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Role of vascular endothelial growth factor receptor 1 in basal adhesion formation and in carbon dioxide pneumoperitoneum-enhanced adhesion formation after laparoscopic surgery in mice

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Objective: To evaluate the role of vascular endothelial growth factor receptor-1 (VEGFR-1) in adhesion formation after laparoscopic surgery.

Design: Prospective, randomized study.

Setting: Academic research center.

Animal(s): Forty female Swiss mice.

Intervention(s): Adhesions were induced by standardized lesions during laparoscopy. The CO_2 pneumoperitoneum was maintained for the minimum time needed to perform the lesions (10 minutes) or for a longer period (60 minutes) to evaluate basal adhesions and pneumoperitoneum-enhanced adhesions, respectively. Mice were treated either with IgG or with antibodies against VEGFR-1.

Main Outcome Measurement(s): Adhesions were quantitatively and qualitatively scored after 7 days during laparotomy.

Result(s): In IgG-treated mice, 60 minutes of CO_2 pneumoperitoneum increased basal adhesions. In VEGFR-1 antibody-treated mice, basal adhesions were similar to the control group and 60 minutes of CO_2 pneumoperitoneum did not increase adhesions. Therefore, in these mice, pneumoperitoneum-enhanced adhesions were lower than in IgG-treated mice.

Conclusion(s): The data confirm that CO_2 pneumoperitoneum is a cofactor in adhesion formation and demonstrate that VEGFR-1 plays a role in pneumoperitoneum-enhanced adhesions, which is consistent with a role of placental growth factor, VEGF-A, and VEGF-B in pneumoperitoneum-enhanced adhesions. These observations give new insight into the pathogenesis of adhesion formation. (Fertil Steril® 2004;82(Suppl 3): 1149–1153. ©2004 by American Society for Reproductive Medicine.)

Key Words: Adhesion formation, laparoscopy, CO_2 pneumoperitoneum, VEGF-A, VEGF-B, PlGF, VEGFR-1, mice

Vascular endothelial growth factor is a family of angiogenic factors including VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PlGF) (1). Placental growth factor, VEGF-A, and VEGF-B bind to VEGFR-1 (Flt-1), whereas VEGF-A binds also to VEGFR-2 (flk-1) (1–4). Both VEGFR-1 and VEGFR-2 are high-affinity transmembrane tyrosine kinase receptors with seven immunoglobulin-like extracellular domains and a kinase intracellular domain. They are selectively but not exclusively expressed on vascular endothelial cells. A truncated soluble form of VEGFR-1, resulting from alternatively splicing and retaining its binding activity is found in serum. VEGFR-1 is, unlike VEGFR-2, also present on inflammatory cells, i.e., macrophages and their progenitors. Therefore, PlGF, VEGF-A, and VEGF-B can stimulate inflammation in addition to angiogenesis (3, 4).

The role of VEGF in adhesion formation after open surgery has already been demonstrated (5–7). Similarly, a crucial role of the VEGF family in adhesion formation after laparoscopic surgery has been recently reported. Indeed, the absence of CO_2 pneumoperitoneum-enhanced adhesion formation in mice defi-

cient for PlGF and in mice treated with antibodies that neutralize the binding of PlGF to its receptor, that is, VEGFR-1 (Flt-1), indicate an up-regulation of PlGF by the CO_2 pneumoperitoneum as a driving mechanism (8). In addition, data from transgenic mice indicate that an up-regulation of VEGF-A and VEGF-B is also involved (8). Since PlGF, VEGF-A, and VEGF-B share a common cellular receptor, a critical role for it can be postulated.

This prospective randomized study was performed to evaluate the role of VEGFR-1, the common receptor for PlGF, VEGF-A, and VEGF-B, in postoperative adhesion formation in a laparoscopic mouse model using monoclonal antibodies against VEGFR-1.

MATERIAL AND METHODS

Animals and Anesthesia

The study was performed in 40 female 10- to 12-week old Swiss mice weighing 30–35 g. Animals were kept under standard laboratory conditions (20°–22°C, relative humidity 50%–60%, 14 hours light and 10 hours dark) at the animal facilities of the Katholieke Universiteit Leuven (KUL) and fed a standard laboratory diet (Muracon G, Carsil Quality, Turnhout, Belgium) ad libitum. They were anaesthetized with pentobarbital (IM 0.07 mg/g), intubated, and ventilated with room air (500 μL at 85 strokes/minute; Rodent Ventilator, Harvard Apparatus, Holliston, MA) as described elsewhere (8–11). The study was approved by the Institutional Review Animal Care Committee.

