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Macrophage colony stimulating factor (CSF-1) is a central growth factor of goldfish macrophages

Patrick C. Hanington [a], Steven J. Hitchen [a], Leigh A. Beamish [a], Miodrag Belosevic [a,b,*]

[a] Department of Biological Sciences, CW-405 Biological Sciences Building, University of Alberta, Edmonton, AB, T6G 2E9 Canada
[b] Department of Public Health Sciences, University of Alberta, Edmonton, Alberta, Canada

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ABSTRACT

We recently characterized macrophage colony stimulating factor (CSF-1) of fish (the goldfish). Here, we report for the first time that goldfish CSF-1 acts through the CSF-1 receptor by showing loss of CSF-1 function in CSF-1R knockdown monocytes using RNAi, and demonstrate that goldfish CSF-1 administration *in vivo* increases the amount of circulating monocytes in blood. We also show that conditioned supernatants from goldfish fibroblast cultures induced the proliferation of goldfish monocytes indicating that, like in mammals, teleost fibroblasts are an important producer of CSF-1. The continuous addition of recombinant CSF-1 to primary goldfish macrophage cultures stabilized and extended their longevity and resulted in a long-term culture of functional macrophages capable of mounting a potent nitric oxide response upon activation with goldfish recombinant TNF-α.

1. Introduction

From an evolutionary perspective, macrophages and their functions are quite conserved and it is not surprising that macrophage-like cells can be found in almost all multicellular organisms. Mammalian macrophage development is under the control of numerous growth factors. Among them, macrophage colony stimulating factor (CSF-1) is critical for proper macrophage development. Until the recent functional characterization of a goldfish CSF-1 molecule [1] and the identification of CSF-1 transcripts in the zebrafish and rainbow trout [2], the only evidence that the CSF-1-regulated pathway of macrophage development existed in organisms other than mammals was the presence of CSF-1 transcripts in the chicken [3,4] and CSF-1-like activity observed in bony fish [5–7]. The hypothesis that CSF-1 existed in lower vertebrates was also supported by the identification of CSF-1 receptor (CSF-1R) in the goldfish [6], puffer fish [8], rainbow trout [9], zebrafish [10] and the gilthead sea bream [11]. Studies focused on characterizing the functional roles of CSF-1 in goldfish macrophage development indicated that like in mammals, goldfish CSF-1 was important in the proper formation of functional monocytes/macrophages.

In contrast to mammalian primary macrophages that require addition of exogenous growth factors (commonly CSF-1) for their growth [12,13], bony fish macrophages secrete their own growth factors and exhibit spontaneous growth *in vitro* [5,7,14]. It has also been demonstrated that goldfish macrophages posses novel mechanisms that control their development. For example, a soluble CSF-1R (sCSF-1R) identified in goldfish has been shown to negatively regulate macrophage proliferation and differentiation [1,6]. These unique features of teleost macrophages coincide with another unique property of teleost immune cells: the ability to become immortal cell lines without transformation or modification [15–18].

In this study, we functionally characterized goldfish CSF-1. We show that *in vivo* administration of recombinant goldfish CSF-1 (rgCSF-1) to goldfish increases the number of circulating monocytes in blood. Using RNA interference (RNAi) to knockdown the expression of the CSF-1R, we demonstrate the specificity of goldfish CSF-1 for the CSF-1R, and provide additional evidence that CSF-1 is

* Corresponding author. Tel.: +1 780 492 1266; fax: +1 780 492 9234.
E-mail address: mike.belosevic@ualberta.ca (M. Belosevic).

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a central growth factor of teleost macrophages. We also report that native CSF-1 is produced in significant amounts by goldfish fibroblast cell line CCL-71, and that conditioned supernatants from CCL-71 promote growth of newly established goldfish primary kidney macrophages. In addition, we demonstrate that addition of rgCSF-1 to primary goldfish kidney macrophage cultures increases their proliferation and longevity *in vitro*.

2. Materials and methods

2.1. Goldfish

Goldfish (*Carassius auratus*), 10–15 cm in length, were purchased from Mt. Parnell Fisheries Inc. (Mercersburg, PA) and maintained in the Aquatic Facility of the Department of Biological Sciences, University of Alberta. The fish were kept at 20 °C in a flow-through water system on a simulated natural photoperiod and fed to satiation daily with trout pellets. The fish were acclimated to this environment for at least three weeks prior to use in experiments. All protocols were carried out in compliance with the guidelines stipulated by the Canadian Council for Animal Care (CCAC) and the University of Alberta.

2.2. Primary goldfish macrophage cultures

Isolation of goldfish kidney leukocytes and the generation of primary kidney macrophages (PKM) were performed as previously described [7,19]. Briefly, goldfish macrophage cultures were established by seeding freshly isolated kidney leukocytes (18–20×10^6 cells from individual fish) into 75 cm^2 tissue culture flasks containing 15 mL of complete medium and 5 mL of cell-conditioned medium (CCM) from previous cultures. The culture medium (MGFL-15) used for cultivation of PKM, has been described previously [7]. Complete medium contained final concentrations of 100 U/mL penicillin, 100 μg/mL streptomycin, 100 μg/mL gentamicin, 10% newborn calf serum (Hyclone), and 5% goldfish or carp serum.

