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Role of vascular endothelial growth factor and placental growth factor in basal adhesion formation and in carbon dioxide pneumoperitoneum-enhanced adhesion formation after laparoscopic surgery in transgenic mice

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Objective: To evaluate the role of vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) in adhesion formation after laparoscopic surgery.

Design: Prospective, randomized study.

Setting: Academic research center.

Animal(s): Female wild-type mice and transgenic mice (n = 110), expressing exclusively VEGF-A_{164} (VEGF-$A^{164/164}$) or deficient for VEGF-B (VEGF-$B^{-/-}$) or for PlGF ($PlGF^{-/-}$).

Intervention(s): Adhesions were induced during laparoscopy. To evaluate "basal adhesions" and "CO_2 pneumoperitoneum-enhanced adhesions," the pneumoperitoneum was maintained for a minimum (10 minutes) or prolonged (60 minutes) period. The role of PlGF was also evaluated by administration of antibodies.

Main Outcome Measurement(s): Adhesions were blindly scored after 7 days.

Result(s): In all wild-type mice, CO_2 pneumoperitoneum enhanced adhesion formation. In comparison with wild-type mice, basal adhesions were higher in VEGF-$A^{164/164}$ mice and similar in VEGF-$B^{-/-}$ and $PlGF^{-/-}$ mice. Pneumoperitoneum did not enhance adhesions in any of these transgenic mice. The effects observed in $PlGF^{-/-}$ mice were confirmed in PlGF antibody-treated mice.

Conclusion(s): The data demonstrate that the VEGF family plays a role in adhesion formation and confirm that CO_2 pneumoperitoneum enhances adhesions. VEGF-A_{164} has a direct role in basal adhesions. Absence of pneumoperitoneum-enhanced adhesions in VEGF-$A^{164/164}$, VEGF-$B^{-/-}$, and $PlGF^{-/-}$ mice indicates up-regulation of VEGF-A_{164}, VEGF-B, and PlGF by CO_2 pneumoperitoneum as a mechanism for pneumoperitoneum-enhanced adhesion formation. (Fertil Steril® 2003;80(Suppl 2):803–11. ©2003 by American Society for Reproductive Medicine.)

Key Words: Adhesion formation, laparoscopy, CO_2 pneumoperitoneum, VEGF-A, VEGF-B, PlGF, transgenic mice

The speed of fibrin degradation after peritoneal injury is a critical process in peritoneal repair and adhesion formation. If the local fibrinolysis is insufficient, the remaining fibrin serves as a scaffold for fibroblast growth, extracellular matrix (ECM) deposition, and capillary growth, leading to adhesion formation. The role of fibrin and other components of the plasminogen system, fibroblasts, and ECM in adhesion formation is well known (1–6). The role of angiogenesis, however, has barely been explored after laparotomy or laparoscopy.

Angiogenesis, the formation of new blood vessels extending from existing vessels, occurs when the distance from the nearest capillary exceeds an efficient diffusion range maintaining adequate supply of oxygen and nutrients to

cells. Angiogenesis is regulated mainly by cellular hypoxia through the modulation of angiogenic factor expression, such as the up-regulation of vascular endothelial growth factor (VEGF)-A (7–10).

A role for angiogenesis and hypoxia in adhesion formation is suspected for several reasons. First, tissue necrosis and the inflammatory reaction after trauma involve cellular hypoxia, which has been recognized as important in adhesion formation (11–13). Second, hypoxia modulates the expression of several molecules involved in different stages of adhesion formation, such as plasminogen activator inhibitor-1 (PAI-1), tissue-type plasminogen activator (tPA), transforming growth factor-β (TGF-β), matrix metalloproteinases (MMPs), and tissue inhibitors of metalloproteinases (TIMPs) (14–23). Third, the CO_2 pneumoperitoneum is a cofactor in adhesion formation after laparoscopic surgery and as adhesions increase in a time- and pressure-dependent manner and decrease with the addition of oxygen, mesothelial hypoxia has been suggested as the driving mechanism (24–27). It is unknown, however, whether the mechanisms of adhesion formation after a peritoneal lesion only ("basal adhesions") or of the increased adhesion formation after a peritoneal lesion with the additional effect of the pneumoperitoneum ("pneumoperitoneum-enhanced adhesions") are different.

Vascular endothelial growth factor is a family of angiogenic factors including VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PlGF). Vascular endothelial growth factor-A is transcribed from a single gene and processed into four isoforms in humans (VEGF-A_{121}, VEGF-A_{165}, VEGF-A_{189}, and VEGF-A_{206}) and three isoforms in mice (VEGF-A_{120}, VEGF-A_{164}, and VEGF-A_{188}). Vascular endothelial growth factor-B is transcribed from a single gene and processed into two isoforms in humans and in mice (VEGF-B_{167} and VEGF-B_{186}). Placental growth factor is also transcribed from a single gene and processed into three isoforms in humans (PlGF-1, PlGF-2, and PlGF-3) and one isoform in mice (PlGF-2) (28–41).

