MARINE BIOTECHNOLOGY

Induction of Japanese Flounder TNF Promoter Activity by Lipopolysaccharide in Zebrafish Embryo

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Abstract

A recombinant plasmid containing the 2381-bp promoter region of Japanese flounder tumor necrosis factor (TNF) and green fluorescence protein (GFP) was introduced into zebrafish fertilized eggs by microinjection. GFP was expressed in 2 transgenic zebrafish lines in the heart and around the pharynx under unstressed condition. When embryos were exposed to lipopolysaccharide (LPS), GFP was expressed in the whole zebrafish embryonic surface, and Western blot analysis also showed that the level of the expressed GFP protein in zebrafish embryo was higher at an LPS concentration of 140 μg/ml than at LPS concentrations of 0 and 70 μg/ml. Stimulation with either concanavalin A or phorbol myristate acetate induced weak GFP expression, but stimulation with both of them induced strong expression similar to that induced by LPS. GFP expression peaked 1 hour after stimulation, then gradually decreased. These results indicate that transcription regulated by the Japanese flounder TNF promoter could be under the control of the LPS-recognition system in zebrafish embryos.

Key words: TNF — promoter — transgenic — zebrafish — LPS induction — GFP

Introduction

Tumor necrosis factor (TNF) is a primary mediator of immune regulation and inflammatory response (Camussi et al., 1991; Fiers, 1991). In mammals TNF is synthesized by different cell types upon stimulation with endotoxic inflammatory mediators or a variety of cytokines including TNF itself (Carswell et al., 1975; Ferrante et al., 1990; Goldfeld et al., 1990, 1991). Japanese flounder TNF gene expression was found to be induced immediately by mitogens, such as lipopolysaccharide (LPS), concanavalin A (Con A), or phorbol myristate acetate (PMA), indicating that it is regulated in a transcription-inducible manner (Hirono et al., 2000). Recently, TNFs from carp (Saeij et al., 2003) and rainbow trout (Laing et al., 2001) have been cloned and shown to be induced in the same manner. In contrast, gilthead seabream TNF showed only basal expression (Garcia-Castillo et al., 2002). Likewise, the activity of the Japanese flounder TNF promoter has been characterized in vitro using fish cell lines. Further studies are needed to confirm its activity in vivo.

The use of zebrafish as an experimental model offers many advantages for transgenic research including short generation times, optical transparency of embryos to visualize internal tissues, and high fecundity. Many transgenic fish studies using green fluorescent protein (GFP) as a reporter gene for developmental analyses are being reported (Gong et al., 2001). GFP can be used in these transgenic fish to visualize specific tissue development and localization. The activity of several tissue-specific promoters in fish tissue has been successfully demonstrated using transgenic technology with GFP as a reporter gene (Gong et al., 2001). This technique has provided a powerful tool to analyze the regulation of gene expression in living fish. GFP-transgenic zebrafish lines have been generated using specific promoters such as erythroid-specific *gata-1* (Long et al., 1997), neuron-specific *islet-1* (Higashijima et al., 2000), and primordial germ-cell-specific *Vas* (Krovel and Olsen, 2002). An inducible gene promoter derived from zebrafish heat shock protein (HSP) has been characterized (Adam et al., 2000; Halloran et al., 2000). The expression of GFP in these transgenic zebrafish reflected the same pattern as the gene from which the promoter was derived.

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In this study we constructed a Japanese flounder TNF promoter linked to GFP as a reporter gene and analyzed its activity using transgenic zebrafish as an experimental model for biomonitoring of immune responses against bacterial infections.

Materials and Methods

Production of Transgenic Zebrafish. Adult zebrafish (*Danio rerio*) and embryos were maintained at 28.5°C on a 14-hour light and 10-hour dark cycle. One-cell-stage embryos were then collected for transgenic studies.

The recombinant plasmid pTNF-GFP (Yazawa et al., 2002) was propagated in *Escherichia coli* JM109 and purified using a GFX Micro Plasmid prep kit (Amersham Pharmacia Biosciences). Purified plasmid DNA was adjusted to 50 ng/μl in distilled water and microinjected into one-cell-stage zebrafish embryos. Living embryos were examined under a fluorescence microscope (Olympus SZX12), and GFP-positive zebrafish embryos were selected. After maturation the GFP-positive fish were mated with wild types to select fish for germline transmission.

