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Therapeutic effect of fucoidan-stimulated endothelial colony-forming cells in peripheral ischemia

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Abstract :

Background: Fucoidan, an antithrombotic polysaccharide, can induce endothelial colony-forming cells (ECFC) to adopt an angiogenic phenotype *in vitro*. *Objectives:* We evaluated the effect of fucoidan on vasculogenesis induced by ECFC *in vivo*. *Methods:* We used a murine hindlimb ischemia model to probe the synergic role of fucoidan-treatment and ECFC infusion during tissue repair. *Results:* We found that exposure of ECFC to fucoidan prior to their intravenous injection improved residual muscle blood flow and increased collateral vessel formation. Necrosis of ischemic tissue was significantly reduced on day 14, to 12.1% of the gastronecmius cross-sectional surface area compared with 40.1% in animals injected with untreated-ECFC. ECFC stimulation with fucoidan caused a rapid increase in cell adhesion to activated endothelium in flow conditions, and enhanced transendothelial extravasation. Fucoidan-stimulated ECFC were resistant to shear stresses of up to 21 dyn cm^{-2}. Direct binding assays showed strong interaction of fucoidan with displaceable binding sites on the ECFC membrane. Bolus intramuscular administration of fucoidan 1 day after surgery reduces rhabdomyolysis. Mice injected with fucoidan (15 mg kg^{-1}) had significantly lower mean serum creatine phosphokinase (CPK) activity than control animals. This CPK reduction was correlated with muscle preservation against necrosis ($P < 0.001$). *Conclusions:* Fucoidan greatly increases ECFC-mediated angiogenesis *in vivo*. Its angiogenic effect would be due in part to its transportation to the ischemic site and its release after displacement by proteoglycans present in the extracellular matrix. The use of ECFC and fucoidan together, will be an efficient angiogenesis strategy to provide therapeutic neovascularization.

Keywords : angiogenesis ; endothelial colony-forming cells ; fucoidan ; ischemia ; revascularization

INTRODUCTION

Cardiovascular disease, due essentially to atherosclerosis, causes about half of all deaths in western countries. Peripheral vascular disease of the lower limbs is characterized by chronic obstruction of the arteries supplying the leg, gradually leading to critical limb ischemia. In end-stage disease, severe hypoperfusion results in ischemic ulceration and gangrene, with a high risk of amputation (10 to 40%) the first years [1]. Limb loss is best prevented by revascularization with endovascular or surgical approaches but this is impossible in 30% of patients with critical limb ischemia. No medical treatments have been shown to reduce the amputation rate at 6 months [2]. In this context, activation of local angiogenesis is a promising approach.

Infusion of *in vitro*-expanded bone marrow progenitor cells can enhance neovascularization in animal models of hindlimb ischemia [3,4]. Among these cells, endothelial progenitor cells are involved in regeneration of injured endothelium and neoangiogenesis after tissue ischemia, and are therefore a candidate cell therapy product [3,5,6]. Different cell populations have been isolated which play a role in angiogenesis, but only one population, called “endothelial colony-forming cells” (ECFC) have been shown to possess all the characteristics of true endothelial progenitor and to form neovessel *in vivo* [3,4,7,8]. However, proangiogenic approaches to critical limb ischemia have so far given disappointing results, owing partly to insufficient homing and survival of transplanted progenitor cells at the ischemic site [2,9-13]. The ability of these cells to mobilize and migrate to ischemic sites appears to be an important factor in the success of such therapy [3,9,14,15]. Proangiogenic potential can be enhanced by activating ECFC prior to their injection and thereby amplifying the biochemical signalling cascades that contribute to the production of cytokines involved in the neovascularization process [3,4,6,16-18].

We have previously reported that fucoidan enhances the proangiogenic properties of ECFC *in* vitro [19]. Fucoidan is a marine sulphated polysaccharide with antithrombotic properties which contrary to heparin does not significantly increase the bleeding risk, as it has a lower capacity to activate antithrombin [20]. Like proteoglycans, fucoidan interacts with a wide range of proteins and thus exhibit other biological properties including anti-inflammatory activity. It may act as a regulator of tissue remodelling [21] and has been shown to promote neovascularization when infused intramuscularly, together with the proangiogenic growth factor FGF-2, in a rat model of hindlimb ischemia [20,22]. Here we studied the interaction of fucoidan with infused ECFC during functional neovascularization in an athymic nude mouse model of hindlimb ischemia. We also investigated the mechanisms by which fucoidan enhances angiogenesis.

METHODS

Cell culture, characterization and pretreatment

Endothelial cells from human umbilical cords (HUVEC) and ECFC from human umbilical cord blood collected from consenting mothers (n=20), were isolated, expanded and characterized as previously described [19]. The study was approved by local ethics committee of “Hôpital des Instructions et des Armées de Begin” (201008043234797) and protocol conformed to ethical guidelines of Declaration of Helsinki. Flow cytometry was used to assess cell surface antigen expression of the endothelial lineage [18]. One day before all experiments, cells were growth-arrested for 24h in EBM-2, 2% FCS and released from growth arrest by adding EBM-2, 5% FCS, with or without 10µg/ml of fucoidan concentrations for 24h or 72h at 37°C, then washed, detached with versene/0.01% collagenase (1/1) and washed twice with buffered Hank’s, 0.5% BSA before use in angiogenesis and adhesion assays as previously described [18]. Supernatants

and cells were separately analyzed after fucoidan stimulation. All the following experiments were performed in triplicate during the first 30 days of culture.

Mouse model of hindlimb ischemia

Animal care conformed to French guidelines (Services Vétérinaires de la Santé et de la Production Animale). The experiments were approved by the IDF Paris Institutional Research Ethics Committee (CREEA, P2.CBV.031.07). Unilateral hindlimb ischemia was induced by femoral artery ligation in seven-week-old male athymic nude mice (Harlan) as previously described [18]. Five hours later the animals received an intravenous injection of normal saline (n=10), ECFC (10^5 cells per animal; n=10) or ECFC incubated in starvation medium overnight then stimulated with fucoidan 10µg/ml for 24 h, and washed (10^5 cells per animal; n=10). To evaluate the effect of fucoidan on critical ischemia, 3 groups of animals underwent the surgical procedure and received two intramuscular injection 1 and 2 days after surgery of normal saline, fucoidan solution (15mg/kg) or low molecular weight heparin (Lovenox, Sanofi Advantis, 2.5mg/kg). Laser Doppler perfusion imaging (Perimed, France) was used to appreciate revascularisation on day 14 [18]. Hindlimb macroscopic necrosis was quantified before sacrifice as previously described [23]. A score was calculated for each animal, as follows: 0: no necrosis, 1: necrosis of one toe, 2: necrosis of two or more toes, 3: foot necrosis, 4: leg necrosis and 5: autoamputation of the entire leg. Vessel density was evaluated by high-definition microangiography (Trophy system) and was expressed as the ratio of the ischemic to non ischemic leg percentage of pixels per image in the quantification area occupied by vessels [18]. Peripheral venous blood samples were collected 14 days after surgery. The quantitative determination of total creatine phosphokinase (CPK) and lactate dehydrogenase (LDH)

concentrations in plasma were determined by enzymatic assays according to procedures provided by Beckman Coulter (Villepinte, France).

