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Isolation and cytochemical characterization of melanomacrophages and melanomacrophage clusters from goldfish (*Carassius auratus*, L.)

Laura Diaz-Satizabal, Brad G. Magor *

Department of Biological Sciences, CW-405 BioSci Bldg., University of Alberta, Edmonton, AB, Canada T6G-2E5

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ABSTRACT

Pigmented or "melano-" macrophages are prominent in lymphoid and non-lymphoid tissues of poikilotherms. Though they have been extensively studied *in situ* only recently has a means to isolate them from other cell types been established. We provide the first *in vitro* characterization of isolated melanomacrophage cytochemistry and survival in culture. Unlike non-pigmented tissue macrophages melanomacrophages do not adhere to polystyrene surfaces making them easy to separate from tissue macrophages. *In vitro* goldfish melanomacrophages are distinguishable from tissue macrophages and neutrophils by being Sudan Black B positive (unlike tissue macrophages) and non-specific esterase positive (unlike neutrophils). Like tissue macrophages they also express acid phosphatase and CSF-1R. As sorted cells melanomacrophages only survive a few days in culture. However in coarsely disaggregated spleen and kidney tissues melanomacrophages survive for at least 3 weeks. Furthermore after 5 days culture disaggregating tissue clumps revealed encapsulated melanomacrophage clusters that remained intact for at least another week. The encapsulated clusters were resilient enough to allow for their isolation for further imaging and isolation of RNA. In some cases the clusters had either melanomacrophages or non-fluorescent cells protruding and in the latter case these could initiate outgrowths onto the plates with subsequent collapse of the cluster.

These approaches for the isolation of melanomacrophages and melanomacrophage clusters should allow further study into specific cell and cluster functions.

1. Introduction

As one of the few distinguishable organized cell masses in fish lymphoid organs, melanomacrophage clusters (MMΦCs) were studied extensively in histological sections over the past 40 years (reviewed in Agius and Roberts, 2003; Wolke, 1992). Our recent finding that B-cells expressing the immunoglobulin gene mutator enzyme AID/Aicda (activation-induced cytidine deaminase) are a part of these clusters (Saunders et al., 2010) has prompted us to re-assess the possible roles of melanomacrophages using modern techniques. In this report we provide the first phenotypic characterization of melanomacrophages isolated by FACS from goldfish spleen and kidney leukocytes, and compare these with non-pigmented tissue macrophages and neutrophils. We also establish that intact melanomacrophage clusters can be isolated from surrounding tissues.

Melanomacrophages (MMΦs) are pigmented and therefore histologically distinguishable macrophages that are fairly widespread in organs of poikilothermic vertebrates. In most fish species they exist in clusters in the spleen and the posterior kidney as well as in less discrete groupings or individually in other tissues including the liver and intestine (reviewed in Agius and Roberts, 2003). Despite their name the dominant pigment in these cells is lipofuscin (reviewed in Agius and Roberts, 2003). Lipofuscins or ceroids, as they are sometimes called, are non-degradable metabolites of non-saturated fatty acid peroxidation that will accumulate in non-dividing cells in the absence of adequate vitamin E (Terman and Brunk, 2004). In mammals this is an age related phenomena that can be replicated in macrophage cell lines 'fed' retinal elements for a period of days (Lei et al., 2012). In fish lipofuscin accumulation is associated with both autophagy and uptake of effete or apoptotic RBCs and leukocytes, and it is thought to be more pronounced than in mammals because of the higher levels of unsaturated fatty acids in cell membranes and because of lower levels of vitamin E (Wolke, 1992). Consistent with this pigment origin past transmission electron microscopy (TEM) studies of melanomacrophages have identified cells containing phagocytosed red blood cells (in spleen; Agius, 1984) and hematopoietic cells (in anterior kidney; Meseguer et al., 1991).

Abbreviations: Aicda, activation-induced cytidine deaminase; CAF, citrate-acetone-formaldehyde; CSF-1R, colony stimulating factor-1 receptor; FSC, forward scatter; FACS, fluorescence-activated cell sorting; MMΦ, melanomacrophage; MMΦC, melanomacrophage cluster; PBL, peripheral blood leukocytes; SSC, side scatter.

* Corresponding author. Department of Biological Sciences, CW-405 BioSci Bldg., University of Alberta, Edmonton, AB, Canada T6G-2E5. Tel.: +1 780 492 5956; fax: +1 780 492 9234.
E-mail address: bmagor@ualberta.ca (B.G. Magor).

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When excited with low wavelengths of light (<500 nm) lipofuscin has autofluorescent emissions in the 500 to 580 nm range, which can be used for FACS sorting melanomacrophages (Saunders et al., 2010).

The second most frequently observed pigment of MMΦs is melanin, which has variously been thought to be derived from exogenous sources (Agius, 1984) and/or to be generated within the cells (Zuasti et al., 1989). Melanin has been suggested to be important in neutralizing free radicals released during the breakdown of phagocytosed cellular membranes (Agius, 1984), and in the production of antimicrobial compounds such as hydrogen peroxide (Wolke et al., 1985). Hemosiderin, the least common of the pigments is a form of intracellular storage of iron that forms during the breakdown of hemoglobin, and serves as an intermediate step in the recycling of iron (Agius, 1979; Kranz, 1989). The presence of these pigments varies between different species (Roberts, 1975), and within cells and tissues of the same organism under different physiological conditions (Wolke, 1992; Wolke et al., 1985).

