Fish & Shellfish Immunology 49 (2016) 442–449

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Development and characterization of a cell line from tilapia head kidney with melanomacrophage characteristics

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ARTICLE INFO

Article history:
Received 21 October 2015
Received in revised form
7 January 2016
Accepted 11 January 2016
Available online 13 January 2016

Keywords:
Cell line
CD markers
Cichlid
Cytochemistry
Hematopoietic
Macrophages
Melanomacrophage
Monocytes

ABSTRACT

A novel cell line THK, derived from the tilapia head kidney, was developed and characterized. The THK cell line comprised fibroblastoid cells that markedly proliferated in Leibovitz L-15 medium containing 2%–15% fetal bovine serum (FBS) at 20 °C-35 °C. Cell proliferation was dependent on the FBS concentration, and the optimal temperature for proliferation ranged between 25 °C and 30 °C. THK cells were characterized for the presence of phagocytic activity, acid phosphatase, alkaline phosphatase, α-naphthyl acetate esterase, lipofuscin, and tyrosinase. Transcripts of CD33, CD53, CD82, CD205, macrophage colony stimulating factor receptor, GATA2, and GATA3 that are specific for leucocytes or monocytes/macrophages or both were detected in the THK cells through PCR. However, THK cells lacked for CD83, a specific marker for dendritic cells. The results indicated that the fibroblastoid THK cells were melanomacrophage-related progenitors. PCR revealed that the THK cells exhibited the transcripts of toll-like receptor 1 (TLR1), TLR2, TLR3, and CD200, of which concern with immunity as well as the transcripts of vascular endothelial growth factor receptor 3, angiomotin, and angiopoietin-like protein 2 that associate with angiogenesis regulation and macrophage proliferation. THK cells were subcultured more than 90 times and can be useful for investigating the development and functioning of the teleostean innate immune system.

1. Introduction

The head kidney (HK) of tilapia (*Oreochromis niloticus*) and other teleost fish is a hematopoietic organ similar to the mammalian bone marrow [1–3]. Teleostean HK comprises a reticulo–endothelial stroma containing endothelial cells, fibroblastoid reticular cells, and macrophage-like reticulum cells and a parenchyma containing blood cells and melanomacrophage (MMΦ) centers [4–6]. The MMΦ centers containing pigmented MMΦs and nonpigmented cells are encapsulated in fibroblastoid reticular cells [4] and are analogous to the mammalian lymphoid germinal centers [7]. MMΦ development is associated with chronic inflammation, infection diseases, and polluted water as well as with tissue breakdown and erythrocyte catabolism [7–9]. In addition to the kidney, MMΦ centers also appear commonly in the teleostean liver and spleen, and occasionally in the gill, brain, and gonad [10].

MMΦs are suspected to be a subpopulation of senile macrophages that have ingested foreign materials and then aggregate to form MMΦ centers [4]. MMΦs contain abundant pigments such as hemosiderin, melanin, and lipofuscin [7], and exhibit ultrastructural and cytochemical features similar to those of fibroblast-like reticular cells [4]. Within MMΦs, lipofuscin generally appears to be the most abundant pigment, and melanin is often—but not always—another major component. However, the appearance may vary among species. Lipofuscin has been reported to be the major pigment in the kidney MMΦs of goldfish (*Carassius auratus*) [11], platyfish (*Xiphophorus maculates*) [12], and blue tilapia (*Oreochromis aureus*) [13]. However, Mozambique tilapia (Oreochromis *mossambica*) MMΦs lack lipofuscin [8], and hemosiderin is the primary pigment in the pearl gourami (*Trichogaster leeri*) MMΦs [12]. Therefore, MMΦs can be Sudan lack B (SB) stain positive [14] or negative [4]. Because the pigment granules are often in vacuoles, they are suggested to be foreign materials that ingested by macrophage. Nevertheless, tyrosinase-associated melanogenesis has been discovered in the MMΦs of Atlantic salmon (*Salmo salar*) [15], gilthead bream (*Sparus auratus*) [16], and barramundi (*Dicentrarchus labrax*) [17]. The SHK-1 cell line derived from the Atlantic salmon HK also exhibits macrophage and

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http://dx.doi.org/10.1016/j.fsi.2016.01.013
1050-4648/

melanogenesis activity [18].

MMΦs, as a subtype of macrophages, share many characteristics with macrophages, e.g. they are phagocytic and exhibit the macrophage colony stimulating factor receptor (MCSFR), acid phosphatase (ACP), and α-naphthyl acetate esterase (ANAE). Despite MMΦs have been reported lack of alkaline phosphatase (ALP) [7,14,19], ALP has shown in the MMΦs of blue tilapia, barramundi and gilthead seabream [3,4,13,20]. Clonal assays show the carp (*Cyprinus carpio*) kidney hematopoietic tissues contain macrophage and T cell common progenitors [21].

CD markers in mammals are useful for identifying hematopoietic cells. CD34 is expressed in hematopoietic stem cells (HSCs), and CD33 expresses specifically in myelomonocytic cells including dendritic cells (DCs), monocytes, and macrophages, and the expression is more prevalent in progenitors than in mature granulocytes or macrophages [22]. CD53 expressing in monocytic and B cells is a proinflammatory marker for macrophages [23]. CD82 is used as an activation marker of mononuclear cells [24]. CD83 expression is primary in $CD34^+$ myeloid and lymphoid progenitor cells [25] and T-cell membranes [26], and has been used as a DC marker [27]. CD205 with a high level of expression can also be a marker for myeloid DCs [28]. CD115 (MCSFR) is the specific marker for monocytes and macrophages [29], whereas CD146 (MUC18) is specific to endothelial cells [30]. By contrast, CD200 is a ubiquitously expressed cell-surface molecule immunoregulatory molecule [31]. Teleostean monocytic cells may have CD expressions similar to those of their mammalian counterparts.