Computed-assisted histomorphometric analysis

The gastronecmius muscles from both hindlimbs were fixed in 4% formol and embedded in paraffin. Histological studies were based on computed-assisted analysis of different area (ICS framework viewer, Tribvn) as previously described [12]. For each muscle, four sections 4µm thick and 300µm apart were stained with H&E then digitized for further analysis. The mean of surface area of each tissue type (necrotic, ischemic or healthy) was quantified in each muscle and reported as a percentage of the entire surface area of the section (n=4). The ratio between the mean entire cross-sectional areas of the ischemic and non ischemic gastronecmius muscles in each animal was used as an index of muscular trophicity.

Shear-flow adhesion assays

Tethering, rolling and flow adhesion experiments were conducted with a parallel-plate flow chamber in physiological shear stress conditions as previously described [18]. To distinguish between the adhesion of pretreated ECFC and that of detached endothelial cells, ECFC were stained with calcein. HUVEC monolayers ($7.5\ 10^5$) were grown on coverslips, maintained at 37°C for 6 days, then placed in the flow chamber and stimulated by exposure to a shear rate of 0.4 dyn/cm^2 for 30min. Calcein-labeled ECFC in adhesion buffer (cation-free HBSS, 10 mM HEPES, 1 mM CaCl2, 1 mM MgCl2, 2 mg/ml BSA, pH 7.4) were then perfused for 15min at 37°C at a shear rate of 0.4 dyn/cm^2 and the coverslips were washed with adhesion buffer for 10min. Adherent cells were visualized by phase-contrast microscopy. All experiments were performed in real time and videotaped for offline analysis. Images from 60 random microscope

fields (1 cm^2) were collected. Data are expressed as the number of adherent cells per cm^2. Detachment of adherent ECFC was studied by increasing the flow rate from 0.4 to 22 dyn/cm^2. In some experiments, fucoidan-stimulated or untreated ECFC were pretreated for 2h at 37°C with 0.5 U/ml heparinase I, 0.1 U/ml heparinase III and 0.2 U/ml chondroitinases ABC (Sigma).

Rat aortic ring assay

Angiogenesis was also studied with aortic explants cultured in three dimensional matrix gels as described by Zhu *et* al [24]. Experiments conformed to the guidelines of the University Paris-Descartes Institutional Animal Care and Use Committee (C75.06.02). The thoracic aorta was isolated from 8-week-old male Wistar rats (Charles River Breeders), immediately transferred to a culture dish containing warm serum-free minimum essential medium, and sliced into 1 to 2mm rings after carefully removing fibroadipose tissue. The aortic rings were rinsed and embedded in rat tail type I collagen gel. The gels were overlaid with DMEM-10% FCS supplemented with 100 U/ml penicillin and 100µg/ml streptomycin. Fucoidan (10µg/ml), FGF-2 (50ng/ml) or VEGF (40ng/ml) was added to the medium in the appropriate wells. The medium was changed on days 3, 6 and 9 of culture. Angiogenesis was scored by counting the number of branching endothelial sprouts.

Statistical analysis

Data are expressed as means ± SEM of at least three independent experiments. Significant differences were identified by ANOVA and Fisher's test. The Statview software package (SAS) was used for all analyses. Differences with probability values below 0.05 were considered significant.

RESULTS

Having previously shown that fucoidan can induce ECFC to adopt a proangiogenic phenotype *in* vitro [19], we first assessed the ability of fucoidan-stimulated ECFC to promote neovascularization in a mouse model of hindlimb ischemia. We focused on ECFC because this cell type is currently proposed as the cell type at the origin of newly formed vessels [7]. Cells were isolated from human umbilical cord blood on the basis of CD34 expression. The presence of Weibel-Palade bodies and combined expression of endothelial markers (CD31,Tie-2,KDR,Flt-1,CD144) unequivocally confirmed the endothelial phenotype of the ECFC thus obtained. Furthermore ECFC do not express leuko-monocytic markers such as CD14 and CD45 [18].

Mice treated with fucoidan-stimulated ECFC are protected from ischemia-induced necrosis.

Respectively 30% and 25% of mice that received normal saline or untreated ECFC exhibited macroscopic necrosis (**Figure 1A**). No mice treated with fucoidan-stimulated ECFC had macroscopic necrosis ($p<0.05$ vs CTRL, n=10).

Fucoidan-stimulated ECFC enhance post-ischemic neovascularization.

Hindlimb perfusion was respectively 34% and 99% higher with fucoidan-stimulated ECFC than with untreated ECFC ($p<0.001$) and normal saline ($p<0.001$) (**Figure 1B**). The ischemic/non ischemic limb angiography score was 11% higher with fucoidan-stimulated ECFC than with untreated ECFC, but the difference was not statistically significant (**Figure 1C**).

Fucoidan-stimulated ECFC protect ischemic tissue against necrosis.

The results of histomorphometric analysis of distal gastronecmius muscle sections on day 14 in mice treated with normal saline, control ECFC and fucoidan-stimulated ECFC are shown in **Figure 2**. Digitized muscle sections were divided into three areas: i) preserved areas with a

normal histology (**Figure 2C**), ii) ischemic areas containing cellular alterations but no necrosis (**Figure 2D**) and iii) necrotic tissue with muscle cell destruction (**Figure 2E**). Animals treated with fucoidan-stimulated ECFC showed less necrosis ($p<0.01$, **Figure 2E**) and better muscle preservation than animals treated with control ECFC ($p<0.05$, **Figure 2C**) or with saline solution alone ($p<0.01$, **Figure 2C**).

CELLULAR AND MOLECULAR MECHANISMS INVOLVED IN FUCOIDAN ENHANCEMENT OF PROANGIOGENIC ACTIVITY.

We next examined the potential mechanisms by which fucoidan-stimulation improved proangiogenic properties of ECFC. We investigated whether ECFC stimulation with fucoidan enhances their homing to ischemic tissue via ECFC adhesion to activated endothelium, their extravasation and differentiation into a vascular network.

Fucoidan-stimulated ECFC show enhanced adhesion to activated HUVEC in flow conditions.

We used a flow-based adhesion assay with HUVEC to investigate the effect of fucoidan stimulation on treated ECFC adhesion to activated endothelium in conditions simulating the shear forces encountered by ECFC when they adhere to vascular endothelial cells. In our experimental conditions, fucoidan-stimulated ECFC adhered much more rapidly than control ECFC. As shown in **Figure 3A**, short ECFC exposure to fucoidan caused a rapid increase in adhesion to HUVEC (1000 treated cells/cm^2 versus 545 control cells/cm^2; $p<0.001$). Fucoidan-stimulated ECFC adhered firmly to the endothelium and were more resistant than control cells to washing at shear rates up to 21 dyn/cm^2 (**Figure 3B**). This property is not correlated to fucoidan stimulation on cell surface integrins $\alpha_L\beta2$ (LFA-1), $\alpha_M\beta2$ (MAC-1) or $\alpha4\beta1$ (VLA4) expression levels and gene expression (data not shown).

Fucoidan-stimulated ECFC show enhanced transmigration through activated HUVEC and enhanced differentiation into vascular tubes.