The VEGF-A, VEGF-B, and PlGF are dimeric glycoproteins that bind to two tyrosine kinase receptors: VEGFR-1 (VEGF-A, VEGF-B, and PlGF) and VEGFR-2 (VEGF-A) (28–30, 41). Heterodimerization of VEGF-A with VEGF-B or PlGF controls the bioavailability of VEGF-A, modulating its mitogenic, chemotactic, and vascular permeability-inducing properties (36–45). In contrast with the marked up-regulation of VEGF-A by hypoxia, the up-regulation of VEGF-B and PlGF by hypoxia is controversial (46–49). The formation of VEGF-A/VEGF-B and of VEGF-A/PlGF heterodimers, however, is clearly modulated by hypoxia (45, 50).

These experiments were designed to investigate the role of the angiogenic factors VEGF-A, VEGF-B, and PlGF in both basal adhesions and CO_2 pneumoperitoneum-enhanced adhesions in a laparoscopic mouse model using transgenic mice and monoclonal antibodies.

MATERIALS AND METHODS

Animals

The study was performed in 110 female, 10–12-week-old mice weighing 30–40 g. For the first study 20 75% Swiss–25% 129SvJ wild-type mice (VEGF-$A^{+/+}$) and transgenic mice deficient for VEGF-A_{120} and for VEGF-A_{188} and overexpressing exclusively VEGF-A_{164} (VEGF-$A^{164/164}$) were used. For the second study 20 100% C57Bl/6J wild-type mice (VEGF-$B^{+/+}$) and transgenic mice deficient for VEGF-B (VEGF-$B^{-/-}$) were used. For the third study 20 50% Swiss–50% 129SvJ wild-type mice (PlGF$^{+/+}$) and transgenic mice deficient for PlGF (PlGF$^{-/-}$) were used. For the fourth study 50 100% Swiss wild-type mice were used.

The VEGF-A and PlGF wild-type mice and transgenic mice were obtained from the Center for Transgene Technology and Gene Therapy of the Katholieke Universiteit Leuven (KUL) and VEGF-B wild-type mice and transgenic mice from the Ludwig Institute for Cancer Research, Stockholm Branch, Sweden. The transgenic mice were generated as described (51–54). No major phenotypic differences were observed between wild-type and transgenic mice of the same strain. Small differences, however, have been reported, such as an atrial conduction abnormality in VEGF-$B^{-/-}$ mice characterized by a prolonged PQ interval in the electrocardiogram, which did not generate arrhythmia or any other malfunction of the heart (54) and a subtle remodeling defect of retinal vessels in PlGF$^{-/-}$ mice (53).

The animals were kept under standard laboratory conditions (temperature 20°–22°C, relative humidity 50%–60%, 14 hours light and 10 hours dark) at the animal facilities of the KUL. They were fed a standard laboratory diet (Muracon.G, Carsil Quality, Turnhout, Belgium) with free access to food and water. The study was approved by the Institutional Review Animal Care Committee.

Anesthesia

After IM anesthesia with pentobarbital (Nembutal, Sanofi Sante Animale, Brussels, Belgium; 0.07 mg/g) the abdomen was shaved and the animal was secured to the table in the supine position. Endotracheal intubation was performed with a 22-gauge catheter (Insyte-W, Vialon, Becton Dickinson, Madrid, Spain) by transillumination of the vocal cords, as described (27). The catheter was connected to a mechanical ventilator (Rodent Ventilator, Harvard Apparatus, Holliston, MA) and the animal was ventilated with room air with a tidal volume of 500 μL and a respiratory rate of 85 strokes/min. In other studies, blood samples from the carotid artery during the pneumoperitoneum showed that this ventilation pattern is enough to maintain oxygen saturation at 98%–99% (Molinas CR, Tjwa M, Binda M, Elkelani O, Vanacker B, Koninckx P, personal communication).

Laparoscopic Surgery for Induction of Intraperitoneal Adhesions

Laparoscopy and induction of adhesions were performed as described (27). A 2-mm endoscope with a 3.3-mm external sheath for insufflation (Karl Storz, Tüttlingen, Germany), connected to a video camera and light source (Karl Storz) and secured in a holder, was introduced into the abdominal cavity through a 3.5-mm midline incision caudal to the xyphoides appendix. Because the mouse abdominal wall is very thin, a variable gas leakage and thus a variable flow through the abdomen occurs. Therefore, the incision was closed gas tight around the endoscope with 6/0 polypropylene suture (Prolene, Ethicon, Johnson and Johnson Intl., Brussels, Belgium).

For the pneumoperitoneum, the gas was insufflated through the main port with the Thermoflator Plus (Karl Storz) using heated (37°C; Optitherm, Karl Storz) and humidified (Aquapor, Dräger, Lübeck, Germany) CO_2 as insufflation gas. An insufflation pressure of 17 mm Hg and a flow rate of 1.5 L/min together with a water valve and an elastic balloon were used to ascertain a continuous insufflation pressure of 20 cm H_2O ($\cong$15 mm Hg). The water valve and the balloon are necessary to adapt the flow rate to a mouse and to dampen the pressure changes during insufflation. Indeed, any excess of CO_2 freely escapes from the water valve, whereas pressure is maintained accurately in the water valve and pressure changes are minimized.

