated hematopoietic-derived cells is distinct from those that are associated with nontumor tissues or organs. Previous studies analyzing the genetic signature of colon cancer and cancer stroma in microdissected tissues from patients (Kitahara et al., 2001; Sugiyama et al., 2005) and fibroblasts associated with melanoma cells (Gallagher et al., 2005) indicated that certain genes are upregulated in stromal cells within the tumor microenvironment but not in stromal cells within normal tissues. We examined the expression of osteopontin in tumor and nontumor associated hematopoietic derived cells and showed that it is expressed abundantly in tumor stromal cells but not in stromal cells associated with normal regions of the intestine or those in tissues such as the spleen or liver. This observation suggests that it is possible to identify therapeutic targets that are specifically expressed in the tumor stroma, as well as genes whose promoters can drive the expression of gene products that, when delivered to the tumor microenvironment, can selectively modulate tumor phenotype or response to therapy. For example, Ferrari et al. (2003) genetically engineered bone marrow derived epithelial precursor cells *ex vivo* to express the thymidine kinase suicide gene by retroviral transduction into hematopoietic stem cells. Upon transplantation into sublethally irradiated mice, these cells homed into the vascular bed of growing tumors and enhanced tumor necrosis upon treatment with ganciclovir with no systemic toxicity observed during treatment (Ferrari et al., 2003). These data underscore the potential use of hematopoietic stem cells for tumor specific targeted therapy to enhance efficacy while minimizing toxicity to normal cells.

Summary

Tumor growth and progression depends on interactions between its neoplastic and nonneoplastic cellular components. While mutation of the *Apc* gene is necessary and sufficient to initiate polyp formation in *Apc^{Min/+}* mice, polyp expansion requires the presence of stromal cells in the tumor microenvironment. Our findings provide evidence that the intestinal tumor compartment is highly vascularized and infiltrated by hematopoietic derived cells. These cells are varied and their genetic signature is distinct from stromal cells associated with nontumor tissues within the intestine and in other organs. Their roles in determining tumor phenotype, progression, and response to therapy are equally diverse, and understanding these is essential to determine the feasibility of selectively targeting these cells as a therapeutic strategy and harnessing their vast potential to enhance therapeutic impact.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Engraftment of eGFP-expressing BMDCs in the intestinal tract of *Apc^{Min/+}* mice. **A–C:** Flow cytometric analyses of peripheral blood from transplanted mice. Erythrocytes were lysed with ammonium chloride. Leukocytes were stained with biotinylated anti-B220 antibodies and streptavidin–PE–Cy5 to detect B-cells. **A:** Nontransplanted control mice. **B,C:** Mouse transplanted with eGFP-expressing marrow. Lower left quadrant represents cells that are negative for both eGFP and PE–Cy5 while upper right quadrant represents cells that are positive for both eGFP and PE–Cy5. **D:** Graphs depicting localization of eGFP-labeled cells in various regions of the gut at 8 weeks (left panel) and 12 weeks (right panel)

post-transplant. Point 1 represents a region of the small intestine adjacent to the stomach. Starting at the junction of the stomach and small intestine, the entire gastrointestinal tract was subdivided into 5 cm sections. The localization and area occupied by cells expressing eGFP were determined by confocal imaging and image analysis. **E:** Confocal images representing each of the numbered sections quantified in the right panel in panel **D** at 12 weeks post-transplant. Four mice were examined for each time point. All mice examined showed an increased number of cells expressing eGFP in regions of the small intestine proximal to the stomach compared to the distal regions and the colon. An increased number of eGFP positive cells were also observed in regions that contained adenomas or tumors (**E**, arrows) compared to adjacent normal regions in the same section of the gut. Scale bar = 2 mm. Red, actin phalloidin; green, eGFP.

Figure 2.

Localization of eGFP-expressing cells in colon tumors. **A:** Colon section from nontransplanted mouse. **B:** Colon section from a wild-type C57Bl/6 mouse transplanted with eGFP expressing HSCs. **C,D:** Colon sections with tumors from mice transplanted with eGFP-expressing HSCs. Although fewer eGFP positive cells were localized in the colon, those that were present were predominantly associated with colonic tumors (**C**, upper right section; **D**, top and lower right region); the number of eGFP positive infiltrating cells decreased with increasing distance from the tumor. Red, actin; green, eGFP positive cells.

Figure 3.

Infiltration of eGFP-expressing cells into the small intestine. **A:** Intestinal regions containing tumors showed enhanced localization of eGFP-expressing BMDCs. **B:** Confocal image of normal regions of intestine. **C:** Confocal image of abnormal regions of the gut. Most eGFP-labeled cells were associated with interstitial tissue in both normal and abnormal regions of the intestine; however, in areas containing tumors or adenomas, occasional eGFP-expressing cells were associated with the epithelial lining of the intestine (arrow in panel C). Scale bar = 70 μ m for panel A and 20 μ m for panels B and C. Red, actin; green, eGFP positive cells; blue, DAPI.

Figure 4.

Engraftment of eGFP-expressing BMDCs in various tissues. Sections from various organs were taken from *Apc^{Min/+}* mice at 12 weeks post-transplantation and analyzed by confocal microscopy. Most eGFP positive cells were found in hematopoietic tissues such as the spleen (A) while few cells were found in tissues such as the heart (B), soleus muscle (C), liver (D), kidney (E), and lung (F). Scale bar = 70 μ m. Red, actin; green, eGFP positive cells.

Figure 5.

Expression of OPN in tumor associated hematopoietic derived stromal cells. Tumor and nontumor regions from the small intestine of *Apc^{Min/+}* mice transplanted with eGFP-expressing hematopoietic stem cells were stained with antibodies against OPN (red). Arrows indicate cells that are positive for both eGFP (green) and OPN (red). **A,B:** Stromal cells in tumor regions of intestines show colocalization of OPN and eGFP positive stromal cells. **C,D:** Stromal cells (green) in nontumor regions do not show staining with OPN antibody. **E,F:** Stromal cells (green) in the liver (**E**) or spleen (**F**) do not express OPN. (Yellow, actin; blue, DAPI; green, GFP; red, OPN.) Stromal derived factor 1 is elevated in tumor regions

(**G**) but not in nontumor regions (**H**) of the intestines, liver (**I**), or spleen (**J**). Red, SDF-1; green, GFP; blue, DAPI.

Figure 6.

Intestinal tumors in *Apc^{Min/+}* mice are highly vascularized. *Apc^{Min/+}* mice were perfused with phosphate buffered saline and then injected with curable yellow silicone latex rubber compound, Microfil MV-122 (Flow Tech, Inc.). After curing and fixation, the intestinal section was dissected and clarified with increasing concentrations of glycerol. Intestinal sections were photographed using a DP71 Olympus digital camera and vascular density was quantified by counting the number of blood vessels in at least five field views of each section at equal magnification. **A:** Normal regions of the intestine. **B:** Nascent tumors less than 0.2 mm in diameter. **C:** Large tumor (diameter = 2 mm).