Award Number: W81XWH-11-1-0656

TITLE: Investigation of the Role of Stress in Inflammatory Bowel Disease Using Zebrafish as an Experimental Model

PRINCIPAL INVESTIGATOR: Dr. Efi Kokkotou

CONTRACTING ORGANIZATION: Beth Israel Deaconess Medical Center, Inc., Boston, MA 02215

REPORT DATE: August 2012

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.
Investigation of the Role of Stress in Inflammatory Bowel Disease Using Zebrafish as an Experimental Model

Dr. Efi Kokkotou
Beth Israel Deaconess Medical Center, Inc.
Boston, MA 02215

W81XWH-11-1-0656

1 Aug 2011 - 31 Jul 2012

Final

Inflammatory bowel disease (IBD) is a chronic debilitating condition affecting 1.4 million Americans, young individuals in particular. Psychological stress and environmental factors such as diet, toxins and the microbiome contribute to disease precipitation and worsen its symptoms. War Veterans appear to be disproportionately affected by these factors. The medical management of IBD remains unsatisfactory and more than half of patients require some type of surgical intervention during their lifetime. The aim of our study was to establish and validate a zebrafish model of IBD, to be used for the development of novel treatments. The advantages of this model include its small size, housing requirements, short gestational period and easy genetic manipulations. We were able to induce chemical enterocolitis in adult zebrafish by intrarectal administration of TNBS. The following similarities with mouse experimental colitis and human IBD were identified: histological hallmarks including disruption of crypt architecture, infiltration by immune cells and mucosal edema; molecular mediators of inflammation such as TNFalpha, IL-1beta, IL-8 and IL-10; microbiome dependence; and disease alleviation in response to 5-aminosalicylic acid treatment. We will use this newly developed to accelerate progress in IBD therapeutics by focusing on evolutionary conserved pathways.

Zebrafish, colitis, inflammatory bowel disease, animal models, intestinal inflammation
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>4</td>
</tr>
<tr>
<td>Body</td>
<td>4</td>
</tr>
<tr>
<td>Key Research Accomplishments</td>
<td>11</td>
</tr>
<tr>
<td>Reportable Outcomes</td>
<td>11</td>
</tr>
<tr>
<td>Conclusion</td>
<td>12</td>
</tr>
<tr>
<td>References</td>
<td>12</td>
</tr>
<tr>
<td>Appendices</td>
<td></td>
</tr>
</tbody>
</table>
INTRODUCTION

It is estimated that more than 1.4 million patients in the US alone suffer from Inflammatory Bowel Disease (IBD), namely Crohn’s Disease and Ulcerative Colitis (1). The impact of the disease to society is disproportionally high since it is a chronic condition affecting primarily young individuals (10-40 years old) and it is characterized by periods of remission and relapse, frequently requiring hospitalization. More than half of patients with IBD have some type of surgical intervention during their lifetime (2). Symptoms like bloody diarrhea, urgency, abdominal pain, general malaise and fever significantly compromise the patient’s quality of life and in 15% of them their ability to sustain a full time job. The pharmaceutical management of IBD remains problematic (3). Only 30% of patients respond to anti-TNFalpha (infliximab) treatment, one of the newest therapies for IBD, making the need to develop more effective and safer drugs for this disease greater than ever (4).

One of the major factors contributing to the lack of effective treatments for IBD is our incomplete understanding the mechanisms of disease pathogenesis. For instance, while stress has for many years been implicated in symptom precipitation, the role of the normal gut flora (microbiome) has only recently been appreciated in IBD pathobiology. Notably, both of these conditions disproportionally affect war Veterans: During field deployment they encounter acute stress, while a significant percentage of them upon their return to home suffer from anxiety, post-traumatic stress disorder and Gulf War illness (5). Moreover, being outside of their country, they are exposed to various environmental and dietary factors that dramatically alter their microbiome.