The kinetics of PKM growth in culture was similar to those reported for mammalian macrophages derived from bone marrow cultures in the presence of conditioned medium from the L929 fibroblast cell line [20]. PKM cultures undergo a lag phase of growth during the first 4 days of cultivation. Both adherent and non-adherent cells were present in these cultures: adherent cells spread extensively on tissue culture flasks and often formed multinucleated giant cells. The non-adherent cells grew in clusters and attained significant cell densities in culture (greater than 5×10^5–1×10^6 cells/mL). PKM cultures were composed of a heterogeneous population of cells, as determined by flow cytometry, morphology, cytochemistry, and function. Three distinct macrophage subpopulations are a feature of PKM cultures which represent macrophage subsets temporally arrested at distinct differentiation junctures in development: the early progenitors, the monocytes and mature macrophages [7,8,19,20]. PKM cultures were incubated at 20 °C until the cells were at a stage of active growth (proliferative phase) or non-proliferative stage (senescence phase) typically 6 and 10 days post-cultivation, respectively.

2.3. Goldfish fibroblast CCL-71 cell line

Goldfish CCL-71 fibroblasts (ATCC) were grown in DMEM supplemented with 15% FCS and 5% carp serum at 20 °C in an atmosphere of air. Cells were grown to confluence at which time the supernatant was removed and 3 mL of 0.25% trypsin–EDTA was added to the cells. After 5 min of gentle agitation, 7 mL of medium was added to the cultures and 4 mL of the cell suspension was added to 16 mL of medium and placed in a new flask. The supernatants from CCL-71 were stored at 4 °C, until used in the experiments.

2.4. Measurement of in vitro cellular proliferation

The effect of goldfish growth factor addition on macrophage proliferation was assessed using the Cell Proliferation ELISA BrdU colorimetric assay (Roche). Macrophages were grown as described above and sorted using a FACS Calibur flow cytometer based on size and complexity. Progenitor cells, monocytes and macrophages were then counted and seeded at a density of 1×10^4 cells/well in 96-well culture plates (Falcon). Cells were seeded in 50 μL of incomplete culture medium and treated with BrdU labelling reagent at a concentration of 15 μM. Cells were incubated with BrdU labelling reagent for 24 h and the medium was replaced to remove excess labelling reagent. After removal of excess BrdU reagent, the cells were treated with 10 ng/mL of rgCSF-1, 10 ng/mL of rgCSF-1 + CSF-1R specific dsRNA, 10 ng/mL of rgCSF-1 + 293fectin, 10 ng/mL of rgCSF-1 after RNAi knockdown of CSF-1R (Fig. 2), 100 ng/mL of recombinant goldfish granulin (rgGrn), 100 ng/mL of rgGrn after RNAi knockdown of CSF-1R (Fig. 3), CCL-71 CCM (Fig. 5), primary goldfish macrophage CCM, or left untreated. Cell proliferation was measured every 2 days from the day 0 time point. The reaction was developed according to the manufacturer's specifications and optical densities determined at 450 nm using a microplate reader. The colorimetric reaction was found to be directly proportional to the number of proliferating macrophages in culture. Recordings from the untreated cells were subtracted from the experimental groups to account for the ability of PKM cells to proliferate in the absence of exogenous growth factor [6].

2.5. RNA interference in goldfish primary kidney macrophages

RNAi transfection procedures were optimized using the Alexafluor (Invitrogen) transfection control oligo in combination with 3 different liposomal transfection reagents: Oligofectamine (Invitrogen), Cellfectin (Invitrogen) and 293fectin (Invitrogen). For the optimization experiments, PKM cultures from 6 fish were pooled together, counted and seeded in 6-well plates at a concentration of 1×10^6 cells/well in 1 mL of complete medium and incubated at 23 °C overnight. A mixture of either 5 μL or 10 μL of transfection reagent in with 10, 50 or 100 nM concentrations of the Alexafluor oligo was prepared in 1.5 mL tubes and incubated for 30 min at room temperature. The cell cultures were centrifuged for 10 min at $300 \times g$, the supernatants removed and the cells re-suspended in 200 μL of Alexafluor mixture and 800 μL of incomplete medium and incubated for 5 h at 23 °C. After incubation, 500 μL of 2× complete medium was added to each well. The cells were incubated at 23 °C for 18 h after which they were centrifuged at $300 \times g$ for 10 min and the cells were re-suspended in 1 mL and washed 3 times in 1 mL of complete medium. After washing, the cells were dislodged from the well by gentle pipetting and then 20 μL of the cell suspension was placed on a microscope slide. Cells were counted in 20 random fields of view (100× objective) and the total number of cells and the number of red fluorescing cells determined. The mean number of fluorescing cells was compared to the mean number of total cells to determine the most effective transfection method for the goldfish macrophages. Ten microlitres of 293fectin was found to be the most effective procedure for transfecting the cells, resulting in an average of 89% transfected cells over the course of 4 independent transfection experiments.

2.6. Confirmation of RNAi-induced knockdown of CSF-1R mRNA

PCR amplicons generated using primers spanning a 500 bp area of the target transcript were ligated into the pCR 2.1 TOPO vector