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Low-Molecular-Weight Fucoidan Promotes Therapeutic Revascularization in a Rat Model of Critical Hindlimb Ischemia

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ABSTRACT

The therapeutic potential of low-molecular-weight (LMW) fucoidan, a sulfated polysaccharide extracted from brown seaweed devoid of direct antithrombin effect, was investigated in vitro and in a model of critical hindlimb ischemia in rat. In vitro results showed that LMW fucoidan enhanced fibroblast growth factor (FGF)-2-induced [^{3}H]thymidine incorporation in cultured rat smooth muscle cells. Intravenous injection in rats of LMW fucoidan significantly increased the stromal-derived factor (SDF)-1 level from 1.2 $\pm$ 0.1 to 6.5 $\pm$ 0.35 ng/ml in plasma. The therapeutic effect of LMW fucoidan (5 mg/kg/day), FGF-2 (1 μg/kg/day), and LMW fucoidan combined with FGF-2 was assessed 14 days after induction of ischemia by 1) clinical evaluation of claudication, 2) tissue blood flow analysis, 3) histoenzymology of muscle metabolic activity, and 4) quantification of capillary density. Both LMW fucoidan and FGF-2 similarly improved residual muscle blood flow (62.5 $\pm$ 6.5 and 64.5 $\pm$ 4.5%, respectively) compared with the control group (42 $\pm$ 3.5%, $p < 0.0001$). The combination of FGF-2 and LMW fucoidan showed further significant improvement in tissue blood flow (90.5 $\pm$ 3%, $p < 0.0001$). These results were confirmed by phosphorylase activity, showing muscle regeneration in rats treated with the combination of FGF-2 and LMW fucoidan. Capillary density count increased from 9.6 $\pm$ 0.7 capillaries/muscle section in untreated ischemic controls to 14.3 $\pm$ 0.9 with LMW fucoidan, 14.5 $\pm$ 0.9 with FGF-2, and 19.1 $\pm$ 0.9 in combination ($p < 0.001$). Thus, LMW fucoidan potentiates FGF-2 activity, mobilizes SDF-1, and facilitates angiogenesis in a rat model. This natural compound could be of interest as an alternative for conventional treatment in critical ischemia.

In recent years, therapeutic angiogenesis has been proposed in the treatment of chronic ischemia. In animals, it was shown that basic fibroblast growth factor (FGF-2) (Gospodarowicz, 1974; Maciag et al., 1984), which is mitogenic for vascular endothelial cells, fibroblasts, and smooth muscle cells, can induce angiogenesis in vivo (Yanagisawa-Miwa et al., 1992; Lefaucheur and Sebille, 1995; Sellke et al., 1996; Yang et al., 1996; Shou et al., 1997). FGF-2 binds to heparan sulfates that stabilize it by protecting it from proteolytic cleavage and enhance its bioavailability (Aviezer et al., 1994; Roghani et al., 1994; Pellegrini, 2001). Tissue heparan sulfates thereby serve as coreceptors for growth factors.

Fucoidans are vegetal sulfated polysaccharides extracted from brown algae. High-molecular-weight (HMW) fucoidans are known to bind growth factors, such as FGFs, and protect them from proteolysis (Belford et al., 1993). HMW fucoidans can release the glycosaminoglycan-bound stromal-derived factor-1 (SDF-1) from its tissue storage sites. SDF-1 mobilizes medullary progenitors (Frenette and Weiss, 2000; Sweeney et al., 2000, 2002), which could participate in angiogenesis with vascular endothelial growth factor and FGF (Salvucci et al., 2002). Therefore, we supposed that fucoidans would have therapeutic potential in critical muscle ischemia.

A fraction of low-molecular-weight (LMW) fucoidan (7 $\pm$ 2 kDa) was obtained by radical depolymerization of HMW extracts from brown seaweed (Nardella et al., 1996) and was devoid of any direct antithrombin effect (Haroun-Bouhedja et al., 2000). In this study, we have tested the ability of LMW fucoidan to potentiate the effect of FGF-2 in vitro and to mobilize SDF-1 in vivo and have assessed the in vivo therapeutic effect using a rat model of critical hindlimb ischemia previously developed in our laboratory (Luyt et al., 2000).

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ABBREVIATIONS: FGF-2, fibroblast growth factor 2; HMW, high-molecular-weight; SDF-1, stromal-derived factor-1; LMW, low-molecular-weight; SMC, smooth muscle cell; EDL, extensorum digitorum longus.

