Fish & Shellfish Immunology 27 (2009) 65–72

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Decreased expression of TGF-β, GILT and T-cell markers in the early stages of soybean enteropathy in Atlantic salmon (*Salmo salar* L.)

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ARTICLE INFO

Article history:
Received 23 January 2009
Received in revised form
28 April 2009
Accepted 28 April 2009
Available online 7 May 2009

Keywords:
Interferon-γ-inducible lysosomal thiol reductase (GILT)
Intestine
Intraepithelial lymphocytes (IEL)
Nutrition
qPCR
CD3
CD8
Transforming growth factor β (TGF-β)

ABSTRACT

This study investigated the early expression of T-cell markers and genes potentially involved in the induction of soybean meal (SBM) enteropathy in the distal intestine (DI) of Atlantic salmon (*Salmo salar* L.). Quantitative PCR was used to study the expression of CD3, CD8β, transforming growth factor β (TGF-β), interferon-γ-inducible lysosomal thiol reductase (GILT) and interleukin-1β (IL-1β) in salmon fed SBM for 1, 3 and 7 days using fish fed fishmeal as controls. In the same tissue, the morphological development of SBM enteropathy was evaluated by routine histology and the presence of T cells was mapped by immunohistochemistry. TGF-β was significantly down-regulated on all days of feeding SBM. GILT was significantly down-regulated on days 3 and 7 compared to day 1. A depression in the expression of T-cell markers was observed on day 3 whereas increased densities of T cells were observed at the base of mucosal folds after 7 days of feeding SBM. Down-regulation of GILT and TGF-β may lead to sensitization of intraepithelial lymphocytes and failure to maintain normal mucosal integrity in the DI. These responses are implicated in the pathogenesis of SBM enteropathy in Atlantic salmon.

1. Introduction

Soy products have long been considered potential substitutes for fish meal (FM) in formulated diets for farmed fish due to their low price, predictable availability and nutritional properties [1]. However, the utilization of soy in diets for salmonids has been limited due to adverse effects on the gastro-intestinal tract [2–4]. Full-fat and defatted (extracted) soybean meal (SBM) induce inflammation, described as sub-acute enteritis, in the distal intestine (DI) exhibiting the following changes: (1) reduced mucosal fold height; (2) loss of normal enterocyte supranuclear absorptive vacuolization; (3) widening of the central stroma within the mucosal folds, with increased amounts of connective tissue; and (4) profound infiltration of inflammatory cells in the lamina propria and submucosa [2,5]. Histological changes have been detected in the DI as early as 2 days after exposure to dietary SBM and after 7 days all characteristics of the enteritis were present [5]. This indicates that the components in SBM responsible for inducing the enteritis exert their effects at an early stage.

In a recent study, mRNA expression of the T-cell markers CD3, CD4 and CD8 were up-regulated in Atlantic salmon (*Salmo salar* L.) fed SBM for 3 weeks [6]. Immunohistochemistry using an antibody against human CD3ε, part of the T-cell receptor (TCR) complex, suggested the presence of putative T cells in the epithelial lining, lamina propria and submucosa of the DI, and that these cells made up a large proportion of the inflammatory cell infiltrate observed in salmon fed SBM. These observations, coupled with the relatively low population of IgM^+ cells in the cell infiltrate [7], indicate that the enteropathy may be T-cell mediated.

Along with the histological changes observed in the DI, increased activities of trypsin have been shown in both the luminal contents and within the wall of the DI [4,8,9]. Trypsin, a serine protease, has also been found to be involved in the pathogenesis of

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doi:10.1016/j.fsi.2009.04.007

inflammatory conditions in the intestine of humans and mammals via protease-activated receptors (PAR). This mechanism is responsible for the induction of inflammation [10], possibly also in salmon with SBM-induced enteropathy [11].

Other signalling substances may be of significance in the modulation of the inflammatory response. Gene expression studies are an important tool in these investigations due to the lack of antibodies available developed specifically against salmon immune factors. Comparative studies on experimental colitis in mice and intestinal enteropathies in humans have identified transforming growth factor β (TGF-β) as an important factor in the control of intestinal immune homeostasis and prevention of mucosal inflammation [12]. TGF-β1 counters T-cell cytokine mediated disruption of the intestinal barrier [13], and thus its involvement during the induction of the SBM-induced enteropathy merits investigation.

Also of interest is interferon-γ-inducible lysosomal thiol reductase (GILT) (also known as IP30 or IFNIP30), which has been reported to suppress the activation of T cells and diminish the magnitude of the T-cell receptor engagement in mice [14]. Furthermore, the induction of GILT may be stimulated by cytokines such as IL-1β [15].

The present study investigated the early stages of SBM-induced enteropathy by characterizing the expression of T-cell markers and the above-mentioned immune-relevant genes that may modulate a T-cell mediated response. In addition, verification of the specificity of the anti-human CD3ε antibody (Dako A0452) to recombinant salmon CD3ε peptide is presented.

