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A novel role of fibroblast growth factor-2 and pentosan polysulfate in the pathogenesis of intestinal bleeding in mice

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Submitted 6 September 2006; accepted in final form 17 October 2006

Jerebtsova M, Wong E, Przygodzki R, Tang P, Ray PE. A novel role of fibroblast growth factor-2 and pentosan polysulfate in the pathogenesis of intestinal bleeding in mice. Am J Physiol Heart Circ Physiol 292: H743–H750, 2007. First published October 27, 2006; doi:10.1152/ajpheart.00969.2006.—Pentosan polysulfate (PPS) is a heparin-like polysaccharide that can antagonize the binding interactions of fibroblast growth factor (FGF-2) with its high-affinity receptors. Patients with angiogenic tumors frequently show high levels of FGF-2 in the circulation. Since FGF-2 is a heparin-binding angiogenic growth factor, PPS has been used successfully to block its activity in patients with angiogenic tumors. However, because of its heparin-like activity, the major toxic effect of PPS is the development of bleeding disorders. The role that circulating FGF-2 plays in the pathogenesis of bleeding disorders in patients treated with PPS is currently unknown. Here we hypothesized that FGF-2 might play a physiological role in the pathogenesis of intestinal bleeding induced by PPS. This hypothesis is supported by previous studies showing that PPS is accumulated in the intestine and that circulating FGF-2 specifically binds to and modulates the angiogenic activity of intestinal submucosal endothelial cells. We used recombinant adenoviral vectors carrying a secreted form of FGF-2 and LacZ control vectors to determine whether high levels of circulating FGF-2 facilitate the development of intestinal bleeding disorders in FVB/N and C57BL/6J mice treated with PPS. We found that PPS, acting together with FGF-2, induced structural changes in intestinal vessels leading to the development of lethal intestinal hemorrhages. These findings might have wider clinical implications for the systemic use of PPS and other heparinoids in the treatment of patients with angiogenic diseases associated with high levels of circulating FGF-2.

heparinoids; abdominal hemorrhages; angiogenesis; coagulation; heparin binding receptors

BASIC FIBROBLAST GROWTH FACTOR (FGF-2) is a heparin-binding growth factor that is involved in several biological processes, including angiogenesis, tumor growth, and healing of gastrointestinal tissues (5, 7). Although FGF-2 lacks a signal peptide for secretion, it can be released into the circulation through nonconventional pathways by injured endothelial cells or angiogenic tumors (5, 7, 13). FGF-2 released into the circulation can be accumulated on the cell surface and basement membranes bound to heparan sulfate proteoglycans (HSPG) (11, 32). In this manner, HSPG can modulate the activity of FGF-2 by preventing its proteolytic degradation and/or affecting the signaling through its high-affinity receptors (18, 27). On the basis of this notion, heparin-like molecules or heparinoids have been widely used to modulate the biological activity of FGF-2 in the treatment of human diseases (3–5, 10).

Pentosan polysulfate (PPS), a semisynthetic sulfated heparinoid polysaccharide, can antagonize the binding of FGF-2 to its cell surface receptors and has been shown to modulate the angiogenic activity of FGF-2 in human and mouse tumors (16, 22, 30, 34). However, its use as an antiangiogenic agent in humans is limited by its heparin-like anticoagulant activity, which leads to the development of bleeding disorders in several tissues, including the intestine (16, 22). To date, the mechanism by which PPS induces intestinal bleeding in humans remains unclear. Thus it is necessary to understand this process, because it may have wider implications for the use of heparinoids in the treatment of patients with angiogenic tumors, gastrointestinal ulcers, and inflammatory disorders (3–5).

Since high levels of FGF-2 are found in the circulation of patients with angiogenic tumors (5), we hypothesized that FGF-2 might play an important physiological role in the pathogenesis of the intestinal hemorrhages induced by PPS. This hypothesis is supported by previous studies showing that PPS has heparin-like activity and accumulates in the gastrointestinal tract (20). In addition, circulating FGF-2 binds to and modulates the growth of intestinal submucosal endothelial cells (21). In this study, we describe a new disease mechanism of intestinal bleeding induced by the combined effects of PPS and FGF-2.

MATeRIALS AND METHODs

Recombinant adenoviral vectors. Recombinant adenoviral (rAd) vectors carrying either the Escherichia coli LacZ gene (rAd–LacZ) or a 700-bp cDNA sequence encoding a secreted form of human FGF-2 (rAd–FGF-2) were generated, amplified, and purified as previously described (8–12, 33). The particle-to-plaque-forming unit (pfu) ratio of the virus stock used in the experiments was 100.