Materials and Methods

LMW Fucoidan Extraction

LMW fucoidan was obtained by radical processing of HMW extracts from brown seaweed (Nardella et al., 1996). Based of previously reported analytical methods (Chevolot et al., 1999), the characteristics of LMW fucoidan were: weight-average molecular mass, 7 ± 2 kDa (polydispersity 1.7); fucose content, 35% (w/w); uronic acid content, 3% (w/w); and sulfate content, 34% (w/w). The anticoagulant activity of the LMW fucoidan was measured by activated partial thromboplastin (Millet et al., 1999); the amount of LMW fucoidan required to obtain an activated partial thromboplastin of 80 s (control, 40 s) was 25 μg/ml, a high concentration providing evidence of the low affinity for thrombin.

Cellular Pharmacology

Primary cultures of Wistar rat smooth muscle cells were used (Battle et al., 1994a). The effects of 10 μg/ml LMW fucoidan or LMW heparin (molecular mass, 5 ± 2 kD; Sigma-Aldrich, St. Louis, MO) on the FGF-2-induced DNA synthesis in SMCs were studied by [^{3}H]thymidine uptake (Battle et al., 1994b).

SDF-1 and MMP-9 Levels in Plasma

HMW fucoidan (Sigma-Aldrich) or LMW fucoidan was injected at 5 mg/kg into the jugular vein in five anesthetized rats. After 1 h, blood was sampled on citrate, centrifuged, and plasma collected. Plasma MMP-9 activities were determined by gelatin zymography, as previously described (Jacob et al., 2002). Similarly, MMP-9 levels were measured in vitro after a 1-h incubation with increasing concentrations (0 to 1 mg/ml) of LMW and HMW fucoidans with citrated rat blood. Plasma concentrations of SDF-1 were determined using an enzyme-linked immunosorbent assay kit (Quantikine, Oxon, UK).

Experimental Model of Critical Hindlimb Ischemia

Surgical Procedure. The surgical procedure has been described elsewhere (Luyt et al., 2000). Male Wistar rats (Iffa-Credo, L'Arbresle, France), weighing 280 to 320 g and aged 10 weeks, were used for this study. The experimental design complied with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the care and use of the laboratory animals (NIH publication no. 86-23, revised 1989; authorization no. 00577, Paris, France). The animals were anesthetized with 50 mg/kg sodium pentobarbital. Under a surgical microscope, the right external iliac and femoral arteries were dissected free from the origin of the external iliac artery (Fig. 1). Because external iliac artery ligation alone did not induce critical ischemia at rest in rats, ischemia was achieved by injection into the internal iliac artery via a retrograde catheter of 10,000 microspheres (Cytodex 2; Amersham Biosciences AB, Uppsala, Sweden) of 150 μm in diameter (Fig. 1). The catheter was removed, the external iliac artery ligated and excised, and the skin sutured. The contralateral hindlimb was sham-operated by an incision of the skin and dissection of the external iliac and femoral arteries.

Therapeutic Design. To evaluate the effect of LMW fucoidan on critical ischemia, four groups of animals underwent the surgical procedure. In group A (control group, $n = 10$), animals received vehicle alone, i.e., phosphate-buffered saline containing 1% bovine serum albumin. In group B ($n = 8$), animals received LMW fucoidan (5 mg/kg/day). In group C ($n = 8$), animals received recombinant FGF-2 (1 μg/kg/day) purified as previously described (Patry et al., 1994). In group D ($n = 8$), animals received FGF-2 (1 μg/kg/day) plus LMW fucoidan (5 mg/kg/day). The treatments or vehicle were administered immediately after surgery and then daily during the 14 days of the study by intramuscular injection in the sham-operated hindlimb. All four groups were evaluated 14 days after induction of ischemia by clinical examination to assess claudication, measurement of tissue blood flow, detection of muscle phosphorylase activity, and capillary count.

Tissue Blood Flow. A Laser-Doppler flowmeter (Perimed, Järfälla, Sweden) was used for evaluation of tissue blood flow (Nilsson et al., 1980; Luyt et al., 2000). Three muscles (tibialis anterior, biceps femoris, and adductor) on each hindlimb were studied. After removal of the skin, the probe was placed on the muscle, and a signal was recorded. The Doppler signal was taken as an index of microvascular perfusion of the muscle area under the probe (6 mm) and at a depth of 1 mm. Three to five measurements on each muscle were recorded and averaged. Results represent "residual blood flow" in the ischemic hindlimb and are expressed as a percentage of the muscular blood flow in the sham-operated hindlimb. For each animal, three determinations were performed: before surgery, immediately after surgery, and 14 days later.