LPS Treatment of Zebrafish Embryos. F_2 transgenic embryos at 30 hours after fertilization were stimulated with LPS (Sigma) at different concentrations (45, 80, 100, 140, and 180 μg/ml) for 30 minutes, then washed in sterilized water. GFP expression in F_2 embryos was examined under the fluorescence microscope 48 hours after fertilization. Survival rate was determined at the same time.

SDS-PAGE and Western Blotting. Ten embryos were collected and homogenized with Sodium dodecylsulfate (SDS) sample buffer (Sambrook et al., 1989) and subjected to 12.5% polyacrylamide gel electrophoresis (PAGE). Separated proteins were transferred to a nitrocellulose membrane. The membrane was immunoblotted with EGFP monoclonal antibody (Clontech) at a 1:5000 dilution as the first antibody, and peroxidase-labeled anti–IgG mouse antibody (Promega) at a 1:5000 dilution as the second antibody. Positive bands were stained with ECL Western blotting detection reagent (Amersham Pharmacia Biosciences).

RT-PCR. Total RNA was extracted from each of the 10 zebrafish embryos using TRIZol reagent (Amersham Bioscience). The purified total RNA (10 μg) was reverse transcribed into complementary DNA using an AMV transcriptase first-strand cDNA synthesis kit (Amersham Pharmacia Biosciences). The reverse-transcribed sample (1 μl) was used in a 50-μl polymerase chain reaction (PCR) mixture. The PCR primers for GFP messenger RNA detection were GFP-F, 5′-AAAGCCACCATGGTGAGCAAGGGCG-3′; and GFP-R, 5′-CCGTACCTGCTCGACATGTTCATTAAT-3′. The β-actin primer set for internal control were zactin-F, 5′-TTTCCCTCCATTGTTGGTCG-3′; and zactin-R, 5′-GCGACTCTCAGCTCGTTGTA-3′. PCR was performed with an initial denaturation of 2 minutes at 95°C, then 20 cycles were run for 30 seconds of denaturation at 95°C, 30 seconds of annealing at 55°C, and 1 minute of extension at 72°C. The reacted products were electrophoresed on a 2.0% agarose gel.

Results

GFP Expression in Transgenic Lines of Zebrafish Embryos. Two transgenic lines were established, TNF-GFP4 and TNF-GFP15. The GFP gene driven by the Japanese flounder TNF promoter was expressed on the surface of F_2 zebrafish embryos at early developmental stages. GFP expression decreased gradually, but remained around the pharynx and heart 72 hours after fertilization (Figure 1).

Mitogen-Inducible Expression of GFP Under Japanese TNF Promoter. GFP gene expression was strongly induced in the epithelial tissues in 2-day-old transgenic embryos after LPS treatment (Figure 2). Western blot analysis showed that the level of the expressed GFP protein in zebrafish embryo was higher at an LPS concentration of 140 μg/ml than at concentrations of 0 and 70 μg/ml (Figure 3).

GFP mRNA expression increased at 1 hour after LPS treatment, then gradually decreased (Figure 4). Expression of GFP was induced in all of the F_2 embryos at LPS concentrations of 140 and 180 μg/μl and in some embryos at even lower concentrations (Table 1). Con A did not induce GFP expression at any concentration, while PMA slightly induced GFP expression in transgenic embryos only at high concentration (300 μg/μl). A combination of Con A and PMA induced GFP expression at a level similar to that induced by LPS induction (Table 1).

Discussion

In this study we established transgenic zebrafish lines expressing GFP driven by the Japanese flounder TNF promoter. The transgenic zebrafish expressed GFP in the heart and around the pharynx under unstressed conditions (Figure 1). This tissue-specific GFP expression was observed in 2 different transgenic strains. This result suggests that this tissue-