Drug development in IBD requires testing in animal models and mice have been primarily used for such purposes. However, experiments in mice are time consuming, costly, and not suitable for large scale screening. Moreover, due to sample size limitations, studies in rodents are often underpowered and inadequate to capture small effects, which nevertheless might be of clinical importance (6).

During the one year support from DOD, we were able to develop an adult zebrafish model of IBD and demonstrate its microbiome dependence. In subsequent studies we will use this model to screen for novel treatments.

RESEARCH BODY

The following tasks were described in the approved statement of work

Task 1: Colitis induction and evaluation in zebrafish (months 1-6)
Task 2: Exposure of zebrafish to stressors (months 6-8)
Task 3. Colitis development in chronically stressed animals (months 8-12)
Task 4: Treatment of established colitis with anti-anxiety drugs using a high-anxiety strain of zebrafish (months 8-12)

Experimental data

a) Dose-response and time-course of TNBS colitis in zebrafish

Methods:
Induction of TNBS colitis: Wildtype zebrafish were purchased from EkkWill Waterlife (Ruskin, FL) and maintained at the BIDMC facility. All studies were approved by the Beth Israel Deaconess Medical Center’s Institutional Animal Care and Use Committee.

Prior to induction of colitis, the zebrafish were transferred to stand-alone tanks and food-deprived overnight. Fish used in the experiments weighed 0.2 – 0.6 grams. Colitis was induced by an intrarectal injection of 1 µl/0.1 g body weight of a TNBS solution (0, 40, 80, 160 or 320 mM) in 30% ethanol. Intrarectal injections were performed in anesthetized fish by placing the fish on its back under a stereomicroscope, pressing gently on the belly so that the rectum protrudes slightly. Survival of fish was monitored for 96 hours.

Data analysis: Results are presented as Kaplan-Meier survival curves and analyzed by the log rank test using Statview 5.0.1. software.

Results:
We found that intrarectal administration of TNBS resulted in acute colitis in zebrafish. The disease severity was proportional to the TNBS dose and reached plateau at 160mM of TNBS (Figure 1A-D). The 160mM TNBS concentration was selected for all subsequent studies.

**Figure 1:** Kaplan-Meier survival analysis of adult zebrafish treated with escalating doses of TNBS (40mM-320mM) (n=10-11 fish per experimental group).

**b) Histological features of TNBS colitis in zebrafish**

**Methods**

*Histological analysis:* At 6hrs post-TNBS treatment, which represents the peak of inflammation in zebrafish (7), fish were euthanized and their gastrointestinal tube was removed en block and fixed in formalin. The severity of colitis was assessed in H&E stained paraffin embedded sections following a previously described scoring system (8). This work is still in progress.

*FACS analysis of neutrophil infiltrates in zebrafish with TNBS colitis.* Myeloperoxidase (mpx) is a marker primarily for neutrophils (7). Colitis was induced in adult transgenic myeloperoxidase-tagged zebrafish (mpx-GFP) as described above. Intestinal tissue was collected at 6 hrs after exposure to TNBS, minced and passed through a 40 μm cell strainer (BD Falcon) followed by a rinse with 3 mL of PBS/2% FBS. The cell pellet was analyzed by flow cytometry (LSRII, BD) for cell size (forward scatter), intracellular complexity (side scatter) and GFP-positive cells. The graph represents the % of mpx-GFP positive cells in control fish and fish with TNBS colitis. Percentages of GFP-positive cells were reported and analyzed by the Mann-Whitney non-parametric test.