Because the peritoneum has a large surface and high exchange capacity, theoretically some oxygen could diffuse from the circulation into the abdominal cavity. To ascertain a constant 100% CO_2 concentration in the abdominal cavity the gas was continuously replaced. This was achieved by inserting a 26-gauge needle (BD Plastipak, Becton Dickinson) through the abdominal wall, giving a continuous flow through the abdominal cavity at 10 mL/min at 20 cm H_2O.

After the establishment of the CO_2 pneumoperitoneum, two 14-gauge catheters (Insyte-W, Vialon, Becton Dickinson) were inserted under laparoscopic vision in both the right and left flanks for the working instruments. The uterus was grasped in the midline with a 1.5-mm grasper and standardized 10-mm by 1.6-mm lesions were performed in the antimesenteric border of both the right and left uterine horns by monopolar or bipolar coagulation (10 W, standard coagulation mode, 10 seconds) (Autocon 350, Karl Storz). In addition, identical lesions were made in both the right and left pelvic sidewalls. The type of lesion in each side was randomly determined. Monopolar coagulation was performed with a homemade 1.6-mm ball electrode, whereas bipolar coagulation was performed with a 1.6-mm probe (Bicap, Circon Corp., Santa Barbara, CA). Although in all previous experiments monopolar lesions induced more adhesions than bipolar lesions, the experimental design to induce adhesions is kept constant to facilitate comparison between the experiments and to detect eventual differences in the mechanism of adhesion formation between both lesions.

To evaluate postoperative basal adhesion formation and pneumoperitoneum-enhanced adhesion formation, the pneumoperitoneum was maintained for the minimum time needed to induce the peritoneal lesions (standardized at 10 minutes) or for a longer period (60 minutes). The secondary ports were removed after finalizing the peritoneal lesions and the incisions were closed. The first incision was closed at the end of the surgery. All incisions were closed in a single layer with 6/0 polypropylene suture (Prolene, Ethicon, Johnson and Johnson Intl.).

PlGF Antibody Preparation and Administration

Mouse monoclonal antibodies were raised against murine PlGF-2 (R&D Systems, Abingdon, U.K.) using $PlGF^{-/-}$ mice and methods as described (55). Purified clones were screened for their ability to inhibit the binding of PlGF to VEGFR-1/Flt-1 in a homemade ELISA assay as described (53). The antibodies varied from complete neutralization of the binding of PlGF to VEGFR-1/Flt-1 to no neutralization. In this in vivo study of adhesion formation, we used one clone that does not neutralize (PLGE1G11: PlGF antibody A), two clones that completely neutralizes (PL17A10F12: PlGF antibody B and PL5D11D4: PlGF antibody C), and one clone that partially neutralizes (PLGH12G5: PlGF antibody D) the binding of PlGF to VEGFR-1/Flt-1.

Animals received four intraperitoneal doses of 20 μg/g of either mouse IgG (Sigma Chemical Company, St. Louis, MO) or one of the four PlGF antibodies (A, B, C, or D) diluted in 200 μL of saline. The first dose was administrated on day 0 at the beginning of the surgery and under direct laparoscopic vision, whereas the subsequent doses were injected on days 2, 4, and 6 after surgery.

Scoring of Adhesions

A xyphopubic midline incision and a bilateral subcostal incision were performed and the whole abdominal cavity was explored during laparotomy 7 days after the induction of adhesions, as described (27). After the evaluation of port sites and viscera, the pelvic fat was carefully removed and adhesions were blindly scored under microscopic vision using a qualitative and a quantitative scoring system.

In the qualitative scoring system the following characteristics were assessed (modified from Leach et al.) (56): *extent* (0: no adhesions; 1: 1%–25%; 2: 26%–50%; 3: 51%–75%; 4: 76%–100% of the injured surface involved), *type* (0: no adhesions; 1: filmy; 2: dense; 3: capillaries present), *tenacity* (0: no adhesions; 1: easily fall apart; 2: require traction; 3: require sharp dissection), and *total* (extent + type + tenacity). In addition, a quantitative scoring system was used as described by Holmdahl et al. (57). This system has the advantage of being devoid of any subjective interpretation. It measures the proportion of the lesions covered by adhesions

using the following formula: adhesions (%) = (sum of the length of the individual attachments/length of the lesion) × 100.

The results are presented as the average of the adhesions formed at the four individual sites (right and left visceral and parietal peritoneum with lesions inflicted by monopolar or bipolar coagulation), which were individually scored.

Experimental Design

All experiments were performed using block randomization by days. Therefore, one block of mice, comprising one animal of each group, was operated during the same day. Within a block the animals were operated on in random order.

In the first experiment (n = 20), basal adhesions and pneumoperitoneum-enhanced adhesions were assessed in VEGF-$A^{+/+}$ mice (n = 5 and n = 5, respectively) and VEGF-$A^{164/164}$ mice (n = 5 and n = 5, respectively).

In the second experiment (n = 20), basal adhesions and pneumoperitoneum-enhanced adhesions were assessed in VEGF-$B^{+/+}$ mice (n = 5 and n = 5, respectively) and VEGF-$B^{-/-}$ mice (n = 5 and n = 5, respectively).