Laparoscopic Surgery and Induction of IP Adhesions

Laparoscopy was performed using heated and humidified CO_2 at 20 cm H_2O for the pneumoperitoneum in a setting as described elsewhere (8–10). Standardized peritoneal lesions in uterine horns and pelvic sidewalls were performed with monopolar and bipolar coagulation to induce IP adhesions as described elsewhere (8–10). The two types of coagulation were used to have the same experimental design and to be consistent with previous studies (8–10). 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) to evaluate basal adhesions and pneumoperitoneum-enhanced adhesions, respectively (8–10).

Vascular Endothelial Growth Factor Receptor-1 Antibodies Generation, Characterization, and Administration

Rat monoclonal antibodies against mouse VEGFR-1 (clone MF1, Imclone, New York), specific against mouse VEGFR-1 without cross reaction with human receptors, were generated and characterized as described in detail elsewhere (4). These antibodies specifically bound VEGFR-1 but not VEGFR-2, inhibiting binding of PlGF and VEGF to VEGFR-1 with IC50 of 0.1 and 0.3 nM, respectively (4).

Animals received four IP injections of 20μg/g (600–700 μg) of either rat IgG (Sigma, St. Louis) or VEGFR-1 antibodies diluted in 200 μL of saline. This dose was determined based on other studies reporting blocking effects between 200 μg and 1,000 μg (4). 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. VEGFR-1 antibodies and IgG were obtained from the Center for Transgene Technology and Gene Therapy of the KUL.

Scoring of Adhesions

Adhesions were qualitatively and quantitatively scored after 7 days during laparotomy as described elsewhere (8–10). The qualitative scoring assesses 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). The quantitative scoring assesses the proportion of the lesions covered by adhesions with the following formula: adhesions (%) = (sum of the length of the individual attachments/length of the lesion) $\times$ 100 (12). The results are presented as the average of the adhesions formed at the four individual sites, which were individually scored.

Experimental Design

The study was performed in two series using block randomization by days. One block of mice, made up of one animal of each group, underwent surgery on the same day. Within a block, the animals were operated on in random order.

In series 1 (n = 20), basal adhesions and pneumoperitoneum-enhanced adhesions were assessed in mice treated either with IgG or with VEGFR-1 antibodies (four groups, n = 5 per group).

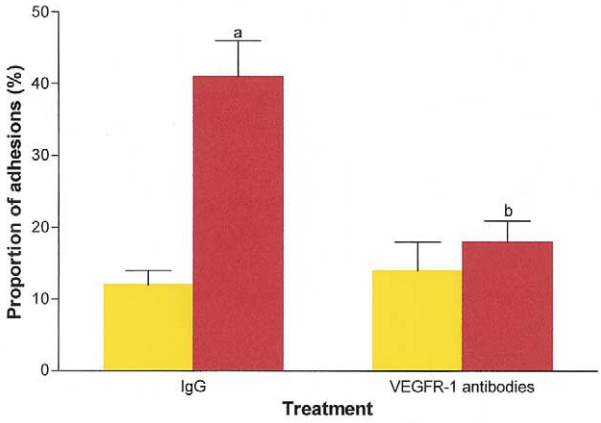
In series 2 (n = 20), a similar experiment was performed and pneumoperitoneum-enhanced adhesions were assessed in mice treated either with IgG or with VEGFR-1 antibodies (two groups, n = 10 per group). In this series, adhesions were scored quantitatively only because the tissues were collected and snap frozen for later investigation by the Center for Transgene Technology and Gene Therapy of the KUL.

Statistics

Statistical analysis was performed with the SAS System (SAS Institute, Cary, NC) using the nonparametric Kruskal-Wallis test. All data are presented as mean $\pm$ SE. To evaluate differences between experimental groups, only the combined scores of the adhesions formed after monopolar and bipolar lesions were used. This was done because in all previous studies (8–10) bipolar lesions induced systematically less adhesions than monopolar lesions and were therefore less sensitive to detect intergroup differences.

