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Isolation, culture and characterization of a primary fibroblast cell line from channel catfish

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Abstract

A primary cell line (designated as CCf) derived from caudal fin tissue of channel catfish, *Ictalurus punctatus*, was developed using explant techniques. The cell line grew fastest in media supplied with FBS and channel catfish serum. The duplication time of the cell line under optimal conditions was ∼56 h at a plating density of 1.1×10^5 cells/ml. The cell line has been propagated continuously for 25 passages (1:4 dilution per passage), cryopreserved, and recovered successfully at different passages. The cultured cells had fibroblastic morphology, and synthesized fibronectin and Type I and III collagens in the cytoplasm. The cell line maintained the normal diploid chromosome number (58) of channel catfish throughout the experiment. Nucleolus organizer regions were located on the short arms of a pair of medium-sized submetacentrics, which is typical for channel catfish. This study provides a method for acquiring a cell line from juvenile catfish without sacrifice, and is especially useful for early screening of valuable fishes.

Introduction

Channel catfish (*Ictalurus punctatus*) has become the most important food fish species cultured in the United States (Stickney, 1993). Research topics for this species have included disease (Thune *et al*., 1993), physiology (Kang and Caprio, 1995) and genetic improvement (Wolters, 1993). Techniques for short-term culture of catfish cells have been reported to support these studies (e.g. Wohlschlag *et al*., 1989), and several cell lines have been developed for long-term use. The first continuous cell line from channel catfish was an ovary cell line that has been used for about 2 decades in the diagnosis of catfish viruses (Bowser and Plumb, 1980). Leukocyte cell lines, including monocyte-like cell lines (Vallejo *et al*., 1991) and B cell lines (Miller *et al*., 1994b), have been developed and used to demonstrate immune functions in catfish.

Compared with the ovary cell and leukocyte lines, fibroblast cells can be sampled at embryonic stages and provide material for rapid and early genetic screening. In addition, fibroblast cells yield high-quality chromosomes in fish species (Amemiya *et al*., 1984). In our laboratory, we have developed a fibroblast cell line to support cytogenetic and molecular genetic studies. The objectives of this study were to develop a fibroblast cell line from caudal fin tissue of channel catfish, to optimize culture conditions, to maintain the cell line by subculture and cryopreservation, and to characterize the cell line by immunocytochemistry and chromosomal analysis.

Materials and methods

Solutions and culture media

The washing solution was composed of Ca^{2+} and Mg^{2+}-free phosphate buffered saline (CMF-PBS), 100 units/ml of penicillin (Gibco BRL, Life Technologies Inc., Gaithersburg, MD), 100 μg/ml of streptomycin (Gibco), and 100 μg/ml of gentamicin (Gibco). Three different basal culture media were prepared in this study. The A/L basal medium was composed of a 1:1 mixture of Leibovitz L15 and AIM V (Gibco), 100 units/ml of penicillin, 100 μg/ml of streptomycin, 10 μg/ml of gentamicin, and 1 mg/ml of $NaHCO_3$. The L15 basal medium was composed of Leibovitz L15 medium, 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 10 μg/ml of gentamicin. The MEM basal medium was composed of Eagle MEM medium (Gibco), 100 units/ml of penicillin, 100 μg/ml of streptomycin, 10 μg/ml of gentamicin, and 25 mM Hepes buffer (Sigma Chemical Company, St. Louis, MO). All solutions and media were adjusted to the osmotic pressure of catfish blood plasma ($\sim$270 mOsm/kg) by addition of distilled H_2O. Preparation of channel catfish serum (CCS) was based on procedures described previously (Miller *et al*., 1994a).

Generation of a primary cell line from fin tissue by the explant technique

Healthy channel catfish (body weight: 1–50 g) were used in this study. The surface of the fish was sterilized by washing with 10% bleach followed by swabbing with 70% ethanol. The terminal edge of the caudal fin was removed, placed in a petri dish filled with washing solution, and cut into 1-mm^2 pieces. The tissue fragments were transferred into a 20-ml glass tube and rinsed 3 times for 20 min each with washing solution. At the end of each rinse, the solution was decanted and discarded. Tissue fragments were digested with 0.25% trypsin solution at 4 °C overnight.