In the third experiment (n = 20), basal adhesions and pneumoperitoneum-enhanced adhesions were assessed in $PlGF^{+/+}$ mice (n = 5 and n = 5, respectively) and $PlGF^{-/-}$ mice (n = 5 and n = 5, respectively).

In the fourth experiment (n = 50), basal adhesions and pneumoperitoneum-enhanced adhesions were assessed in mice treated with mouse IgG (n = 5 and n = 5, respectively) or with PlGF antibodies A (n = 5 and n = 5, respectively), B (n = 5 and n = 5, respectively), C (n = 5 and n = 5, respectively), or D (n = 5 and n = 5, respectively).

Statistics

Statistical analysis was performed with the SAS System (SAS institute, Cary, NC) using the nonparametric Kruskal-Wallis test to compare individual groups. All data are presented as the mean ± SE.

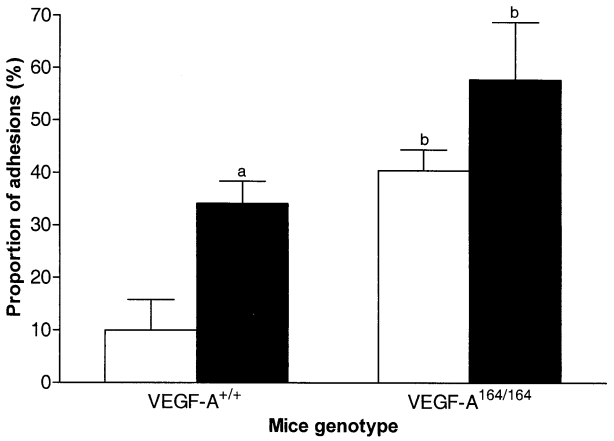
RESULTS

All animals survived the surgical procedures and all of them were available for adhesion scoring after 7 days. Adhesions only formed between the injured visceral site and the pelvic fat or between the injured parietal site and the pelvic fat. No adhesions were observed at the site of the laparoscopic ports or at other sites.

In all experiments monopolar lesions systematically induced more adhesions than bipolar lesions (data not shown) for both basal and pneumoperitoneum-enhanced adhesion formation. The effect of the pneumoperitoneum, however, was equally observed with both monopolar and bipolar lesions separately analyzed. To maximize statistical significance, only the means of both lesions are discussed.

FIGURE 1

Proportion of postoperative intraperitoneal adhesions in wild-type mice (VEGF-$A^{+/+}$) and transgenic mice deficient for VEGF-A_{120} and for VEGF-A_{188} isoforms and expressing exclusively VEGF-A_{164} isoform (VEGF-$A^{164/164}$). Adhesions were induced during laparoscopy and scored after 7 days during laparotomy. Basal adhesions (□) and pneumoperitoneum-enhanced adhesions (■) were assessed maintaining the pneumoperitoneum for the minimum time needed to perform the peritoneal lesions (10 minutes) or for a longer period (60 minutes), respectively. Means ± SE are indicated. $^{a}P \leq .05$: pneumoperitoneum-enhanced adhesions vs. basal adhesions. $^{b}P \leq .05$: VEGF-$A^{164/164}$ mice vs. VEGF-$A^{+/+}$ mice.



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In VEGF-A wild-type mice, pneumoperitoneum enhanced adhesion formation (proportion: $P=.03$; extent: $P=.04$; total: $P=.05$). In comparison with VEGF-A wild-type mice, basal adhesions were higher in VEGF-$A^{164/164}$ mice (proportion: $P=.01$; extent: $P=.02$; type: $P=.02$; tenacity: $P=.02$; total: $P=.01$). In VEGF-$A^{164/164}$ mice, pneumoperitoneum enhance adhesion formation slightly (P=NS). In comparison with VEGF-A wild-type mice, pneumoperitoneum-enhanced adhesions were higher in VEGF-$A^{164/164}$ mice (proportion: $P=.05$; extent: $P=.02$; tenacity: $P=.04$; total: $P=.05$) (Fig. 1, Table 1).

In VEGF-B wild-type mice, pneumoperitoneum enhanced adhesion formation (proportion: $P=.02$; type: $P=.04$; total: $P=.05$). In comparison with VEGF-B wild-type mice, basal adhesions were similar in VEGF-$B^{-/-}$ mice (P =not significant [NS]). In VEGF-$B^{-/-}$ mice, pneumoperitoneum did not enhance adhesion formation. Therefore, in comparison with VEGF-B wild-type mice, pneumoperitoneum-enhanced adhesions were obviously lower in VEGF-$B^{-/-}$ mice (proportion: $P=.05$; type: $P=.03$; total: $P=.05$) (Fig. 2, Table 1).

