Author's response to reviews

Title: Fucoidan present in brown algae induces apoptosis of HT-29 human colon cancer cells

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Author's response to reviews: see over
Dear Dr. Shipley

Thank you for reviewing the manuscript referenced above. We have conducted additional experiments using HCT116 human colon cancer cells to validate our results and have revised the manuscript in accordance with the reviewers’ recommendations. Detailed responses to each of the specific points raised by the reviewers are outlined below. We have also had the paper copyedited by a professional copyediting service in order to improve the style of written English. All these changes have been highlighted in yellow throughout the text and in this letter. For your information, the reviewers’ comments are typed in Arial font and our responses are typed in Comic Sans MS.

Reviewer: Suguru Fukahori

<Major Compulsory Revisions>

1. Materials
(p8, line3-4) The reagents employed in this study were purchased from the indicated suppliers: fucoidan. The author did not explain the details of fucoidan employed in this study, so should explain how fucoidan is prepared from the seaweed and the details of major components of fucoidan such as L-fucose and sulfate content if known as the previous literature showed that the proportion of these components in the fucoidan plays a great role in the anti-tumor effect.

We have added the following sentence with a reference to explain the details of the fucoidan employed herein (page 9, line 13):

The fucoidan (Sigma) was prepared from Fucus vesiculosus via a modified version of the method described by Black et al. [27] and a crude polysaccharide composed predominantly (> 95%) of sulfated fucose.

2. Discussion
(p16, line 21-22, p17, line 6-9)

The author noted that fucoidan induced apoptosis in HT-29 cells at low concentration, so should add descriptions how the mechanism of apoptosis in
this study differ from previous reports in terms of the components of fucoidan. Since this study and the study of Hyun et al. used the same kind of fucoidan, the difference in results from the two studies cannot be attributed to any difference in the components of fucoidan. We conducted additional experiments using HCT116 human colon cancer cells under conditions identical to those used for HT-29 cells, and noted that the degree of apoptosis-inducing responses to fucoidan was lower in the HCT116 cells than in the HT-29 cells (Figures 2C and 2D, Figures 2E and 2F). Together, these results indicate that the degree of response to fucoidan varies among these colon cancer cell lines. We have added the following sentences (page 17, line 8):

We also noted that fucoidan induced the apoptosis of HCT116 cells. However, the degree of response to fucoidan was smaller in HCT116 cells than in HT-29 cells. Hyun et al. [41] previously reported that high concentrations of fucoidan (100 µg/mL) induced apoptosis in HCT-15 cells. These observations suggest that fucoidan induces apoptosis in human colon cancer cells, but that the efficacy of fucoidan in inducing apoptosis varies among different types of colon cancer cells.

<Discretionary Revisions>

3. In results, all obtained data is from only HT-29 human colon cancer cells except Cell culture and cell viability assay. To increase a reliability of remaining studies' data, the author should obtain the data from FHC human normal colon epithelial cells, too if it is physically possible.

→ We have conducted additional experiments to increase the reliability of the present data using HCT116 human colon cancer cells and FHC cells. The results have been added (Figures 1B, 2D, 2F, and 2G) and the following sentences have been added in the Results section (page 12, line 7; page 13, line 4; page 13, line 9).

Fucoidan also inhibited the growth of HCT116 cells. However, the degree of inhibition was smaller in HCT116 cells than was noted with the HT-29 cells. The treatment of HCT116 cells with 20 µg/mL of fucoidan for 72 h resulted in a 36.7 ± 2.0% reduction in the viable cell numbers (Figure 1B).

In HCT116 cells, the proportions of apoptotic cells were increased significantly by
treatment with 10 µg/mL of fucoidan. However, the proportions of apoptotic cell numbers were lower in HCT116 cells than in HT-29 cells (Figure 2D).

Furthermore, fucoidan treatment resulted in increases in the levels of cleaved PARP in both HT-29 (Figure 2E) and HCT116 cells (Figure 2F). Fucoidan exerted no detectable effects on PARP cleavage in FHC cells (Figure 2G).

<Minor Essential Revisions>
4. Cell culture and cell viability assay
   (p8, line10) …with various concentrations of fucoidan…………
   Exact concentrations should be described.
5. (p8, line22) …with various concentrations of fucoidan…………
   Same comment.
6. Flow cytometry measurement of mitochondrial membrane potential
   (p10, line5) …with various concentrations of fucoidan…………
   Same comment.
7. Western blot analysis
   (p10, line13-14) We cultured the cells in 100mm dishes and administered fucoidan to them, as described above.
   Same comment.
→ We have provided the exact concentrations of fucoidan in the Methods section (page 9, line 11; page 10, line 10; page 10, line 20; page 11, line 6).