After digestion, the tissue was centrifuged (Beckman® model TJ-6 centrifuge, Palo Alto, CA) at 350 × g for 10 min, and the supernatant removed. Cold A/L complete medium, composed of A/L basal medium and 10% fetal bovine serum (FBS) (Gibco), was added to the tube, and digested tissue fragments were placed into each well of a 6-well culture plate (Falcon Plastics, Becton Dickinson Inc., Franklin Lakes, NJ). A coverslip (22 × 22 mm) was placed on the tissues to enhance attachment. Prewarmed A/L complete medium (2.5 ml) was added drop by drop to each well. Cultures were incubated at 27 °C in a humidified environment (VWR 1820 water jacket incubator, VWR Scientific, Sugarland, TX) supplied with 5% CO_2.

Epithelial-like and fibroblast-like cells grew from the tissue fragments 48 h to 72 h after seeding. Monolayers were rinsed with washing solution at day 5, and fresh medium was added. Once cells grew to $\sim$50% confluence, monolayers were trypsinized and the cells pooled and cultured in 25-cm^2 flasks (Falcon). After the first passage, the cultures were digested with 0.05%, 0.01%, or 0.25% trypsin when they reached 95% confluence.

Cell line subculture

The cell line developed as described above was designated as CCf (channel catfish fin tissue) and maintained as follows. The cultures were divided 1:4 into subcultures once cells reached confluence. After examination for sterility, flasks were placed in a laminar flow hood and the outer surfaces of the flasks were sterilized with 70% ethanol. Spent medium was poured off, the monolayer rinsed twice briefly with 3 ml CMF-PBS, and 5 ml of 0.25% trypsin solution (27 °C) was added. Four ml of trypsin solution was withdrawn, leaving 1 ml in the flask. Most cells detached within 5 to 15 min at room temperature. Three ml of L15 complete medium (27 °C) were added, and cell clumps were dissociated by pipetting. One ml of cell suspension was transferred to a fresh flask, and 4 ml of fresh L15 complete medium was added. Flasks were sealed and maintained at 27 °C. The cells reached confluency within 4 d. A sterility test was conducted at passage 20, in which cells were treated as described above except antibiotic-free solution or medium was used.

Effects of basal medium and serum supply

After transfer to 25-cm^2 flasks, cells were passed two more times before use in an optimization study. A 3 × 5 factorial arrangement was designed for identifying optimal growth conditions, with basal media and serum supplementation as the two factors. Each treatment included four replicates. Cells were seeded (at 2.4×10^4 cells/well) in each well of three 24-well plates, one for each medium type. Cells were grown at 27 °C in a humidified environment with 5% CO_2 (for A/L and MEM medium) or without CO_2 (for L15 medium), and cultures harvested 5 d after plating.

Viability and cell concentration were calculated

by counting live and dead fibroblasts using a dye-exclusion method. Cell samples from each treatment were diluted and stained with 0.1% trypan blue, and counted within 20 min in a hemacytometer (Fisher Scientific, Pittsburgh, PA) at 100-x magnification using phase-contrast microscopy (Optiphot-2, Nikon Inc., Garden City, NY).

Cell concentrations were analyzed by two-way analysis of variance with basal medium (L15, A/L, or MEM) and serum type (5% CCS, 10% CCS, 5% CCS, 5% FBS, 5% FBS, or 10% FBS) as the two factors. Duncan's multiple means comparison was used to identify differences among treatments, which were considered significantly different at $P < 0.05$.

Growth at different plating cell densities

Cells at passage 3 were cultured in L15 medium supplied with 5% CCS and 5% FBS as a result of the optimization study. Fibroblasts were trypsinized at confluence, diluted to 1.1×10^5 cells/ml, 0.7×10^4 cell/ml, or 1.0×10^3 cell/ml, and seeded into three 24-well plates (Costar Corp., Cambridge, MA), one at each cell density, with 1 ml per well. After 24 h at 27 °C, cells in three wells of each plate were counted. The plates were returned to the incubator immediately after removing cell samples. Samplings were repeated at 24-h intervals for 5 d, and thereafter once every 2 to 4 d until day 14. Medium was changed when pH dropped below 7.