In PlGF wild-type mice, pneumoperitoneum enhanced adhesion formation (proportion: $P=.01$; extent: $P=.01$; type: $P=.01$; tenacity: $P=.02$; total: $P=.01$). In comparison

TABLE 1

Adhesion scores in wild-type mice (VEGF-$A^{+/+}$, VEGF-$B^{+/+}$, and $PlGF^{+/+}$) and transgenic mice deficient for VEGF-A_{120} and for VEGF-A_{188} isoforms and expressing exclusively VEGF-A_{164} isoform (VEGF-$A^{164/164}$) or deficient for VEGF-B (VEGF-$B^{-/-}$) or for PlGF ($PlGF^{-/-}$).

		Scores			
Genotype	Adhesions	Extent	Type	Tenacity	Total
VEGF-$A^{+/+}$ (n = 10)	Basal	0.6 ± 0.3	0.6 ± 0.3	0.5 ± 0.3	1.7 ± 0.9
	PP-enhanced	1.6 ± 0.2[a]	1.6 ± 0.2	1.4 ± 0.2	4.6 ± 0.5[a]
VEGF-$A^{164/164}$ (n = 10)	Basal	2.0 ± 0.2[b]	2.0 ± 0.2[b]	1.9 ± 0.2[b]	5.9 ± 0.5[b]
	PP-enhanced	2.8 ± 0.4[b]	2.1 ± 0.3	2.3 ± 0.2[b]	7.1 ± 0.8[b]
VEGF-$B^{+/+}$ (n = 10)	Basal	0.4 ± 0.2	0.5 ± 0.3	0.6 ± 0.3	1.5 ± 0.8
	PP-enhanced	0.7 ± 0.2	1.1 ± 0.2[a]	0.9 ± 0.2	2.7 ± 0.6[a]
VEGF-$B^{-/-}$ (n = 10)	Basal	0.5 ± 0.2	0.7 ± 0.2	0.7 ± 0.2	1.9 ± 0.6
	PP-enhanced	0.4 ± 0.2	0.6 ± 0.3[b]	0.5 ± 0.2	1.5 ± 0.6[b]
$PlGF^{+/+}$ (n = 10)	Basal	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.2	1.3 ± 0.4
	PP-enhanced	1.3 ± 0.2[a]	1.3 ± 0.2[a]	1.2 ± 0.1[a]	3.8 ± 0.5[a]
$PlGF^{-/-}$ (n = 10)	Basal	0.3 ± 0.1	0.4 ± 0.1	0.5 ± 0.2	1.2 ± 0.4
	PP-enhanced	0.2 ± 0.1[b]	0.3 ± 0.1[b]	0.3 ± 0.1[b]	0.8 ± 0.4[b]

Adhesions were induced during laparoscopy and scored after 7 days during laparotomy. Basal and pneumoperitoneum (PP)-enhanced adhesions were evaluated maintaining the pneumoperitoneum for the minimum time needed to perform the peritoneal lesions (10 minutes) or for a longer period (60 minutes), respectively. Means ± SE are indicated.

[a] $P \leq .05$: PP-enhanced adhesions vs. basal adhesions.

[b] $P \leq .05$: transgenic mice vs. wild-type mice.

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with PlGF wild-type mice, basal adhesions were slightly lower (P=NS) in $PlGF^{-/-}$ mice. In $PlGF^{-/-}$ mice, pneumoperitoneum did not enhance adhesion formation. Therefore, in comparison with PlGF wild-type mice, pneumoperitoneum-enhanced adhesions were obviously lower in $PlGF^{-/-}$ mice (proportion: P=.01; extent: P=.01; type: P=.01; tenacity: P=.01; total: P=.01) (Fig. 3, Table 1).

In the experiment with PlGF antibodies two control groups were used: mice treated with IgG and mice treated with the non-neutralizing PlGF antibody A, and therefore all other groups were compared with both control groups. In mice treated with IgG, pneumoperitoneum enhanced adhesion formation (proportion: P=.01; extent: P=.01; total: P=.03). In comparison with mice treated with IgG, basal adhesions were similar in mice treated with the PlGF antibody A (P=NS). In mice treated with PlGF antibody A, pneumoperitoneum enhanced adhesion formation (proportion: P=.02; extent: P=.04; tenacity: P=.02; total: P=.05). In comparison with mice treated with IgG, pneumoperitoneum-enhanced adhesions were similar in mice treated with PlGF antibody A (P=NS) (Fig. 4, Table 2).

In mice treated with the neutralizing PlGF antibody B, basal adhesions were lower than in mice treated with IgG or with PlGF antibody A (proportion: P=.03, P=NS; type: P=.02, P=NS; tenacity: P=.05, P=NS; total: P=.04, P=NS, respectively). In mice treated with PlGF antibody B, pneumoperitoneum did not enhance adhesion formation. Therefore, in these mice pneumoperitoneum-enhanced adhe-

FIGURE 2

Proportion of postoperative intraperitoneal adhesions in wild-type mice (VEGF-$B^{+/+}$) and transgenic mice deficient for VEGF-B (VEGF-$B^{-/-}$). Adhesions were induced during laparoscopy and scored after 7 days during laparotomy. Basal adhesions (□) and pneumoperitoneum-enhanced adhesions (■) were assessed maintaining the pneumoperitoneum for the minimum time needed to perform the peritoneal lesions (10 minutes) or for a longer period (60 minutes), respectively. Means ± SE are indicated. [a]$P \leq .05$: pneumoperitoneum-enhanced adhesions vs. basal adhesions. [b]$P \leq .05$: VEGF-$B^{-/-}$ mice vs. VEGF-$B^{+/+}$ mice.

