Features of fascin expression in the small intestine of rats exposed to processed Eucheuma seaweed

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Abstract
This study focuses on the assessment of fascin expression in the small intestinal tissue of rats orally administered a food additive E407a (processed Eucheuma seaweed), which is widely used to improve the texture of food products. The issue of its safety is under debate nowadays. Small intestinal expression of fascin, an actin-bundling cytoskeletal protein involved in the formation of filopodia and microspikes, was evaluated immunohistochemically in 9 rats exposed to 140 mg of E407a per kg of weight daily during two weeks and 8 control animals. Fascin was found to be upregulated both in the lamina propria and epithelia of the small intestine in rats administered processed Eucheuma seaweed compared with the control group. Thus, oral consumption of E407a is associated with overexpression of fascin in the small intestine of rats.

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Introduction

Carrageenans (CGNs) are hydrophilic, sulfated polysaccharides composed of galactose derivatives linked with α-1,4 and β-1,3-glycosidic bonds [1]. CGNs are known as food additives E407 (food-grade or refined) and E407a (semi-refined CGN also known as processed Eucheuma seaweed (PES)). Compared to PES, refined CGN contains small amounts of acid insoluble matter (AIM, primarily algal cellulose), while the content of AIM in E407a varies from 8% to 15% [2]. CGNs have found extensive application in food industry, since they show gelling, stabilizing, protein-suspending, emulsifying and thickening properties [3]. CGNs are categorized into several types, including λ, κ, τ, ε, μ, depending on the sulfation degree, which can reach 35% [4].

The share of CGN market is the fourth largest among hydrocolloid additives and approximately 70,000 tones of this food additive is produced annually [2]. In addition to its role of a food additive, CGNs are added to meat as a cheap non-meat ingredient. This is considered an illegal food fraud [5].

According to different estimates, the daily intake of CGNs in Western diet may reach up to 7.7 g and it has been increasing since the appearance of the first data in 1970s [6,7]. However, the average consumption of CGN in industrialized countries is estimated to reach 250 mg per day [8,9].

According to numerous studies, CGN consumption poses serious health-related risks [4,10]. Food additives E407 and E407a have been demonstrated to promote intestinal inflammation in laboratory animals [4, 10-14]. Furthermore, CGNs have been shown to induce generation of reactive oxygen species (ROS) and pro-inflammatory cytokines, including in colonocytes [15-18]. However, the findings outlined above are challenged and refuted by the studies in which it is stated that CGNs are non-toxic compounds, which have neither ROS-generating nor pro-inflammatory properties [19,20]. Such multidirectional conclusions in evaluating the toxicity of CGNs may be due to confusion in terms, since polysaccharides with different structures and molecular weights may be referred to as CGNs in research papers: food-grade CGN (200-800 kDa), degraded CGN (20-40 kDa), and poligeenan (10-20 kDa). Food-grade CGN is officially recognized safe by major international regulatory authorities, including the Food and Drug Administration, while degraded CGN and poligeenan are prohibited to be used in food industry due to their well characterized toxic effects [19].

To add some insight to the mechanisms by which food-grade CGN-containing food additives may promote intestinal inflammation, we evaluated the influence of PES on the expression of fascin protein. Fascin is a cytoskeletal protein, which is responsible for the formation of cell protrusions, filopodia, and microspikes providing cell motility. Its expression is reported to be limited to cells of mesenchymal origin, and this protein is not expressed in epithelial cells under normal circumstances [21]. There is strong evidence that fascin may induce epithelial-mesenchymal transition (EMT), which is a process of losing epithelial features by cells and gaining properties of mesenchymal cells, inter alia, the ability to move [22, 23]. It is important to emphasize that EMT plays a significant role in intestinal inflammation [24], making promising the assessment of fascin expression for evaluating the impact of E407a on the intestine.

The aim of this study was to evaluate features of fascin expression in the small intestine of rats orally exposed to a common food additive E407a during two weeks.

Material and methods

1. Study design

WAG rats weighing 160-190 g were used in this study. Group 1 consisted of 9 animals orally administered a water solution of E407a containing 140 mg of PES per kg of weight on a daily basis during a fortnight. Group 2 served as control and included 8 intact rats. The rats were maintained in standard laboratory conditions. Access to food was provided ad libitum. The animals of both groups were sacrificed with the subsequent collection of fragments of small intestine for immunostaining.

This study was approved by the local Ethics and Bioethics Committee at Kharkiv National Medical University (Kharkiv, Ukraine).

All the experimental procedures were carried out following the guidelines of EU Directive 2010/63/EU on the protection of animals used for scientific purposes, which is based on the Council of Europe Convection for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS123).

2. Immunostaining

Tissue samples were washed with ice-cold saline after their collection. They were fixed in formalin solution for 24 hours. Then samples were dehydrated in alcohol of ascending grades and cleared in xylene. Four-μm-thick sections from paraffin-embedded small intestine samples were immunostained using mouse monoclonal antibodies to fascin. Ultra Vision Quanto Detection System HPR DAB Chromogen manufactured by Thermo Fischer Scientific (UK) was used visualization.

According to the staining protocol, the microslides were incubated with the primary anti-fascin antibodies and secondary horseradish peroxidase-conjugated antibodies. The appearance of brown coloration was considered a marker of positive staining, since 3,3′-diaminobenzidine (DAB) was used for visualization.

