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Fucoidan ingestion increases the expression of CXCR4 on human CD34$^+$ cells

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Objective. **Transplantation of hematopoietic progenitor stem cells (HPC) is an important treatment modality for a variety of neoplastic diseases. HPC collection for transplantation with granulocyte colony-stimulating factor may be unsuccessful in patients who have received prior chemotherapy or for other reasons. Methods to improve mobilization of HPCs are required. Disruption of the interaction between the cell surface receptor CXCR4 and its ligand stromal derived factor-1 (SDF-1) is a mechanism for HPC release from the bone marrow into the peripheral blood (PB).**

Methods. **We carried out a clinical trial to evaluate the effects of ingestion of a fucoidan, galactofucan sulfate (a putative HPC mobilizing agent) on circulating CD34$^+$ cells, CXCR4 expression, and levels of SDF-1, interferon gamma (IFN-γ) and interleukin 12.**

Results. **Following ingestion of fucoidan, CD34$^+$ cells increased significantly in the PB from 1.64 to 1.84 cells/μL after 4 days. The proportion of CD34$^+$ cells that expressed CXCR4 increased from 45 to 90% after 12 days, the plasma level of SDF-1 increased from 1978 to 2010 pg/mL, and IFN-γ level increased from 9.04 to 9.89 pg/mL.**

Conclusion. **Oral fucoidan significantly amplified the CXCR4$^+$ HPC population. The ability to mobilize HPC using sulfated polysaccharides and mobilize more HPC with high levels of CXCR4 could be clinically valuable.** © **2007 International Society for Experimental Hematology. Published by Elsevier Inc.**

Autologous transplants of hematopoietic progenitor stem cells (HPC) are used to treat a variety of neoplastic and other diseases. HPC can be mobilized from the bone marrow (BM) niche into the peripheral blood (PB) via the administration of granulocyte colony-stimulating factor (G-CSF) or other agents such as AMD3100 or fucoidan. The mechanisms for the mobilization may include modulation of serine proteases, metalloproteases, and of stromal derived factor-1 (SDF-1)-CXCR4 interactions [1].

The success of subsequent engraftment may be associated with the expression of the receptor CXCR4 on CD34$^+$ HPC. This effect can be observed both clinically [2] and experimentally [3,4]. Mobilization of adequate amounts of HPCs including CD34$^+$ CXCR4$^+$ is not always successful. Failure to achieve sufficient mobilization can occur in patients who have received multiple cycles of chemotherapy or for other, unknown reasons.

Previous research has shown that intravenous (IV) fucoidan has a pronounced and extended mobilizing effect on HPC in mice and on nonhuman primates [5–8]. This effect is postulated to be the result of dissociation of the chemokine SDF-1 from the BM stroma, creating an attractive gradient into the peripheral circulation. Disruption of the interaction between the CXCR4 and its ligand SDF-1 is one of the mobilizing mechanisms also common to G-CSF [9] and newer agents such as AMD3100 [10,11].

Fucoidan is a generic term for the sulfated, fucose-rich polysaccharides derived from brown macroalgae [12] or echinoderms [13]. In animal models, ingestion of fucoidan has inhibitory effects on tumors, which appear to be associated with a rise in interferon-gamma (IFN-γ), interleukin-12 (IL-12), and stimulation of innate immunity [14–17]. In vitro treatment of BM mononuclear cells (MNCs) with IFN-γ can upregulate the expression of CXCR4 granulocyte precursors and monocytes [17].

Despite the available literature evidence that IV fucoidan had an effect in tumor animal models and mobilization of HPC, there are no reports to date about the clinical use of oral fucoidan to modulate or mobilize HPC. Although

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fucoidans synergize with G-CSF to increase mobilization 11 times over G-CSF alone in primates, to date no clinical trials in patients have been reported. In this study, we examined the effects of orally ingested *Undaria pinnatifida* fucoidan on the PB stem cells, the expression of CXCR4, and plasma levels of SDF-1, IL-12, and IFN-γ.

