# 魚類鱗片培養法を用いた免疫反応の評価

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Madoka MURATA[1], Yoichiro KITANI[1]: Immune response evaluation method using fish scale culture

**【Background】**

The body surface of fishes is covered with the vulnerable epidermal cells because of their habitat –living in the water. The surrounding water contains a high density of microorganisms including pathogenic bacteria, viruses and parasites. Under this situation, fishes defend themselves from those pathogens by their host-defense system. On the fish body surface, physical and physiological host-defense system –consist of the scales and bioactive substances– could synergically prevent the injury and the invasion of the pathogens. However, the body surface immunity of the fishes is still unclear because of the complexed system and the species-dependent variety of the system.

**【Purpose of this work】**

To understand the fish body surface immune system, I focus on the fish scales –one of the unique component of the fish body surface. Fish scales have a potential as a biological materials to investigate the defense system because a variety of cells are attaching on the scales. In addition, it is possible to obtain some tens of scales from one fish; this point will be an advantage to increase the biological replication without any variation of the specimen. In this research, I try to establish the fish scale culture method for understanding the fish body surface immunity.

**【Optimization of fish scale culture】**

Goldfish (*Carassius auratus*) was used for this work because this fish is easy to handle and it has adequate gene information. The fish was anesthetized using 0.03 % of MS-222, and the scales are collected. The scales immediately transferred to the wells of the microtiter culture plate containing 500 μL of L15 medium with antibiotics. The optimal culture condition was decided by temperature and duration as follows; scales were cultured at 5 °C, 15 °C, 25 °C and 37 °C and the term was 1 day, 2 days, 4 days and 7 days. Optimal culture condition was judged by the cell viability using XTT (N-methyl dibenzopyrazine methyl sulfate) method. In the 5 °C culture, the reaction of XTT was the lowest in each condition because of the low temperature. In the 25 and 37 °C culture, the media became turbid after 4 days culture. Based on those results, I decided that the scale culture conditions were 15 °C for 4 days.

**【Immune responses by the inducers】**

The chemical inducer exposure trial was carried out to understand the fish scale immune responses. I used the chemical inducers as follows; 4-Ethoxymethylene-2-Phenyl-2-Oxazoline-5-on (oxazolone, chronic inflammation inducing reagent, 1.0 to 100 μg/mL in this work), lipopolysaccharide (LPS, the component containing in gram-negative bacteria, 0.1 to 10 μg/mL in this work) and polyinosinic-polycytidylic acid (poly I:C, similar to virus dsRNA, 0.1 to 10 μg/mL in this work). The preparation of scales was followed by the method described above and cultured with chemical inducers at 15 °C for 1 or 3 days. The immune responses were evaluated by the gene expression analysis using quantitative PCR instrument. The amplification of the

PCR reaction was detected by the SYBR Green method. The immune-related genes as follows; interleukin-1-beta (*il1b*, proinflammatory cytokine), Interleukin-8 (*il8*, chemokine), tumor necrosis factor alpha (*tnfa*, inflammatory regulator), interferon gamma (*ifng*, viral infection maker), ribosomal protein S30 (*rps30*, reference gene) and elongation factor 1-alpha (*ef1a*, reference gene). The expression levels of those genes were normalized by the expression of the internal reference genes. In the LPS treatment group, *il1b* expression level was increased at 1-day incubation according to LPS concentration-dependency (Table 1). The highest value reached 16 times greater than the negative control. In the poly I:C treatment group, *ifng* expression was 4 times increased at 1-day incubation. The oxazolone treatment did not cause alteration of any target gene expression. From the facts described above, the cultured scales showed immune responses by the inducers and it was different by the addition of each inducer.

**Table 1 Gene expression changes by chemical inducers**

| | Oxazolone 1d | Oxazolone 3d | LPS 1d | LPS 3d | Poly I:C 1d | Poly I:C 3d |
|---|---|---|---|---|---|---|
| *il1b* | ↗ | ↘ | ↗↗↗ | ↗↗ | → | ↗↗ |
| *tnfa* | - | - | → | → | → | → |
| *il8* | → | ↘ | → | → | → | ↗ |
| *ifng* | ↘↘ | ↘ | → | ↗ | ↗ | → |

Arrows indicate expression change, →; <2 fold; ↗; 2 fold, ↗↗; 5 fold, ↗↗↗; 10 fold. Hyphen indicate the value did not detect.

**【Application of this assay】**

In the last section, the inflammatory response in culture scales was strongly induced by LPS exposure. Therefore, I tried to apply this assay method for the screening of anti-inflammatory substances. First, I examined that hydrocortisone (HC) could suppress the inflammatory response in this assay method. Hydrocortisone is a well-known steroid hormone that work as an immune suppressor. I cultured the scales together with 10 μg/mL of LPS and 0 to 50 μg/mL of HC at 15 °C for 1 day and 3 days. The alteration of *il1b* expression was measured by the quantitative PCR method described above. At 3 day incubation, *il1b* was induced 9 times higher than the negative control. On the other hand, the addition of HC decreased the *il1b* to a same expression level of the negative control. This result suggested that this method could detect anti-inflammatory substances. Second, I searched anti-inflammatory substances from unutilized marine lives (two of starfishes; *Patiria pectinifera* and *Astropecten polyacanthus*, one of sponge; *Fusinus ferrugineus* attached sponge). The samples were disrupted by the homogenizer with 4 times volume of water. The homogenates were centrifuged (18,000 x *g*, 30 min, 4 °C) and the supernatants were collected as the test extracts. To measure the anti-inflammatory effect, the test extracts were added instead of HC in the previous experiment. The activity was judged by the *il1b* expression level compared with the negative control and LPS control. The investigation showed that the test extracts did not suppress the *il1b* expression at 1-day incubation. Subsequent 3-day incubation, *P. pectinifera* starfish extract slightly suppressed the *il1b* expression. Taken together the results in this work, the test extracts did not show the potent anti-inflammatory activity. Interestingly, some of the test extracts showed that the inflammation stimulation activity.

**【Conclusion】**

The cultured fish scales showed the immunological responses by the immunological inducers. This may be possible to apply the other fish immunological studies such as *in vitro* infection study and/or the molecular marker survey. Also, I would suggest that this work have a possibility to the novel drug screening method such as immune suppressor and inducer.

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