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Development of a Simple Culture Method for the Tissues Contaminated with Microorganisms and Application to Establishment of a Fish Cell line

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ABSTRACT—Cell cultures are good in vitro model systems in place of using living animals. In the present study, we developed a simple culture method in which tissues were pretreated with a low concentration of sodium hypochlorite solution (NaClO) to prevent not only bacteria but also fungi. Scales removed from a goldfish (*Carassius auratus*) body were treated with 70% ethanol and then with 0.3% of sodium hypochlorite solution, and cultured in vitro in an atmosphere containing 0.5% CO_2. The doubling time of the established cells (GAKS)† was 24 hr. The GAKS cells contained alkaline phosphatase activity (8.3±1.1 nmol/min/mg protein) and secreted 0.32±0.07 pg/ml endothelin during a 3 day culture of a full monolayer sheet.

INTRODUCTION

Cell culture systems have been used for investigations on genetic analysis, hormonal regulation, cytokine secretion, virus titration and drug sensitivity in place of living animals, because cultured cells mimic living whole organisms in certain experiments. Therefore, the usefulness of the cell culture system will be increased in the future. In particular, this system in which animals are not needed is excellent in the assray for cytotoxic compounds.

A large number of cell lines were established from mammalian tissues such as those of the rat, mouse and human, because they have been used conventionally in the laboratory. In addition, many biochemical reactions were investigated precisely using cultured cells.

Recently, the effects of environmental hormones (endocrine disrupters) or dioxin on organisms have been discussed not only from scientific viewpoints but also from social perspectives. To evaluate these effects, other animals except for mammals should be also examined, because they are exposured directly to environmental pollutants. Thus, fish are one of the best animals to investigate the effects of these compounds on living organisms (Babich and Borenfreund, 1987). In addition, many fish cell lines derived from gill, fin, gonad, testis, kidney, *etc*. were established (Wolf and Mann, 1980; Kuroda, 1991). However, primary cell cultures of fish are not so easy, because the growth of bacteria and/or fungi contaminating original tissues easily occurs. In this study, we tried to establish a fish cell line, and developed a simple culture method which can prevent bacterial and fungal growth in cell cultures, and in which fish scales are used as the initial tissue. In our developed method, as fish scales could be obtained without the sacrifice of fish, the fish cell line was established easily. In addition, this method is applicable for the cell culture of tissues contaminated with microorganisms.

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† The name of this cell line was registered initialy as GF-Scale (Cell No. RCB0082) in RIKEN CELL BANK (Tsukuba, Japan), but changed here to GAKS by the cell-established authors.

MATERIALS AND METHODS

Cell culture

Goldfish (*Carassius auratus*) were fed in normal water. Several scales removed from a fish's body were treated with 70% ethanol for 30 sec, and then with a 0.3% sodium hypochlorite solution (NaClO) for 30 sec. Sodium hypochlorite solution (6%), Purelox-S, was purchased from Ohyarax Comp. Ltd. (Tokyo, Japan). Scales were then washed with phosphate buffered saline (PBS), cut into two pieces and cultured in DM 160 (Katsuta and Takaoka, 1976) containing 100 U/ml of penicillin, 100 μg/ml of streptomycin and 10% fetal bovine serum. Recently, we have modified this method for human samples such as oral tissues and digestive organ tissues, which are highly contaminated with microorganisms.

Alkaline phosphatase assay

Cells were homogenized in H_2O with a Physicotron, NITI-ON I Rikagaku Seisakusho (Tokyo, Japan) at 20,000 rpm for 30 sec (Sorimachi, 1999). Aliquots of homogenates (50–100 μl) were used for the enzyme assay. The alkaline phosphatase assay was carried out according to the previously reported method (Sorimachi, 1987), in which the Kind and King method (1954) was slightly modified. The cellular protein concentration was estimated by the method of Lowry

et al. (1951) using bovine serum albumin as the standard.

Endothelin assay

The amount of endothelin secreted from GAKS cells was measured by an enzyme-linked immunosorbent assay kit, purchased from the Cayman Chemical Company (Ann Abor, MI, USA). A small amount of endothelin was detected in the conditioned medium of the GAKS cells without condensation of the medium. However, to obtain an accurate value, the conditioned medium was condensed by a freeze-dried method, and the sample was dissolved into a small volume of H_2O.