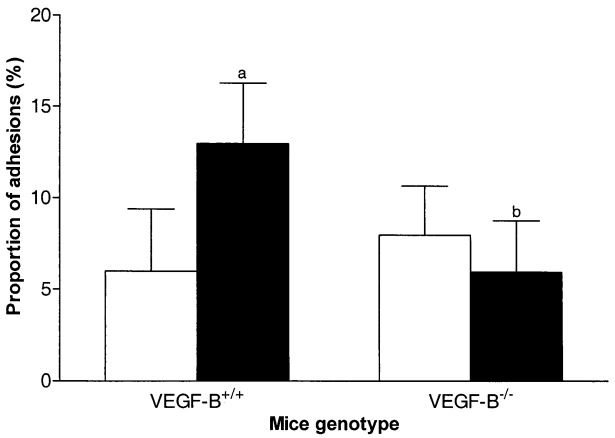

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FIGURE 3

Proportion of postoperative intraperitoneal adhesions in wild-type mice ($PlGF^{+/+}$) and transgenic mice deficient for PlGF ($PlGF^{-/-}$). Adhesions were induced during laparoscopy and scored after 7 days during laparotomy. Basal adhesions (□) and pneumoperitoneum-enhanced adhesions (■) were assessed maintaining the pneumoperitoneum for the minimum time needed to perform the peritoneal lesions (10 minutes) or for a longer period (60 minutes), respectively. Means ± SE are indicated. $^{a}P \leq .05$: pneumoperitoneum-enhanced adhesions vs. basal adhesions. $^{b}P \leq .05$: $PlGF^{-/-}$ mice vs. $PlGF^{+/+}$ mice.

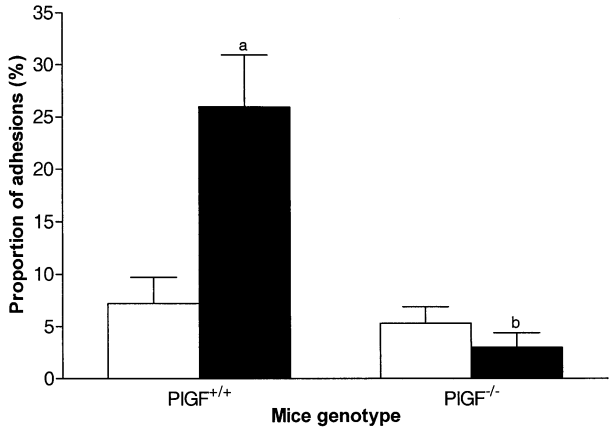

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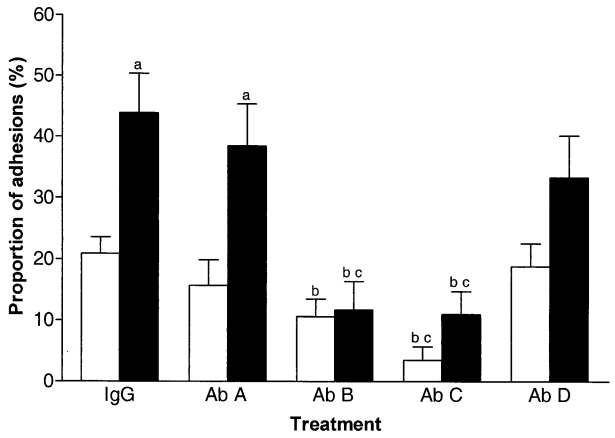
FIGURE 4

Proportion of postoperative intraperitoneal adhesions in wild-type mice treated with IgG or with PlGF antibodies with different neutralizing capacity according to their ability to inhibit the binding of PlGF to VEGFR-1 (Ab A: no neutralizing, Ab B: neutralizing, Ab C: neutralizing, Ab D: semineutralizing). Adhesions were induced during laparoscopy and scored after 7 days during laparotomy. Basal adhesions (□) and pneumoperitoneum-enhanced adhesions (■) were assessed maintaining the pneumoperitoneum for the minimum time needed to perform the peritoneal lesions (10 minutes) or for a longer period (60 minutes), respectively. Means ± SE are indicated. $^{a}P \leq .05$: pneumoperitoneum-enhanced adhesions vs. basal adhesions. $^{b}P \leq .05$: Ab B-C-D vs. IgG. $^{c}P \leq .05$: Ab B-C-D vs. Ab A.



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sions were lower than in mice treated with IgG or with PlGF antibody A (proportion: $P=.01$, $P=.02$; extent: $P=.01$, $P=.02$; tenacity: $P=.04$, $P=$NS; total: $P=.02$, $P=.05$, respectively) (Fig. 4, Table 2).

