Immunomodulatory activity of fucoidan against aspirin-induced gastric mucosal damage in rats

Hanumantha Rao Balaji Raghavendran a,⁎,1, Periasamy Srinivasan b,1, Sathyanath Rekha a

a Liver and Immunology Research Center, Daejeon Oriental Hospital of Daejeon University, 22-5 Daehung-dong, Jung-gu, Daejeon, Republic of Korea 301-724
b Department of Environmental and Occupational Health, College of Medicine, National Cheng Kung University, Tainan, Taiwan

Article history:
Received 7 August 2010
Received in revised form 10 October 2010
Accepted 1 November 2010
Available online 26 November 2010

Keywords:
Fucoidan
Aspirin
Anti-inflammatory
Cyclooxygenase
Cytokines
Immunomodulatory

A R T I C L E   I N F O

A B S T R A C T

Gastric ulcers and related complications associated with the use of non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin, represent a major global health problem. In the present study, we investigate the immunological activity of fucoidan against aspirin-induced gastric mucosal damage in rats. Thirty-six rats were randomly divided into the following, normal (Carboxy methyl cellulose 0.05 %), aspirin (Asp—400 mg/kg) treated, fucoidan alone (Fu—0.02 g/kg, daily for 14 days) and Fu + Asp. Cytokines, total nitrite and nitrate (NOx) analysis and tissue localization of Cyclooxygenase 1, 2 and epidermal growth factor receptor (EGFR) were done using Elisa and immunohistochemistry respectively. Histopathology of gastric tissue, collagen deposition was performed using Hematoxylin and Eosin and Masson’s trichrome were performed. Treatment of rats with a single dose of aspirin (400 mg/kg, orally) led to significant alterations in the levels of total nitrite and nitrate (NOx), interleukins (IL-4, 6, 10, 12), tumor necrosis factor (TNF-α), and interferon gamma (IFN-γ). Notably, collagen deposition in glandular tissue and localization of cyclooxygenase 1, 2, and epidermal growth factor were considerably affected in aspirin-treated rats. These severities were prevented to a significant extent in rats pretreated with fucoidan (0.02 g/kg/day for two weeks orally). Our findings collectively indicate that the gastroprotective effect of fucoidan against aspirin-induced ulceration in rats is mediated through its immunomodulatory properties.

© 2010 Elsevier B.V. All rights reserved.

1. Introduction

The possible mechanisms through which non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin, represent a major global health problem. In the present study, we investigate the immunological activity of fucoidan against aspirin-induced gastric mucosal damage in rats. Thirty-six rats were randomly divided into the following, normal (Carboxy methyl cellulose 0.05 %), aspirin (Asp—400 mg/kg) treated, fucoidan alone (Fu—0.02 g/kg, daily for 14 days) and Fu + Asp. Cytokines, total nitrite and nitrate (NOx) analysis and tissue localization of Cyclooxygenase 1, 2 and epidermal growth factor receptor (EGFR) were done using Elisa and immunohistochemistry respectively. Histopathology of gastric tissue, collagen deposition was performed using Hematoxylin and Eosin and Masson’s trichrome were performed. Treatment of rats with a single dose of aspirin (400 mg/kg, orally) led to significant alterations in the levels of total nitrite and nitrate (NOx), interleukins (IL-4, 6, 10, 12), tumor necrosis factor (TNF-α), and interferon gamma (IFN-γ). Notably, collagen deposition in glandular tissue and localization of cyclooxygenase 1, 2, and epidermal growth factor were considerably affected in aspirin-treated rats. These severities were prevented to a significant extent in rats pretreated with fucoidan (0.02 g/kg/day for two weeks orally). Our findings collectively indicate that the gastroprotective effect of fucoidan against aspirin-induced ulceration in rats is mediated through its immunomodulatory properties.

© 2010 Elsevier B.V. All rights reserved.

2. Materials and methods

2.1. Drugs and chemicals

Fucoidan (Sigma Chemical Co., St. Louis, MO) subjected to Limulus amebocyte lysate (LAL) assay and the levels of endotoxin were less than 10 EU/mg. A polysaccharide composed predominantly of sulfated fucose. Aspirin and all other chemicals were procured from Sigma Chemical Co., St. Louis, MO. For ELISA, the capture and detection antibodies for cytokines were acquired from e-Biosciences, San Diego, USA. Primary and biotinylated secondary antibodies for cyclooxygenase (COX-1 and 2) and epidermal growth factor receptor (EGFR), employed for immunohistochemical analysis, were obtained from Cell Signaling Technology Inc., Beverly, USA.
2.2. Experimental animals

The study was conducted using Albino rats of the Wistar strain weighing 130–160 g (Animal Feeding Center of our institute). All rats were housed (six per cage) in clear polycarbonate cages in the animal care facility, and fed a standard animal diet and water ad libitum under controlled temperature conditions with 12 h light–dark cycles. Animals received proper care, in accordance with methods approved under institutional guidelines, and experiments were conducted in keeping with the principles specified in the ‘Animal Care Act’.