Phosphorylase Activity. Ischemic and nonischemic extensorum digitorum longus (EDL) muscles were excised in all groups at day 14, frozen in liquid nitrogen-cooled isopentane, and stored at −80°C (Luyt et al., 2000). The muscles were sectioned transversally at 8 μm using a cryostat and stained with hematoxylin and eosin for topographical examination. Additional sections were

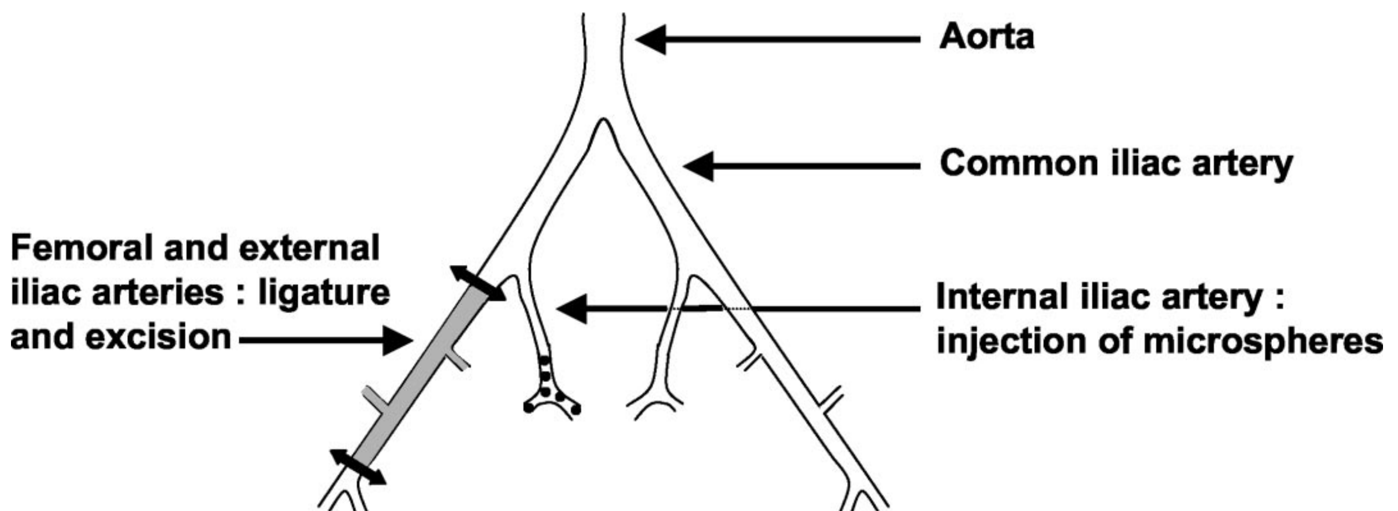

Fig. 1. Experimental model of critical hindlimb ischemia. A catheter is introduced into the femoral artery and microspheres are injected into the internal iliac artery. The femoral and external iliac arteries are then excised (Luyt et al., 2000). Animals were treated during 14 days by intramuscular injections of LMW fucoidan (5 mg/kg/day), FGF-2 (1 μg/kg/day), a combination of both, or vehicle alone.

stained with 1% lugol substrate to study phosphorylase activity (Carlson and Gutmann, 1975). Phosphorylase activity indicates muscle glycogenolysis, and metabolically active skeletal muscle fibers stain brown, whereas a yellow stain indicates absence of metabolic activity (Carlson and Gutmann, 1975).

Capillary Count. To detect angiogenesis, we performed a capillary count in ischemic and control muscles. Frozen sections of the EDL muscles (see above) were used. Sections were incubated with lectin from *Bandeiraea simplicifolia* (Sigma-Aldrich), as previously described (Alroy et al., 1987). The reaction was amplified using extravidin-peroxidase (Sigma-Aldrich) and revealed by diaminobenzidine (Sigma-Aldrich). Sections were counterstained with methylgreen, dehydrated, and coverslipped before examination. The total number of capillaries on the area of the entire muscle cross-section was counted.

Statistical Analysis

All results are expressed as the means ± S.E.M. Comparisons of muscle perfusion in different groups were performed using a two-way ANOVA. Differences were analyzed using a one-factor ANOVA with post hoc comparisons by Fisher's test. A χ^2 test was performed to compare the positive phosphorylase activity between groups. A p value of <0.05 was considered statistically significant.