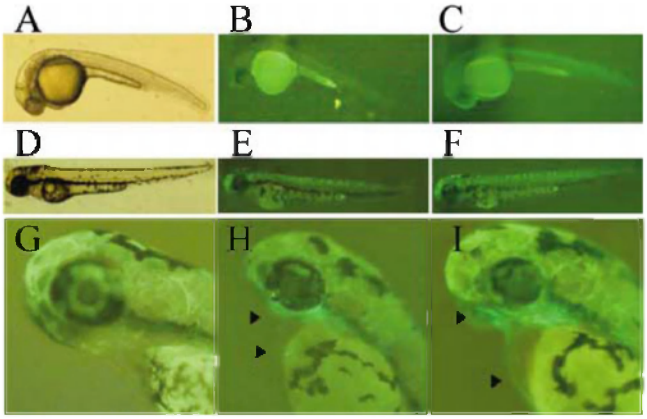

Fig. 1. GFP expression in developmental stages of transgenic zebrafish embryos. **A**: Larval embryos at 36 hours after fertilization in bright field. **B**: Wild-type embryo at 36 hours under fluorescence microscopy. **C**: F_2 transgenic line, at 36 hours, fluorescence. **D**: Larval embryo at 72 hours after fertilization in bright field. **E**: Wild-type, at 72 hours, fluorescence. **F**: F_2 transgenic line, at 72 hours, fluorwscence. **G**: Wild-type 4-day embryo, fluorescence. **H**: Transgenic strain TNF-4 4-day embryo, Fluorescence. **I**: Transgenic strain TNF-15 4-day embryo, fluorescence. Arrowheads indicate GFP expression in 4-day embryos and TNF-15 (I) strains.

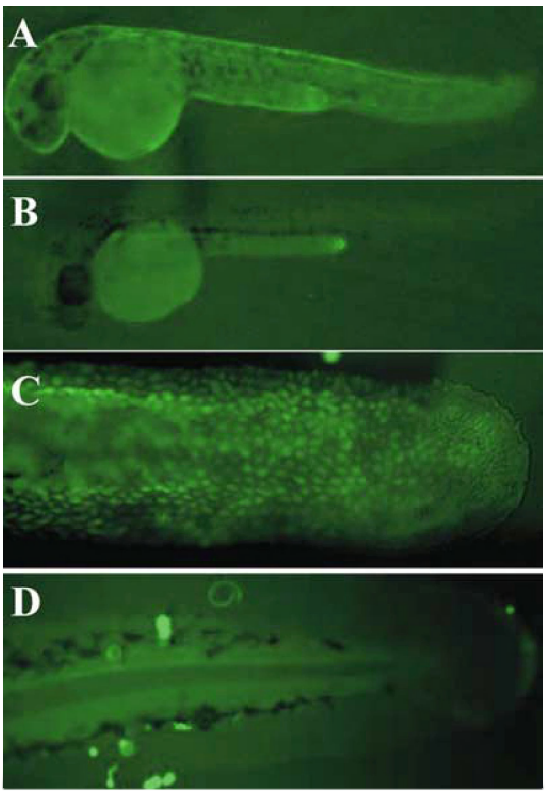

Fig. 2. LPS-inducible GFP expression in transgenic zebrafish embryos. Two-day embryos of F_2 transgenic generation with LPS treatment (**A**) and without LPS treatment (**B**) under fluorescence microscopy. Enlarged view of tail portion with LPS treatment (**C**) and without LPS treatment (**D**).

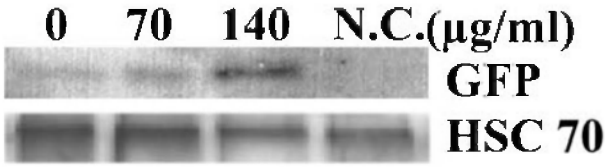

Fig. 3. GFP expression level after LPS stimulation at various concentrations. Lane N.C. is protein extracted from wild-type zebrafish embryos.