Fluorescence microscopy of labelled fucoidan-stimulated ECFC that migrated through the activated endothelium showed that fucoidan enhanced ECFC motility by a factor of 2.5 compared to 40 ng/ml VEGF ($p<0.01$) (**Figure 3C**). Similar results were obtained when VEGF was present in the bottom well of the chamber. Fucoidan enhanced ECFC transmigration through activated HUVEC monolayers towards VEGF ($p<0.01$). Fucoidan also enhanced the proangiogenic activity of heparin growth factors present in EGM-2 culture medium ($p<0.05$) (**Figure 3D**). Real-time RT-PCR showed no change in mRNA levels of the proangiogenic cytokines VEGF-A, FGF-2, angiopoietin and SDF-1 (data not shown). In the same experimental conditions, no significant increase in proangiogenic cytokine release was observed in media conditioned by fucoidan-stimulated ECFC, as measured by ELISA. However, gelatine zymography of the same conditioned medium revealed that tube formation coincided with increased secretion of pro-MMP-9 (2-fold, $p<0.05$ **Figure 4A-B**).

Fucoidan binds to the ECFC surface.

Fluorescent DTAF-fucoidan that retains its full proangiogenic activity bound rapidly to the ECFC outer membrane in a time-dependent manner (**Figure IA** available online) and was internalized within 15 min at 37°C, probably by endocytosis. After 24h of incubation, DTAF-fucoidan was found in endocytotic vesicles that localized in the perinuclear region (**Figure IB** available online). DTAF-fucoidan interacted with displaceable binding sites on ECFC, as heparin and cold fucoidan both induced concentration-dependent displacement of labelled fucoidan when added to the culture medium ($p<0.001$ **Figure IC** available online).

Bound fucoidan acts as a glycosaminoglycan

Because fucoidan is a sulphated polysaccharide and as heparan-sulphate proteoglycans (HSPG) play key roles in the angiogenic process, presenting chemokines to circulating cells, we investigated the role of fucoidan bound to the ECFC surface in ECFC adhesion and differentiation processes. ECFC were treated for 120 min with enzymes that selectively degrade either heparan sulphate or chondroitin sulphate, and were then incubated with 10 µg/ml fucoidan at 37°C overnight. Enzyme treatment abolished ECFC adhesion to the endothelium in flow conditions (-65% $p<0.001$; **Figure 3A**) and also reduced tube formation in Matrigel (-50%, $p<0.01$). Preincubation of enzyme-treated cells with fucoidan enhanced ECFC adhesion by 82% (342 cells/cm^2 with fucoidan-stimulated ECFC without HSPG (HSPGd) versus 187 cells/cm^2 with HSPGd-ECFC ($p<0.001$)) (**Figure 3A**). These fucoidan-pretreated HSPGd-ECFC showed poor resistance to detachment when tested at shear stresses up to 20 dyn/cm^2 (**Figure 3B**). This fucoidan pretreatment had no effect on HSPGd-ECFC extravasation or tube formation (data not shown).

Fucoidan promotes angiogenesis in the aortic ring model.

Rings of rat aorta embedded in collagen gel immediately after excision generate branching microvessels, and this process was enhanced by FGF-2 and VEGF in a concentration-dependent manner, as previously reported [24]. Unstimulated control rings exhibited only rare and short endothelial sprouts. As shown in **Figure 5**, aorta rings treated with FGF-2 (**Figure 5A**) and VEGF (**Figure 5B**) showed approximate 2.5- and 2-fold increases in microvessel outgrowth, respectively, after 9 days of culture. Fucoidan significantly increased the number of neovessel sprouts induced by FGF-2 (**Figure 5A**, $p<0.01$) but not by VEGF (**Figure 5B**). The distance between the ring and the edge of the cell front around the ring, which reflects both cell

proliferation and migration, was 1.4-fold longer in the presence of both fucoidan and FGF-2 than in the presence of FGF-2 alone (**Figure 5C**).

Fucoidan protects ischemic tissue against necrosis better than heparin.

Histomorphometric analysis of distal gastronecmius muscle sections on day 14 in mice treated with intramuscular injection of normal saline, low molecular weight heparin (2.5 mg/kg) and fucoidan (15mg/kg) are shown in **Figure 6**. Animals treated with fucoidan showed less necrosis and better muscle preservation than animals treated with saline solution alone and heparin treated animals ($p<0.001$ and $p<0.01$ respectively, **Figure 6A-C**). This correlates with significant decrease of blood levels of CPK 14 days after surgery (**Figure 6D**). Serum CPK and LDH are enzymes whose levels can increase significantly when muscle damage or inflammation has occurred. In the control animals, a serum CPK activity of 2200 ± 600 UI^{-1} was observed. Fucoidan pretreatment markedly attenuated the CPK activity (≈70% p=0.002, ≈60% p=0.0320) compared with respectively the control and heparin groups. No significant changes were observed regarding the serum lactate dehydrogenase activity.

DISCUSSION

Fucoidan, an antithrombotic polysaccharide, can induce ECFC to adopt an angiogenic phenotype *in vitro* [19]. Human ECFC treatment with fucoidan induced proangiogenic phenotype, involving both an early angiogenic event (proliferation and migration) and a late event (differentiation into vascular cords). This study shows that the beneficial effect of ECFC infusion in a mouse model of hindlimb ischemia is amplified significantly by fucoidan pretreatment, preventing necrosis. This tissue protection is associated with enhanced neoangiogenesis and reduction of rhabdomyolysis.

The angiogenic process involves ECFC rolling and adhesion to activated endothelium and their extravasation to target sites. Our results provide strong evidence that fucoidan prestimulation enhanced each of these steps, by inducing pro-MMP-9 secretion involved in cell homing [25]. These actions were associated with direct fucoidan interaction with the ECFC surface. Fucoidan bound to the cell membrane and was internalized. We have previously shown that fucoidan stimulation leads to ECFC actin cytoskeleton changes, which may be involved in extravasation [19]. Experiments underway in our laboratory indicate that fucoidan interaction is partially inhibited by blocking antibodies against P-selectin, CD11b (α_M) and CD18 ($\beta 2$) (30 to 40%, $p<0.001$). This is in keeping with the reported high affinity of selectin and integrin $\alpha_M\beta_2$ for a high-molecular-weight commercial fucoidan preparation [26,27]. Because of its spatial structure, fucoidan can mimic the clustering of sulphated, sialylated and fucosylated oligosaccharides on the cell surface and can provide the appropriate structural backbone for selectin binding. Fucoidan interaction with P-selectin does not affect the ECFC arrest on activated endothelium in flow conditions, as VLA4 ($\alpha 4\beta 1$) can mediate both tethering and firm adhesion [28]. The possible interaction of $\alpha_M\beta 2$ or other integrins with fucoidan remains to be addressed. We found that fucoidan interacted with displaceable binding sites on ECFC, allowing it to be released in the ischemic tissue environment after displacement by heparan sulphate present in extracellular matrix. There, it can interact with heparin-like growth factors and enhance neoangiogenesis through the transduction of intracellular signals required to induce the angiogenic phenotype. Its effect on signalling pathways is under investigation.