Cell storage and recovery

The monolayers were trypsinized when they reached ∼90% conflunce, diluted with 5 ml of cold L15 complete medium, and cell suspensions transferred to 15-ml tubes. After centrifugation, pellets were resuspended in 2 ml of cryopreservation medium (70% L15 basal medium, 20% FBS, and 10% DMSO), and transferred to sterile cryovials (Corning Inc., Corning, NY) in 1-ml aliquots. Cryovials were wrapped with cotton, covered with foil, and placed in a –80 °C freezer overnight. Frozen cryovials were transferred to liquid nitrogen or left at –80 °C until use.

For recovery, the cryovials were removed from the liquid nitrogen or freezer, dipped immediately into a beaker filled with 37 °C water, and shaken until ice crystals disappeared. Vials were opened aseptically and the cell suspensions transferred to 15-ml tubes. Cells were diluted with 10 ml of pre-cooled L15 basal medium (4 °C) with constant stirring. After centrifugation, the pellet was resuspended in 1 ml of pre-warmed L15 complete medium, transferred immediately to a 25-cm^2 culture flask, and incubated with additional 4 ml of pre-warmed (27 °C) L15 complete medium.

Light microscopy

Cells in culture were observed with a phase-contrast inverted microscope (Diaphot-TMD, Nikon Inc.). Cells were grown on sterile, coated slides (SuperCell™ Slide, Erie Scientific Company, Portsmouth, NH), fixed in 10% neutral buffered formalin and stained for 3 to 5 min with hematoxylin.

Immunocytochemistry

The second passage of cells was grown on coverslips to ∼70% confluence, rinsed twice with 0.01 M CMF-PBS (pH 7.4), and fixed for 10 min with 10% buffered formalin (pH 7.0). The cells were rinsed twice with PBS and incubated in 0.2% Triton X-100 for 5 min. Coverslips were rinsed twice with PBS, immersed in 0.3% H_2O_2 for 5 min, rinsed twice with PBS and incubated in 20% colostrum-free bovine serum (CFBS) (Sigma) for 30 min at room temperature.

Coverslips were incubated with 100 μl of diluted (1:40) rabbit anti-rat monoclonal antibody against the following components: Type I collagen (Chemicon, Temecula, CA), Type III collagen (Chemicon) and fibronectin (ICN Immuno Biologicals, Costa Mesa, CA). For control treatments, either the primary antibody was omitted or non-immune serum was substituted for the primary antibody. Incubations were performed at room temperature for 1 h. Coverslips were rinsed 3 times with PBS for 5 min each, and the secondary antibody, peroxidase-conjugated goat anti-rabbit antibody (ICN), was added to each coverslip, and incubated at room temperature for 1 h. The coverslips were rinsed 3 times with PBS for 5 min each. The coverslips were finally stained with diaminobenzidine (DAB), prepared by adding 0.6 mg DAB and 10 μl of 3% H_2O_2 to 1 ml of PBS.

Chromosome analysis

Cultured cells, maintained under the same conditions as described above for subcultures, were used for preparation of chromosomes at passages 3, 7, 11, 17, and 21. Twenty μl of colchicine solution (100 μg/ml in CMF-PBS) were added to each 25-cm^2 flask when cultures reached exponential growth (about 70% confluence), and cultures were incubated for 1 h. The

monolayer was trypsinized and cell masses were dissociated by repeated pipetting. Hypotonic treatment (0.56% KCl) and cold fixation (3:1 ethanol-acetic acid) were based on procedures used for cultured leukocytes (Wolters *et al.*, 1981).

