ANIMAL MODELS

Orthotopic Animal Model of Pseudomyxoma Peritonei *An in Vivo Model to Test Anti-Angiogenic Drug Effects*

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Pseudomyxoma peritonei (PMP) is an uncommon peritoneal mucinous carcinomatosis confined to the peritoneal cavity. The rarity of PMP in humans makes evaluation of the disease biological features and new therapeutic strategies difficult. Accordingly, there is a need for animal models of PMP. Human PMP tissue was i.p. grafted and grown into nude mice, then constituted into reliable and reproducible orthotopic models. Histological and immunostaining analysis was performed. Bevacizumab was injected twice a week either during tumor growth or after cytoreductive surgery. *In vivo* imaging of tumor angiogenesis was performed using barium sulfate or isolectin microangiography and Doppler ultrasonography of the superior mesenteric artery. Tumor angiogenesis was confirmed by the presence of tortuous vascular networks with high levels of expression of CD31, vascular endothelial cadherin, and desmin. Doppler ultrasonography of the superior mesenteric artery revealed a twofold increase in blood flow velocity compared with tumor-free mice ($P < 0.001$). Bevacizumab administration was correlated with the normalization of tumor vascularity when injected during tumor growth and with the stabilization of the histological and hemodynamic findings when injected after cytoreductive surgery. Our PMP models mimic human PMP. Our results confirmed the presence of tumor angiogenesis related to PMP growth. Our murine model allows researchers to actually bench test and evaluate, in preclinical studies, the efficacy of new therapeutic strategies and anti-angiogenic therapies. (*Am J Pathol* 2014, 184: 1920–1929; <http://dx.doi.org/10.1016/j.ajpath.2014.03.004>)

Pseudomyxoma peritonei (PMP) is a rare malignant disease characterized by the progressive accumulation of mucinous tumor tissue in the peritoneal cavity without extraperitoneal growth, leading to intra-abdominal compression and a fatal outcome. The consensus is that PMP has an intestinal origin and results from the perforation of a mucinous appendiceal neoplasm.^{1–4} Complete cytoreductive surgery with hyperthermic i.p. chemotherapy is now a well-established treatment option for patients with PMP.^{5–10} Despite the

improved survival rate achieved with the combined treatment, this therapeutic option is not applicable to all patients, especially for those with coexisting causes of

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morbidity. Moreover, treatment failure and recurrence are not rare.¹¹

Histopathologically, PMP is graded from disseminated peritoneal adenomucinosis (DPAM or low grade) to peritoneal mucinous carcinomatosis (PMCA or high grade), with an intermediate histological type.¹² PMP presents as large patches of mucinous deposits that contain a few cells organized as a low-proliferative epithelium. The paucity of tumor cells may explain the relative lack of efficacy of the common cytotoxic chemotherapy regimens on PMP. To date, we are aware of only two case reports that have studied the potential impact of an anti-angiogenic agent (ie, bevacizumab) in PMP and reported a partial response.^{13,14} To improve the overall survival and recurrence-free survival rate, the development of new therapeutic strategies that combine chemotherapy and/or an anti-angiogenic drug with cytoreductive surgery is of major importance.

Animal models of PMP should mirror the peritoneal growth pattern of PMP in humans and should also mirror the histopathological classification used in humans. In addition, such animal models should be appropriate for testing new anti-angiogenic therapies and evaluating the tumor response. To our knowledge, there are only three published animal studies that describe the development of orthotopic xenografted PMP in animal models.^{15,16}

To date, the evaluation of patients with PMP is based on computed tomography and no functional imaging modality has a proven efficacy in the follow-up of these patients. Doppler ultrasonography (DUS) has been successfully used to monitor the tumor blood supply in primary and secondary liver cancers.^{17–21} The main blood supply of the PMP tumor arises from the superior mesenteric artery (SMA). We, thus, hypothesized that DUS of the SMA would provide an evaluation of the blood flow supplying the tumor vascular network. Indeed, it has been previously demonstrated that measurement of the blood flow velocity in the feeding artery upstream of the tumor vascular network allows for the semiquantitative analysis of the development or the involution of the tumor vasculature.^{17,21,22}

The goals of our study were threefold. First, we wanted to develop a murine model of PMP to investigate tumor angiogenesis in PMP. Second, we wanted to evaluate the capabilities of DUS in depicting and quantifying the development or the involution of the tumor vasculature in this experimental murine model. Third, we wanted to investigate the effects of anti-angiogenic therapies alone or as adjuvant treatments of PMP after incomplete surgical resection.