Tissue ischemia is also accompanied by inflammation and fucoidan has been shown to possess anti-inflammatory properties [20]. Parenchymal tissue protection against inflammatory processes and myofibroblastic remodelling have been observed after daily subcutaneous injection of

fucoidan in a rat cardiac allograft model [29]. During ischemia, hindlimb muscle destruction causes permeabilization of muscle fibbers and the release of their contents into the bloodstream including muscle enzyme such as CPK. Serum CPK activity will thus reflect the magnitude of acute muscle damage due to ischemia. Bolus administration of fucoidan injected directly in the quadriceps muscle of ischemic mice was shown to induce 70% and 60% reduction in elevated CPK activities compared to respectively control and heparin mice. This could account for the anti-necrotic effect observed here, although further investigations are needed. This effect seems to be specific to the polyfucose; intramuscular injection of low molecular weight heparin in the same experimental conditions does not protect the muscle from necrosis. This is in keeping with previous studies showing the important role of the fucosyl backbone in fucoidan properties [20]. Finally in the aortic ring model, the angiogenic activity of fucoidan in the presence of FGF-2 stems from its ability to potentiate FGF-2 proangiogenic activity. These results corroborate the ones observed by Luyt *et al.* showing that fucoidan can induce angiogenesis *in vivo* by modulating the proangiogenic properties of heparin-binding growth factor FGF-2 [22]. However regeneration of ischemic tissue was only observed when fucoidan was associated with FGF-2 [22 and unpublished data, 2010]. Therefore, the angiogenic potency of the fucoidan-stimulated ECFC is likely attributable to both the beneficial effects of fucoidan on the proangiogenic potential of transplanted ECFC and the influence of fucoidan and ECFC on the ischemic hindlimb.

So far, efforts to stimulate angiogenesis have been limited to the introduction of exogenous growth factors such as FGF-2 or VEGF by means of protein delivery or gene transfer. Our results indicate that a similar response could be obtained with a sulphated polysaccharide. Of particular interest, fucoidan partially restored the adhesion of ECFC to activated endothelium after their treatment with enzymes that remove HSPG located at the cell surface or in the extracellular

matrix and that play crucial roles in physiological processes and tissue remodelling [30]. Fucoidan shares several characteristics with HSPG, including sulphate groups and short polysaccharide chains. Owing to its ionic structure, fucoidan also mimics some properties of heparan sulphate and can thus bind proangiogenic heparin-binding growth factors, thereby thus increasing local concentrations at the cell surface or protecting these factors against enzymatic degradation [20,31]. We cannot rule out the possibility that free and cell-associated fucoidan may bind proangiogenic growth factors and confer a stable, receptor-compatible conformational change upon growth factor, which might increase their affinity for their receptors and improve angiogenesis process. Fucoidan can modulate their biological activity and disrupt heparan sulphate-growth factor/cytokine complexes, thus releasing chemokines sequestered by heparan sulphate [19,20,32]. Intramuscular fucoidan injection increases plasma levels of SDF-1 and is thus able to promote ECFC mobilization [20,32]. Fucoidan can also act on surrounding cells such as smooth muscle cells, neutrophil and/or macrophages attracted by cytokines released by the ischemic site.

Figure 7 summarizes the multiple roles of fucoidan in therapeutic angiogenesis and in the proangiogenic activity of transplanted ECFC. Fucoidan immobilization on transplanted ECFC allows these cells to home rapidly to ischemic sites, by enhancing ECFC adhesion to activated endothelium and extravasation. These effects involve upregulating the secretion of pro-MMP-9 involved in stem cell homing. Locally released fucoidan binds heparin-like growth factors and regulates their mitogenic activity, leading to increased angiogenesis. Fucoidan can also specifically displace sequestered SDF-1 from its HSPG anchors and thus contribute to its release into the circulation promoting ECFC mobilization.

In conclusion, our results show that the therapeutic effects of fucoidan-stimulated ECFC are due to both individual and synergistic effects of the two agents. The fucoidan-ECFC tandem warrants further studies as a means of promoting revascularization of ischemic tissues and as potential candidate to cell therapy on peripheral ischemia.

Addendum

G. Sarlon, F. Zemani and C. Boisson-Vidal designed and performed the research, analyzed the data and wrote the manuscript.

L. David: rat aortic ring experiments, data collection and image analysis.

JP Duong Van Huyen: computed-assisted histomorphometric analysis, data collection and interpretation of the results.

B. Dizier: *in vivo* experiments, data collection and doppler analysis.

F. Grelac: zymography, data collection and image analysis.

S Colliec-Jouault: obtained funding

I. Galy-Fauroux: cell culture supervisor

P. Bruneval: Histomorphometric study supervisor

J Emmerich, AM Fischer: Critical revisions of the manuscript for important intellectual content.

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FIGURE LEGENDS

Figure 1: **Stimulation of ECFC by fucoidan increases neovascularization in hindlimb ischemia**. Cumulative incidence of clinical necrosis (**A**), foot perfusion (**B**), and quantitative analysis of angiographic score (**C**) of normal-saline (CTRL, ■), untreated ECFC (ECFC, □) and fucoidan-stimulated ECFC (ECFC + Fuc, ■) transplanted mice 14 days after femoral artery ligation. Values are expressed as means ± SEM (n=10 per group). *p<0.05, **p<0.01, ***p<0.001 vs normal saline-injected mice, # # p<0.01 vs ECFC-injected mice. Representative photomicrographs are shown.

Figure 2: **Representative histological analysis showing tissue protection in nude mice treated with fucoidan-stimulated ECFC**. Macroscopic aspects of ECFC-injected mice (**A**) and fucoidan-ECFC-injected mice (**B**). H&E staining of the same distal gastronecmius muscle sections on day 14 and quantification of histologically preserved area (**C**), ischemic infiltrated area (**D**) and necrotic area (**E**) types surface. The surface of each area type is reported as a percentage of the entire histological section surface. Values are expressed as means ± SEM (n=15). **p<0.01 vs normal saline-injected mice (CTRL), # p<0.05 vs ECFC-injected mice. NI: non ischemic hindlimb, isch: ischemic hindlimb.

Figure 3: **Calcein labelled fucoidan-stimulated ECFC showed enhanced adhesion to activated endothelium at 0.4 dyn/cm^2, and enhanced motility and migration through activated endothelium**. (**A)** Adherence of calcein-stimulated ECFC to activated HUVEC under dynamic conditions. When indicated (enzymes +), ECFC were treated with appropriate enzymes (heparinase I and III, chondroitinases) to selectively degrade heparan sulphate and chondroitin sulphate on cell surface. (**B)**

Resistance to detachment by incremented shear stresses from 0.4 to 22 dyn/cm^2. Control ECFC (□), fucoidan-stimulated ECFC (■), ECFC devoid of HSPG (▲), fucoidan-stimulated ECFC devoid of HSPG (△). (**C)** Representative calcein-labelled – untreated ECFC (ECFC,□,■) and -fucoidan-stimulated ECFC (ECFC+Fuc,■,▨) accumulated under the membrane (objx4). Extravasation assay were performed in a chemotaxis chamber with (VEGF,■,▨) or without (EBM-2,□,■) VEGF 40ng/ml as a chemoattractant. **Fucoidan pretreatment enhances heparin-like growth factors-induced tubular morphogenesis by ECFC in Matrigel.** (**D)** Tubular morphogenesis induced by control ECFC (ECFC,□,▨) and fucoidan-stimulated ECFC (ECFC+Fuc,■,▨) in EMB-2 5% FCS (EBM-2,□,■) and EGM-2 (▨,▨) on matrigel. Phase-contrast micrograph, original x4. Data are presented as percentages versus untreated ECFC. Values are expressed as mean± SEM: *p<0.05, **p<0.01, ***p<0.001, n=3.