Results

Thymidine Incorporation by Cultured SMCs

Because heparin-like molecules can modulate SMC proliferation by potentiation of FGF-2, we tested the effect of LMW fucoidan with FGF-2 on [^{3}H]thymidine uptake by rat aorta SMCs. In agreement with previously published data (Bjornsson et al., 1991; Goncalves, 1998), FGF-2 stimulated the [^{3}H]thymidine uptake in SMCs (Fig. 2). The addition of LMW heparin had no effect on the growth of FGF-2-stimulated SMCs, whereas the adjunction of LMW fucoidan potentiated FGF-2-induced [^{3}H]thymidine incorporation into SMCs (Fig. 2).

MMP-9 and SDF-1 Levels in Plasma

To test whether fucoidan can directly stimulate release of MMP-9 by leukocytes, we studied the effect in vitro of HMW and LMW fucoidans at different doses (0 to 1 mg/ml). HMW and LMW fucoidans were incubated with total citrated rat blood for 1 h. Blood was then centrifuged, and MMP-9 release in plasma was measured by zymography. HMW and LMW fucoidans did not modify MMP-9 levels in vitro (data not shown). In contrast, in vivo intravenous injection in rats of HMW fucoidan increased both MMP-9 ($p < 0.01$) and SDF-1 concentrations ($p < 0.01$) (Fig. 3). Interestingly, LMW fucoidan did not modify MMP-9 levels in vivo but significantly increased SDF-1 concentration ($p < 0.01$) (Fig. 3).

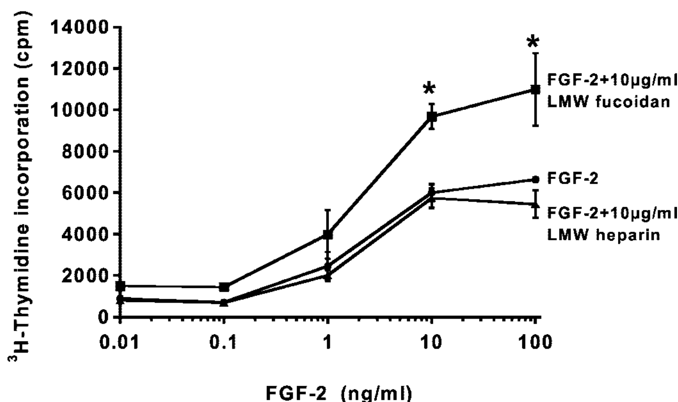

Fig. 2. Proliferation of vascular smooth muscle cells. [^{3}H]Thymidine incorporation in aortic rat smooth muscle cells after addition of various concentrations of FGF-2 alone or associated with 10 μg/ml LMW fucoidan or LMW heparin. *, indicates significant differences between FGF-2 and FGF-2 + LMW fucoidan treatment ($p < 0.001$).

Critical Hindlimb Ischemia

To evaluate the ability of LMW fucoidan alone or combined with FGF-2 to modulate rat hindlimb ischemia, we assessed its ability to improve clinical status, muscle blood flow, and activity.

Clinical Evolution. Fourteen days after surgery, all animals presented ischemia at rest and muscular atrophy. All the animals in the control group presented claudication without skin necrosis. In the group B (FGF-2-treated group) and C (LMW fucoidan-treated group), rats had less critical ischemia at rest than those in the control group. In group D (combined treatment of FGF-2 and LMW fucoidan), three of eight animals showed a complete resolution of their claudication after 14 days of treatment ($p < 0.05$ in comparison with the control group).

Muscle Perfusion. Functional evaluation before the induction of ischemia showed that muscular blood flow was similar in the two hindlimbs for all animals. Immediately after surgery, the residual blood flow in the ischemic hindlimb (compared with the sham-operated hindlimb) decreased from 100% to approximately 15% (Fig. 4).

Fourteen days after induction of ischemia, the mean residual blood flows of the control group had risen from 15 ± 5.1 to 42 ± 3.5%. In the FGF-2-treated group and the LMW fucoidan-treated group, mean residual blood flows at day 14 were 64.5 ± 4.5 and 62.5 ± 6.5%, respectively ($p < 0.0001$ in comparison with the control group). In the combined-treatment group, this value reached 90.5 ± 3% ($p < 0.0001$ in comparison with other groups) (Fig. 4).