In mice treated with the neutralizing PlGF antibody C, basal adhesions were lower than in mice treated with IgG or with PlGF antibody A (proportion: $P=.01$, $P=.03$; extent: $P=.03$, $P=$NS; type: $P=.01$, $P=$NS; tenacity: $P=.01$, $P=.03$; total: $P=.01$, $P=$NS, respectively). In mice treated with PlGF antibody C, pneumoperitoneum did not enhance adhesion formation. Therefore, in these mice pneumoperitoneum-enhanced adhesions were lower than in mice treated with IgG or with PlGF antibody A (proportion: $P=.01$, $P=.02$; extent: $P=.01$, $P=.01$; type: $P=.01$, $P=.01$; tenacity: $P=.01$, $P=.01$; total: $P=.01$, $P=.01$, respectively) (Fig. 4, Table 2).

In mice treated with the semineutralizing PlGF antibody D, basal adhesions were similar than in mice treated with IgG ($P=$NS) or with PlGF antibody A ($P=$NS). In mice treated with PlGF antibody D, pneumoperitoneum enhanced adhesion formation but not significantly. In these mice, pneumoperitoneum-enhanced adhesions were similar than in mice treated with IgG ($P=$NS) or with PlGF antibody A ($P=$NS) (Fig. 4, Table 2).

Comparing the effects of the neutralizing PlGF antibodies B and C with the effect of the semi neutralizing antibody D, the following differences were significant: basal adhesions were higher in mice treated with PlGF antibody D than in mice treated with PlGF antibody C (proportion: $P=.01$) and pneumoperitoneum-enhanced adhesions were higher in mice treated with PlGF antibody D than in mice treated with PlGF antibody B and C (proportion: $P=.05$, $P=.02$; extent: $P=$NS, $P=.01$; type: $P=$NS, $P=.01$; tenacity: $P=$NS, $P=.02$; total: $P=$NS, $P=.02$, respectively). These data are consistent with PlGF antibody D having a partial neutralizing effect. The effects of antibodies B and C were comparable (Fig. 4, Table 2).

DISCUSSION

In this study, a laparoscopic mouse model permitting evaluation of basal adhesion formation and pneumoperitoneum-enhanced adhesion formation was used. We fully recognize that the so-called basal adhesions not only result from the peritoneal lesion inflicted with the electrocautery but also from the effect of the CO_2 pneumoperitoneum that was present albeit for 10 minutes only. Basal adhesions without any additional effect of CO_2 pneumoperitoneum would require the shortest duration possible, the minimum insufflation pressure, and some 3% of oxygen added to the CO_2

TABLE 2

Adhesions scores in wild-type mice treated with IgG or with PlGF antibodies.

Treatment (n)	Adhesions	Scores			
		Extent	Type	Tenacity	Total
IgG (n = 10)	Basal	1.1 ± 0.1	1.4 ± 0.1	1.5 ± 0.2	4.0 ± 0.4
	PP-enhanced	2.2 ± 0.2[a]	1.8 ± 0.2	2.0 ± 0.1	6.0 ± 0.5[a]
Ab A (n = 10)	Basal	1.0 ± 0.2	1.0 ± 0.3	1.1 ± 0.2	3.1 ± 0.7
	PP-enhanced	1.9 ± 0.3[a]	1.8 ± 0.1	1.9 ± 0.2[a]	5.6 ± 0.6[a]
Ab B (n = 10)	Basal	0.7 ± 0.1	0.7 ± 0.2[b]	0.8 ± 0.2[b]	2.2 ± 0.5[b]
	PP-enhanced	0.7 ± 0.2[bc]	0.9 ± 0.4	1.0 ± 0.3[b]	2.6 ± 1.0[bc]
Ab C (n = 10)	Basal	0.3 ± 0.2[b]	0.3 ± 0.2[b]	0.3 ± 0.2[b]	0.9 ± 0.6[bc]
	PP-enhanced	0.5 ± 0.1[bc]	0.5 ± 0.1[bc]	0.8 ± 0.1[bc]	1.8 ± 0.4[bc]
Ab D (n = 10)	Basal	0.9 ± 0.2	1.0 ± 0.2	1.1 ± 0.2	3.0 ± 0.6
	PP-enhanced	1.6 ± 0.3	1.5 ± 0.2	1.6 ± 0.2	4.7 ± 0.7

Adhesions were induced during laparoscopy and scored after 7 days during laparotomy. Basal and pneumoperitoneum (PP)-enhanced adhesions were evaluated maintaining the pneumoperitoneum for the minimum time needed to perform the peritoneal lesions (10 minutes) or for a longer period (60 minutes), respectively. PlGF antibodies have different neutralizing capacity according to their ability to inhibit the binding of PlGF to VEGFR-1 (Ab A: no neutralizing, Ab B: neutralizing, Ab C: neutralizing, Ab D: semineutralizing). Means ± SE are indicated.

[a] $P \leq .05$: PP-enhanced adhesions vs. basal adhesions.

[b] $P \leq .05$: Ab B-C-D vs. IgG.

[c] $P \leq .05$: Ab B-C-D vs. Ab A.