For evaluation of general morphology, metaphase spreads were stained for 10 to 15 min with 5% Giemsa solution (freshly prepared in 0.01 M phosphate buffer, pH 6.8). Silver staining (Howell and Black, 1980) was used to reveal the nucleolus organizer regions (NOR). A 50% silver nitrate and 2% gelatin solution was added (2:1; v/v) to each slide, which was covered with a coverslip (22 × 60 mm), and incubated at 50 °C until a bright, golden color developed (about 6 to 10 min). Slides were rinsed briefly with deionized water, and dried at room temperature.

Fifty metaphase spreads for each passage were used to develop frequency distributions of diploid numbers. A chi-square test of homogeneity was used to analyze the percentage of cells with a modal diploid number (2N = 58) at different passages. Treatments were considered significantly different at $P < 0.05$.

Results

Cells began to grow out from explanted fin tissue on day 3. Cultures reached ∼40% to 60% confluence between days 7 and 12, and did not expand significantly after this period. Therefore, cells were not left in culture for more than 15 d. Coverslips facilitated the attachment of tissue fragments, although the coverslips interfered with subsequent rinsing steps, and may have reduced monolayer expansion. Thus, coverslips were removed after 7 d. Differential detachment separated fibroblast-like cells from epithelial-like cells; fibroblast cells generally came off first after trypsinization. However, the epithelial-like cells disappeared after 3 passages without differential digestion with trypsin.

Cells were confluent 3 to 5 d after being transferred to culture flasks. Cell attachment and propagation in fresh flasks were enhanced when the subcultures were carried out immediately prior to confluence. However, cells could remain viable for more than a month without a change of medium. The cell line was found to be sensitive to trypsinization. Prolonged exposure (30–45 min) to trypsin caused the failure of subcultures. The trypsinization of the monolayers was most successful at room temperature. Bacterial and fungal infections were not detected in the cell line at passage 20.

Table 1. Cell densities of viable cells[a] of each treatment in different basal media and serum types[b].

Media	Number of replicates	Concentration[c] ($\times 10^4$cells)
Basal medium		
A/L	46	9.8 ± 13.4 A
L15	45	9.9 ± 13.3 A
MEM	51	9.2 ± 13.4 A
Serum type		
5% CCS	27	9.4 ± 1.3 D
10% CCS	27	0 D
5% CCS + 5% FBS	27	34.6 ± 8.0 A
5%	30	3.5 ± 0.7 C
10% FBS	31	10.3 ± 4.0 B

[a] Viability was 100% in all treatments.
[b] Cells were cultured for 5 d.
[c] Mean ± SD; values sharing a letter were not significantly different ($P > 0.05$).
Abbreviations: FBS, fetal bovine serum; CCS, channel catfish serum.

Cell viability was 100% for all media, and no significant differences were observed in cell densities among the three different basal media ($P = 0.95$). However, significant differences were observed in cell densities among cultures grown in media with different serum types ($P = 0.0001$). There was no interaction between basal medium and serum supply ($P = 0.77$). Cells grown in media with no FBS did not attach after culture for 5 d (Table 1). The cultures in media supplied with 5% CCS and 5% FBS showed the fastest increase in cell number.

Cultures at an initial density of 1.0×10^5 cells/ml had a doubling time of between 48 h and 72 h (Figure 1). Exponential growth was observed between days 2 and 5, followed by a brief decline between days 5 and 6. Growth was resumed at day 7, followed by another period of exponential growth between days 10 and 13. Cultures with an initial density of 0.7×10^4 cells/ml did not show an increase in cell numbers until day 6. Lag-phase growth was observed between days 6 and 13. Exponential growth began on day 14. No growth was observed in cultures with an initial density of 1.0×10^3 cells/ml during the examination period (14 d).

The cryopreserved cells had a recovery of as high as 100% viability after frozen storage for 1 week. Survival was ∼50% to 70% after 3 months at –80 °C. Cells stored in liquid nitrogen had a recovery of 70% to 100% after 3 months. Successful recovery was indicated by cell attachment in flasks within 2 h.