Materials and methods

Human volunteers

In a single-blind, randomized, placebo-controlled clinical trial, 37 nonsmoker volunteers of either sex were divided into three groups after giving informed consent and after human ethics approval was obtained from the Southern Tasmania Health & Medical Human Research Ethics Committee. As placebos, six volunteers took 3 g of guar gum. Another six volunteers took 3 g of whole *Undaria* containing 10% w/w fucoidan, and another 25 volunteers took 3 g of 75% w/w fucoidan daily for 12 days. All volunteers took three capsules (0.33 g each) three times a day. During the study time, volunteers were asked not to eat any seafood, seaweed-derived products, drugs, or food supplements. Blood samples were collected as described later.

Preparation of capsules

Fucoidan is a highly sulfated, polyanionic soluble fiber derived from Tasmanian *Undaria pinnatifida* by Marinova Pty. Ltd. (Hobart, TAS Australia). We chose a neutral nonsulfated dietary fiber derived from guar gum as a placebo (Novartis Pty. Ltd., Mulgrave, VIC Australia). The capsules were prepared as described previously [15]. The structure and the therapeutic characteristics of these compounds have been described previously [12,14,15].

Collection of blood samples

Venous blood from the antecubital vein from the three groups of volunteers was collected using ethylenediamine-tetraacetic acid (EDTA) tubes. Plasma samples (platelet poor for SDF-1 assay) were collected and stored in aliquots at −80°C within 30 minutes of collection for later analysis. Complete blood counts were obtained using an automated cell counter (CELL-DYN-4000 System, Abbott Lab, IL, USA).

Flow cytometry analysis

The expression of different surface membrane markers on normal human PB HPCs was evaluated by fluorescein-activated cell sorting (FACS) direct immunofluorescence. Cells were Fc-blocked with 1 μg of human immunoglobulin G (IgG)/10^5 cells (Zymed Lab., San Francisco, CA, USA) for 15 minutes at room temperature, and then stained with the designated monoclonal antibody. FITC-CD34, Cy5-CD45 (Becton Dickinson, San Jose, CA, USA) and PE-CXCR-4 (R&D Systems Inc., Minneapolis, MN, USA) were used in the study. A negative control tube was prepared identically but contained isotype controls IgG_1, or IgG_{2a} antibodies (BD). FACS-fixed cells were analyzed using FACScan flow cytometer and the Cell Quest software package (BD).

Preparation of MNCs and colony-forming unit granulocyte assays

Human PB MNCs were isolated from healthy subjects using Histopaque-1077 kit and protocol from Sigma-Aldrich Co. (St. Louis, MO, USA). Briefly, 3 mL of EDTA blood was layered onto the Histopaque-1077 and centrifuged at 400*g* for 30 minutes. The opaque interface was mixed with 10 mL isotonic phosphate-buffered saline (PBS) then centrifuged at 250*g* for 10 minutes. The cell pellet was washed with 5 mL PBS twice and resuspended in 0.3 mL PBS. Peripheral blood mononuclear cells were plated at densities of 1×10^5 viable nucleated cells per 35-mm plate using growth medium (MethoCult GFH4534, StemCell Technologies, Vancouver, BC, Canada), then colony-forming unit granulocyte (CFU-GM) was performed. Petri dishes were incubated for 14 days at 37°C in a humidified atmosphere containing 5% CO_2. CFU-GM colonies defined as clusters of ≥30 cells were then counted using an inverted microscope and recorded as the mean of quadruplicate counts. Plates were neglected if contaminated or >50 colonies were counted.

Plasma cytokines and cytokine assays

PB was collected from volunteers according to the designated schedule in tubes containing EDTA. Platelet-poor plasma was prepared within 30 minutes of blood collection and stored at −80°C for later analysis. Three different cytokines, SDF-1, IFN-γ, and IL-12 levels were analyzed directly after thawing plasma samples gradually on the day of test by enzyme-linked immunosorbent assay using kits and protocols from R&D Systems Inc.