specific GFP expression was regulated by the Japanese flounder TNF promoter. There has been no report of the TNF gene expressed in the heart and around the pharynx in any species. The use of a heterogeneous promoter caused this unexpected transcription regulation in these transgenic strains. GFP expression in the transgenic zebrafish was induced immediately with LPS treatment (Figure 2). GFP mRNA transcription was induced at 1 hour after LPS treatment, and decreased gradually at 3 and 6 hours after LPS treatment (Figure 4). Such an immediate induction of mRNA transcription is similar to that of Japanese flounder TNF expression in LPS-stimulated peripheral blood lymphocytes (PBLs; Hirono et al., 2000). Moreover, stimulation with either ConA or PMA induced Weak GFP expression. This GFP expression, but stimulation with both ConA and PMA induced strong expression. This GFP expression was detected in epithelial tissues. The mammalian TNF gene is also expressed in epithelial tissue after LPS stimulation and is suggested to have an important role as a first trigger in the innate immune system and as a first barrier to bacterial infection. These results suggest that the Japanese flounder TNF promoter used in this study is sufficient to regulate the faithful expression of a downstream gene. This is the first report to establish that induction of GFP expression could be observed by LPS stimulation in living embryos of a transgenic fish.

Recently, several fish TNF genes have been induced mainly in peripheral blood lymphocytes by mitogens, an exceptions being gilthead seabream TNF-α gene (Garcia-Castillo et al., 2002). TNF gene expression of gilthead seabream is constitutively

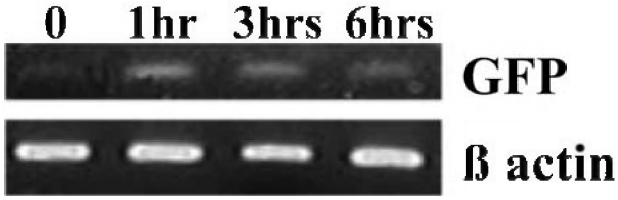

Fig. 4. Time-course expression of GFP in transgenic zebrafish embryo after LPS stimulation.

Table 1. Induction of GFP Expression in Transgenic Zebrafish Embryos by Various Mitogens

	Concentration (μg/ml)	*Total No. of treated embryos*	*No. of surviving embryos*	*No. of GFP positive embryos*
LPS	45	200	186 (93.0)*	72 (38.7)*
	80	204	184 (90.1)	91 (49.5)
	100	202	142 (70.2)	104 (73.2)
	140	194	112 (57.7)	112 (100.0)
	180	201	73 (36.3)	73 (100.0)
Con A	0.02	96	80 (83.3)	3 (3.8)
	0.04	104	72 (69.2)	5 (6.9)
	0.08	96	48 (50.0)	6 (12.5)
	75	100	96 (96.0)	5 (5.2)
	150	104	80 (76.9)	9 (11.3)
	300	96	56 (58.3)	15 (26.8)
Con A/PMA	0.013/15.5	100	80 (80.0)	42 (52.5)
	0.025/31.0	100	64 (64.0)	53 (82.8)
	0.05/62.5	96	56 (58.3)	56 (100.0)
Control		100	98 (98.0)	2 (2.0)

*Numbers in parentheses indicate percentages.

expressed in several organs. In this study we confirmed that the Japanese flounder TNF gene promoter could be induced by LPS stimulation. We speculate that the function of the Japanese flounder TNF gene is more similar to that of the mammalian TNF gene than to that of the gilthead TNF-α gene. These results also suggest that the mechanism for recognition of bacterial infection in zebrafish is similar to that in mammalian species. In mammalian species TNF plays an important role as the first trigger molecule in inflammatory response and innate immunity (Camussi et al., 1991; Fiers, 1991). Secretion of TNF induced the expression of cytokines including interleukins 1 and 6 chemokines (Cunha et al., 1994; Vandevoorde et al., 1991). Recently, much progress has been made in identifying cytokines, including TNF in fish (Hirono and Aoki, 2003; Secombes et al., 2001). However, the function and interaction of the cytokine network system are poorly understood in fish. Our results show that the Japanese flounder TNF promoter and the transgenic zebrafish lines used in this study are useful tools for analyzing the signal pathway in fish inflammatory and innate immune mechanisms.

Transgenic technology is useful and efficient not only for basic biology but also for producing fish with commercially important traits. Development of promoters is indispensable for transgenic fish research. In this study we showed that the Japanese flounder promoter has mitogen-inducible activity. The Japanese flounder TNF gene promoter with GFP as an indicator may be useful for monitoring bacterial infection in live fish. Furthermore, it is useful for the production of disease-resistant transgenic fish lines. Under the control of Japanese flounder TNF promoter, it is possible to generate transgenic fish expressing an antibacterial or antiviral peptide only in cases of infection with pathogens.

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