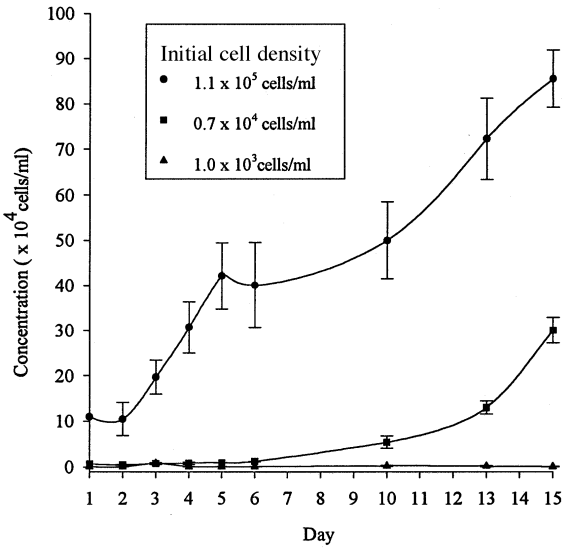

Figure 1. Growth of cultured fibroblast cells at three plating densities. Vertical bars indicate standard deviations.

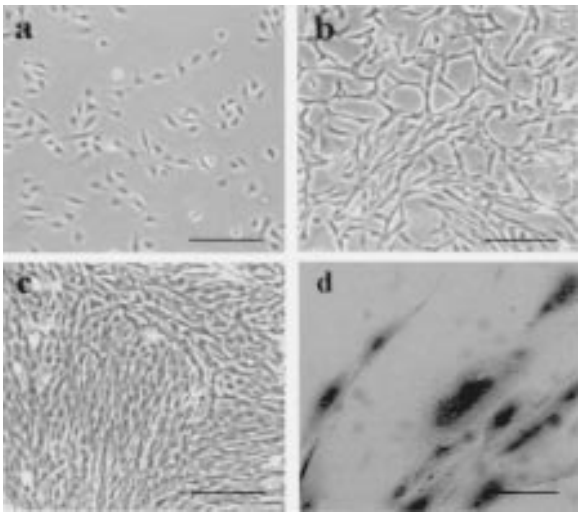

Figure 2. Phase-contrast microscopy of CCf cells examined after culture for 2 h (a), 36 h (b), and 84 h (c) (Bar = 100 μ). Cells were fixed with 10% neutral buffered formalin and stained with hematoxylin. Nuclei of fibroblast cells were located at the centers of cell bodies (d) (Bar = 50 μ).

After the line was established, cells appeared spindle-shaped (Figures 2a, 2b and 2c) when the cultures were not confluent. Cells were oriented randomly; the elongation of processes at each pole developed gradually until contact with processes of adjacent cells. The egg-shaped nuclei were located at the center of the cell bodies (Figure 2d).

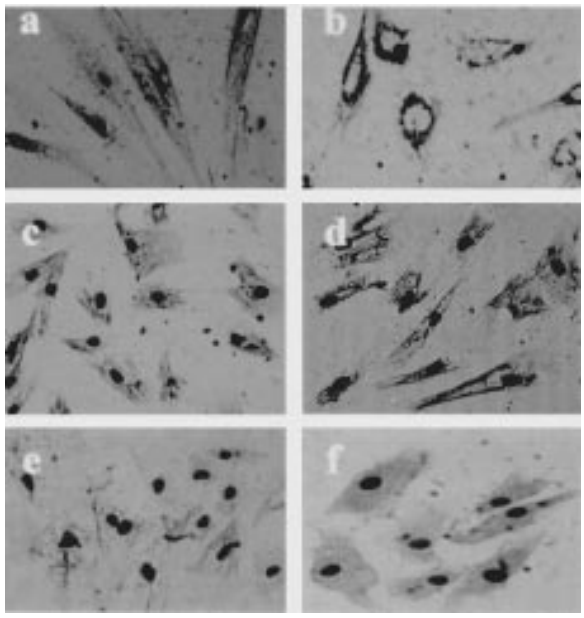

Figure 3. Immunochemical staining for fibronectin (a and b), Type I collagen (c) and Type III collagen (d). Negative staining for these cell components was observed when non-immune anti-sera were used to replace the specific antibody in the experiment (e), and when antibodies were omitted (f) (x250).