Phosphorylase Activity. Fourteen days after surgery, no phosphorylase activity was detectable in the ischemic muscles of the control group. A diffuse yellow staining of ischemic muscle sections was observed, whereas the normal muscles of the sham-operated side all stained brown, indicating ATP-dependent phosphorylase activity. On the hematoxylin and eosin-stained cross-sections of the ischemic muscles, there was evidence of severe diffuse cellular ischemia, shown by a loss of architecture of the muscle, disappearance of the muscle nuclei, and inflammatory cell infiltration compared with sections from normal muscle (Fig. 5).

In the LMW fucoidan-treated group, all animals had partial regeneration of the ischemic muscle. In the FGF-2-treated group, one rat showed complete regeneration of the muscle, and the seven others had partial regeneration of the muscle. In the combined-treatment group (group D), three animals had complete regeneration of the EDL muscle, observed both with hematoxylin and eosin-staining and with phosphorylase activity, and the five others showed partial regeneration of the EDL muscle (Fig. 5).

Capillary Count. Figure 6 shows the results of quantification of capillary density. In the control group, there were significantly less capillaries than in the other groups ($p < 0.001$ in comparison with all groups). FGF-2 or LMW fucoidan alone significantly increased the number of capillaries

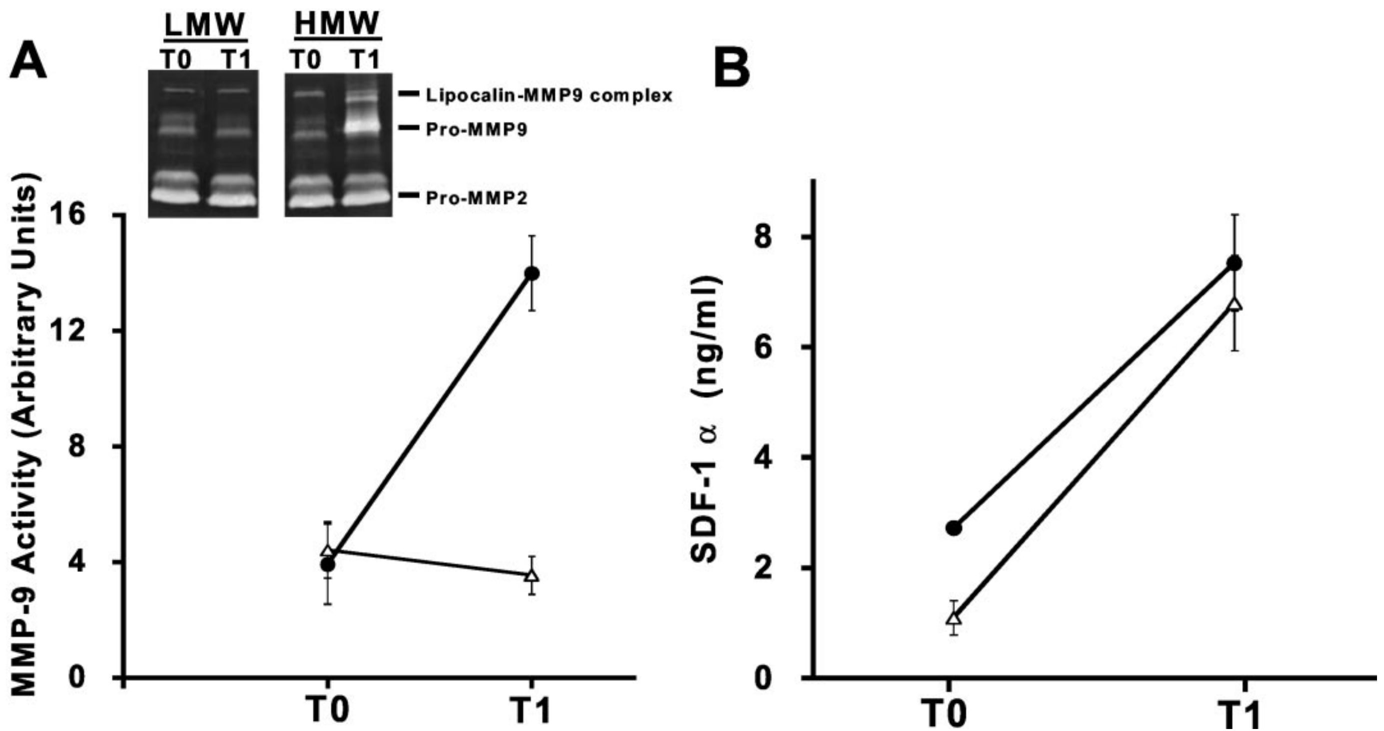

Fig. 3. Metalloproteinases and SDF-1 in plasma after intravenous injection of fucoidans. Five milligrams per kilogram HMW fucoidan (●) or LMW fucoidan (△) were injected intravenously in five Wistar rats. After 1 h, plasma samples were analyzed for MMP-9 activities (Fig. 3A) by gelatin zymography (inset A) and pro-MMP-9 activities were quantified using NIH software analysis. Concentration of SDF-1 in plasma (Fig. 3B) was quantified using an enzyme-linked immunosorbent assay test (Quantikine). T0, HMW fucoidan or LMW fucoidan injection; T1, 1 h after HMW fucoidan or LMW fucoidan injection. Results are expressed as the means ± S.E.M.

per section compared with untreated ischemic muscles ($p < 0.001$). Combined treatment with FGF + LMW fucoidan further enhanced the capillary density ($p < 0.001$) (Fig. 6).

Discussion

FGF-2 is mitogenic for vascular cells (Burgess and Maciag, 1989) and enhances the migration of vascular cells both in

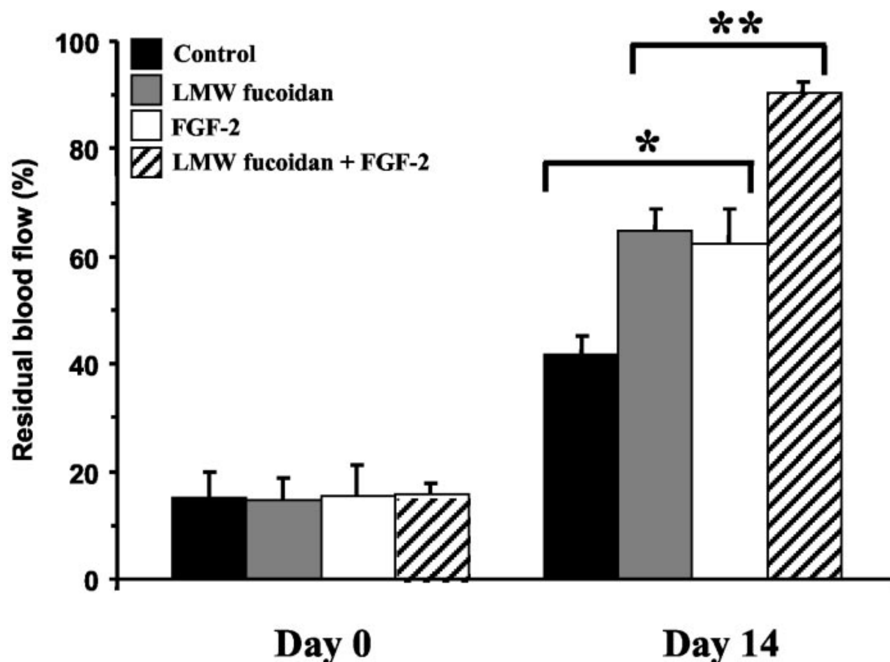

Fig. 4. Effects of LMW fucoidan treatment with or without FGF-2 on blood flow. Residual blood flow (% of contralateral values) observed immediately (Day 0) and 14 days (Day 14) after induction of ischemia. Results are expressed as the means ± S.E.M. ANOVA was used to compare the LMW fucoidan or FGF-2 groups with the control group (∗, $p < 0.0001$) and the LMW fucoidan + FGF-2 groups with the other groups (∗∗, $p < 0.0001$).