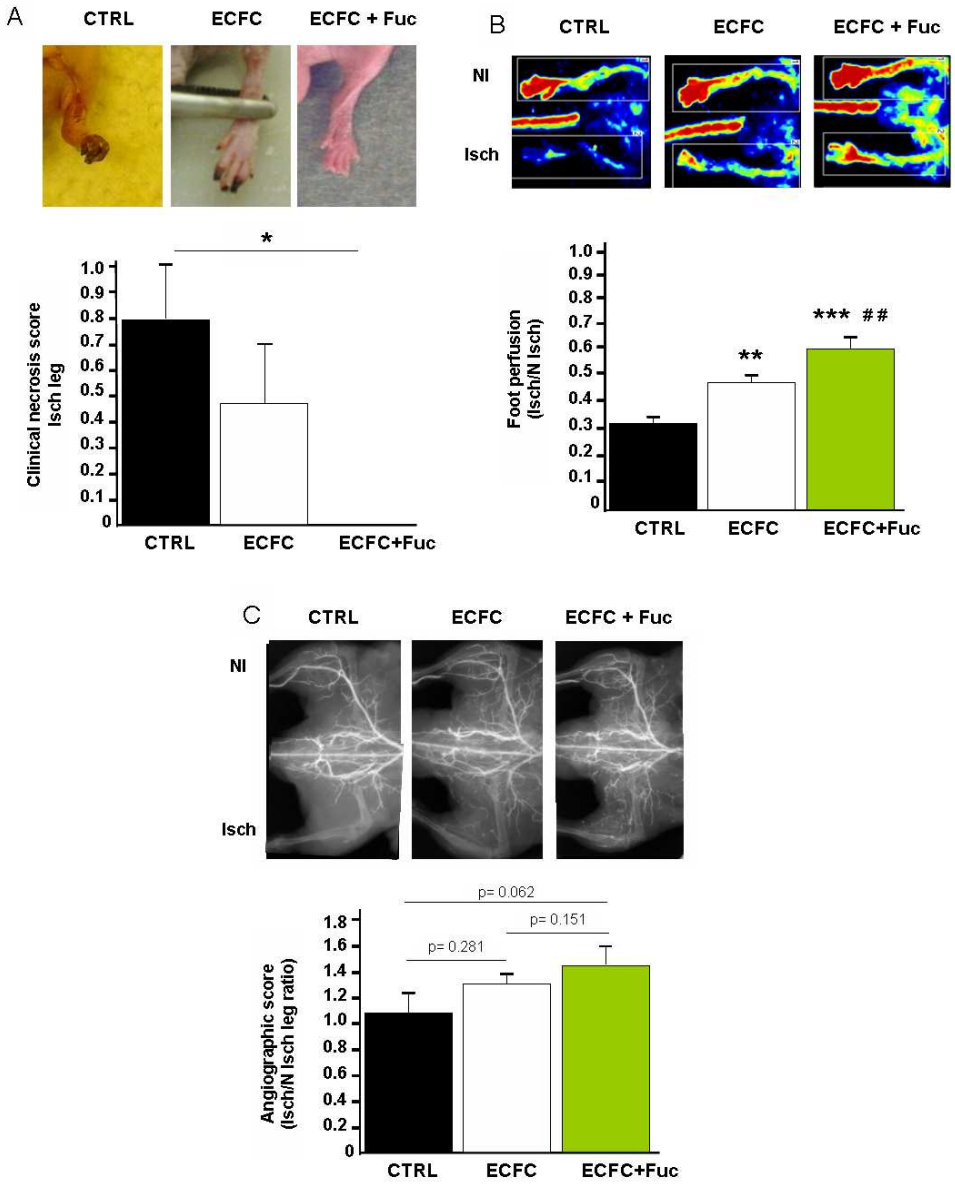
Figure 4: **Fucoidan-stimulated ECFC (FUC) showed enhanced pro-MMP9 secretion**. (**A)** Gelatinolytic activities of metalloproteinases detected in culture supernatants by zymography. HT1080 cell conditioned medium was used as standard. This established cell line constitutively releases proMMP-2 and proMMP-9 into the culture medium. **(B)** Quantitative analysis of gelatinolytic activities 24h (□,■) after prestimulation with (■) or without (□) fucoidan. Results referred to gelatinolytic activities of HT1080 media. Values are expressed as means ± SEM versus untreated-ECFC: *p<0.05, **p<0.01, n=3.

Figure 5: **Fucoidan promotes capillary outward sprouting of rat aorta rings.** (**A–B**) Microvessels sprouts were counted after 9 days of culture in DMEM 10% FCS for untreated control rings (CTRL,■) or DMEM 10% FCS supplemented with 10 µg/ml

fucoidan alone (□), 50 ng/ml FGF-2 (**A**), 40 ng/ml VEGF (**B**) in the presence (▨) or in the absence (■) of fucoidan. (**C**) Effect of fucoidan on fibroblasts proliferation and migration around rings. Values are expressed as means ± SEM: *p<0.05, **p<0.01.

Figure 6: **Fucoidan protects ischemic tissue against necrosis**. Quantification of histologically preserved area (**A**), ischemic infiltrated area (**B**) and necrotic area (**C**) types surface 14 days after surgery and intramuscular bolus administration of fucoidan (15mg/kg Fuc), heparin (2.5 mg/kg Hep) or normal saline (CTRL) at day 1 and 2. ***p<0.01 vs normal saline-injected mice (CTRL). (D) Serum levels of creatine phosphokinase (CPK, □) and lactate dehydrogenase (LDH, ■) among Fucoidan, Heparin and Control groups measured 14 days after surgery.

Figure 7: **Proposed mechanisms involved in beneficial effects of fucoidan on ECFC proangiogenic function in peripheral ischemia**. Fucoidan induces a proangiogenic phenotype in ECFC via pro-MMP-9 secretion promoting their adhesion to activated endothelium (**a**), extravasation (**b**) and migration (**c**) in the ECM. Released in the tissue, it binds heparin-like growth factors (○) (**d**) and activates their mitogenic activity leading to increased angiogenesis (**e**). PG: proteoglycans, EC: endothelial cells, EPC : endothelial progenitor cells, Fuc : fucoidan.



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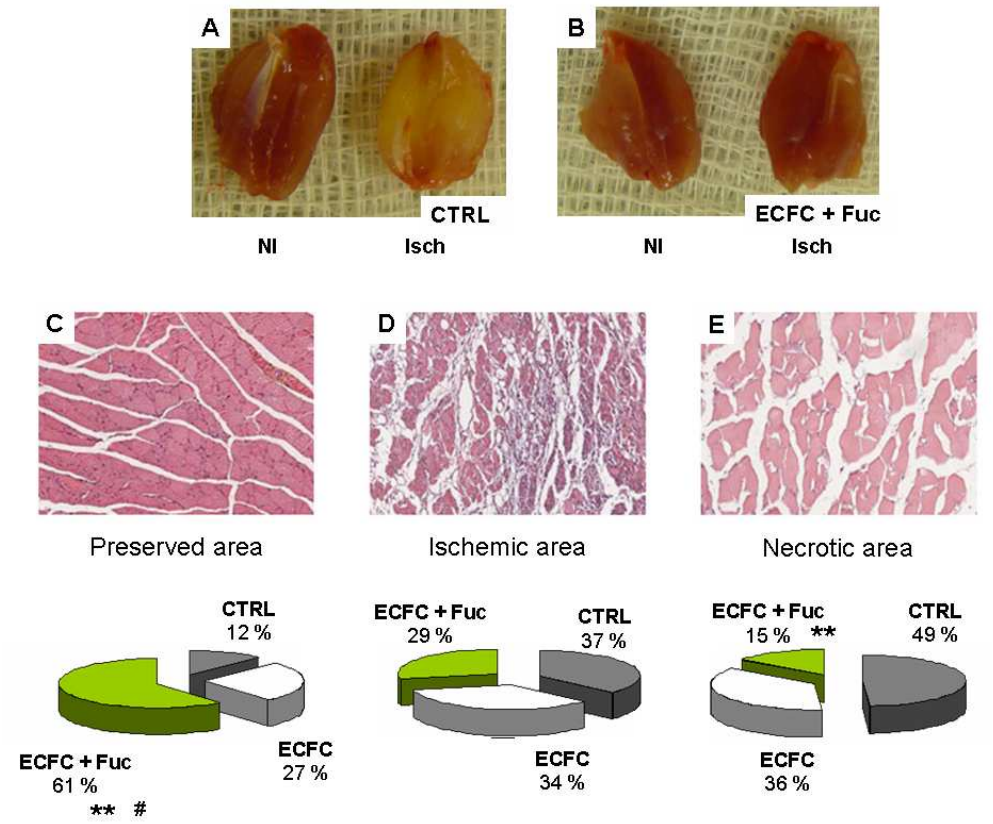