RESULTS

Primary culture

When the fish scales without pretreatment with sodium phypochlorite solution were cultured, the growth of certain bacteria and fungi was not prevented, even if antibiotics such as penicillin, streptomycin and amphotericin B (Fungizon) were added to the culture media. To develop a culture method for primary fish cell cultures, we treated fish scales with 70% ethanol and then a 0.3% sodium hypochlorite solution before starting cell cultures, as described in the Materials and Methods. This treatment completely inhibited the growth of bacteria and fungi, and the newly proliferated cells spread from the cut place, as shown in Fig. 1.

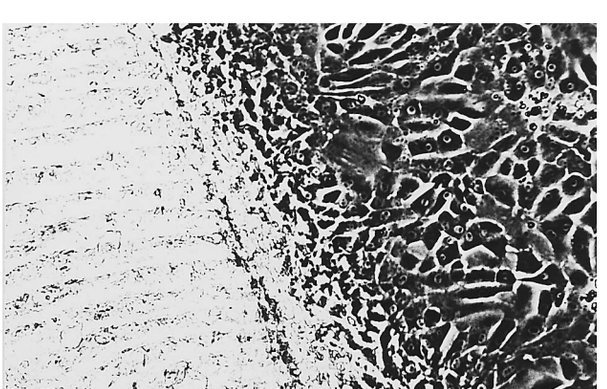

Fig. 1. Phase-contrast micrograph of a primary culture of goldfish scales. Goldfish scales were cut into two pieces and cultured for 3 weeks.

Cell culture

After 5 weeks, as the proliferated cells formed a monolayer, the cells were trypsinized and newly inoculated in other culture dishes. In the early stage the cell growth was slow, but after 6 months cell growth became stable and the passage was carried out constantly once a week.

Cell characterization

Fig. 2 shows the phase-contrast micrograph of the fish cells from the skin tissue on scales (GAKS). The morphology of cells is an epithelial type.

Fig. 3 shows the growth curve of the GAKS cells. The doubling time was 24 hr. The distribution of the chromosomal number of the GAKS cells was measured by RIKEN CELL BANK (Tsukuba, Japan), when the cells were deposited. The chromosomal numbers were distributed in the range of 108–264, and the maximum distribution was observed at 142.

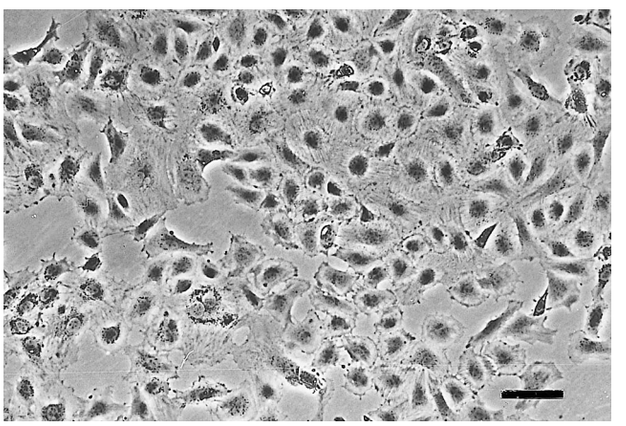

Fig. 2. Phase-contrast micrograph of goldfish scale (GAKS) cells. Bar = 30 mm.

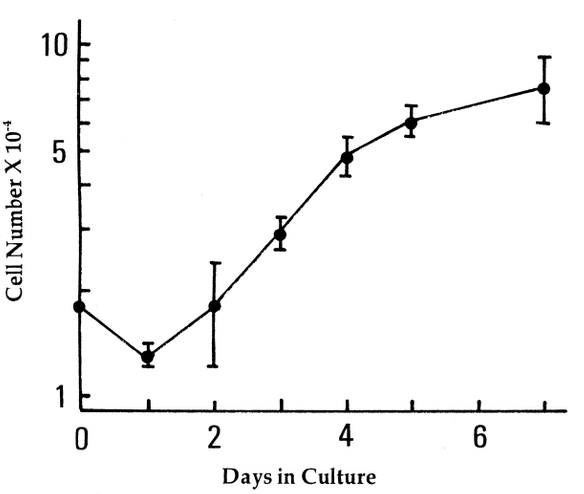

Fig. 3. Growth curve of goldfish scale (GAKS) cells. Cells (1.8×10^4) were inoculated in 35-mm plastic culture dishes. The values are the means ± S.D. for 3 experiments.