Statistical analysis

Student's *t*-test and analysis of variance were used to analyze data. A *p* value of 0.05 was chosen as the limit of statistical significance. Triplicate readings for each sample were averaged. All other statistical parameters were calculated using Microsoft Office, Excel and SPSS, version 12.

Results

No side effects were reported, and none of the volunteers exhibited toxicity when 3 g of guar gum, 10% fucoidan, or 75% fucoidan extracts were taken orally three times a day for 12 days.

Fucoidan ingestion caused mild leukopenia and lymphopenia but had no effect on neutrophils.

We observed a nonsignificant decrease in the total number of leukocytes in the PB when 10% fucoidan was ingested, but when 75% fucoidan was ingested, the decrease was significant after 12 days (Table 1). There was a decrease in leukocytes from 5.74 cells/nL at baseline to 5.37 after 12 days ($p = 0.05$). Of the leukocyte fractions, lymphocytes were most affected. Ingestion of either 10% or 75% fucoidan decreased the lymphocyte count but the decrease was only significant with the 75% fraction. The absolute number of lymphocytes decreased from 2.18 cells/nL at baseline to 1.98 after 12 days ($p = 0.03$; Table 1). Neutrophil count was not affected after ingesting guar gum or 10% or 75% fucoidan. Furthermore, there was no effect on the expression of CD16 in the $CD45^+$ population (results not shown).

Table 1. Average readings of all of the tests at four time points

Treatment[§]	Test	0 days*	4 days	8 days	12 days	n
Guar gum	Leukocyte (cells/nL)	7.37 ± 0.23	7.38 ± 0.61	7.03 ± 0.87	7.22 ± 0.71	6
	Lymphocyte (cells/nL)	2.80 ± 0.13	2.82 ± 0.16	2.65 ± 0.15	2.83 ± 0.18	6
	Neutrophil (cells/nL)	3.63 ± 0.44	3.52 ± 0.38	3.45 ± 0.32	3.43 ± 0.26	6
	SDF-1 (pg/mL)	1977 ± 11	1978 ± 8	1981 ± 2	1974 ± 16	3
	IFN-γ (pg/mL)	9.03 ± 0.18	9.14 ± 0.15	9.2 ± 0.15	9.17 ± 0.08	3
10% fucoidan	Leukocyte (cells/nL)	7.48 ± 0.35	6.98 ± 0.29[†]	7.00 ± 0.51	6.95 ± 0.59	6
	Lymphocyte (cells/nL)	2.87 ± 0.12	2.73 ± 0.16	2.6 ± 0.30	2.62 ± 0.26	6
	Neutrophil (cells/nL)	3.73 ± 0.28	3.42 ± 0.18	3.42 ± 0.24	3.35 ± 0.31	6
	SDF-1 (pg/mL)	1973 ± 8	1977 ± 5	1980 ± 25	1979 ± 15	3
	IFN-γ (pg/mL)	9.01 ± 0.13	9.01 ± 0.38	9.05 ± 0.19	8.97 ± 0.27	3
75% fucoidan	Leukocyte (cells/nL)	5.74 ± 0.28	5.62 ± 0.33	5.48 ± 0.30	5.37 ± 0.37[†]	25
	Lymphocyte (cells/nL)	2.18 ± 0.14	2.06 ± 0.13	1.95 ± 0.14[‡]	1.98 ± 0.14[†]	25
	Neutrophil (cells/nL)	2.94 ± 0.19	2.97 ± 0.22	2.90 ± 0.19	2.82 ± 0.22	25
	SDF-1 (pg/mL)	1978 ± 26	1996 ± 31	2101 ± 33[‡]	2059 ± 47	23
	IFN-γ (pg/mL)	9.04 ± 0.42	9.41 ± 0.49	9.89 ± 0.39[‡]	9.82 ± 0.57[†]	20

Table shows the mean readings observed on baseline (0 day) and on 4th, 8th and 12th day after ingesting 3 g of each treatment. Guar gum is used as a placebo control. All values are average ± mean standard error. n = number of volunteers, different volunteers have been used for each treatment.