Materials and Methods

Orthotopic Murine Model of PMP

Patients

All of the tissue samples were collected after Institutional Review Board approval and after informed consent was obtained from all of the patients. Tumor samples were obtained from 18 patients with histologically proven PMP that

originated from an appendiceal mucinous neoplasm; patients were treated with surgery at two different institutions between November 2009 and May 2012.

Animals

Five-week-old female athymic Swiss nude mice (Charles River Laboratories International Inc., Arbresle, France), weighing between 16 and 18 g, were acclimatized for 1 week before tumor transplantation. The animals were maintained under specific pathogen-free conditions, and irradiated food and water were supplied *ad libitum*. During surgical procedures, the animals were anesthetized with a mixture of 80 mg/kg ketamine and 16 mg/kg xylazine delivered *i.p.* (Virbac, Carros, France). All of the experimental protocols and the animal procedures for the care and use of laboratory animals were conducted according to the guidelines of our institution and the Federation of European Laboratory Animal Science Association.

Tumor Collection and Implantation

The tumor samples were collected and immediately brought to the animal facility. The fresh tumor tissue was cut into 3 × 3 × 3-mm pieces. The tumor fragments were *i.p.* implanted in two mice for each sample. A 1-cm midline laparotomy was performed, and six tumor pieces were placed in the peritoneal cavity in both sides of the subdiaphragmatic area, the flank, and the pelvis. After implantation, the peritoneum and muscle were closed with one layer of Monocryl 5/0 (Ethicon, Somerville, NJ), and the skin was closed with staples.

Sampling and Evaluation

The mice were carefully monitored and weighed twice a week. The mice were sacrificed when they weighed >40 g. After sacrificing the mice, the macroscopic assessment of PMP was performed, during which a search for primary and metastatic tumors was performed; these tumors were sampled for further analyses. The tumor samples were embedded in optimal cutting temperature compound or fixed in formalin.

Cytoreductive Surgical Procedure

Cytoreductive surgery was performed at 33 g. Each mouse was anesthetized with 1.5% isoflurane in 100% O₂. A large midline incision was made, and maximum resection was performed with a clip applier. Lesions adjacent to the digestive tract were not resected because of the high risk of a lethal wound. The peritoneum and muscle were closed with one layer of Monocryl 5/0, and the skin was closed with staples. Immediately after surgery, mice were given an analgesic drug (buprenorphine; Schering-Plough Europe, Brussels, Belgium) in a single *s.c.* dose of 0.1 mg/kg.

Ex Vivo and in Vivo Characterization of Orthotopic Murine Models

Histological and Immunohistological Analyses

Tissue from the mouse tumor was explanted after sacrifice, fixed with formalin, and stained with H&E-safran and Alcian Blue. Histological classification was performed according to the three

groups, as described by Winer and Buckanovich¹⁴: 1, DPAM; 2, PMCA; and 3, intermediate histological type PMCA.

Archival paraffin-embedded tissue blocks were cut into sections (4 to 5 μ m) and transferred to glass slides. After deparaffinization, antigen retrieval was performed using citrate solution at pH 6.0. The slides were stained on an automated Benchmark Ultra immunostainer (Ventana Medical Systems, Tucson, AZ). The monoclonal anti-intestinal mucin (MUC)-2 antibody (cloner Ccp58; Novocastra, Newcastle, UK) was used at 1:100 dilutions. The epitope specifically recognized by the anti-MUC-2 antibody is human MUC-2 glycoprotein (no cross-reaction with MUC-1- or MUC-3-derived peptides).

Slides were incubated with the previously described primary antibody for 1 hour at room temperature; thereafter, goat anti-mouse secondary antibody was applied, and the reaction was developed using diaminobenzidine.