Reviewer: Hee-Kyoung Kang
<Minor Essential Revisions>
1. The present study seems to be similar to the previous report (Hyun et al.,2009) except cell line. The differences and advanced points of yours study compared to the previous study need to be mentioned.
→ We have added and revised the following sentences to explain the differences and advanced issues in the present study from the previous report by Hyun et al. (page 18, line 1).

Additionally, Hyun et al. [41] reported that 100 µg/mL of fucoidan induced apoptosis in HCT-15 cells via the activation of caspase-9 and -3 accompanied by changes in Bcl-2 and Bax, as well as changes in the phosphorylation of ERK, p38 kinase, and Akt. In this study, we noted that fucoidan at a concentration of 5 - 20 µg/mL 1) increased the
activation of caspases, 2) reduced the protein levels of IAPs, 3) increased mitochondrial membrane permeability and cytochrome c and Smac/Diablo release, 4) increased the levels of Bak and t-Bid but reduced the levels of Mcl-1, and 5) increased the levels of Fas, DR5, and TRAIL in HT-29 human colon cancer cells. We also noted that the inhibitors of caspase-8 and caspase-9 reduced fucoidan-induced apoptosis. The results of this study show that fucoidan induces apoptosis through the activation of caspases via both death receptor-mediated and mitochondria-mediated apoptotic pathways.

2. In Hyun’s study, fucoidan at 100 µg/mL induces the apoptosis of HCT-15, whereas 5-20 µg/mL fucoidan can induce the apoptosis of HT-29 in this study. HT-29 and HCT-15 are same human colorectal carcinoma cells. In regard to apoptosis induction, what can exhibit the concentration difference between the two cells? Please comment that in the discussion

→ We have added the following sentences to explain the difference between the two cells in the Discussion sections (page 17, line 8).

We also noted that fucoidan induced the apoptosis of HCT116 cells. However, the degree of response to fucoidan was smaller in HCT116 cells than in HT-29 cells. Hyun et al. [41] previously reported that high concentrations of fucoidan (100 µg/mL) induced apoptosis in HCT-15 cells. These observations suggest that fucoidan induces apoptosis in human colon cancer cells, but that the efficacy of fucoidan in inducing apoptosis varies among different types of colon cancer cells. The potent in vitro efficacy of fucoidan in colon cancer cells indicates that fucoidan may potentially prove useful in the prevention of colon carcinoma. However, it remains to be determined whether or not fucoidan suppresses the development of colon cancer in both animal cancer models and humans. Additionally, it will also be necessary to determine why the degree of response to fucoidan varies among different types of colon cancer cells.

Reviewer: Arun Rishi
A minor concern is an apparent discrepancy in the data in figures 1A and 2C. In figure 1A, treatment with 20 microgram per ml dose of fucoidan results in 25% viable cells while in figure 2C the histogram indicates 62% live cells following similar treatment. Authors need to clarify this discrepancy in the results section.

→ In Figure 1, viable cell numbers were estimated by the MTT assay and in Figure 2C, the living cells were estimated by counting Annexin V-negative cells. We and other investigators have frequently observed the differences
between the data from the two methods. The degree of changes was consistently greater with MTT assay (mitochondrial succinate dehydrogenase activity) than Annexin-V staining (phosphatidylserine at the outer leaflet of plasma membrane (Kim et al., J Med Food 12: 943-951, 2009; Kim et al., Food Chem Toxicol 46: 3651-3658, 2008; Guan et al., Acta Pharmacol 28: 1984-1990, 2007; Jung et al., J Nutr Biochem 17: 689-696, 2006; Kim et al., Am J Physiol Gastrointest Liver Physiol 283: G357-G367, 2002). The results in Figure 1 reflect changes in cell numbers owing to changes in proliferation and cell death occurring during the 3-day period. However, the results in Figure 2C are reflective of the proportion of living cells and early apoptotic cells present at 72 h after the addition of fucoidan. The description of results in Figure 2C has been revised. It now reads as follows (page 12, line 23).

In HT-29 cells, the proportions of apoptotic, Annexin V-positive/7-AAD-negative cells increased in a time-dependent manner in cells that had been treated with 20 µg/mL of fucoidan (Figure 2B). Additionally, a concentration-dependent increase in the proportions of apoptotic cells was noted after the cells were treated for 72 h with increasing concentrations of fucoidan (Figure 2C).

<Quality of written English>
Needs some language corrections before being published
→ We have edited the paper by a professional copyediting service to improve the style of written English.