2.3. Animal grouping

Rats were randomly divided into four groups. Group I rats (Normal) received carboxymethyl cellulose (0.05%) as the vehicle. After 24 h fasting, Group II (Asp) rats were administered an oral dose of aspirin (400 mg/kg body weight) on day 14. Group III (Fu) alone and IV (Fu + Asp) rats were pretreated with fucoidan (0.02 g/kg body weight, p.o.) for two weeks daily, and Group IV alone was post-treated with aspirin (400 mg/kg body wt suspended in 0.05% carboxymethyl cellulose) following overnight fasting. After 8 h of aspirin treatment, rats were anesthetized under mild ether, and sacrificed via cervical decapitation. Blood samples collected from experimental animals without anticoagulant were centrifuged at 3500×g for 10 min to obtain clear serum.

2.4. Cytokine assay

Gastric tissue samples were homogenized with a Physcotron NS-310E mechanical micro homogenizer (Niti-on, Tokyo, Japan) in 1 ml of Phosphate Buffer Saline containing complete protease inhibitor cocktail (Roche, Indianapolis, IN, USA). The homogenate was centrifuged for 30 min at 20 000 rpm (4 °C) in a Hitachi Hmac CF 1502 centrifuge (Hitachi High-Technologies, Tokyo, Japan) and the supernatant was used for the determination of cytokines. The levels of cytokines (IL-4, 6, 10, 12, IFN-α and TNF-α) were evaluated using the e-Biosciences ELISA kit reagent, according to the manufacturer's instructions. A standard curve was run on each assay plate using recombinants of the respective cytokines in serial dilutions.

2.5. Determination of total nitrite and nitrate (NOx) concentrations

Protein-free serum samples were used for total nitrite and nitrate determination. An aliquot (100 μl) of supernatant was applied to a microtiter plate well, and 100 μl vanadium (III) chloride (8 mg/ml) added to each well (for reduction of nitrate to nitrite), followed by the Griess reagents, 50 μl sulfanilamide (2%) and 50 μl N-(1-Naphthyl) ethylenediamine dihydrochloride (0.1%). After 30 min of incubation at 37 °C, absorbance was read at 540 nm using the ELISA reader. A linear standard curve was generated using 0–200 μmol/l sodium nitrite.

2.6. Histopathology findings

Stomach tissue samples were fixed in 10% neutral buffered formalin, dehydrated, and embedded in paraffin. Samples were subsequently sectioned at 5 μm thickness, and stained with H&E. Unstained sections were subjected to Masson’s trichrome staining for collagen deposition and immunohistochemical analysis. Stomach tissue samples were homogenized with a Physcotron NS-310E mechanical micro homogenizer in 1 ml of 4°C. The homogenate was centrifuged at 3500×g for 10 min to obtain clear serum.

2.7. Immunohistochemistry (IHC)

To establish the immunolocalization of COX 1, 2 and EGF (antigen), a mouse polyclonal antibody (Cell signaling technology, USA) was used at a working dilution of 1:20. The antibody was applied directly to sections, and slides were incubated overnight at 4 °C in a humidified chamber. Immune complexes were subsequently treated with the secondary antibody (containing anti-rabbit and anti-mouse immunoglobulins) and detected via application of streptavidin peroxidase treatment for 20 min at room temperature. After rinsing sections with three changes of PBS, immunoreactivity was visualized with diamine benzidine (DAB)-hydrogen peroxide (20 min). Sections were gently rinsed in distilled water, counterstained with H&E, and photomicrographs taken under a microscope (Olympus Optical Co., Tokyo, Japan).

2.8. Statistical analysis

All results are expressed as means ± standard deviations for 6 rats in each group. Statistical analyses were processed according to conventional procedures using the Statistical Program of Social Sciences (SPSS) software for Windows, Version 12.0 (Productivity, LSD, One-way ANOVA). A P value < 0.05 was considered statistically significant.

3. Results

3.1. Effects of fucoidan on cytokine and NOx levels in aspirin-induced ulcer

Aspirin (400 mg/kg body weight) induced lesions in stomach tissue, characterized by glandular erosions and blood spots in rugae (macroscopic observations). Rats pretreated with fucoidan did not display blood spots in the rugae of gastric tissue, but some glandular erosion was evident. Notably, aspirin stimulated significant elevation of the pro-inflammatory cytokines, IL-6 and IL-12 (Fig. 1a). Additionally, TNF-α and IFN-γ levels were relatively higher than normal, following aspirin treatment (Fig. 1b). These changes were prevented upon pretreatment with fucoidan, with the exception of aspirin-induced increase in IL-6. Levels of the anti-inflammatory cytokines, IL-10 and IL-4, as well as total nitrite and nitrate (Fig. 1d) were additionally affected in rats treated with aspirin. Interestingly, pretreatment with fucoidan significantly attenuated the aspirin-promoted decline in NOx and IL-10 levels, while the decrease in IL-4 was relatively unaffected (Fig. 1c).