In addition, tumor samples from each passage through passage 5 were embedded in optimal cutting temperature compound for the preparation of frozen tissue sections (5 μ m thick), which were then fixed in acetone at 4°C for 10 minutes and immunostained as described. After washing, the non-specific sites were saturated for 20 minutes with bovine serum albumin (10 g/L in PBS–1% Tween). The sections were then incubated for 1 hour with the following primary antibodies: anti-pan-cytokeratin antibody (1:100; Abcam, Cambridge, MA) and anti-cytokeratin 20 (CK20) antibody (prediluted) (Abcam), which are specific for colon cancer; an antibody against Ki-67 (1:100; Neomarker Inc., Fremont, CA), which is a nuclear protein expressed in proliferating cells; antibodies against platelet endothelial cell adhesion molecule (CD31) (1:50; BD Pharmingen, Le Pont-de-Claix, France); an antibody against vascular endothelial (VE) cadherin (1:50; Santa Cruz Biotechnology Tebu SA, Le Perray-en-Yvelines, France), which is a protein localized at the intercellular junctions of endothelial cells; and an antibody against desmin (1:200; Neomarker Inc.), which is a protein specific for smooth and striated muscle. After washing, the sections were incubated for 30 minutes with the appropriate secondary antibody (1:200) [Alexa Fluor 555 goat anti-mouse, goat anti-rabbit, or donkey anti-goat, or Alexa Fluor 488 goat anti-mouse, donkey anti-rat, or donkey anti-goat (Interchim, Asnieres, France)] and then washed with PBS–1% Tween. The primary antibody was omitted on sections that were used as negative controls. To perform double immunostaining, the sections were initially incubated with the first primary antibody, then with the second primary antibody, and, finally, with the appropriate secondary antibody. The histological sections were analyzed with a fluorescence microscope equipped with the appropriate filters (Observer.Z1; Carl Zeiss MicroImaging GmbH, Göttingen, Germany).

TP53 Functionality and KRAS Mutation Analysis

Mutational analysis of *TP53* was performed using functional analysis of separated alleles in yeast, in accordance with the method described by Flaman et al.²³

This method requires frozen tumor samples and evaluates the transactivation activity of p53 on a p53-responsive promoter stably integrated in the yeast genome. RNA was extracted by the phenol-chloroform method and reverse transcribed, and p53 transcripts were amplified by PCR and transfected into yeast. Yeast colonies transformed with wild-type or mutated TP53 sequences appear white and large or red and small, respectively. TP53 status was considered mutated when >10% of the yeast colonies were red and analysis using the split versions of the test could identify the defect in the 5' or 3' part of the gene.

The seven most frequent *KRAS* mutations on codons 12 and 13 were analyzed by allelic discrimination after DNA extraction from selected formalin-fixed, paraffin-embedded sections (>20% tumor cells) of the tumor samples using the QIAamp DNA minikit (Qiagen, Hilden, Germany), according to manufacturer's instructions. Specific probes for each allele (mutated or not) were labeled with fluorescent reporter dyes at their 5' end and analyzed by real-time PCR on an LC480 instrument (Roche Diagnostics, Meylan, France).²⁴

Microangiography

Conventional microangiography was performed in five grafted mice to characterize the tumor vascular network. Under anesthesia, a longitudinal laparotomy was performed, and the abdominal aorta was exposed by moving the tumor to the left side and then ligating upstream of the iliac bifurcation. A polyethylene catheter with monitored pressure and volume was introduced into the abdominal aorta to inject a contrast material (barium sulfate, 1 g/mL). Image acquisition was performed with a digital X-ray transducer (model 2100; Kodak Dental Systems, Atlanta, GA).²²

Alternatively, performing the same surgical procedure in another five mice, we perfused 0.5 mL of heparinized saline and 0.8 mL of *bandeiraea simplicifolia* isolectin B4 (IB4) conjugated to fluorescein isothiocyanate (Sigma, Saint Quentin Fallavier, France), diluted with PBS to a concentration of 0.25 mg/mL. This tracer binds selectively to terminal α -galactose (IB4)²⁵ and is an endothelial cell-specific marker that was used to identify and characterize the neovessels in the tumor. Fifteen minutes later, the mice were sacrificed, and the tumors were harvested. After fixation, the tumor samples were analyzed with a computer-assisted Nikon fluorescence microscope with a digital camera (Eclipse TE200; Nikon Inc., Melville, NY). Because of the heterogeneous distribution of the vasculature and the presence of wide ranges of mucinous content, we did not quantify the density of the vessels.

Ultrasonographic Examination

Ten Swiss, nude, PMCA tumor-grafted and 10 age-matched normal mice had ultrasonographic measurements using a DUS instrument (Vivid 7; GE Medical Systems Ultrasound, Horten, Norway) equipped with a 12-MHz linear transducer, as previously described.²² Briefly, after sedation, the mice were placed on a heating blanket (38°C) in the left lateral prone position to avoid placing pressure on the

abdomen with the transducer. The color Doppler mode was activated with an anterior longitudinal view of the abdomen crossing the longitudinal axis of the aorta. The SMA and celiac trunk were localized on the screen by their color-coded blood flow. A pulsed Doppler sample was then placed on the longitudinal axis of each vessel, and pulsed Doppler velocity waveforms were recorded. Peak-systolic, end-diastolic, and time-averaged mean blood flow velocities (BFVs) were measured according to the pulsed Doppler spectrum. The DUS recordings were repeated every 2 weeks from the initiation of the tumor graft up to 6 weeks.