[§]Volunteers ingested 3 g of each treatment three times daily.

*The mean value at 0 day (baseline) was used in the *t*-test as first set of data to which other groups are compared.

[†]$p < 0.05$ using paired Student's *t*-test.

[‡]$p < 0.01$ using paired Student's *t*-test.

Increase in CD34$^+$ cell count in PB after fucoidan ingestion

A slight increase in the circulating CD34$^+$ was observed after ingesting fucoidan. When 10% fucoidan (3 g/d) was ingested, a nonsignificant increase in CD34$^+$ in PB, from 1.07 to 1.29 cells/μL ($p = 0.06$, n = 6) after 12 days, was observed. However, when 75% fucoidan was ingested, the CD34$^+$ count increased from 1.64 cells/μL to 1.84, 1.80, and 1.79 cells/μL at 4, 8, and 12 days, respectively. This increase was significant at day 4 ($p = 0.04$). Some volunteers presented a large increase in the CD34$^+$ on days 8 and 12 but were considered by the statistical program as outliers and were not included in the calculated median.

Increase in the expression of CXCR4 on CD34$^+$ cells after fucoidan ingestion

When 3 g/d of 10% fucoidan was ingested, a nonsignificant increase in the CD34$^+$CXCR4$^+$ was observed. However, when 3 g/d of the 75% fucoidan was ingested, the CD34$^+$CXCR4$^+$ count increased significantly ($p < 0.0002$) from 0.75 cells/μL at baseline to 1.65 cells/μL after 12 days (Fig. 1). The proportion of CD34$^+$CXCR4$^+$ increased from 45 to 90% after 12 days of treatment (Table 2). A few volunteers showed a large increase in the CD34$^+$ cell count but were considered by the statistical program as outliers and were not included in the calculated median.

Fucoidan ingestion has no effect on PB MNCs in CFU-GM

Generally, there was a nonsignificant decrease in the number of CFU-GM per microliter of l blood after ingesting the 75% fucoidan. The mean CFU-GM count at baseline was 1.87/μL (±mean standard error = 0.29) and decreased to 1.71 (±0.26), 1.4 (±0.29), and 1.31 (±0.35) after 4, 8, and 12 days ($p = 0.50, 0.19, 0.14$; n = 13) of ingesting 75% fucoidan, respectively.

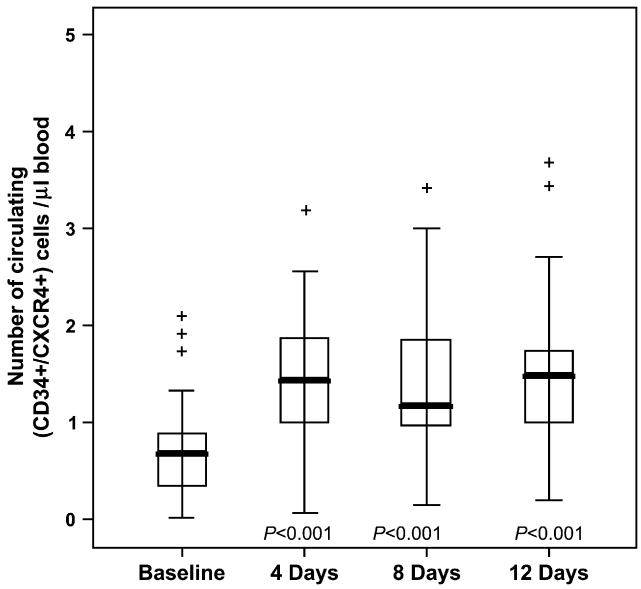

Figure 1. Total number of PB CD34$^+$/CXCR4$^+$ cells at baseline and after 4, 8, and 12 days of taking 75% fucoidan. Black lines represent medians for 23 duplicate experiments representing 23 volunteers. The boxes represent the median (50% of the population), and the error bars represent the mean standard error. +, outliers or high responders.