**Results**

TNBS-induced enterocolitis in zebrafish was characterized by disruption of the epithelial architecture and mucosa thickening and edema, goblet cell depletion and infiltration by immune cells, including granulocytes, eosinophils, macrophages and lymphocytes (Figure 2A). By FACS analysis, we detected a ten-fold increase in the intestinal influx of mpx positive cells (primarily granulocytes) in animals with TNBS-induced colitis compared to the control, vehicle treated group (0.614% ± 0.202% vs 0.052% ± 0.014%, respectively; p=0.0269; n=6-7 per group; Figure 2B).
Figure 2: A. Representative H&E stained intestinal sections from zebrafish treated for 6hrs with either vehicle or 160 mM TNBS. B. The % of GFP+ cells infiltrating the intestine was significantly increased in transgenic mpx-GFP fish with TNBS-induced colitis.

b) Molecular analysis of TNBS colitis in zebrafish

Methods
Quantitative real-time PCR: Zebrafish were treated with 160mM TNBS or vehicle and intestinal tissue was harvested after 6hrs. Total RNA was extracted using Trizol (Invitrogen) and purified using the RNeasy mini-kit (Qiagen). One microgram (µg) of RNA was reverse-transcribed into cDNA using the Advantage RT for PCR reagents with oligo(dT) (Clontech Inc). Quantitative gene expression was assessed by real-time PCR using Sybr Green PCR Master Mix (Applied Biosystems) and the following zebrafish gene-specific primers (7): IL-1beta-f: 5’TGCGGGCAATATGAAGTCA-3’, IL-1beta-r: 5’-TTGCGCATGACATGCTCC-3’, IL-8-f: 5’TGTGTTATTGTTTTCTGGCATTTT-3’, IL-8-r:5’GCGACAGGTGGATCTACAG-3’, IL-10-f: 5’-AGGGCTTTCTTTAAGA-3’, IL-10-r: 5’-ATATCCCCGTTGAGTCC-3’, TNFalpha-f: 5’-AGACCTTAGACTGAGAGAATGAC-3’, TNFalpha-r: 5’-CAAGACACCTGGCTGGTGTAGAC-3’, tbp-f: 5’-ACCAGTCTCTTTGTTTGC-3’, and tbp-r: 5’-TTCTGGTTCTCGTATTTCAT-3’ (Applied Biosystems, Carlsbad, CA). Data were captured in a ABI PRISM 7700 Sequence Detection System and results are expressed as arbitrary mRNA units (AU) relative to control (control =100), after normalization to expression of the TATA- binding protein (TBP) housekeeping gene. For statistical analysis, we used the Mann-Whitney non-parametric test (n=9-10 fish per group).

Results
For the molecular analysis of the TNBS-induced inflammatory response in zebrafish, we measure intestinal mRNA expression of the pro-inflammatory cytokines, IL-1beta, IL-8, TNFalpha and the anti-inflammatory cytokine IL-10. We found a significant upregulation of IL-1beta expression (100 ± 28.202 arbitrary units (AU) for vehicle vs. 6,935.674 ± 2,454.183 AU for TNBS-treated; p=0.0179; Figure 3A ), IL-8 (100 ± 21.345 AU for vehicle-treated vs. 246.479 ± 62.831 AU for TNBS-treated; p=0.0500; Figure 3B) and IL-10 (100 ± 7.996 AU for vehicle-treated vs. 384.933 ± 84.408 AU for TNBS treated; p=0.0055; Figure 3D), while it was a trend of TNFalpha upregulation (100 ± 12.285 AU for vehicle treated vs. 421.123 ± 242.747 AU for TNBS-treated; Figure 3C; p=0.2282). Notably, the same cytokines are upregulated in human IBD and in mice with experimental colitis, underscoring the clinical relevance of the zebrafish model of IBD.
4) Similarities of zebrafish experimental colitis with mouse and human IBD

e.1) Induction of TNBS-colitis in zebrafish depends on the microbiome

Rationale
Several of the mouse models do not develop colitis under germ-free conditions, suggesting a microbiome dependence of the intestinal inflammation (9,10). The importance of the microbiome has also been demonstrated in human IBD (11).