Serum Angiogenesis Markers

After sacrificing the mice, blood was collected into chilled tubes containing heparin (Sarstedt, Marnay, France). The plasma concentrations of vascular endothelial growth factor (VEGF), placental growth factor (PIGF), and transforming growth factor β (TGF- β) were determined with ELISA tests (MMV00, DPG00, and MB100B, respectively), according to the manufacturer's instructions.

Treatment

In another set of experiments, we applied an anti-angiogenic treatment in two models of PMCA (PMCA-1 and PMCA-2). Four weeks after the PMCA surgical graft, the mice were randomly assigned to two different groups. The mice in the treated groups were i.p. injected twice a week with 5 mg/kg bevacizumab ($n = 10$ for PMCA-1 and $n = 10$ for PMCA-2), according to prior publications. The mice in the control groups ($n = 10$ for each experiment) received an equivalent volume of PBS. The mice were sacrificed when they weighed >40 g. The serum angiogenesis markers (VEGF, PIGF, and TGF- β) were quantified in these two groups 6 weeks after the tumor graft and compared with five age-matched, non-grafted mice. In another set of experiments, after cytoreductive surgery was performed at the weight of 33 g, the grafted mice were submitted to an anti-angiogenic treatment with bevacizumab ($n = 11$) versus PBS ($n = 9$), i.p. injected twice a week in an adjuvant setting. The mice were carefully monitored for 4 weeks with DUS and weighed weekly. The development of tumor growth was evaluated by following the increase in weight. Serum angiogenesis markers were also quantified.

Statistical Analysis

Results were expressed as the means $\pm$ SD. Heart rate, blood flow velocities, and concentrations of serum angiogenesis markers were compared using an analysis of variance. Post hoc paired and unpaired Student's *t*-tests were performed to identify which time point or which group differences accounted for the significant analysis of variance. The survival probabilities were estimated using the Kaplan-Meier product-limit method (MedCalc, version 10.4.3.0; MedCalc Software, Ostend, Belgium). $P < 0.05$ was considered to be significant.

Results

Tumor Growth in the Animal Models

Among the 18 i.p. grafted, patient-retrieved PMP samples, nine succeeded in growing, but growth occurred only once for one of the samples. Among the eight remaining samples, seven were similar to a PMCA (PMCA-1 to PMCA-7), and one was representative of a DPAM. These eight models were considered to be reliable and reproducible orthotopic mouse models of PMP. The delay between successive tumor growths decreased from 136 days (range, 116 to 166 days) after the first transplant to 78 days (range, 42 to 115 days) after the fifth transplant. The take rates ranged from 80% to 100% for PMCA-1 to PMCA-7 and from 40% to 90% for DPAM. As observed in humans, the mice presented abdominal distension due to tumor growth. PMCA-1 to PMCA-7 were considered as more aggressive compared with DPAM because these tumors grew more quickly.

The typical initial i.p. growth pattern was characterized by mucinous ascites, accompanied by a variable number of solid mucinous tumor lesions of varying sizes. The solid tumor components were attached to the peritoneum and serosa of all of the i.p. organs. No animal presented any metastatic lesions.

Microscopic Assessment

The animal models of PMP reflect the histopathological classification after each transplant generation. In the mice, we confirmed with H&E-safran staining that DPAM developed from a low-grade appendiceal mucinous tumor. This subtype was characterized by widespread peritoneal deposits of abundant pools of extracellular mucin with strips of proliferative mucinous columnar epithelium with little cytological atypia or mitotic activity (Supplemental Figure S1, A and B). PMCA consisted of a juxtaposition of glands formed by an abundant mucinous epithelium and/or signet ring cells and commonly had severe cytological atypia. This subtype had poorly differentiated architecture (Supplemental Figure S1, C and D). The pathological characteristics were conserved through the successive tumor passages, with no change in the proportion of mucinous ascites and solid mucinous tumor lesions.

High levels of expression of pan-cytokeratin and CK20, as indicated with immunostaining, were observed in the tumor through the successive tumor passages from mouse to mouse, which indicates that the human and digestive origin was maintained (Supplemental Figure S2, A and B). Low levels of DAPI immunostaining and the presence of only a few isolated Ki-67-positive tumor cells were observed, which verified the paucicellular and low proliferative characteristics of the tumor (Supplemental Figure S2, C and D).