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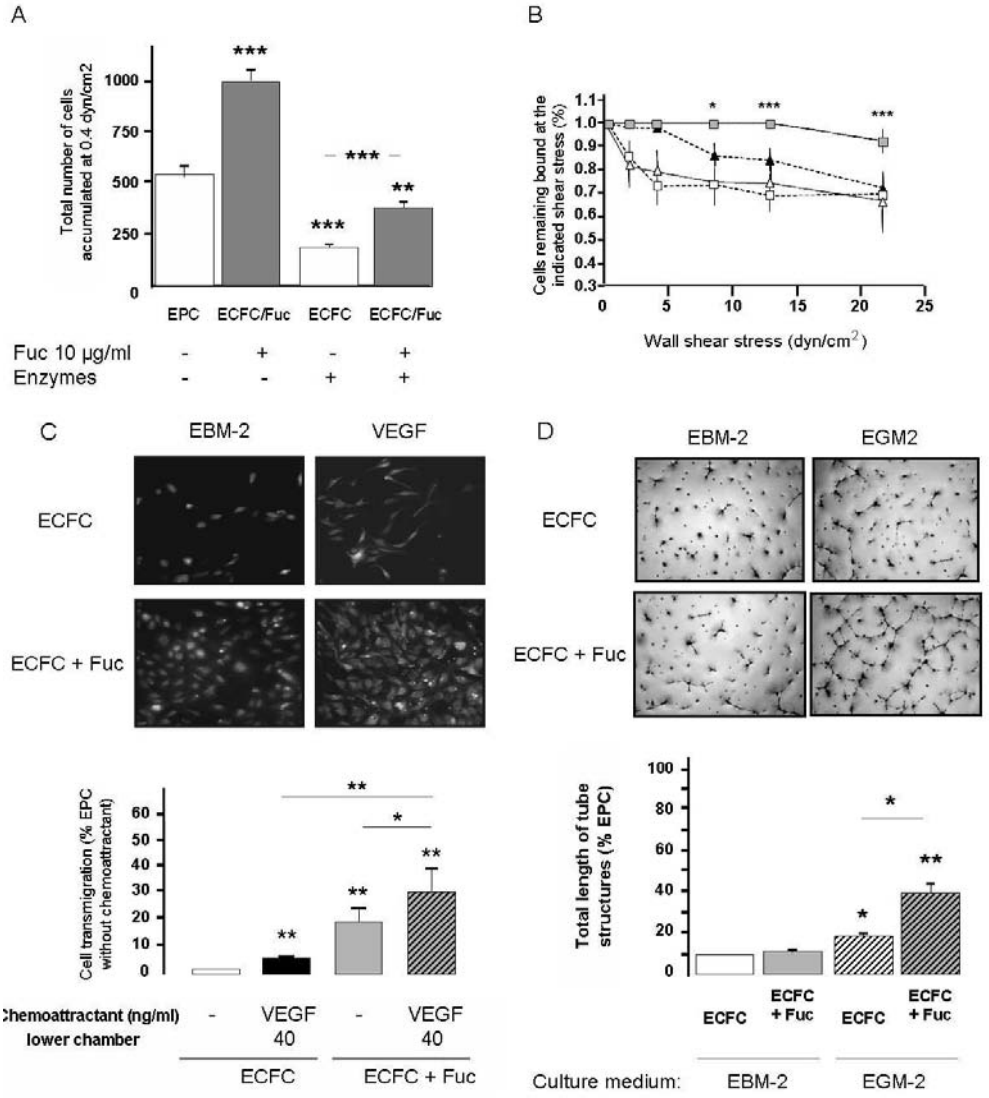

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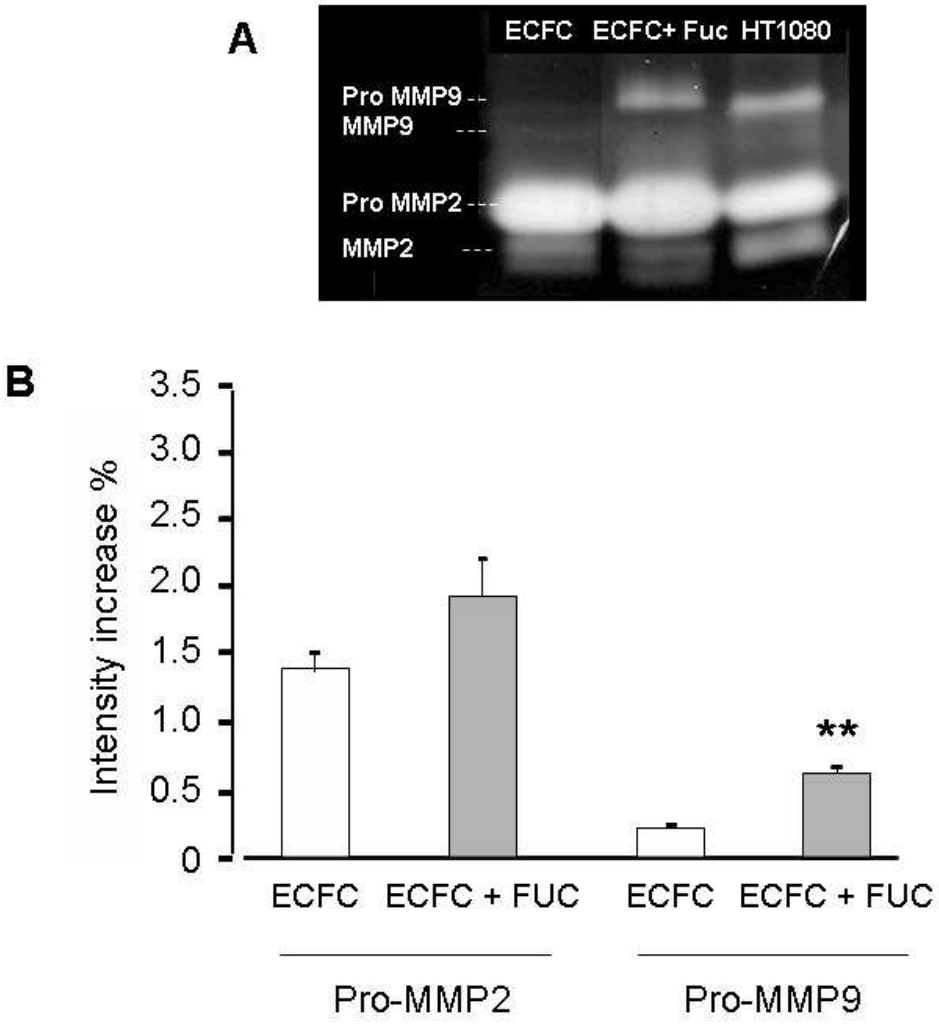