Methods
Vancomycin treatments: Vancomycin hydrochloride (Sigma) was added to the fish water at different concentrations (10, 50, or 100 mg/L) for 18 hours prior to intrarectal administration of 160 mM TNBS as described above. Tank water with vancomycin was replaced every 24hrs. A separate group of fish without TNBS colitis was treated with vancomycin to access drug toxicity. Kaplan-Meier survival analysis followed by log-rank test was used to estimate the effect of vancomycin treatment on TNBS colitis (n=10-25 per group).

Results
Treatment with vancomycin can reduce mortality associated with TNBS colitis: Indeed, in the zebrafish model as well, we observed decreased susceptibility to TNBS-induced colitis associated with antibiotic (vancomycin) treatment (Figure 4). Specifically, treatment of zebrafish with 100 mg/L vancomycin prior exposure to exposed to TNBS resulted in 50% survival compared to less than 20% survival in fish with intact microbiome (p= 0.0263, Figure 4D).
Figure 4: TNBS colitis in zebrafish depends on the microbiome. Vancomycin treatment increases survival of zebrafish following intrarectal TNBS administration in a dose-dependent manner.

2) MCH/MCHR1 expression analysis in zebrafish

Rationale: Melanin-concentrating hormone (MCH) is an evolutionary conserved neuropeptide with almost identical amino acid sequence in fish, rodents and humans. In human and fish two MCH receptors have been identified, while rodents have only one (12). Our previous work has demonstrated an upregulation of MCH and its receptors in the affected mucosa of patients with IBD and in mice with TNBS induced colitis (13). Most importantly, MCH-deficient mice develop attenuated intestinal inflammation, suggesting a proinflammatory role of MCH (13,14). To further validate our zebrafish model, we examined MCH expression in zebrafish in response to TNBS-induced colitis and found similar regulation as in humans and mice.

Methods

Analysis of MCH/MCHR1 expression by real-time PCR: TNBS-colitis was induced in zebrafish as described above and at different time-points (3hrs and 6hrs) intestinal tissue was harvested for mRNA extraction followed by quantitative PCR using the following primers: 

- pmch-f: 5'-TGCGGACACAGGAATTAAAGG-3', pmch-r: 5'-ATCCATCGTGCTGAATCCATC-3', pmchl-f: 5'-ATCATCGTGGTGGCTGACTCC-3', pmchl-r: 5'-GCTTTTCGGTGCGTGGAGTG-3', mchr1a-f: 5'-AAATGCCAGGCTAAACAAACA-3', mchr1a-r: 5'-GCTTTCGGTGCGTGGAGTG-3', mchr1b-f: 5'-TTGTTGGAACCTCCTCCTAGG-3', mchr1b-r: 5'-CCGGATGGAACAAATAAAACT-3', mchr2-f: 5'-TTGCAATCGTCCATCCTACA-3', mchr2-r: 5'-CTGGTGGAGTGCATCCTACT-3'.

Results are expressed as mean±SE of arbitrary mRNA units (n=9-10 per group).

Immunofluorescence analysis: Zebrafish were treated with TNBS (160mM) for 3hr and intestinal tissue was fixed in 4% paraformaldehyde and then frozen in O.C.T. (Optimal Cutting Temperature, Sakura Finetek 4583) media. Cryosections (5 µm) were incubated with a rabbit polyclonal antibody against MCH (dilution 1:300) as previously described (13) followed by incubation with Alexa Fluor 594 Donkey anti-rabbit (Molecular Probes, A-12074, dilution 1:300) secondary antibody. Slides were treated with Prolong Gold 4',6-diamidino-2-phenylindole (DAPI) mounting solution (Invitrogen, P-36931) and visualized under a Zeiss LSM510 META Confocal System.
For negative control, the same procedure was followed but incubation with the primary anti-MCH antibody was omitted.