1 This paper was presented at the 9th Cardiovascular-Kidney Interactions in Health and Disease Meeting at Amelia Island Plantation, Florida, on May 26–29, 2006. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Animal model. The animal care was approved by the Children's Research Institute Animal Care and Use Committee, FVB/N or C57BL/6J mice, 6–8 wk old, were purchased from Taconic (German-town, MD). Mice were injected retro-orbitally with $5 \times 10^8$ pfu/mouse of each rAd vector as described before (33). Pentosan polysulfate (Bene Arzneimittel; Munich, Germany), was injected intra-peritoneally every week. The PPS dosing schedule was selected based on the results of previous studies (30, 34), which showed that PPS was effective and safe in the treatment of experimental mouse tumors. In addition, we did not give multiple daily injections of PPS to avoid prolonging the anticoagulant activity of PPS. For the single bolus experiments, recombinant human (rh) FGF-2 was purchased from Biosource (Camarillo, CA). The level of endotoxin contamination of this preparation was below <0.1 ng/µg protein. The size of the hemorrhages was assessed by using the following arbitrary score: grade 1, no macroscopic bleeding visible; grade 2, congestion of vessels and minor capillary blood leaks (<10 mm); grade 3, localized hemorrhages (10–50 mm); grade 4, multiple and/or larger hemorrhages (>50 mm); and grade 5, lethal hemorrhages.

RESULTS

PPS increases the recruitment of FGF-2 in the intestine. To determine whether PPS increased the recruitment of circulating FGF-2 in the intestine, mice were injected intravenously with $^{125}$I-FGF-2 (0.1 ng/100 µl) in the presence or absence of PPS (Fig. 1A). In agreement with previous studies (31), $^{125}$I-FGF-2 was rapidly cleared from the circulation within minutes and was accumulated predominately in the liver and kidney (Fig. 1, A–C). In contrast, preincubation of $^{125}$I-FGF-2 with PPS, to allow the formation of a PPS-FGF-2 complex, changed the pattern of FGF-2 distribution, increasing its accumulation in the intestine and kidney and decreasing its accumulation in the liver (Fig. 1B). Moreover, by immunohistochemistry, FGF-2 was localized in the extracellular matrix and submucosal intestinal vessels of these mice (Fig. 2B).

PPS and FGF-2 induce lethal intestinal hemorrhages in mice. Since patients with angiogenic diseases can be exposed to high levels of circulating FGF-2 for prolonged periods of time (4, 5), we developed a mouse model to mimic this condition. Because FGF-2 injected intravenously is rapidly cleared from the circulation, all mice were injected with rAd vectors carrying the DNA sequence encoding a secreted form of human FGF-2 (rAd-FGF-2) (8). As shown in Fig. 1C, the peak levels of circulating FGF-2 were detected 7 days after the initial infection. Next, to determine whether FGF-2 and PPS acting together induced intestinal bleeding disorders in mice, five groups of 5-wk-old FVB/N male mice (n = 5 mice per group) were treated with either 1) rAd-FGF-2 (5 $\times 10^8$ pfu/mouse); 2) rAd-FGF-2 + PPS (60 mg/kg weekly, ip); 3) rAd vectors carrying the LacZ gene (18) (5 $\times 10^8$ pfu/mouse); 4) rAd-Lacz + PPS (60 mg/kg weekly, ip) or 5) PPS alone (same dose). This PPS dose was selected because it did not induce adverse effects in mice (30, 34), and our preliminary studies showed no significant bleeding disorders in 26 mice followed for 2–4 wk. Thus, as expected, all mice injected with rAd-FGF-2 alone, rAd-Lacz alone, or PPS alone and those mice injected with rAd-Lacz plus PPS did not develop hemorrhages and remained in good health throughout the 3 wk of study (Fig. 1D). However, in remarkable contrast, all mice injected with rAd-FGF-2 in combination with PPS developed intestinal hemorrhages and died within the first 2 wk of treatment (Fig. 1D). These results were reproduced in female FVB/N and C57BL/6J mice (n = 5 mice per group; data not shown), confirming that this phenomenon was neither sex nor strain specific. Finally, these experiments were repeated in a larger number of FVB/N male mice. From a total of 38 mice injected with rAd-FGF-2 + PPS (60 mg/kg per week), 12 mice (~32%) died within 48 h, showing mean serum FGF-2 levels of 150 ± 50 pg/ml (mean ± SD). This concentration is within the biological range of the FGF-2 levels detected in the circulation of children at high risk of developing intestinal bleeding (23, 24). The remaining mice died after they received the second dose of PPS, showing higher serum levels of FGF-2 (14 ± 4 ng/ml; mean ± SD).