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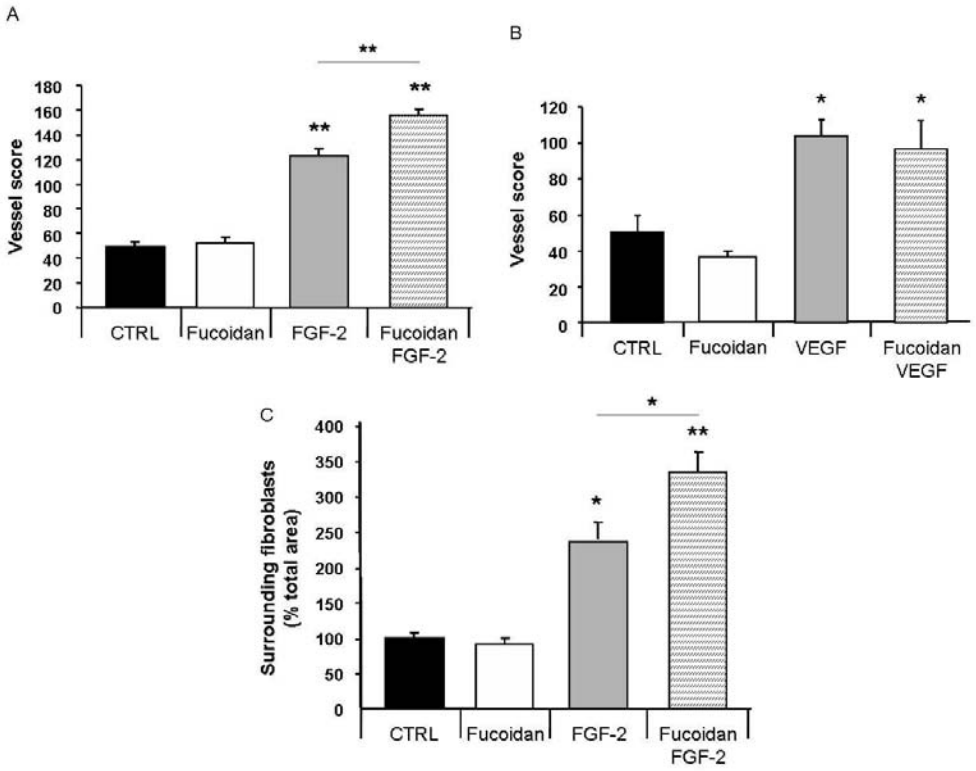

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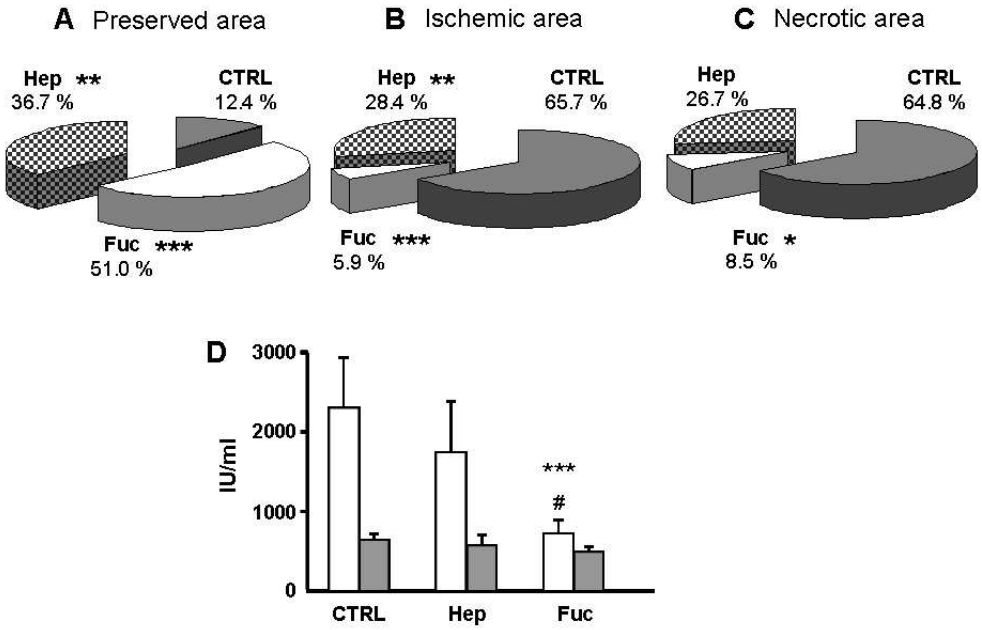

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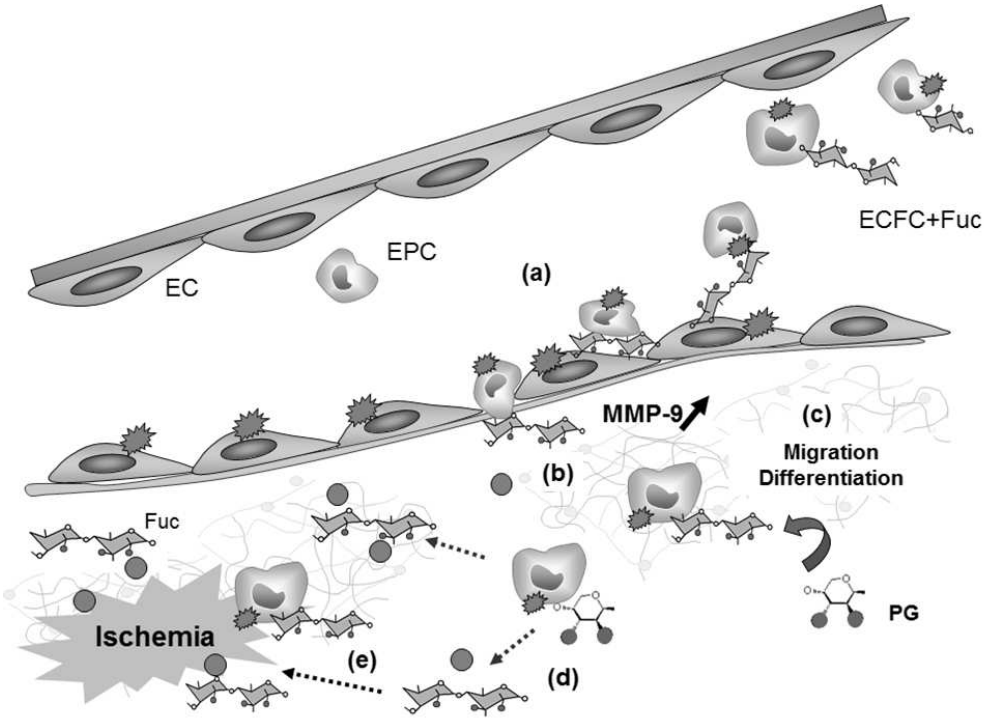

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255x185mm (96 x 96 DPI)

THERAPEUTIC EFFECT OF FUCOIDAN-STIMULATED ENDOTHELIAL COLONY-FORMING CELLS IN PERIPHERAL ISCHEMIA

Gabrielle Sarlon, **Faouzia Zemani**, Laurent David, Jean-Paul Duong Van Huyen, Blandine Dizier, Françoise Grelac, Sylvia Colliec-Jouault, Isabelle Galy-Fauroux, Patrick Bruneval, Anne-Marie Fischer, Joseph Emmerich and Catherine Boisson-Vidal.