**Results:**
We found that intestinal MCH mRNA expression was upregulated more than 9-fold in TNBS-treated fish at three hours post-treatment (100 ± 29.444 vs. 909.444 ± 235.556; control vs TNBS, p=0.0049) and remained elevated after 6hrs (100 ± 24.914 vs. 652.235 ± 172.313; p=0.0079; Figure 5A). Furthermore, expression of MCHR1b was also upregulated at both the 3 hr (100 ± 18.75 vs. 411.25 ± 137.5; p=0.0495) and 6 hr time points (100 ± 40.209 vs. 451.537 ± 150.964; p=0.0469; Figure 5B) in TNBS-treated fish. Interestingly, we found that MCHR2 was downregulated by 6 hours post TNBS-treatment (100 ± 33.748 vs. 28.014 ± 9.219; p=0.0456; Figure 6C). Overexpression of MCH in the zebrafish intestine in response to TNBS treatment was also confirmed by immunofluorescence (red staining) (Figure 5D). Taken together with cytokine expression patterns described in Figure 3 these results identify common mediators of intestinal inflammation in zebrafish, mice and humans.

Figure 5: A-C) Time course of mRNA expression of MCH (pmch) and its receptors mchr1b and mchr2 in the intestine of zebrafish treated with TNBS.
D) Immunoflourescence staining of MCH in intestinal sections of zebrafish exposed to TNBS for 3 hours (magnification 63x).

d) Treatment of TNBS colitis in zebrafish

**Methods**

*Injection im:* Anesthetized zebrafish were injected i.m. with 0.5mg/g prednizolone or 0.5-1mg/g dexamethasone immediately prior to intrarectal administration of TNBS (160mM) (n=10-11/group)

*Treatments in the tank water:* Zebrafish were transferred to new tanks containing 25 mg/L prednisolone, 100mg/L 5-Aminosalicylic Acid or vehicle for 18hrs followed by intrarectal administration of TNBS (160mM) as above (n=9-10/group). Tank water was replaced every 24 hrs in the presence of each treatment till the end of the experiment.

**Results**
We tested classes of anti-inflammatory agents that are routinely used to treat human IBD, such as corticosteroids (prednizolone and dexamethasone) and non-steroid anti-inflammatory drugs (5-aminosalicylic acid) at various concentrations and via different routes of administration to treat TNBS-induced colitis in zebrafish (Figure 6). This work is still in progress and so far only treatment with 5-aminosalicylic acid showed a trend of improved the survival of zebrafish with colitis (Figure 6D).
Challenges and negative results

- In the experiments described above, the intestine was removed from anesthetized fish under a magnifying microscope and processed for histology or molecular analysis. However, initially, whole fish were fixed in formalin, decalcified and processed for histological analysis of sections cut at different planes (sagital and coronal) and at different levels of the intestinal tube. Besides being laborious and time consuming, this process resulted in poor preservation of tissue architecture and rarely the sections covered similar anatomical sites, thus making comparisons between samples with colitis and controls almost impossible.

- This we believe might be one of the reasons that we failed to demonstrate histologically colitis in fish treated with DSS. Various concentrations of DSS added in the fish tank water were used and fish were monitored for up to 48hrs for signs of disease. Fish in moribund state were not allowed to die, but they were euthanized (see table below)

<table>
<thead>
<tr>
<th>DSS (mg/L)</th>
<th># surviving 24 hours</th>
<th>48 hours</th>
<th>72 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>19/19</td>
<td>19/19</td>
<td>19/19</td>
</tr>
<tr>
<td>0.25</td>
<td>21/21</td>
<td>21/21</td>
<td>20/21</td>
</tr>
<tr>
<td>0.5</td>
<td>18/19</td>
<td>18/19</td>
<td>18/19</td>
</tr>
<tr>
<td>0.75</td>
<td>21/21</td>
<td>21/21</td>
<td>20/21</td>
</tr>
<tr>
<td>1</td>
<td>22/23</td>
<td>15/23</td>
<td>3/23</td>
</tr>
</tbody>
</table>
Relationship of the most recent findings with that of previously reported findings

To our knowledge, there are only three previous studies reporting colitis in zebrafish (7,8,15).