Immunohistochemistry showed high levels of cytoplasmic staining of tumor cells by the antibody anti-MUC-2 and in the human tumor as after passages from mouse to mouse, confirming that tumor kept its biochemical

characteristics with a high production of mucin (Supplemental Figure S3, A and B).

Molecular characteristics were also analyzed in the PMCA-2 model for initial patient and xenografted PMP in two mice. No alteration of the p53 functionality (*TP53* wild type) was detected in the initial human sample or in the xenografted tumors. Nevertheless, an activating p.G12V mutation of the *KRAS* gene was detected in the human sample and two xenografted mice, highlighting that the xenografted tumors reflect the human origin at the molecular level.

Angiogenesis Analysis

Tumor angiogenesis was visualized with anti-CD31 and anti-VE-cadherin antibodies, which are specific for endothelial cells (Supplemental Figure S2, E–G), and an anti-desmin antibody, which stained pericytes (Supplemental Figure S2H). Double staining confirmed the colocalization of the VE-cadherin and desmin immunoreactivity.

Microangiography

After *in vivo* intra-aortic injection of barium sulfate (Figure 1A) or IB4 (Figure 1B), microangiography revealed the arterial vascular network and showed the abdominal aorta, the left and right renal arteries, the gastric arteries, the hepatic arteries, and the superior mesenteric artery. The vascular networks downstream of the SMA presented many functional tortuous and irregular newly developed vessels going toward and through the i.p. tumor.

Ultrasonographic Examination

The tumor growth and development of ascites were observed by DUS (Figure 2A). In the control mice, time-averaged mean BFV measurements in the SMA and in the celiac trunk remained stable: 6.9 ± 1.9 cm/second and 12.5 ± 4.4 cm/second, respectively (Figure 2, B–E and F). In contrast, in the tumor group, the mean BFV in the SMA increased from the time of the tumor graft to the time of sacrifice (from 6.9 ± 1.9 to 13.8 ± 2.1 cm/second; $P < 0.001$) (Figure 2, C and E), although the mean BFV remained stable in the celiac trunk (Figure 2, D and F). The 1.75-fold increase in the mean BFV in the SMA compared with the control values ($P < 0.001$) was representative of the development of the vascular network downstream from the artery (ie, the number of opened and dilated vessels, thus indicating tumor angiogenesis of the peritoneal tumor).

Treatments

Treatment with an Anti-Angiogenic Agent (Bevacizumab) Alone without Cytoreductive Surgery

Survival was defined by the time required to reach 40 g weight. Mice in the PMCA-1-grafted and PMCA-2-grafted mouse models, treated with bevacizumab, had a significantly greater survival time than the mice in the control groups without treatment (Figure 3, A and B). For the PMCA-1 model, the median survival time was 79.8 days (range, 45 to 103 days) in the treated group ($n = 10$) and 58.4 days (range, 45 to 68 days) in the control group ($n = 10$) [$P < 0.001$; hazard ratio (HR), 0.12; 95% CI, 0.03 to 0.47]. For the PMCA-2 model, the median survival time

Figure 1 **A:** Representative computed photo-microangiography performed with a digital X-ray transducer in a 12-week-old mouse with PMP. **B:** Representative images of neoangiogenesis in PMP after *in vivo* intra-aortic injection of IB4 (green fluorescence) in untreated control mice. **C and D:** After 6 weeks of bevacizumab treatment, the tumor vessels appeared more regular, straight, and organized. Original magnification: $\times 10$ (B–D). A.A., abdominal aorta; F., femur; G.&H., gastric and hepatic; L.K., left kidney; M., mesenteric; R.K., right kidney.

Figure 2 **A:** Two-dimensional DUS of the abdomen of a 12-week-old mouse with PMP showing solid tumor and ascites. **B:** Anterior longitudinal view of the abdomen crossing the longitudinal axis of the aorta, with the SMA and celiac trunk revealed by color-coded Doppler ultrasonography. Representative BFV waveforms recorded in the SMA in ungrafted (**C**) and PMP-grafted (**D**) mice. The blue line represents the time-averaged mean BFV. **E** and **F:** Changes in the mean BFV in the SMA and in the celiac trunk from the time of the tumor graft to 6 weeks afterward ($n = 10$). The mean BFV remained constant in the celiac trunk but increased markedly in the SMA. * $P < 0.05$, *** $P < 0.001$ versus the beginning of the recordings just after tumor graft; † $P < 0.05$, †† $P < 0.01$ versus healthy mice ($n = 10$). Gray bars, PMP; white bars, controls.

was 81.6 days (range, 54 to 126 days) in the treated group ($n = 9$) and 58.2 days (range, 51 to 70 days) in the control group ($n = 9$; $P = 0.002$; HR, 0.16; 95% CI, 0.05 to 0.57).