To determine the relative contribution of the PPS dose in this process, additional groups of mice were injected with a lower dose of PPS (20 mg/kg weekly, ip) in combination with
Fig. 1. Clearance of human recombinant FGF-2 and pentosan polysulfate (PPS) and induction of lethal intestinal hemorrhages in mice. A: $^{125}$I-labeled FGF-2 ($^{125}$I-FGF-2; specific activity 1 μCi/ng) was incubated with and without PPS (5 mg/ml) and injected retro-orbitally in the right eye (0.1 ng/100 μl PBS). Blood samples (20 μl) were collected by retro-orbital bleeding of the left eye and analyzed in a gamma counter ($n = 3$ mice per group). B: accumulation of FGF-2 in tissues harvested 3 h after the $^{125}$I-FGF-2 injection. Values are expressed as counts per million per gram (cpm/g) of fresh tissue. *Two-tailed $P < 0.0003$ ($n = 3$ mice per group). GI, gastrointestinal. C: FGF-2 levels in the circulation of mice infected with recombinant adenoviral (rAd)-LacZ or FGF-2 vectors ($n = 5–10$ mice per group). The inset (arrow) shows a Western blot for FGF-2 in samples collected on day 7. Std, human recombinant FGF-2 (1 ng). All values in A–C are expressed as means ± SD. D: survival of mice treated with rAd-LacZ and FGF-2 + PPS (PPS-20 = 20 mg/kg per week; PPS-60 = 60 mg/kg per week). Log-rank test comparison of survival curves $P < 0.0001$ ($n = 5–21$ mice per group). E–G: representative pictures of the abdominal cavity of mice injected with rAd-LacZ + PPS-60 (E) or rAd-FGF-2 + PPS-60 (F and G). SI, small intestine; L, liver; C, colon. These pictures were taken from mice that were killed on day 8 after the second dose of PPS was given. Bars, 0.5 cm. All experiments were repeated at least two times.
Fig. 2. FGF-2 + PPS induce dilatation and bleeding of small intestinal submucosal vessels. All samples were harvested from mice that were treated for 1 wk and received two doses of PPS as described in MATERIALS AND METHODS. A: section stained with the control antibody. B: representative immunohistochemistry staining for FGF-2 (brown color) in mice injected with FGF-2. The white arrow points to a submucosal intestinal vessel. C and D: hematoxylin and eosin-stained sections harvested from mice infected with rAd-FGF-2 and treated with PPS for 1 wk. These sections demonstrate leakage of red blood cells (RBC) in the intestinal submucosa. The arrow points to leakage of RBC and edema in a small intestinal villae. E and F: immunohistochemistry staining for von Willebrand factor (vWF) antigen (red color) in representative small intestinal samples of mice infected with rAd-Lac Z (E) or rAd-FGF-2 (F) (5 x 10^8 plaque-forming units/mouse) and treated with PPS (60 mg/kg per week). Bars, 100 μm. G: graph demonstrating that FGF-2 + PPS induced dilatation of submucosal intestinal vessels. LacZ and FGF-2 indicate mice infected with these adenoviral vectors for 1 wk. PPS− and PPS+, groups of mice not treated (−) or treated (+) with PPS. Values are expressed as means ± SE. *One-way ANOVA, P = 0.0002 (n = 6 mice per group).
rAd-FGF-2 (n = 21) or rAd-LacZ (n = 5). In a similar manner, we found that 12% of the mice injected with rAd-FGF-2 plus PPS died of lethal intestinal hemorrhages (Fig. 1D). In contrast, all mice injected with a similar dose of PPS in combination with rAd-LacZ remained in excellent health and did not develop intestinal hemorrhages during the study period (Fig. 1D). A detailed histological analysis of hematoxylin and eosin-stained sections harvested from the liver, spleen, kidney, and gastrointestinal tissues of mice injected with rAd-FGF-2 plus PPS showed that the bleeding was initially localized to the intestinal submucosa; however, the structure of intestinal epithelial cells of the crypts or villae remained intact (Fig. 2, C and D). Furthermore, during the early bleeding stages, we did not find evidence of gastrointestinal ulcers and/or histological changes in the liver, spleen, or systemic vasculature that could explain the development of intestinal hemorrhages. In the least severe cases, the hemorrhages were small and remained encapsulated in the intestinal submucosa of mice that survived. In contrast, in the lethal cases, large hemorrhages resulted from the leakage of blood from large mesenteric vessels as well as the disruption of the intestinal mucosa and the accumulation of blood in the intestinal lumen and peritoneal cavity (Fig. 1, F and G). These findings indicate that both FGF-2 and PPS are essential for the development of submucosal intestinal hemorrhages and that the rAd vectors per se, alone or in combination with PPS, did not induce intestinal hemorrhages in our mouse model system. To determine whether a single bolus of FGF-2 was sufficient to induce lethal intestinal hemorrhages, two groups of FVB/N male mice (n = 5 mice each) were injected through the retro-orbital venous plexus with either PPS alone (60 mg/kg) or in combination with rh-FGF-2 (5 μg/mouse). This dose of FGF-2 (200 μg/kg per day) is extremely high in relation to any likely human exposure and resulted in very high circulating levels of FGF-2 (156 ± 19 ng/ml; mean ± SD) 5 min after the initial injection. As expected, FGF-2 was rapidly cleared from the circulation and could not be detected in the serum 24 h. None of the mice injected with PPS alone or PPS + FGF-2 showed evidence of intestinal bleeding or died after a single bolus injection. Two additional groups of male FVB/N mice (n = 5 mice per group) were injected via the peritoneal route with PPS (60 mg/kg) alone or in combination with rh-FGF-2 (5 μg/mouse). The peak levels of circulating rh-FGF-2 were detected ~2 h after the initial injection (27 ± 2.3 ng/ml; mean ± SD). However, once again, rh-FGF-2 was rapidly cleared from the circulation and remained undetectable in the serum 24 h after the initial injection. In a similar manner to the previous group, none of these mice showed evidence of intestinal bleeding or died after the single bolus injection of PPS + FGF-2. Thus it appears that high and prolonged steadystate levels of FGF-2 are required to induce lethal intestinal bleeding in mice treated with PPS.