2. Materials and methods

2.1. Fish, diets and sampling

Farmed Atlantic salmon (Salmobreed strain, mean weight 216 g) were stocked in two fibreglass tanks (1 m^3; $n = 20$ fish per tank) containing running seawater (salinity 32–34 g L^{-1}) at 8–10 °C under 24 h light. Fish were fed a fishmeal (FM) based diet for a 27-day acclimation period after which time fish in one of the tanks were switched to a diet containing 46% extracted SBM (Table 1). Fish were sampled after 1, 3 and 7 days' of exposure to the SBM diet. Fish maintained on the FM diet during the experimental period served as controls. The experiment was performed at Nofima Marine's (formerly AKVAFORSK – The Institute of Aquaculture Research) research station in Sunndalsøra (Norway).

On each day of sampling fish were randomly selected, anaesthetized with tricaine methanesulphonate (MS-222), weighed, measured and killed with a sharp blow to the head followed by abdominal evisceration. Only fish that had digesta throughout the intestinal tract were sampled to ensure intestinal exposure to SBM as well as to avoid results caused by anorexia. The intestines were cleaned of all fatty tissue and intestinal content prior to collection of tissue samples from the distal intestine (DI, defined as the intestinal region from the increase in intestinal diameter and appearance of transverse luminal folds to the anus). For extraction and quantification of RNA approximately 300 mg of the DI were collected from 2 fish fed FM on days 1, 3 and 7 (i.e. 6 in total), and 6 fish fed SBM on each of the same days (18 in total). Samples were stored in RNAlater® (Ambion Inc., Austin, TX, USA) at −20 °C until further analysis. For histology and immunohistochemistry samples of the DI (approx. 5 mm × 5 mm) from the same SBM-fed individuals (18 in total) and from 6 FM-fed fish (4 in addition to the 2 sampled for RNA) on days 1, 3 and 7 (18 in total), were collected and placed in 10% buffered formalin for approximately 24 h and subsequently transferred to 70% ethanol for storage prior to processing.

Table 1
Formulation and composition of the diets.

	Fishmeal (FM) diet	Soybean meal (SBM) diet
Formulation (g kg^{-1})		
Fm[a]	794.6	321.6
SBM[b]	0	463
Fish oil	87.2	109
Starch	111.1	100
Vitamin and mineral mixture	7.02	6.29
Dry matter (g kg^{-1})	924	914
Chemical composition (g kg^{-1} dry matter)		
Crude protein	629.8	465.6
Lipid	142.5	158.5
Starch	110.3	116
Dietary fibre	29.4	110.4
Ash	131.9	81.6

[a] NorsECO, egersund Sildeoljefabrikk AS, Egersund, Norway.
[b] Deno-soy F®, hexane extracted and toasted soybean meal with hull, Denofa, Fredrikstad, Norway.

2.2. Cloning of TGF-β

Based on a TGF-β sequence from rainbow trout (*Oncorhynchus mykiss*) available in GenBank (Accession number AJ007836) primers were designed to amplify Atlantic salmon TGF-β (forward primer GTG GAC AGA TAC TGA GCA AGC; reverse primer AAC AAT CAT ATT GGA CAA CTG CT). The resulting PCR product was ligated into a TOPO TA vector (Invitrogen Ltd, Paisley, UK), cloned and sequenced. A 954 bp sequence was obtained and has been submitted to GenBank with accession number EU082211. The Atlantic salmon TGF-β showed 90% similarity to the rainbow trout sequence at the amino acid level. On the basis of the attained sequence, primers for real-time PCR were designed (Table 2).

2.3. RNA extraction of DI samples

RNA was extracted using Trizol® Reagent (Invitrogen Ltd) according to the manufacturer's instructions and the concentrations measured using the NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The 2100 Bioanalyzer in combination with an RNA Nano Chip (Agilent Technologies, Inc., Santa Clara, CA, USA) was used to evaluate the RNA integrity.

2.4. DNase treatment

Prior to reverse transcription, total RNA from all samples were subjected to DNase treatment using the TURBO DNA-freeTM kit (Ambion Inc., Austin, USA) in accordance with the manufacturer's recommendations.

2.5. First-strand cDNA synthesis

From 0.8 μg total RNA, first-strand cDNA from each sample was synthesized in duplicates using Superscript III (Invitrogen Ltd) and Oligo$(dT)_{20}$ primers (Invitrogen Ltd) in accordance with the manufacturer's instructions.

2.6. Relative quantification

Real-time PCR amplifications were performed to examine the relative expression of selected genes (Table 2) using the LightCycler 2.0 (Roche Diagnostics, Mannheim, Germany). PCR reactions were run in duplicates using the LightCycler FastStart DNA MasterPLUS SYBR GREEN I kit (Roche Diagnostics) and a total volume of 10 μl comprising 4.5 μl PCR-grade water, 0.5 μl of each PCR primer (10 μM), 2 μl master mix and 2.5 μl template.