The plasma concentrations of VEGF, PlGF, and TGF- β in the five healthy ungrafted mice were 17.9 ± 2.7 , 20.9 ± 4.1 , and 4.5 ± 2.9 ng/L, respectively. In the PMCA-1 model, the control mice ($n = 5$) presented dramatically increased plasma concentrations of 140.7 ± 19.5 , 203.4 ± 55.2 , and 28.1 ± 5.4 ng/L for VEGF, PlGF, and TGF- β , respectively ($P < 0.001$, compared with the healthy mice). In the bevacizumab-treated mice ($n = 5$), the serum levels were lower compared with the untreated grafted mice, and were decreased to 50.6 ± 5 ng/L ($P = 0.005$), 54.2 ± 13.4 ng/L ($P = 0.014$), and 14.1 ± 3.5 ng/L ($P = 0.047$), respectively, for VEGF, PlGF, and TGF- β . However, the serum levels remained elevated compared with the healthy mice ($P < 0.01$) (Figure 4). After treatment with bevacizumab, the vessels were more organized, regular, and straight, as indicated by microangiography using IB4 in conjunction with fluorescein isothiocyanate (Figure 1, C and D).

Treatment with an Anti-Angiogenic Agent (Bevacizumab) after Cyto-reductive Surgery

After surgery, all of the mice presented lower weights ($P < 0.01$). The PBS-treated mice had a rapid recurrence of

the PMP, with an increase in weight and abdominal volume (Figure 5A). Moreover, all of the PBS-treated mice reached the cutoff weight of 40 g within the first 4 weeks after resection. The mean BFV recorded in the SMA increased to 190% 4 weeks after surgery ($P < 0.01$), without any decrease immediately after surgery (Figure 5B). The mean BFV recorded in the celiac trunk remained unchanged (Figure 5C). In contrast, the bevacizumab-treated mice did not present any increase in their weight or in their abdominal volume until the fourth week (ie, at the end of the follow-up). Moreover, they had lower weights at 4 weeks compared with the untreated mice ($P < 0.05$). At the fourth week, the mean BFVs in the SMA decreased to 75% of the value recorded before surgery ($P < 0.05$). Moreover, they were lower compared with those recorded in the untreated mice at the fourth week of follow-up ($P < 0.001$) (Figure 5B). The mean BFV remained unchanged in the celiac trunk during the first 3 weeks after surgery and the introduction of anti-angiogenic treatment, but decreased at the fourth week compared with the untreated mice ($P < 0.01$) (Figure 5C). The plasma concentrations of VEGF, PlGF, and TGF- β in the mice treated with surgery alone were 178.6 ± 42.4 , 12.82 ± 3.79 , and 65.6 ± 7.3 ng/L, respectively, whereas the levels of these markers decreased in the mice treated with surgery in combination

Figure 3 Kaplan-Meier analysis of survival in i.p. tumor-grafted mice injected twice a week with 5 mg/kg bevacizumab compared with mice injected with vehicle (control mice). **A:** First model of PMCA (PMCA-1). **B:** Second model of PMCA (PMCA-2). Mice treated with bevacizumab had a significantly longer survival time than the mice in the control groups in both models. $P < 0.001$ (HR = 8.7318 in **A** and HR = 7.0511 in **B**).

with bevacizumab to 41.5 ± 17.4 ng/L ($P < 0.001$), 4.6 ± 1.6 ng/L ($P < 0.001$), and 23.4 ± 7.0 ng/L ($P < 0.001$) for VEGF, PLGF, and TGF- β , respectively. However, the serum levels remained elevated compared with the healthy mice ($P < 0.01$) (Figure 4B).

Discussion

We established eight reliable and reproducible *in vivo* models of PMP in immune-compromised nude mice, including one DPAM model and seven PMCA models. The tumors resulting from the i.p. implantation of PMP retained histological, immunohistological, molecular, and topographical characteristics similar to those in humans. The immunostaining analysis highlighted the presence of vessels within the tumor associated with a few isolated Ki-67–positive tumor cells, thus confirming the low proliferation characteristic of the tumor and this tumor's low sensitivity to standard chemotherapy.²⁶

By using our models, we were also able to demonstrate with microangiography and DUS the actual *in vivo* development of tumor angiogenesis. Moreover, we also demonstrated a correlation between the mean BFV, as measured in the SMA with DUS and the body weight. Finally, we illustrated the anti-angiogenic effect of bevacizumab, which manifested as a slower tumor progression.