FIGURE 1

Proportion of adhesions in mice treated either with IgG or with antibodies against VEGFR-1. Adhesions were induced during laparoscopy and scored after 7 days during laparotomy. Basal adhesions (yellow squares) and pneumoperitoneum-enhanced adhesions (red squares) 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 together with significances (Kruskal-Wallis) are indicated. [a]$P \leq .05$: pneumoperitoneum-enhanced adhesions vs. basal adhesions. [b]$P \leq .05$: VEGFR-1 antibodies treatment vs. IgG treatment.



Molinas. VEGFR-1, laparoscopy, and adhesion formation. Fertil Steril 2004.

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 series 1, in IgG-treated mice, 60 minutes of CO_2 pneumoperitoneum increased adhesion formation (proportion: $P = .01$; extent: $P = .01$; total: $P = .05$). In comparison with these IgG-treated mice, basal adhesions were similar in VEGFR-1 antibody-treated mice (P = not significant). In these VEGFR-1 antibody-treated mice, 60 minutes of CO_2 pneumoperitoneum did not increase adhesion formation (P = NS). Therefore, in these mice, pneumoperitoneum-enhanced adhesions were lower than in IgG-treated mice (proportion: $P = .01$; extent: $P = .01$; type: $P = .02$; tenacity: $P = .01$; total: $P = .01$) (Fig. 1, Table 1).

In series 2, the proportions of pneumoperitoneum-enhanced adhesions were 32% ± 5% in IgG-treated mice and 8% ± 2% in VEGFR-1 antibody-treated mice ($P = .001$), confirming the effects observed in series 1.

DISCUSSION

In this study, the CO_2 pneumoperitoneum increased adhesion formation in animals used as controls, confirming previous observations (8–11, 13–16). The CO_2 pneumoperitoneum, however, did not increase adhesions in mice treated with antibodies against VEGFR-1. This is consistent with the reported role of PlGF, VEGF-A, and VEGF-B in adhesion formation after laparoscopic surgery, indicating an up-regulation of these factors by the CO_2 pneumoperitoneum as the driving mechanism (8). Indeed, CO_2 pneumoperitoneum did not increase adhesions in mice deficient for PlGF ($PlGF^{-/-}$) or in mice treated with neutralizing antibodies against PlGF. Similarly, it did not increase adhesions in mice deficient for VEGF-B ($VEGF\text{-}B^{-/-}$), whereas it slightly increased adhesions in mice expressing exclusively $VEGF\text{-}A_{164}$ ($VEGF\text{-}A^{164\text{-}164}$) and deficient for $VEGF\text{-}A_{120}$ and $VEGF\text{-}A_{188}$ (8).

Since PlGF, VEGF-A, and VEGF-B have a common receptor, i.e., VEGFR-1, and since antibodies against VEGFR-1 prevent CO_2 pneumoperitoneum-enhanced adhesions, our data indicate that the effects of the VEGF family in adhesion formation are mediated, to a large extent, by this

TABLE 1

Adhesion scores in mice treated either with IgG or with antibodies against VEGFR-1.

		Scores			
Treatment	Adhesions	Extent	Type	Tenacity	Total
IgG	Basal	0.9 ± 0.1	1.4 ± 0.2	1.3 ± 0.2	3.6 ± 0.5
	Pneumoperitoneum-enhanced	2.0 ± 0.2[a]	1.8 ± 0.2	1.8 ± 0.2	5.6 ± 0.6[a]
VEGFR-1 antibodies	Basal	0.8 ± 0.1	1.0 ± 0.1	0.8 ± 0.1	2.6 ± 0.3
	Pneumoperitoneum-enhanced	1.0 ± 0.1[b]	1.0 ± 0.2[b]	1.1 ± 0.1[b]	3.1 ± 0.3[b]

Note: Adhesions were induced during laparoscopy and scored after 7 days during laparotomy. CO_2 pneumoperitoneum was maintained for the minimum time needed to perform the peritoneal lesions (10 minutes) or for a longer period (60 minutes) to evaluate basal and pneumoperitoneum-enhanced adhesions, respectively. Means ± SE together with significances (Kruskal-Wallis test) are indicated.

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

[b] $P \leq .05$: VEGFR-1 antibodies treatment vs. IgG treatment.

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receptor. It remains unclear, however, whether these effects are mainly related to stimulation of angiogenesis and/or inflammation. Indeed, PlGF, VEGF-A, and VEGF-B stimulate angiogenesis and inflammation since their receptors are expressed on endothelial cells (VEGFR-1 and VEGFR-2) and on inflammatory cells (VEGFR-1), that is, macrophages and their progenitors (3, 4).