To characterize the GAKS cells from biochemical aspects, the cells were examined as to whether alkaline phosphatase activity was retained, as many cell lines retain this enzyme activity (Sorimachi, 1987). Alkaline phosphatase activity was detected, although it was low (Table 1). In addition, it was reported that keratinocytes produced and secreted endothlins (Imokawa *et al.*, 1992, 1995; Ahn *et al.*, 1998). Therefore, we examined as to whether GAKS cells secrets endothelins into culture medium. The secretion of endothelin was clearly

Table 1. Alkaline phosphatse activity and endothelin secretion from GAKS cells.

Alkaline phosphatase	8.3 ± 1.1 nmol/min/mg protein
Endothelin	0.32 ± 0.07 pg/ml

The values are the means ± S.D. for 3 or 4 experiments.

observed, as shown in Table 1.

DISCUSSION

Our method can easily obtain fish cells from the scale, and establish cell lines. Thus, our method can provide us with fish cells for biochemical experiments and phylogenetic studies in the laboratory without the sacrifice of fish.

Contamination by bacteria or fungi is a serious problem in tissue cultures. In general, the growth of bacteria can be inhibited by antibiotics, but its antibiotic activity is limited. Moreover, we do not have suitable drugs which can specifically inhibit fungal growth at present. However, the treatment of tissues with a sodium hypochlorite solution improved fish scale cell cultures, because this compound functions not only as an anti-bacterial, but also as an anti-fungal reagent. As another important point, tissues were directly treated with a sodium hypochlorite solution. In this case, the surface cell of tissues died in the presence of hypochlorite at 0.3%, as well as microorganisms contaminating the tissue. However, the dead cell layer protects the inner cells from sodium hypochlorite. Indeed, when monolayer cells were treated with a 0.3% sodium hypochlorite solution, all cells died. The modified method (described in the Materials and Methods) showed excellent results in the cell cultures of human samples such as oral tissues and digestive organ tissues (unpublished data). In these cases, an aproximately 3 mm piece of cubic tissue was washed with a 0.6% sodium hypochlorite solution, and then with a solution of hypochlorite whose concentrations were reduced to 0.06% and 0.006% in a stepwise manner. Finally, the tissue was washed with PBS. The hypochlorite solution at different concentrations was stirred to wash tissues by a vortex mixer for 30 sec. After that, the tissue was minced, trypsinized and inoculated in culture dishes. These results indicate that our methods can be applicable for the cell cultures of animals which live with microorganisms.

When tissue cultures of aquatic animals are started in vitro, microorganisms should be removed from the tissues. To remove bacteria, the animals are usually kept in the water containing antibiotics before tissue sampling, although fungi can not be removed by antibiotics. On the other hand, our developed method using NaClO can start cell culture without a long time pretreatment of aquatic animals with antibiotics.

GAKS cells have already been used to investigate the effects of cytotoxic compounds on fish cells (Saito *et al*., 1991a; Saito *et al*., 199b; Saito *et al*., 1993). Therefore, GAKS cells are useful as a preliminary tool for the assessment of chemical risks to aquatic environments, and as a detecting tool of pollutants in aquatic enviroments. In addition, we used this cell line to investigate changes in the cellular amino acid composition during biological evolution (Sorimachi, 1999). The fish scale cell line (GAKS) established in the present study secrets endothelin. Endothelins produced by keratinocytes play an essential role in the maintenance of melanocyte proliferation and pigmentation in human (Imokawa *et al*., 1992, 1995). Thus, endothelin production from GAKS cells suggests that the similar effect of endothelins on melanocytes occurs in fish. To our knowledge, this is the first report that shows endothelin secretion from cultured fish cells.

Thus, the GAKS cell line would be a good model system to investigate the biochemical functions of fish cells.

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