Molinas. VEGF and PIGF in adhesion formation. Fertil Steril 2003.

pneumoperitoneum, because adhesion formation decreases with shorter duration, lower pressure, and the addition of oxygen (24–27). In these experiments, for the evaluation of basal adhesions, the pneumoperitoneum was maintained for the minimum time required to perform the lesions (standardized at 10 minutes). We used, however, 100% CO_2 at 20 cm H_2O because a lower pressure and the addition of oxygen, although theoretically better, would introduce additional variables, such as pressure and oxygen. Doing both controls was not feasible because of limited availability of transgenic animals.

This study confirms our previous finding that the pneumoperitoneum is a cofactor in adhesion formation (24–27) because pneumoperitoneum-enhanced adhesions were observed in all wild-type mice used as control animals.

To the best of our knowledge this is the first study demonstrating directly a role of VEGF-A, VEGF-B, and PlGF in postoperative adhesion formation. Our results can be explained by postulating that pneumoperitoneum enhances adhesion formation, at least in part, through an up-regulation of VEGF-A_{164}, VEGF-B, and PlGF. This is consistent with the reported up-regulation of VEGF-A and VEGF-A/PlGF heterodimers by hypoxia (21, 22, 28–30, 45, 50) and confirms the hypothesis that CO_2 pneumoperitoneum-enhanced adhesion formation is mediated by mesothelial hypoxia (27).

In VEGF-$A^{164/164}$ mice, basal adhesions were higher than in wild-type mice, demonstrating a direct role of VEGF-A_{164} in basal adhesion formation. This was not unexpected as VEGF-A was found in peritoneal adhesions by immunohistochemistry (58) and by reverse transcriptase-polymerase chain reaction (59). Moreover, a reduction in adhesion formation after open surgery was reported in mice after the administration of polyclonal antibodies against VEGF-A (60). The slight but not significant increased in adhesion formation after 60 minutes of CO_2 pneumoperitoneum in VEGF-$A^{164/164}$ mice does not rule out an up-regulation of VEGF-A_{164} by the CO_2 pneumoperitoneum, as the effect of VEGF-A on adhesion formation could already be near-maximal in mice overexpressing this VEGF-A isoform.

In VEGF-$B^{-/-}$ mice, basal adhesions were comparable with those in wild-type mice, suggesting that VEGF-B has no major role in basal adhesion formation. In these VEGF-$B^{-/-}$ mice, adhesion formation did not increase after 60 minutes of CO_2 pneumoperitoneum, demonstrating that the mechanism of CO_2 pneumoperitoneum-enhanced adhesions involves VEGF-B, which obviously cannot be up-regulated in these mice.

In PlGF$^{-/-}$ mice, basal adhesions were identical or possibly slightly lower than in wild-type mice, suggesting that PlGF does not play a major role in basal adhesion formation. It should be recognized, however, that the design of the study and the number of animals involved do not permit to detect minor changes in basal adhesions. Adhesion formation clearly did not increase after 60 minutes of CO_2 pneumoperitoneum, demonstrating that the mechanism of CO_2 pneumoperitoneum-enhanced adhesions involves PlGF. This was confirmed by the absence of pneumoperitoneum-enhanced adhesions in mice treated with PlGF-neutralizing antibodies.

We recently evaluated the role of the plasminogen system, that is, PAI-1, urokinase-type plasminogen activator (uPA), and tPA, in adhesion formation in transgenic mice using the same model (61), confirming the major role of these factors in basal adhesions. The up-regulation of PAI-1 by the CO_2 pneumoperitoneum was shown to be involved in pneumoperitoneum-enhanced adhesions. To fully understand the exact importance of the plasminogen system and of the VEGF family on basal adhesions and pneumoperitoneum-enhanced adhesions more detailed investigations in the normal and in the damaged peritoneum are obviously required (e.g., by studying the effects of the duration of pneumoperitoneum, the insufflation pressure, and the addition of oxygen). The available data, however, show clearly that the mechanisms involved in basal adhesions and in pneumoperitoneum-enhanced adhesions are at least partially different.

An effect of the mouse strain in adhesion formation was also observed. Indeed, the more Swiss genetic background, the more adhesions were observed. These strain differences were obvious for both basal adhesions and pneumoperitoneum-enhanced adhesions. This observation was not surprising as strain differences have been reported for fibrosis and healing responses, for example, for hepatic fibrosis (62), lung fibrosis (63), colorectal fibrosis (64), ear wound healing (65), myocardial healing (66), and bone regeneration (67).

In conclusion, our data confirm the role of the CO_2 pneumoperitoneum as a cofactor in adhesion formation and demonstrate a direct role of VEGF-A in basal adhesions and of VEGF-B and PlGF in pneumoperitoneum-enhanced adhesions, suggesting VEGF-A_{164}, VEGF-B, and PlGF up-regulation as a mechanism for this pneumoperitoneum-enhanced adhesion formation. This is fully consistent with the up-regulation of these angiogenic factors by hypoxia and with the concept that CO_2 pneumoperitoneum causes mesothelial hypoxia. These observations open insights in the pathophysiology of adhesion formation. Because the mechanisms involved in basal adhesions and in pneumoperitoneum-enhanced adhesions are at least partially different, new methods for adhesion prevention after laparoscopic surgery could be developed.

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