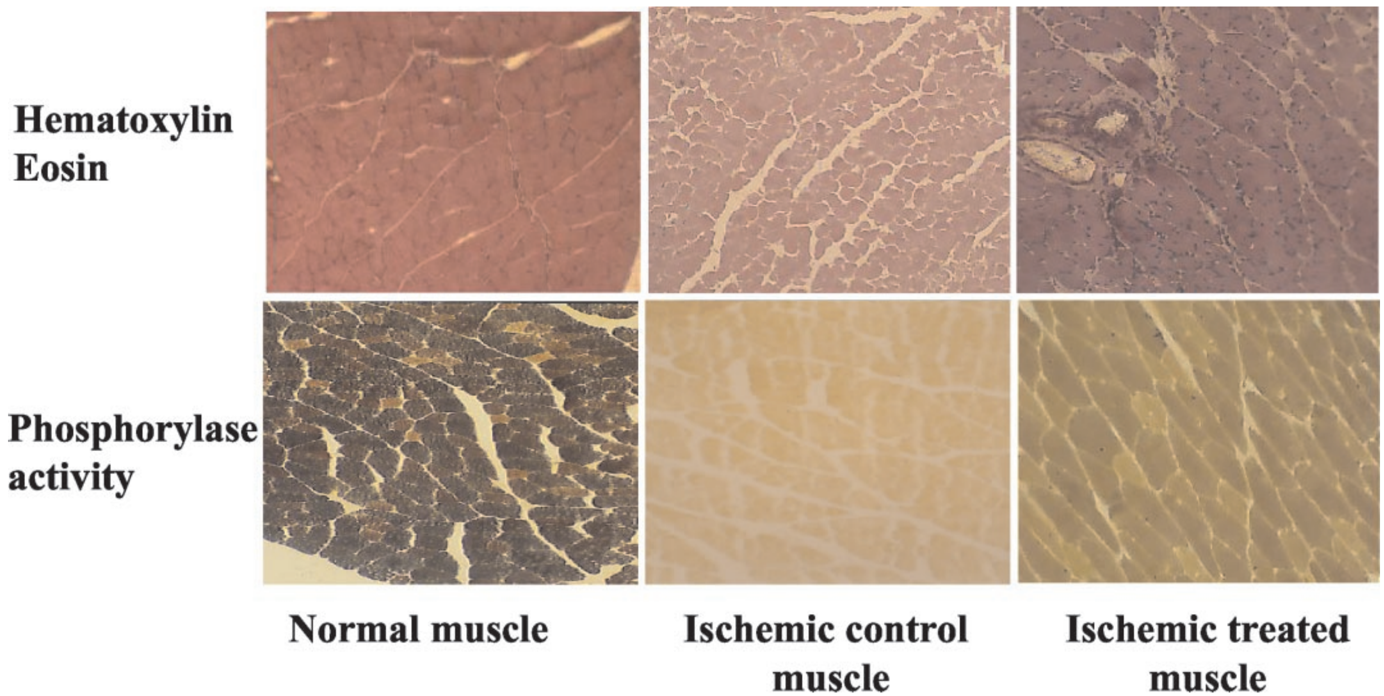

Fig. 5. Morphological studies of muscle sections after 14 days of treatment. Transverse sections of control (sham-operated animal), ischemic, and treated muscle (LMW fucoidan) were stained with hematoxylin-eosin and assessed for phosphorylase activity. In treated animals, partial regeneration was observed. Magnification: 10×.

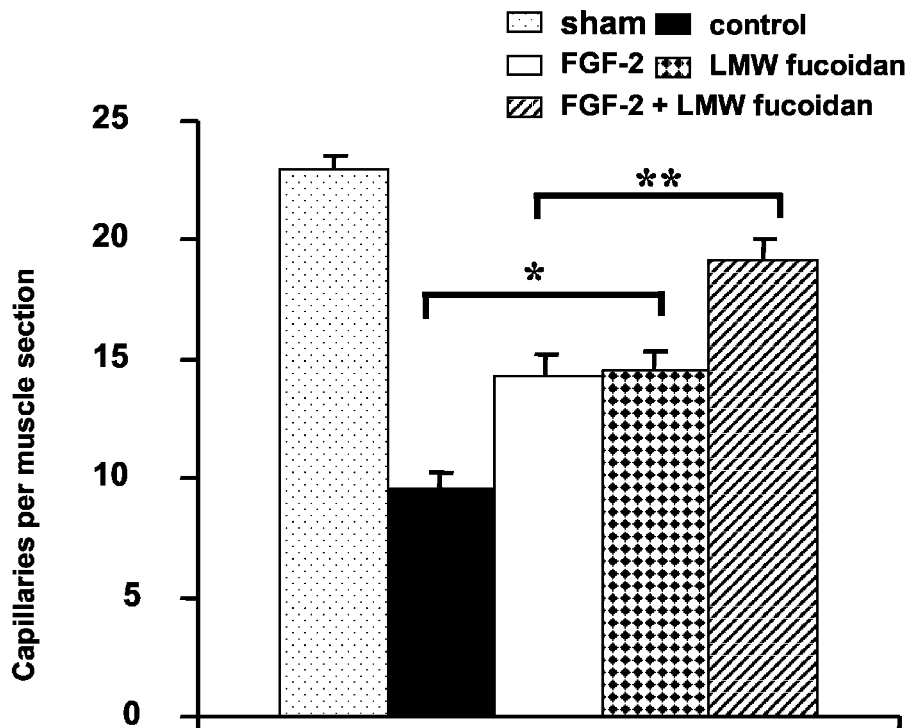

Fig. 6. Effects of LMW fucoidan treatment with or without FGF-2 on capillary density. The capillary count per muscle section was performed after 14 days of treatment. Statistical analysis was performed using ANOVA test and compared the control group with the LMW fucoidan and with the FGF-2-treated group (*, $p < 0.001$) or the control group with the LMW fucoidan + FGF-2-treated group (**, $p < 0.0003$).