Results

We evaluated the expression of fascin in the small intestinal mucosa of a total of nine rats consumed a common food additive PES whose major ingredient is kappa-carrageenan during a fortnight and eight control animals. In the control samples, fascin expression was limited to the lamina propria. The small intestinal epithelia of rats not exposed to PES showed no signs of fascin staining (Figure 1).

Figure 1 - Representative microphotograph of fascin staining in the small intestine of a rat from the control group. Fascin expression is not found in the epithelial cells, while moderate staining in observed in the lamina propria. 100x.
However, fascin-positive cells, including presumably fibroblasts, some types of leukocytes and endothelia of stromal microvasculature, were observed in the lamina propria of intact small intestine (Figure 1). Thus, fascin immunostaining was detected in cells of mesenchymal origin in the control group and was absent in apparently normal epithelial cells.

The pattern of fascin expression in the small intestine of rats administered E407a differed from samples of the control animals. Some areas of epithelia demonstrated no fascin staining. In other regions, staining varied from low to high. Moderate fascin staining was mainly found in the epithelial lining, which was not observed in controls (Figures 2, 3). It is worth emphasizing that fascin expression was stronger in areas with the damaged villi in comparison with the regions less affected by the inflammation. Fragments of desquamated villous epithelia with fascin overexpression could be even noticed in the intestinal lumen (Figure 3). In the intestinal stroma, a significant increase in fascin immunoreactivity was determined in animals after exposure to E407a (Figures 2, 3). The intense positivity was observed in regions where villous epithelial lining was damaged.

**Discussion**

In several studies it has been shown that fascin is virtually absent in the intestinal epithelial lining in normal conditions [25,26]. This fact was corroborated by our findings. However, inflammatory processes in the intestine result in upregulation of this protein. It is of huge importance to mention that fascin overexpression in inflammatory bowel disease (IBD) is associated with regions of intense repair and regeneration, since fascin is involved in repairing gaps in the damaged epithelial barrier [25]. The pattern of fascin expression observed in this study confirms this hypothesis, since its extremely strong expression was found in the damaged areas with the desquamated epithelia located in the lumen of the small intestine. Thus, fascin upregulation in the epithelial cells of rats exposed to E407a can be considered a response to the CGN-induced tissue damage.

It is believed that fascin overexpression is a subject to an inflammation-mediated regulation. It has been reported that inflammation-related cytokines such as tumor necrosis factor-alpha (TNF-α), IL-1β and transforming growth factor-beta (TGF-β) may upregulate fascin [27, 28]. Thus, it can be assumed that E407a-induced tissue damage and destruction resulted in the development of inflammation and subsequent overexpression of fascin in the most damaged areas due to the pro-inflammatory microenvironment.

Furthermore, strong expression of fascin in rats exposed to PES may promote EMT, since fascin is known to be involved in EMT and fascin knockdown suppresses this process in experiments [23]. It is worth noting that after exposure to E407a some intestinal epithelial cells lose the ability to produce E-cadherin and start expressing vimentin (unpublished data), which is typical for EMT. In particular, these key events in EMT, namely loss of E-cadherin, a cell-cell adhesion molecule that provides normal connection of cells in the epithelial layer, and expression of vimentin, an intermediate filament protein involved in cell motility, are mediated by Snail1, a member of Snail transcription factors [29]. Fascin is known to be actin-bundling protein required for the formation of actin-based cell protrusions. It is important to mention that fascin is overexpressed in various tumors providing their motility and invasion [21, 30]. Fascin expression in epithelial cells changes significantly their morphology, including the appearance of lamellipodia and induction of an increase amount of microvilli on the apical surface of cells. Such fascin-induced morphological alterations provide epithelial cells with the ability to move [31, 32]. Thus, our findings of fascin upregulation observed primarily in the regions of damaged epithelia under the influence of CGN-containing food additive E407a indicate that its overexpression may have reparative functions. In agreement with previous studies, we can assume that the fascin-expressing epithelial cells after the intake of E407a acquire the ability to move and may cover areas of deep epithelialized villi to try to maintain the integrity of epithelial barrier and prevent the flow of luminal bacteria deeper in the intestinal mucosal layer.

We believe that a higher amount of fascin-expressing cells in the stroma of small intestine collected from E407a-consuming animals can be due to an increase in the number of motile fibroblasts and dendritic cells that can express this protein [33]. To some degree, EMT may contribute to an increase in the number of fibroblasts in rats exposed to E407a, since this process has been identified to provide fibroblasts involved in the emergence of intestinal fibrosis in experimental models of IBD in mice. In particular, Flier et al reported that approximately...
30% of fibroblasts in 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced experimental colitis are former epitheliocytes undergone EMT [34]. This hypothesis was supported by other studies indicating that EMT can be considered one of the major contributors to the formation of activated fibroblasts [35,36].

Our findings of fascin overexpression both in the epithelial layer and stroma of the small intestine of rats exposed to E407a suggest that CGN may upregulate fascin. However, it is now clear how the induction occurs. We can speculate that fascin overexpression develops in response to CGN-induced intestinal inflammation.

Conclusion

Oral administration of a κ-carrageenan-containing food additive E407a during two weeks results in damage to the small intestine and the appearance of compensatory fascin expression in small intestinal epithelial cells to repair the regions affected and fascin upregulation in the lamina propria.

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