Reviewer: Nancy Turner

<Major compulsory revisions>
1. The use of serum-deprivation medium in order to stabilize cells in the cell cycle is completely understandable. However, the authors must describe why they continued to use serum-deprivation medium during the treatment phase of the experiment. This alone will severely limit cell growth and promote apoptosis. How are you able to establish and compare the effect that fucoidan would have in vivo, in a non-limiting condition, to the effects reported here?
→ We were also concerned that cell growth might be severely limited and apoptosis would be promoted in serum-deprivation medium. However, in preliminary studies, we noted that HT-29 and HCT116 cells grow similarly in
We employed the serum deprivation medium containing 10 mL/L of charcoal-stripped FBS in order to minimize the possible effects of various growth factors and phytochemicals present in the FBS.

2. Hopefully, the final figures will be of higher quality than those included for review. As they are currently presented it is very hard to read the text and numbers.
→ We apologize for the fact that our figures were so low-resolution. We have replaced the figures with new ones with improved resolution.

<Minor essential revisions>
1. The protein levels of survivin do not decrease in a dose-dependent manner (p13, l 13-14).
→ We have revised the sentences as follows (page 13, line 23). They read as follows:

The levels of XIAP protein were reduced significantly by treatment with increasing concentrations of fucoidan. Additionally, fucoidan at a concentration of 10 µg/mL effectively reduced the levels of survivin protein (Figure 3B).

2. According to the author’s citations, this isn’t the first paper documenting the effect of fucoidan on colon cancer cell lines and newer references for in vivo data, and there is information concerning its effects on colitis. As such, they need to remove their assertion that it is the first throughout the paper.
→ We have removed the assertion that this study is the first throughout the paper.

3. What are the differences in the data shown in figure 2, panel C for living cells
and the data in figure 1?

In Figure 1, viable cell numbers were estimated by the MTT assay and in Figure 2C, the living cells were estimated by counting Annexin V-negative cells. We and other investigators have frequently observed the differences between the data from the two methods. The degree of changes was consistently greater with the MTT assay (mitochondrial succinate dehydrogenase activity) than with Annexin-V staining (phosphatidylserine at the outer leaflet of plasma membrane (Kim et al., J Med Food 12: 943-951, 2009; Kim et al., Food Chem Toxicol 46: 3651-3658, 2008; Guan et al., Acta Pharmacol 28: 1984-1990, 2007; Jung et al., J Nutr Biochem 17: 689-696, 2006; Kim et al., Am J Physiol Gastrointest Liver Physiol 283: G357-G367, 2002). The results in Figure 1 reflect changes in cell numbers due to changes in proliferation and cell death during the 3-day period. However, the results in Figure 2C reflect the proportion of living cells and early apoptotic cells present at 72 h after the addition of fucoidan. The description of results in Figure 2C has been revised. It now reads as follows (page 12, line 23):

In HT-29 cells, the proportions of apoptotic, Annexin V-positive/7-AAD-negative cells increased in a time-dependent manner in cells that had been treated with 20 µg/mL of fucoidan (Figure 2B). Additionally, a concentration-dependent increase in the proportions of apoptotic cells was noted after the cells were treated for 72 h with increasing concentrations of fucoidan (Figure 2C).

<Discretionary revisions>
Specific comments:
Page Line
5 3 It is the second leading cause of cancer death when the data for both genders are combined, which is what the sentence suggests the authors are referring to.

→ Dr. Turner is correct. Colorectal cancer is the third leading cause of cancer-related mortality in males as well as in females. However, this cancer is the second most frequent cause of cancer-related mortality when the data for both genders are combined. Therefore, we have changed ‘third’ to ‘second’ in the Background section.
I am not sure what the authors mean by “reverse progression to invasive cancer”.

→ We have revised this sentence as follows (page 5, line 11).

Recently, the development of cancer chemoprevention protocols employing natural or synthetic agents for the prevention or suppression of progression to invasive cancer has been recognized as a field with enormous potential to reduce cancer burden [5].

You need to indicate the FBS was charcoal-stripped.

→ We have changed the ‘FBS’ to ‘charcoal-stripped FBS’ (page 9, line 9).

Instead of saying “as described above” throughout the paper, give us the specifics of the treatment regimen for each purpose.

→ We have added the specifics of the treatment regimen rather than saying “as described above” throughout the paper (page 9, line 22; page 10, line 7; page 10, line 17; page 11, line 3).

You need to indicate the concentration of fucoidan used, time of administration, etc.

→ We have indicated the concentration of fucoidan used and the time of treatment throughout the paper.

This is true, except for caspase 3, in which 1 is set at 5 µg/ml.

→ We have revised the legend for Figure 3 in accordance with your comment. It now reads as follows (page 32, line 12):

The relative abundance of each band to its own β-actin was quantified, and the control levels (0 µg/mL fucoidan) were set to 1 except for caspase-3, for which 1 is set to 5 µg/mL of fucoidan.

We appreciate the thoughtful comments of the reviewers and hope that this manuscript will now be accepted for publication in BMC Gastroenterology. We look forward to hearing from you regarding its status.

Sincerely,
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