**DISCUSSION**

In the present study, we have generated a new mouse model of lethal intestinal hemorrhages and described a new role of FGF-2 in the pathogenesis and treatment of this disorder. More specifically, we showed that PPS increases the recruitment of circulating FGF-2 in the intestine and that both factors, acting together, induce structural changes in intestinal vessels leading to the leakage of blood in the intestinal submucosa and peritoneum.

The notion that FGF-2 induces specific proangiogenic changes in submucosal intestinal vessels is consistent with the results of Paris et al. (21). They showed that FGF-2 prevented radiation-induced apoptosis of submucosal intestinal endothelial cells by acting specifically through FGF high-affinity receptors located in these cells (21). Although it could be argued that PPS should antagonize, rather than increase, the action of FGF-2 on intestinal endothelial cells by blocking its interaction with its cell surface receptors, previous studies have shown that the inhibitory effects of PPS could be fully reversed by adding more FGF-2 (30). In addition, the presence of HSPG in vascular structures with specific inhibitory or stimulatory...
angiogenic activity can regulate the activity of heparinoids and FGF-2 in a tissue-specific manner (1, 14, 19). For example, in experimental rat models, low doses of heparin improved the outcome of colitis and decreased the binding of FGF-2 to intestinal cells (14). In contrast, high doses of heparin induced the opposite effects (14, 15). In our mouse model, we postulate that PPS induces the recruitment of circulating FGF-2 in the intestine. Thus FGF-2 accumulates at a relative higher concentration than does PPS, and the balance between the antiangiogenic versus the proangiogenic activity of PPS and FGF-2, respectively, is shifted toward the latter, causing significant structural changes in intestinal endothelial cells. In this case, PPS might act like high doses of heparin, facilitating the recruitment of FGF-2 and enhancing its angiogenic activity. Both the angiogenic and anticoagulant activities of PPS + FGF-2 seem to be required to induce the leakage of red blood cells in the intestinal submucosa and peritoneal cavity. This process might be further facilitated by the capacity of anion polysaccharides to inhibit the adhesion of red blood cells to the endothelium (2). In summary, we propose that the ability of PPS and FGF-2 to induce intestinal bleeding depends on 1) the relative concentration of both factors in the intestinal submucosa; 2) the type of HSPG and FGF high-affinity receptors expressed on intestinal endothelial cells; and 3) the anticoagulant activity of PPS in the intestinal submucosa.