To our knowledge, our models are among the first mouse models of PMP. We found in the literature only three publications that reported animal models of PMP.^{15,27,28} Flatmark et al^{16,27} published five mouse models of human PMCA. Indeed, three of the five models consisted of mucinous peritoneal carcinomatosis from various origins. In addition, two of the five models were classified as PMCA-I, according to Ronnett's classification, which corresponded to tumors with a moderate degree of aggressiveness.³ In their last study, Flatmark et al²⁸ successfully tested several i.p. chemotherapies and a new immunotoxin immediately after the graft. Although their model is interesting, they i.p. inject mucinous ascites and not solid tumor tissue; therefore, their model is more liquid. Moreover, no study has tested any therapy in an adjuvant setting or after cytoreductive surgery.

Chua et al¹⁵ have generated three models of histologically proven DPAM in rats. Although they reported a take rate of 100% within 3 months after the grafts, they did not provide any information regarding the conservation of their model, which makes their model difficult to reproduce.¹⁵ In addition, similar

Figure 4 Analysis of serum angiogenesis markers (VEGF, PLGF, and TGF- β). The expression levels of VEGF, PLGF, and TGF- β were significantly increased in PMP-grafted mice ($n = 5$) compared with healthy mice ($n = 5$). **A:** The expression levels were decreased in the PMP-grafted mice that were injected twice a week with 5 mg/kg bevacizumab ($n = 5$), but remained elevated compared with the control group. Analysis of serum angiogenesis markers (VEGF, PLGF, and TGF- β) in healthy mice ($n = 5$), in PMP-grafted mice treated with cytoreductive surgery and PBS ($n = 9$), and in grafted mice treated with cytoreductive surgery and 5 mg/kg bevacizumab injected twice a week ($n = 11$). **B:** The expression levels were decreased in the bevacizumab-treated group, but remained elevated compared with the control group. $^{**}P < 0.01$, $^{***}P < 0.001$ versus healthy mice; $^{ii}P < 0.01$, $^{iii}P < 0.001$ versus the vehicle group.

to Flatmark et al,²⁷ the models of Chua et al⁵ corresponded to a benign subtype of PMP, precluding the meaningful assessment of anti-angiogenic treatments. The histopathological subtype of a tumor is an independent predictor of poor progression-free survival and poor overall survival. Consequently, the assessment of anti-angiogenic drugs should be performed on the most

aggressive models of PMP. In our study, we developed PMCA animal models of the most aggressive subtypes, which are the most useful for evaluating anti-angiogenic treatments.

Angiogenesis has become a new area for intensive research in imaging. The quantification of angiogenesis requires new models and more refined tools or the implementation of functional applications of well-established techniques for monitoring the response to newly developed anti-angiogenic drugs. The development of techniques to quantify tissue activity may help predict tumor sensitivity to a specific treatment and allow for the early detection of the absence of a response to new and expensive therapies that are not free from adverse effects.

Noninvasive functional imaging modalities, such as magnetic resonance imaging, dynamic contrast-enhanced computed tomography, and positron emission tomography, have been used for the assessment of the vessels and microcirculation in animals. However, the limitations of these techniques include a high cost and limited availability. For preclinical studies in small animals, we require noninvasive methods that ideally would be reliable, rapid, reproducible, easy to repeat during a prolonged follow-up, and affordable. Two-dimensional, color-coded, pulsed DUS for the measurement of BFV in tumor-feeding vessels provides a powerful tool for the *in vivo* evaluation of tumor growth and the assessment of neovascularization in preclinical studies and can be a useful technique to assess treatment efficacy.^{19–21}

We used the SMA mean BFV recordings to assess the functionality and density of the downstream vessels. In our study, we found greater mean BFVs in the SMAs of mice with peritoneal tumors containing abnormal vessels. The peritoneal vasculature is mainly supplied by the SMA. According to our results, mean BFV measurements in the SMA could be an indirect indication of the development of vessels in the peritoneal tumor and constitute a useful tool for the assessment of neovascularization in PMP in mice. The results of the DUS, along with an increased abnormal vessel density observed with microangiography and an increase in the blood markers of angiogenesis, reinforce our hypothesis that our orthotopic murine models of PMP are relevant to test angiogenesis and the putative beneficial effects of anti-angiogenic therapies.