Table 2. Average percentage of cells that are $CXCR4^+$ out of the total $CD34^+$ cells at four time points

Treatment*	0 days†	4 days	8 days	12 days	n
Guar gum	62.33 ± 4.64	64.15 ± 2.81	66.43 ± 3.18	63.66 ± 3.09	6
10% fucoidan	67.27 ± 8.33	65.49 ± 9.13	71.78 ± 9.01	71.96 ± 13.09	6
75% fucoidan	49.75 ± 7.19	84.57 ± 4.74‡	84.4 ± 5.57‡	91.15 ± 3.63‡	23

The table shows the average readings observed on baseline (0 day) and on 4, 8, and 12 days after ingesting 3 g of each treatment. Guar gum is used as a placebo control. Values shown are average % of $CD34^+/CXCR4^+$ ± mean standard error. n = number of volunteers; different volunteers have been used for each treatment.

*Volunteers ingested 3 g of each treatment three times daily.

†The mean value at 0 days (baseline) was used in the *t*-test as first set of data.

‡$p < 0.01$ using paired Student's *t*-test.

Increase in plasma levels of SDF-1 and IFN-γ with fucoidan ingestion

Ingestion of guar gum and 10% fucoidan did not affect the levels of SDF-1 and IFN- γ in plasma (Table 1). Volunteers who ingested 3 g of the 75% fucoidan had an elevation in the plasma level of SDF-1 after 8 days from 1978 to 2101 pg/mL ($p = 0.00005$).

Further, the plasma level of IFN-γ was assessed because this cytokine has been associated with the upregulation of CXCR4. Volunteers who ingested 3 g of the 75% fucoidan showed a significant elevation ($p = 0.04$) in the plasma IFN-γ, from 9.04 to 9.82 pg/mL (Table 1). However, there was no detectable change in the IL-12 plasma (results not shown).

Discussion

We carried out this clinical study to determine the effects of ingested *Undaria*-derived fucoidan on PB. We found that there was a small increase in $CD34^+$ cells and a profound increase, from 40 to 90%, in the proportion of $CD34^+/CXCR4^+$ when 75% fucoidan was ingested. A smaller increase was noted when 10% fucoidan was ingested. We also observed a significant increase in IFN-γ and SDF-1 in the 75% fucoidan group but not in the control or 10% groups. We did not observe an increase, but rather a nonsignificant decrease in CFU-GM, despite the increase in the $CD34^+$ cell count. This slight increase after oral intake contrasts with the large and sustained response elicited by IV fucoidan [5–8], AMD3100 [10], or G-CSF [2].

Previous studies have shown that IV fucoidan produces rapid mobilization of murine HPCs with long-term BM repopulating potential in mice and nonhuman primates [6–9]. CXCR4 was not assessed in those studies. Fucoidan has also been used as a tool to examine the effects of binding SDF-1. Mavier and colleagues [16] demonstrated that after experimental hepatic destruction, IV fucoidan blocked the SDF-1 expression of liver stem cells and markedly decreased their accumulation. Ingestion of fucoidan has been shown to inhibit tumors of various kinds [17], an effect that may be attributable to a stimulation of the nonspecific immune system [14,18]. Fucoidans are well-known experimental selectin blockers. In vitro, the binding of L-selectin on lymphocytes by fucoidan enhanced the expression of CXCR4 in lymphocytes [19]. Clinical use of a fucoidan preparation was made in the 1960 s. Claudio and Stendardo [20] reported favorable results from patients with leukopenia and leukocytosis with increases in the general condition of patients.