Fig. 3. Light and electron microscopy (EM) pictures showing the structural changes induced by FGF-2 and PPS (60 mg/kg) in vessels harvested from the small intestinal submucosa of mice. All samples were harvested from mice that were treated for 1 wk and received two doses of PPS as described in MATERIALS AND METHODS. A and B: a representative hematoxylin and eosin staining of small intestinal vessels harvested from mice infected with rAd-LacZ (A) or rAd-FGF-2 (B) and treated with PPS. Bars, 100 μm. The black arrows point to RBC trapped in dilated vessels. C and D: representative EM pictures allowing the direct comparison of the changes detected in small intestinal vessels between control LacZ + PPS mice and FGF-2 + PPS-infected mice. Bars, 2 μm. E and F: representative EM pictures of small intestinal vessels allowing the direct comparison of the changes detected in endothelial cells and basement membranes between control LacZ + PPS mice and FGF-2 + PPS-infected mice. Bars, 2 μm. The black arrows show ruffling of endothelial cell membranes. The white arrows show partial detachment and loosening of endothelial cells from the basement membranes.
It should be noted, however, that, at the present time, we do not know the exact basic mechanisms by which both FGF-2 and PPS induce the development of intestinal bleeding. Nevertheless, the light and electron microscopy examination of the intestinal vessels shows that PPS and FGF-2 induced capillary and endothelial abnormalities in mice undergoing bleeding disorders. These alterations include the dilatation of intestinal submucosal capillaries and ruffling of endothelial cell membranes, as well as the detachment of endothelial cells from the basement membranes. Thus the disruption of the normal structure of intestinal capillaries and endothelial cells acting together with the anticoagulant effect of PPS may explain, at least partially, the leakage of red blood cells. In support of these findings, experimental evidence implicates FGF-2 in the formation of vascular lesions (25). FGF-2 induces specific autocrine effects on several endothelial cell types, including intestinal endothelial cells (21). For example, when FGF-2 is injected subcutaneously into nude mice, it causes the appearance of leaky vascular lesions that mimic the early-stage lesions of Kaposi’s sarcoma (4). FGF-2 is also upregulated and released by endothelial cells during the proliferation phase of humans hemangiomas (5, 25). Furthermore, McDonnell et al. (17) found that overexpression of a secreted fibroblast growth factor-binding protein enhances the activity of FGF-2 and induces lethal vascular hemorrhages in developing chicken embryos (17). In a similar manner, overexpression of FGF-1 (17) or FGF-2 (25) in chicken embryos also induces significant vascular leakage and hemorrhages. It is possible that FGF-2 may act indirectly by inducing the synthesis or release of other angiogenic or proteolytic factors (i.e., VEGF and matrix metalloproteinase-2), which are known to induce structural changes in endothelial cells and vascular leakage (5, 6, 29). In any case, further studies are needed to elucidate the basic mechanism by which FGF-2 in combination with PPS induced intestinal bleeding.

In summary, we have identified a new disease mechanism of intestinal bleeding, as well as a new role for FGF-2 in the pathogenesis of this disorder. Overall, both high and steady-state expression levels of circulating FGF-2, plus the anticoagulant activity of PPS, are needed to induce lethal intestinal bleeding in mice. It could be argued that the high peak levels of circulating FGF-2 detected in our study would not be achieved in human disease states. Nevertheless, patients with intestinal angiogenic tumors, ulcers, or other inflammatory intestinal diseases can express, release, and accumulate FGF-2 in the intestine, even in the presence of much lower levels of circulating FGF-2 (5). In addition, children affected with the hemolytic uremic syndrome show high levels of circulating FGF-2 (23, 24) and are at high risk of intestinal bleeding. Thus these findings might have wider clinical implications for patients treated systemically with PPS and other heparinoids for angiogenic tumors, ulcers, and intestinal inflammatory-angiogenic disorders (5, 16, 30, 34). In contrast, patients treated orally with PPS for cystitis are not likely to be at higher risk of bleeding, since FGF-2 is not accumulated in the bladder, and PSS given orally should not induce anticoagulant changes in the bladder. We speculate that the plasma levels of FGF-2 might be a useful tool to identify patients at high risk of intestinal bleeding when treated with systemic infusions of PPS.

GRANTS
Support was provided by National Institutes of Health Grants R01-DK-4919; R01-HL-55605, and R21-CA-109011 (to P. E. Ray) and the Fundación Argentina para el Desarrollo Infantil (Buenos Aires, Argentina).

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