Immunochemical staining for fibronectin demonstrated positive cells (Figure 3a and 3b), with staining localized to filamentous structures, but mostly concentrated around the nuclei. The immunochemical staining for Type I collagen (Figure 3c) and Type III collagen (Figure 3d) was heavily positive, and in each case the staining was cytoplasmic and concentrated on filamentous structures. No staining was observed on slides treated with non-immune primary antibody (Figure 3e), which demonstrated that the staining was specific to fibronectin, and Type I and Type III collagens. No staining was found on slides prepared without anti-sera (Figure 3f).

The diploid chromosome numbers of cells from individual passages were counted, and the data combined for analysis of frequency distribution (Figure 4, top; Figure 5a). The modal diploid (2N) number of the cultured fibroblast cells was 58. The percentage of cells with the modal chromosome number was between 57% and 64% at different passages. No significant difference ($\chi^2 = 0.5$, df = 4; $P > 0.05$) was found among the passages examined (Figure 4, bottom). Nucleolus organizer regions were located on the short arms of a pair of medium-sized submetacentric chromosomes (Figure 5b).

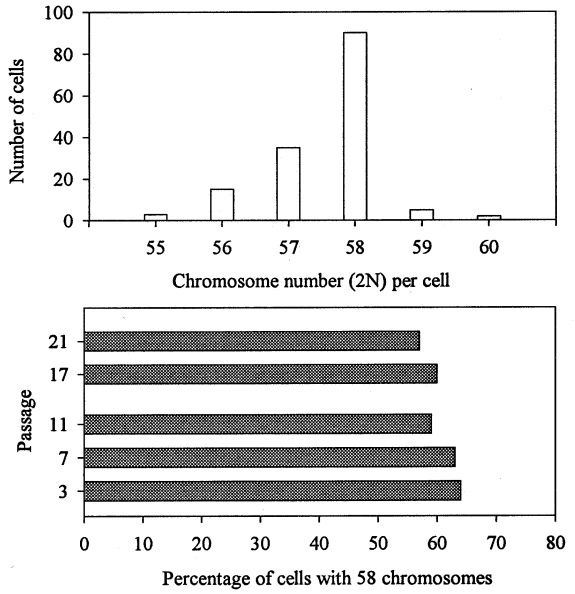

Figure 4. Chromosome analysis of the CCf cell line. Top, frequency distribution of diploid numbers (2N) of cells from all of the passages examined. Bottom, percentage of cells with modal (2N = 58) chromosome number at different passages (χ^2 = 0.5, df = 4; $P > 0.05$).

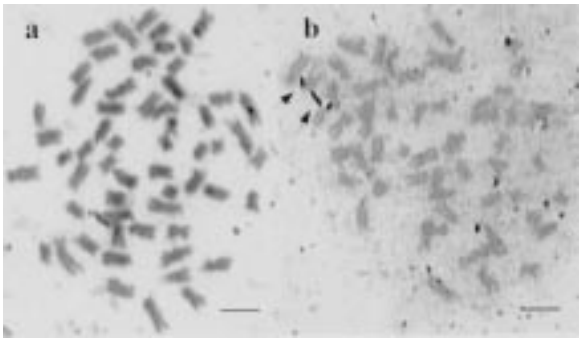

Figure 5. Representative metaphase spread prepared from the CCf cell line: Giemsa staining (a) and silver staining (b). Arrowheads indicate the location of the nucleolus organizer regions (NOR). Bar = 5μ.

Discussion

In this study, we present methods for generating a primary fibroblast cell line from channel catfish, and the conditions for long-term maintenance. The cell line was characterized by immunochemical and cytogenetic techniques. With minor modifications of this method, we were able to culture fibroblast cells from other catfish species of the family Ictaluridae such as flathead catfish (*Pylodictis olivaris*), black bullhead catfish (*Ameiurus melas*), and the hybrid offspring of these species with channel catfish (data not shown).