In a prior work, two preparations of fucoidan to mobilize HPCs have been used; 100 mg/kg IV of sulfated linear fucan from the sea urchin *Lytechinus variegatus* [6,8] and 25 mg/kg intraperitoneally of branched fucoidan fraction from *Ascophyllum nodosum* (from Fluka) [5]. Despite the differences in fucoidans, similar mobilizations were seen and attributed to the creation of SDF-1 gradient into the PB.

The fucoidan used in our studies is derived from *Undaria pinnatifida*. Our previous work using an antibody-based detection method indicated that when 3 g of 75% fucoidan was ingested daily, plasma concentration was elevated up to 4 mg/L after 4 days, and then 13 mg/L after 12 days despite the fact that fucoidan is a large-molecular-weight material [15]. Acidic conditions in the stomach may cause a limited hydrolysis of the fucoidan. Humans do not produce enzymes capable of breaking down fucoidans, and the latter also appear to be unaffected by human fecal flora [21]. We hypothesized that small quantities of fucoidan may cross the intestinal wall as whole molecules probably by endocytosis. The slight but significant changes in HPCs shown here do not indicate stand-alone utility of this substance as a clinical entity but instead indicate a need for further investigation of its potential. Further work is needed to investigate the repopulating potential of the cells, and the timing of peak values for CXCR4 and total numbers of cells.

AMD3100, which is a reversible inhibitor of the binding of SDF-1α to its cognate receptor CXCR4, is currently in clinical trials as a mobilizing agent. It significantly improves the mobilization capacity of G-CSF when used in combination with it in mice [22]. We postulate that fucoidan may have a similar mechanism of action on SDF-1/CXCR4 that could play a role in the mobilization of $CD34^+$ cells from BM to PB especially if fucoidan is used IV.

The increase in $CD34^+CXCR4^+$ cells was marked in this study, although the increase in the $CD34^+$ cells was small. Although we observed no significant change in SDF-1 levels at 4 days, when CD34 levels increased, SDF-1 levels were increased by day 12. Either the rise in SDF-1 is not correlated with the enhanced $CD34^+$ cell numbers or CXCR4 expression, or the effect is only at the BM level at the 4 day stage.

Previously, CXCR4 expression on HPCs has been shown to increase after administration of G-CSF within both human and murine BM, reaching peak levels at the time of mobilization [9], although SDF-1 levels did not rise. Interestingly, we observed a small decrease in CFU-GM over 12 days in the 75% fucoidan group. This effect may perhaps be attributed to the rise in IFN-γ. Constitutive expression of low levels of IFN-γ by stromal cells has been noted to have a profound inhibitory effect on hematopoiesis [23].

CXCR4 plays an important role in regulating the trafficking of HPCs and their homing/retention in BM, and it modulates several biologic processes in more differentiated cells [24].

In this study, a small, significant decrease in leukocytes and lymphocytes was observed after 12 days of ingesting the 75% fucoidan, although this decrease was within normal clinical range. In previous studies, there was a fall in circulating leukocytes immediately after G-CSF was given [25–27].

The presence of normal number of functional neutrophils is important for mobilization of HPCs [28,29]. In this study, we have demonstrated that 3 g of oral fucoidan has no effect on neutrophil count and does not cause neutropenia.

We observed that there was an increase in the plasma level of IFN-γ, consistent with a previous study that showed an increase in HPCs accompanied by an increase in the level of SDF-1, IFN-γ, and IL-12 [8]. The level of IL-12 in our study did not change (results not shown). It was shown previously that in vitro treatment of BM MNCs with IFN-γ can upregulate the expression of CXCR4 on granulocyte precursors and monocytes [30]. This may, in part, reflect our observation of increased expression of CXCR4 on $CD34^+$ cells. It could be more relevant to look at the BM cytokine levels as well and compare the changes between PB and BM levels especially as PB SDF-1 may originate from the BM pool.

In summary, oral administration of fucoidan significantly amplified the $CXCR4^+$ HPC population. The ability to mobilize HPCs with high levels of CXCR4 expression could be clinically valuable. However, the effect of IV fucoidan in humans remains to be determined.

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