The potentially beneficial effects of anti-angiogenic therapies have led to the development of various drugs that require preclinical studies in animals to evaluate their efficacy and toxicity before clinical trials can be initiated. The role of angiogenesis and vascular remodeling in the development of cancer has been largely demonstrated.^{29,30} Consequently, tumor vessels have become an important target in cancer therapy with the development of novel targeted therapies.^{31–33} However, these clinical developments appear to have more challenges to controlling tumor growth, such that preclinical studies in small animals with the evaluation of tumor angiogenesis are required. We postulated that PMP models could be used for testing the efficacy of new anti-angiogenic drugs because PMP tumors grow slowly. Because of the associated antitumor effect of most anti-angiogenic agents, testing only the anti-angiogenic function, apart from the antitumor

Figure 5 Treatment with an anti-angiogenic agent (bevacizumab) after cytoreductive surgery. All of the mice had reached 35 to 40 g before surgery. After resection, the weight decreased significantly in both groups. **A:** In the PBS-treated group, the weight was significantly higher at 4 weeks compared with the preresection weight, whereas no significant difference was found in the bevacizumab-treated group. **B:** In the PBS-treated group, the mean BFV in the SMA increased progressively after surgery, whereas the BFV decreased in the bevacizumab-treated group. **C:** In the PBS-treated group, the mean BFV in the celiac trunk remained stable after surgery, whereas the BFV decreased at the fourth week in the bevacizumab-treated group. [‡] $P < 0.05$, ^{††} $P < 0.01$, and ^{†††} $P < 0.001$ versus PBS-treated mice.

function, is difficult with common cancers. Indeed, the low proliferative profile of PMP associated with neo-angiogenesis allows for the evaluation of the efficacy of anti-angiogenic drugs without any bias induced by an anti-proliferative effect. PMP is a rare disease, but this animal model could offer a new possibility to replace the Matrigel test that is usually proposed. Indeed, bevacizumab induced a significant delay in tumor growth, according to our experiments.

Recent studies have revealed that anti-VEGF therapy may have a limited effect on tumor vessels,^{34–36} highlighting the importance of developing other anti-angiogenic strategies for cancer treatment. Other therapeutic targets may be explored through identifying cytotoxic drugs that target the atypical cells or disturb the mucinous excretion component of the tumor cells. More important, there is a need to recognize why conventional DNA-damaging cytotoxic drugs have a limited efficacy in PMP. Indeed, most PMPs contain large amounts of mucin but are paucicellular. Anti-inflammatory drugs could inhibit extracellular mucin production in PMP by targeting inflammatory cascades and may increase the disease-free interval or reduce the extent or frequency of morbid cytoreductive surgical procedures.³⁷

Our study has several limitations. First, in xenograft models, tumor vessel formation originates from the host mouse. The loss of human stromal cells was reported in a large colon cancer xenograft panel that was recently published.³⁸ For this reason, it could be postulated that some drugs may be ineffective, or less effective, because the drugs are targeted to human vessels and not to mouse vessels. Although theoretically possible, we did not observe such an occurrence in our models. Second, DUS is an operator-dependent technique. Therefore, sampling of the mean BFV was repeated five times and averaged to limit intraobserver variability. Moreover, the investigator performing the DUS (P.B.) was blinded to the treatment for each mouse. Third, although we demonstrated angiogenesis in PMP, because of the poor cellularity inside the tumor and the amount of nonvascularized mucinous component, this angiogenesis does not account for the global increase in blood flow in the SMA. However, isolectin microangiography indicated irregular and tortuous neovessels in the tumor. It is likely that further experimentation with *in vivo* functional imaging of the tumor will help to understand the changes in tumor vascularity in terms of permeability and changes in the size of the vessels.

In conclusion, we established eight reproducible murine models of PMP, which are similar to human PMP, particularly regarding the local peritoneal development and the absence of metastasis. Moreover, the human histological features of the tumor are retained in the mouse. In this model, we highlighted the role of tumor angiogenesis, even for this paucicellular tumor. The anti-angiogenic drug (bevacizumab) has proven benefit with respect to the reduction of tumor microvessel density, as revealed with DUS and the normalization of the microvessels and when this drug is introduced as an adjuvant therapy after cytoreductive surgery. This promising adjuvant therapy will help to avoid a recurrence of the disease and the re-accumulation of mucin,

which characterizes the failure of surgical treatments and is responsible for repeated treatment and increased morbidity.

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Supplemental Data

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