In two similar studies, one from a group in the study by from New Zealand (Oehlers et al (15)) and the other form UK (Fleming et al (8)) exposure of zebrafish larvae to TNBS resulted in enterocolitis characterized by damage of intestinal crypts, leukocyte infiltration and marked induction of proinflammatory cytokines (IL-1beta, TNFalpha, IL-8 and CCL20). Most importantly, treatment with antibiotics (ampicillin/kanamycin) prevented the development of colitis and treatment with .5-aminosalicylic acid, prednisolone or NOS inhibitors could rescue the phenotype. These findings are very similar to ours, with the major difference that in our study we induced colitis in adult zebrafish, which we believe is more relevant to human IBD.

The group form the Netherlands (Brugman et al (7)) induced chemical enterocolitis in adult zebrafish via intrarectal administration of oxazolone. In mice, oxazolone elicits primarily Th2 immune responses in contrast to TNBS, which has been associated with Th1 responses. However, comparing the oxazolone and TNBS (our study) models in zebrafish, they seem quite similar based on the following: a) microbiome dependence; b) peak of inflammation between 5-6 hrs post chemical exposure and upregulation of IL-1beta, TNFalpha and IL-10. with our study, it appears that they share many common characteristics, including microbiome peak of inflammation between 5-6 hrs and upregulation and our study, does not appear to this does not seem to be the case in zebrafish, since in the oxazolone model Again, this model was also dependent on the microbiome and

In addition to confirming and extending previous findings, our study points to a different class of inflammatory mediators, namely neuropeptides like MCH, which are evolutionary conserved and play an immunomodulatory role, while in parallel regulate central pathways affecting energy balance, motivational behavior and mood. Indeed, blocking MCH using an antibody or an antagonist in mice, not only attenuates colitis (13) but also exhibits anxiolytic and anti-depressant effects, (16-18). In the future, such treatments might particularly benefit Veterans with IBD and co-morbidities such as anxiety, depression and post-traumatic stress disorder. In summary, the newly established adult zebrafish enterocolitis model will allow us to further study the effects of stress mediating neuropeptides including the CRH/urocortins (19) and NPY (20,21) in intestinal inflammation. Most importantly, it will allow us to incorporate endocrine, psychological, behavioral and environmental factors in our search of novel treatments for IBD.

KEY RESEARCH ACCOMPLISHMENTS

- Induction of enterocolitis in adult zebrafish by intrarectal administration of TNBS
- Description of the histological features of chemical enterocolitis in zebrafish
- Identification of evolutionary conserved, from human to fish, mediators of intestinal inflammation
- Demonstration of microbiome-dependence of TNBS-colitis in zebrafish, as is the case with the mouse models of experimental colitis.

The following Investigators contributed to the design of the study, performed the experiments, analyzed data and interpreted results: E Kokkotou, P. Fraenkel, B. Geiger, B.Gras, A. Bai, A. Karagiannis

REPORTABLE OUTCOMES

- A manuscript describing these findings is under preparation, with the goal to be submitted within one month of the completion of this award

- We have provided the scientific community, and in particular IBD research and therapeutics, with a robust and reproducible model of acute experimental colitis, which exhibits striking similarities to the respective mouse model, in terms of precipitating factors, histological manifestations and molecular mediators.
CONCLUSIONS

The zebrafish model of enterocolitis is a robust one that captures several important aspects of IBD pathogenesis, including immune mediators, microbiome dependence and response to treatment with corticosteroids and non-steroid anti-inflammatory drugs. The study of adult zebrafish offers advantages by allowing the study of complex psychological, behavioral and environmental interactions that can be targeted toward development of new therapeutic approaches for the treatment of IBD.

REFERENCES