Attachment of explanted tissue to culture vessels is a critical step for primary cultures (Freshney, 1994). Common treatments include repeated trypsinization for increasing surface adhesion of tissue fragments (Noga, 1980), or the application of physical force such as with a coverglass (Avella *et al*., 1994). Each treatment was helpful in the present study, although channel catfish fibroblast cells were found to be sensitive to excessive trypsinization. Tissue fragments digested at 37 °C for 1 h did not yield viable cells for attachment and growth. The same phenomenon was observed in subsequent propagation of the cell line, in which monolayer cells treated with warm trypsin solution (37 °C) failed to attach and grow.

All of the basal media used were modifications of media designed for use with cells of other animals, because medium specific for culture of catfish cells has not been developed. The most widely used media, such as Dulbecco's modified Eagle medium (DMEM), have been demonstrated to be effective for culture of catfish ovary cells (Bowser and Plumb, 1980) and hepatocytes (Wohlschlag *et al*., 1989). More recently, a serum-free medium, AIM V, has been used successfully with Leibovitz's L15 medium for culture of different leukocyte cell lines of channel catfish (Miller *et al*., 1994a). In our study, we found L15 medium could also support the growth of fibroblast cells. The cells were cultured with L15 medium in sealed flasks, and did not require CO_2 or a humidified environment. Therefore, L15 medium would seem to be most suited for culture of the CCf cells because of convenience and low cost.

Serum supplementation is critical for attachment and growth of the CCf cells. Fetal bovine serum is commonly used in the establishment of continuous fish cell lines derived from tissue explants (Fryer and Lannan, 1994), including ovary cells of channel catfish (Bowser and Plumb, 1980). However, FBS was not found to support the proliferative response of catfish leukocytes (Miller and McKinney, 1994), while CCS did. In the present study, FBS and CCS were each found to be important for culture of CCf cells: FBS was indispensable for attachment of tissue fragments and subsequent propagation of the CCf cell line, and CCS provided enhanced cell growth. Other factors such as flask type did not influence the attachment of cells. The CCf cells grew most effectively at plating concentrations of 1.0×10^5 cells/ml, while cells dispensed at 1.0×10^3 cells/ml failed to grow.

Fibroblasts can synthesize and secrete fibronectin (Hynes and Yamada, 1982) and collagen (Porter and Pappas, 1959). When CCf cells were permeabilized with Triton X-100, there was an intense staining of fibronectin and collagen in the cytoplasm surrounding the nucleus. Perinuclear staining observed for fibronectin in CCf cells was similar to that seen in chick fibroblast cells (Yamada, 1978), and the perinuclear staining for collagen parallels that seen in permeabilized cells of rat dental follicles (Wise *et al*., 1992) and human fibroblasts (Gay *et al*., 1976).

The diploid chromosome number of channel catfish has been reported to be 58 (Wolters *et al*., 1981), and has been verified by intergeneric hybridization (LeGrande *et al*., 1984; Zhang and Tiersch, 1997). The CCf cells maintained a modal diploid chromosome number of 58. The frequency of cells with this modal number varied from 64% at passage 3 to about 58% at passage 21, percentages typically observed in karyotyping of fish from natural populations (LeGrande *et al*., 1984; Zhang and Tiersch, 1997). More significantly, each metaphase spread had one pair of submetacentric NOR-bearing chromosomes, which was found to be a representative feature of the channel catfish genome (Zhang and Tiersch, 1997). The ploidy and chromosome morphology of the CCf cell line could change after more passages, as observed in the channel catfish ovary cell line (Bowser and Plumb, 1980); however, this remains to be determined.

The CCf cell line has been used in genetic studies including replication banding in which cultured cells provided control of timing for blockage and release of DNA synthesis, and convenience for subsequent removal of chemicals and rinsing (Zhang, 1996). The CCf cell line was used to prepare chromosomes for study of sister-chromatid exchange (Zhang, 1996). Another use of this cell line included providing a model for *in vitro* expression of transgenes for channel catfish. Fibroblast cells are less differentiated, and therefore can accomodate and express foreign genes more easily than specialized cell lines such as B-cells (Bouchard *et al*., 1989).

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