vitro and in vivo (Lindner and Reidy, 1991). Previous studies in animals (Lefaucheur and Sebille, 1995; Sellke et al., 1996; Shou et al., 1997) demonstrated the ability of FGF-2 to improve revascularization in vivo. HMW fucoidan in association with FGF-2 was previously reported to improve endothelial cell proliferation in vitro (Giraux et al., 1998). Despite the direct in vitro and in vivo inhibitory effects of HMW and LMW fucoidan on vascular smooth muscle cell growth (McCaffrey et al., 1992; Logeart et al., 1997a,b; Deux et al., 2002), our results indicate that LMW fucoidan potentiates the effect of FGF-2 on [^{3}H]thymidine uptake. We have extended these concepts to experimental therapeutics, showing

that LMW fucoidan promotes FGF-2 effects in vivo, suggesting its potential interest for use in vascular tissue repair (Deux et al., 2002) and angiogenesis (Religa et al., 2000; Matou et al., 2002).

Effects of FGF-2 in vivo are multiple and complex including an arterial vasodilatory effect (Cuevas et al., 1991) and mitogenic properties on vascular cells (Gospodarowicz, 1974; Maciag et al., 1984). The half-life of FGF-2 is short, yet is prolonged when sulfated polysaccharides are coinfused (Whalen et al., 1989; Lazarous et al., 1997). Unfractionated HMW and LMW heparins alone, however, were reported to have no therapeutic effects in angiogenesis (Rosengart et al., 1997). Contrasting with these results, we demonstrate here, for the first time, a beneficial effect in revascularization with of LMW fucoidan in vivo.

At the site of the injury, tissue repair is in part mediated by growth factors such as FGFs, which are released from their extracellular or cellular glycosaminoglycan storage sites. As already described for HMW fucoidans (Belford et al., 1993) and for other natural and synthetic heparan sulfates (Belford et al., 1993; Aviezer et al., 1994; Roghani et al., 1994; Meddahi et al., 1995, 1996; Rusnati and Presta, 1996), LMW fucoidan may act in vivo by trapping and protecting endogenously released FGFs from deactivation and proteolytic cleavage and may also displace endogenous FGFs from their tissue heparan sulfate storage sites thus increasing their bioavailability.

Another effect of fucoidan is the ability to promote progenitor stem cell mobilization via the release of SDF-1 from heparan sulfate storage sites (Amara et al., 1999; Sadir et al., 2001). SDF-1 is a heparin binding cytokine (Lortat-Jacob et al., 2002) involved in angiogenesis (Mirshahi et al., 2000). It has been recently shown that SDF-1 regulates endothelial cell branching morphogenesis (Salvucci et al., 2002) and, conversely, that FGF-2 and vascular endothelial growth factor up-regulate the expression of SDF-1 receptors (CXCR4) on endothelial cells (Salcedo et al., 1999). Thus, SDF-1 mobilization could be one of the molecular effectors of therapeutic revascularization. Our results indeed show an increased SDF-1 concentration in plasma after a single bolus injection of LMW fucoidan. This effect was previously described by Sweeney et al. (2002) for HMW fucoidan and other glycosaminoglycans, such as dextran sulfates and chondroitin sulfates. Sweeney et al. (2002) also indicated that plasma MMP-9 significantly increased in response to intravenous injection of HMW fucoidan. In contrast, LMW fucoidan did not induce an increase in MMP-9 level in vivo. These results suggest that sulfated polysaccharides from the same family may exhibit different properties depending on their electrical charges, their degree of sulfation, and their molecular weight.

In conclusion, this study demonstrates for the first time, the therapeutic potential of LMW fucoidan in experimental critical hindlimb ischemia, providing a promising new tool for the promotion of revascularization. In addition, this polysaccharide of natural origin has no direct antithrombin effect, allowing clinical applications without hemorrhagic side effects.

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