GATA transcription factors play a key role in controlling hematopoietic cell differentiation. GATA1 is associated with the differentiation of erythrocytes, megakaryocytes, mast cells, DCs, basophils, and eosinophils; by contrast, GATA2 maintains and promotes the proliferation of early hematopoietic progenitors, and GATA3 is confined to specific lymphocyte populations [32]. GATA6 has a marked association with the proliferative renewal ability of macrophages [33], and GATA4 induces GATA3 expression and T cell development [34]. GATA4 and GATA6, but not GATA3, proteins have been observed in monocytes [35]. GATA2 and GATA3 expressions are higher in macrophage progenitor cells than in macrophages in goldfish [36]. GATA markers could be useful for clarifying the identity of culture cells from teleostean HK.

After cyprinids, tilapia and other cichlids are the most productive and internationally traded freshwater fish, with Nile tilapia being the most crucial tilapia [37]; however, no myeloid cell line has been developed. The present study reports the establishment and characterization of a continuous myeloid cell line (THK) from Nile tilapia HK. The cells are associated with MMΦs, because they exhibit macrophage characteristics, lipofuscin, and tyrosinase. Adiponectin [38] and the toll-like receptor (TLR) [39] are associated with cell innate immunity; therefore, transcripts of adiponectin receptor protein 2 (ADIPOR2), TLR1, TLR2, and TLR3 in THK cells were examined. Moreover, expressions of endothelial growth factor receptor 3 (VEGFR3), angiomotin (AMOT), and angiopoietin-like protein 2 (ANGPTL2) were tested because they can regulate angiogenesis [40,41]. Furthermore, the effect of temperature and serum on cell growth was analyzed.

2. Materials and methods

2.1. Cell culture

Nile tilapias, approximately 10 cm in length, were obtained from a local farm. The fish were anesthetized with MS-222 (Sigma) and aseptically decapitated. The HK was aseptically removed and finely sliced using scissors in phosphate-buffered saline (PBS). Subsequently, the tissue fragments were washed several times with PBS containing streptomycin (500 μg/mL) and penicillin (500 IU/mL). The fragments were transferred to 25-cm^2 cell culture flasks (Nunc). In each flask, 2 mL of Leibovitz L-15 medium supplemented with 15% fetal bovine serum (FBS, HyClone) was added, and the cells were incubated at 25 °C. Every 4–6 d, half the medium was removed and replenished with fresh medium until passage. The primary culture cells were subcultured 1 mo after culture initiation using trypsin-EDTA. Subsequent subculturing was performed at intervals of 5–6 d. The cell line was designated THK. The FBS concentration in the growth medium was reduced to 10% after 20 passages, and the cell line was cultured for more than 90 passages since its initiation in January, 2012.

2.2. Effect of temperature and FBS on cell growth

The effect of temperature and FBS concentration on THK growth was studied at the 50^{th} passage level. The cells (2.5×10^5/mL) were inoculated in cell culture flasks and incubated at 15 °C, 20 °C, 25 °C, 30 °C, and 35 °C. The number of cells from triplicate flasks at each temperature was counted using a Scepter cell counter (Millipore) after 5-d incubation. Similarly, the effect of various FBS concentrations (2%, 5%, 10%, and 15%) on cell growth was assessed at 25 °C.

2.3. Phagocytosis

The phagocytic activity of THK (65th passage 2 d incubation) was examined using deep blue-dyed latex beads (mean diameter, 0.8 μm; Sigma) under a Zeiss inverted fluorescent microscope with phase optics. A bead suspension was prepared by adding 2 μL of the commercial bead preparation (10% solid latex) to 5 mL of growth medium. The bead suspension replaced the regular growth medium in the investigated cultures. At various time points after adding beads (up to 24 h), the cells were rinsed 4 times with PBS, detached by trypsin-EDTA, suspended in the growth medium, and placed on coverslips to remove noningested beads. On the next day, the cells were fixed in 4% formaldehyde solution and counterstained with DAPI (Sigma–Aldrich) and visualized under an Axiovert 200 fluorescence microscope using ApoTome and AxioVision software (Carl Zeiss).

2.4. Lipofuscin staining and enzyme cytochemistry

For lipofuscin staining, THK cells at passage level of 85th were incubated at 30 °C for 1 and 10 d after subculture, fixed in 4% formaldehyde solution, and stained for 2 h in saturated SB solution.

ACP, ALP, β-glucuronidase (BG), and ANAE staining kits (Sigma–Aldrich) were used for the enzyme analyses. THK cells at passage levels 54th and 56th were cultured on 24-well plates. After 2 d incubation at 30 °C, the cells were fixed in citrate-acetone-formaldehyde fixative solution (25 mL citrate solution, 65 mL acetone, and 8 mL 37% formaldehyde) for 30 s, and the plates were rinsed thoroughly in distilled water. The enzyme substrate solution was prepared according to the staining procedure inside the kit box. THK cells were incubated with the substrate solution at 30 °C for 1 h. To test the tartrate resistance of ACP, tartrate solution was added to the ACP substrate solution. Tyrosine-DOPA reaction [42] was used to examine tyrosinase in the THK cells. THK cells (75th passage and 10 d incubation) after fixation were incubated in 0.1% L-DOPA (Sigma–Aldrich) in PBS at 30 °C for 3 h for producing melanin (by tyrosinase). The DOPA medium was refreshed every 0.5 h to avoid auto-oxidation. GBC1, a transform cell line derived from grouper brain [43], was used as negative control.