Expanded methods and Methods

Materials

VEGF, FGF-2 were purchased from Valbiotech (Paris France). Calcein was purchased from Fluoroprobes. Growth factor-reduced (GFR) Matrigel were from Becton-Dickinson (Le Pont de Claix, France). Heparinase I (heparin lyase), heparinase III (HS lyase; EC 4.2.2.8) and chondroitinases ABC (chondroitinases ABC lyase; EC 4.2.2.4) were purchased from Sigma.

In vitro studies

Preparation and labelling of low-molecular-weight fucoidan.

Low-molecular-weight (LMW) fucoidan was provided by Therapol (France). It was obtained by radical depolymerization of high-molecular-weight fucoidan extracted from *Ascophyllum nodosum* using procedures adapted from Nardella et al. [*Nardella A et al. Carbohydr Res 1996,289:201-208*]. The molecular weight was 4 ± 1 kDa and the chemical composition was as follows: 34% fucose, 4% galactose, 3% xylose, 3% uronic acid and 4% sulphate. LMW fucoidan was labelled with 5-([4,6-dichlorotriazin-2-yl]-amino) fluorescein hypochloride (DTAF) as described by Ellouali et al. [*Ellouali M Anticancer Res 1993,13:2011-2020*].

Analysis of fluorescent fucoidan binding and internalization.

ECFC were incubated for 1 h at 4°C in HANKS buffer, 2% FCS, 0.5% BSA containing various concentrations of DTAF-fucoidan. After two washes in cold buffer the cells were resuspended in 500 µl of ice-cold buffer and analysed in a flow cytometer. Non specific binding was determined with a 100-fold excess of unlabelled fucoidan. Fucoidan internalization by ECFC was studied with DTAF-fucoidan as described by Ellouali et al *Ellouali M Anticancer Res 1993,13:2011-2020*]. Displacement assays were performed at 4°C as previously described [*Logeart D et al. Eur J Cell Biol. 1997,74:385-390]*.

Cell culture, characterization and pretreatment

Endothelial cells (HUVEC) and ECFC were isolated from respectively the human umbilical cords and from the human umbilical cord blood of healthy consenting mothers (n=20), by density-gradient centrifugation with Histopaque-1077 (Sigma-Aldrich; France) as previously described [*Zemani F et al. Biochem Pharmacol. 2005,70:1167-1175*]. The study was approved by local ethics committee of "Hôpital des Instructions et des Armées de Begin" (France – n°201008043234797) and protocol conformed to ethical guidelines of Declaration of Helsinki. The endothelial cell phenotype was shown by double positivity for DiI-Acetylated LDL uptake (6µg/ml; Molecular Probes) and BS-1 *Ulex europaeus* lectin binding (10µg/ml; Sigma-Aldrich). Further endothelial characterization was obtained by flow cytometry analysis (FACSCan flow cytometer, BD Biosciences) of combined expression of cell-surface antigens of the endothelial lineage, namely CD31, KDR, Tie-2, CD144, CD34 and Flt-1. One day before all experiments, cells were growth-arrested for 24h in EBM-2, 2% FCS and released from growth arrest by adding EBM-2, 5% FCS, with or without 10 µg/ml of fucoidan concentrations for 24h or 72h at 37°C, then washed, detached with versene/0.01% collagenase (1/1) and washed twice with buffered Hank's, 0.5% BSA before use and used in angiogenesis and adhesion assays. Supernatants and cells were separately analyzed after

fucoidan stimulation. All the following experiments were performed in triplicate during the first 30 days of culture.

Flow cytometry

Expression of cell-surface antigens on fucoidan-treated and untreated ECFC was analysed by measuring immunofluorescence with a FACSCan flow cytometer (BD Biosciences). Labelling with mouse anti-human monoclonal KDR (Sigma), Flt-1 (BD Biosciences), Tie-2 (Becton-Dickinson), VE-cadherin (CD144, Santa Cruz Biotechnology – Tebu-Bio), α5 (CD49, clone VC5, BD Biosciences), αL (CD11a, clone 38, Abcys), αM (CD11b, clone 44, Abcys), β2 (CD18, clone MEM 48, Abcys), and ICAM-1 (CD54, clone 15.2, Abcys) was visualised by using RPE-conjugated goat anti-mouse $F(ab')_2$ (Beckman Coulter). Anti-human α4 (CD49d, clone 9F10, BD Biosciences), α6 (CD49f, clone NKI-GoH3, Abcys), β4 (CD54 Pharmingen International) and VCAM (CD106, clone P3C4, Abcys), CD34 (Immunotech) and PECAM-1 (CD31, Immunotech) were used directly PE-conjugated. Mouse monoclonal antibodies against α2 (CD49b, clone AK-7, BD Biosciences) and β1 (CD29, clone 353, Valbiotech) were used directly FITC-conjugated. In each immunofluorescence experiment isotype-matched mouse IgG1 or IgG2 antibodies (from the same manufacturer as the immune antibodies) were used as a negative control and the fluorescence intensity of stained cells was gated according to established methods. Data were analysed with CellQuest™ software (BD Biosciences). Each analysis included at least 12,000 events of mononuclear gated cells.

Real-time (RT) polymerase chain reaction

Total RNA was extracted either directly with the RNABle® technique (Eurobio GEXEXTOO-OW) or following pre-treatment with fucoidan (10µg/ml) for 4 and 24 hours. Total RNA (250ng/µl) was reverse-transcribed. Transcripts of the TBP gene, an endogenous RNA control coding for the TATA box-binding protein, were quantified and target gene

expression was normalized on the basis of its TBP content [*Bieche I et al. Cancer Res. 2001,61:1652-1658*]. PCR was performed with the SYBR® Green PCR Core Reagents kit (Perkin-Elmer Applied Biosystems), in duplicate for each data point. Primers for TBP and the target genes (sequences available on request) were chosen with the assistance of Oligo 5.0 software (National Biosciences, USA).

In vitro angiogenesis assays

Cell adhesion, transendothelial migration and matrigel tube formation were measured as previously described [*Zemani F Arterioscler Thromb Vasc Biol. 2008,28:644-650*]. Gelatin zymography of culture medium conditioned by fucoidan-treated and untreated ECFC was performed as described elsewhere [*Senni K et al. Arch Biochem Biophys. 2006,445:56-64]*. HT1080 cell conditioned medium was used as standard. This established cell line constitutively releases proMMP-2 (72kDa) and proMMP-9 (92kDa) into the culture medium. Analysis was performed on culture supernatants by semi-automated image analysis expressed as grey level x surface of lysis band for 1000 cells and referred to gelatinolytic activities of HT1080 media.

Legends for supplementary figures

Figure I : Binding and internalization of fluorescent DTAF-fucoidan in ECFC. (**A**) Time course of DTAF-fucoidan binding to ECFC membrane realized at 4°C. **(B)** Fixation and internalization of DTAF-fucoidan in ECFC after 1h of incubation at 37°C observed by fluorescent microscopy. **(C)** Displacement experiments of ECFC cell-bound DTAF-fucoidan (DTAF Fuc) by 100-fold excess of unlabelled fucoidan and unlabelled-heparin. Values are expressed as means ± SEM (n=15). *p<0.05, **p<0.01, ***p<0.001 vs CTRL. Scale bar = 30 µm.

Figure I : available online