Several complementary and nonexclusive mechanisms have been proposed for VEGF-driven angiogenesis and inflammation (3, 4, 17). Vascular endothelial growth factor-A induces angiogenesis by activating VEGFR-2, while VEGFR-1 might function as an inert "decoy" regulating the availability of VEGF-A to activate VEGFR-2. Placental growth factor stimulates angiogenesis by several mechanisms. First, PlGF displaces VEGF-A from VEGFR-1, increasing the fraction of VEGF-A available to activate VEGFR-2. Second, PlGF up-regulates the expression of VEGF-A by fibroblasts and inflammatory cells. Third, PlGF transmits its own intracellular angiogenic signals through VEGFR-1. Fourth, PlGF activates receptor cross-talk between VEGFR-1 and VEGFR-2, leading to enhanced VEGFR-2–driven angiogenesis. Fifth, PlGF forms heterodimers with VEGF-A. VEGF-A and PlGF stimulate inflammation through VEGFR-1 by increasing mobilization of bone marrow–derived myeloid progenitors into peripheral blood, by increasing myeloid cell differentiation, mobilization, and activation, and by increasing cytokine production by macrophages (3, 4, 17).

To what extent these processes, that is, angiogenesis and inflammation, contribute to adhesion formation remains to be elucidated. Independently of the main mechanism of action, the available data point to peritoneal hypoxia as the trigger factor (8–10). Indeed, we have recently demonstrated that the CO_2 pneumoperitoneum does not increase adhesions in mice partially deficient for hypoxia inducible factors (HIFs) 1α or 2α (10). The role of hypoxia and HIFs is consistent with the results presented in this study since VEGF-A is up-regulated at the transcriptional level by HIF-1α (18). It is also consistent with the absence of pneumoperitoneum-enhanced adhesions in mice deficient for plasminogen activator inhibitor-1 (PAI-1) (9), since PAI-1 is up-regulated by hypoxia through HIF-1α (19–21).

The therapeutic potential of these mechanisms, that is, hypoxia, induction of HIFs, PlGF, VEGF-A, and VEGF-B; and stimulation of inflammation and/or angiogenesis, for inhibition of adhesion formation is unclear. Prevention of CO_2 pneumoperitoneum-enhanced adhesions has been reported with neutralizing antibodies against PlGF (8). This study indicates that this can also be achieved with antibodies against VEGFR-1. This is consistent with the reported effects of VEGFR-1 antibodies for inhibition of prototypic angiogenic disorders such as cancer, retinal ischemia, arthritis, and atherosclerosis in animal models. Indeed, antibodies against VEGFR-1 suppress neovascularization in the cornea and in the ischemic retina and reduce angiogenesis and tumor growth in mice (4). Furthermore, these antibodies reduce atherosclerotic-plaque growth and vulnerability and suppress autoimmune arthritic joint destruction in mice (4). The safety and efficacy of antibodies against PlGF and VEGFR-1 to prevent adhesion formation, as well as the clinical significance, obviously has to be evaluated in detail in time- and dose-response experiments in this and in other models.

The relevance of the mouse data for human surgery still has to be proven. There is evidence that the mechanisms involved in adhesion formation after laparoscopic surgery are much more complex than anticipated. The mouse model has the advantage of being suitable and cheap enough for a thorough investigation of the roles of different pneumoperitoneum-related factors, such as duration, pressure, gas, temperature, and humidity. Furthermore, the mouse model allows the evaluation of the relative importance of all factors involved such as cells, for example, mesothelial cells, fibroblasts, and macrophages; molecules, for example, the plasminogen system, VEGF family, and HIFs; and processes, for example, inflammatory reaction, fibrin deposition/degradation, extracellular matrix deposition/degradation, and angiogenesis. The use of this model in adhesion formation studies will permit a more comprehensive understanding of the mechanisms involved to design more specific experiments in larger animal models and later in humans.

In conclusion, our data confirm the role of the CO_2 pneumoperitoneum as a cofactor in adhesion formation and demonstrate a direct role of VEGFR-1 in pneumoperitoneum-enhanced adhesions. The data are consistent with previous observations on PlGF, VEGF-A, and VEGF-B up-regulation as mechanisms in CO_2 pneumoperitoneum-enhanced adhesions. These observations are one more step in the understanding of the pathogenesis of adhesion formation. These new insights might lead to new methods for adhesion prevention after surgery, especially laparoscopic surgery.

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