3.2. Histopathological changes

Histopathological examination revealed that aspirin-induced severe degenerative changes in the stomach tissue, characterized by gastric pit damage and vacuolization of the glandular portion, particularly in mucus-secreting cells (Fig. 2a and b). Pretreatment with fucoidan considerably attenuated, but did not completely prevent the severity of these histopathological changes, and some erosion in sub-glandular and epithelial necks was evident (Fig. 2c and d).

3.3. Deposition of collagen in glandular tissue

Collagen deposition in the glandular connective septa is considered an index of gastric damage. Accordingly, we determined the intensity of Mason's trichrome dye staining in stomach tissue. Ulcerated rats displayed intense positive staining for Mason's trichrome dye (arrows) around the glandular region (Fig. 3a and b). Conversely, the intensity of dye staining in fucoidan-treated samples was considerably reduced (Fig. 3c and d).

3.4. Immunolocalization of COX 1, 2 and epidermal growth factor in gastric tissue

Immunohistochemical analysis was performed to ascertain the localization of COX 1, 2 and EGFR in gastric mucosa tissue. Gastric mucosal tissue of rats treated with aspirin did not display significant immunoreactivity for COX-1, 2 or EGFR (Fig. 4a–c, Fig. 5a–c and Fig. 6a–c). In contrast, the group of rats pretreated with fucoidan presented partial COX 1 and 2-like immunoreactivity around the glandular regions, mucoid cells and neck gland cells of gastric tissue.
Fig. 1. Effects of fucoidan on cytokines and NO\textsubscript{x} levels in aspirin-induced ulcer. (a) Interleukin 6 and 12; (b) Tumor necrosis factor alpha (TNF-\(\alpha\)) and Interferon gamma (IFN-\(\gamma\)); (c) Interleukin 10 and 4; (d) NO\textsubscript{x} (Micromole). Aspirin-induced significant alterations in the pro-inflammatory cytokines and NO\textsubscript{x}, were suppressed by fucoidan.

Fig. 2. Histopathological changes in aspirin-induced stomach ulcer tissue (a and b), with severe degenerative changes in glandular region, epithelial folds, lamina propria, proventriculus and connective septa (100× and 200× magnifications). A representative photograph (c and d) of fucoidan and aspirin challenge (400 mg/kg, p.o.) display some changes around the epithelial folds and connective septa (100× magnification).
Fig. 3. Deposition of collagen in glandular tissue. Aspirin-induced rats (a and b) tissue display intense staining of Masson's trichrome (positive stain for collagen deposition) around the connective septa and muscularis region of the stomach (100× and 40× magnifications). Fucoidan + aspirin (c and d) treated rat tissue display a pale staining of Masson's trichrome dye (positive stain for collagen deposition) in the connective septa and muscularis region of stomach (100× and 40× magnifications).

Fig. 4. Immunolocalization of COX-1, in gastric tissue. Photomicrographs a, b and c display weak COX-1 immunoreactivity in sub-glandular region of aspirin-induced rats (100× and 40× magnifications). Photomicrographs d, e and f display scanty and mild COX-1 immunoreactivity around the glandular region and epithelial folds of fucoidan + aspirin-treated stomach tissue (40× and 100× magnifications).
Fig. 5. Immunolocalization of COX-2, in gastric tissue. Photomicrographs a, b and c show a vague COX-2 immunoreactivity in sub-glandular region of aspirin-treated rats (100× and 40× magnifications). Photomicrographs d, e and f—partial display of COX-2 immunoreactivity in the glandular region and epithelial necks of stomach tissue of rats those received fucoidan + aspirin. (40× and 100× magnifications).

Fig. 6. Immunolocalization of epidermal growth factor receptor (EGFR) in ulcer untreated and fucoidan-treated tissue. Photomicrographs a, b and c—EGFR very weak positive signal in glandular region of aspirin-induced ulcerated tissue (100× and 40× magnifications) and d, e and f displays a significant EGFR immunoreactivity around oxyntic cells of stomach tissue of rats administered with fucoidan + aspirin (40× and 100× magnifications).
induction of endogenous IL-10. We propose that the apparent ability inhibiting the production of pro-inflammatory cytokines through T-cell activation, specifically modulator of mucosal secretive function in response to aspirin-induced stress. However, rats treated with fucoidan and aspirin displayed moderate COX-1 immunoreactivity signals in the glandular regions and necks of epithelial folds.

In gastric mucosa of the ulcer margin, epithelial cells form a characteristic “recovery zone”. Dilated gastric glands undergo dedifferentiation and express epidermal growth factor receptor (EGFR), a key growth factor involved in repair mechanisms [36,37]. Aspirin-treated rats display a weak positive EGF immunoreactive signal, confirming that the epidermal growth factors produced locally activate re-epithelialization and migration of epithelial cells from the ulcer margin to restore mucosal epithelial continuity. Conversely, rats treated with fucoidan show significant EGFR immunoreactivity around the submucosal connective tissue. These findings collectively indicate that fucoidan improves the resistance of gastric tissue against aspirin-induced ulcer scars to accelerate the healing process.

In conclusion, while our results provide a limited explanation of the comprehensive mechanisms of fucoidan action in ulcer protection, this study presents a constructive platform for further research.

References