In addition to a scavenging role for melanomacrophages MMΦCs have also been observed to be sites of long-term antigen retention (Agius, 1981; Ellis, 1980; Fulop and McMillan, 1984) possibly in antibody–antigen complexes (Press et al., 1996). This, along with reports of MMΦCs having clusters of adjacent B-cells (Falk and Dannevig, 1995; Fournier-Betz et al., 2000), led to the hypothesis that MMΦCs may be germinal center-like aggregates in fishes. Consistent with this hypothesis we recently used *in situ* hybridization and laser capture microdissection to determine that channel catfish MMΦCs are interspersed with B-cells expressing Aicda, the immunoglobulin gene mutator that drives germinal center affinity maturation in mammals. These clusters also appear to contain CD4 and TcRβ expressing cells (Saunders et al., 2010) again consistent with a germinal center-like cell composition.

In a bid to better understand and characterize functions of melanomacrophages we have gone to the goldfish model in which non-pigmented macrophage characteristics have and continue to be extensively studied. As we report here goldfish melanomacrophages can be distinguished from their non-pigmented counterparts and from peritoneal neutrophils by a number of features that facilitate their isolation from one another. We also examine some of the heterogeneity in size, internal complexity and cytochemical staining that exists within and between spleen and kidney melanomacrophages. Finally we observed that during culture of spleen and kidney tissue explants that seemingly encapsulated clusters would emerge and that these could be easily isolated for further investigations.

2. Materials and methods

2.1. Fish and tissues

Goldfish (*Carassius auratus* L.) were obtained from Mt. Parnell Fisheries Inc. (Mercersburg, PA) and kept at the aquatic facility of the Department of Biological Sciences, University of Alberta. Fish were maintained in tanks in a continuous flow water system at 17 °C, simulated natural photoperiod (Edmonton, Alberta), and fed daily to satiation with trout pellets. Fish were acclimatized and monitored for health for at least 3 weeks before use. Fish housing and procedures done on fish were in accordance with Canadian Council for Animal Care guidelines and were vetted by the University of Alberta Animal Policy and Welfare Committee.

2.2. Tissue preparation for cell isolation, sorting, or generation of primary kidney macrophages

Goldfish (60 to 80 g) were anesthetized with Tricaine methanesulfonate (TMS, Syndel Laboratories), blood was collected from the caudal vein and diluted 1:5 with MFGL-15 media (Neumann et al., 1998, 2000) containing heparin and antibiotics. After blood collection fish were sacrificed. The kidneys and the spleens were aseptically removed, placed in ice-cold MGFL-15 with heparin (50,000 U/mL, Sigma) and antibiotics (100 U/mL penicillin/100 μg/mL streptomycin, Gibco). Immediately after collection, tissues were passed first through a 0.5 mm metal mesh then through sterile 40 μm cell strainers (BD Falcon) and MGFL-15 containing heparin and antibiotics. Tissue clumps were separated from the strainer and put into culture. Subsequent isolation of encapsulated melanomacrophage clusters was by washing the clusters over 75 μm nitex screens. Clusters were either stained live with propidium iodide (Sigma) for 30 minutes and then fixed in neutral formalin or first fixed and then stained with Hoechst 33342 (Molecular Probes) for 30 minutes. The strained single cell suspensions and the diluted blood were layered on 51% Percoll (Sigma) and centrifuged for 25 minutes at 400 × *g* for removal of erythrocytes. The buffy layer was removed and washed twice with MGFL-15 by centrifugation for 10 minutes at 230 × *g* and 4 °C.

For collection of tissue macrophages, freshly isolated leukocytes from the spleen and the kidney of the goldfish were seeded into polystyrene tissue culture flasks (Corning) MGFL-15 media as previously described (Neumann et al., 2000). Cells were incubated overnight at 25 °C to allow tissue macrophages to adhere to the tissue culture flasks. Supernatants were removed and the flasks were washed twice with 0.9 X PBS to remove all non-adherent cells including melanomacrophages, which are relatively non-adherent. Multiple flasks containing adherent spleen and kidney cells were checked under fluorescence for the presence of contaminating melanomacrophages. We never observed more than a handful (<20 autofluorescent cells) of melanomacrophages in the flasks.

For comparative staining peritoneal macrophages and neutrophils were also obtained from these fish by peritoneal lavage using ice cold 0.9X PBS.

2.3. Melanomacrophage cell sorting and survival in culture

Melanomacrophages from the spleen and the kidney of goldfish were isolated by flow cytometry based on forward and side light scatter and their auto-fluorescence properties. Sorting parameters and compensation were based on the autofluorescence emission spectrum previously established for channel catfish melanomacrophages (Saunders et al., 2010). Peripheral blood leukocytes (PBLs), which contain few if any melanomacrophages, were used to set the background fluorescence parameters (<0.05% fluorescent events). Cells were sorted using a FACSAria flow cytometer (BD Biosciences). A sorting gate was set based on the forward and side scatter profile of all PBLs. From this gate, cells with fluorescence intensity higher than the background in both the green and the red channels were isolated as MMΦs. Cells were sorted into tubes coated with 5% BSA containing 5 mL of ice-cold MGFL-15 medium.

Sorted cells were transferred to MGFL-15 media and placed 25 °C to allow cells to recover from any sorting perturbations and/or to assess their survival *in vitro*. For the latter the sorted melanomacrophages from the spleen and kidneys of each of 3 fish were divided into 24 culture aliquots. Total cell counts (FACS and hemocytometer) and cell viability (by trypan blue exclusion) was assessed daily for three of the 24 replicate cultures for the kidney and spleen melanomacrophages of each of the 3 fish.

For mixed cell cultures tissue clumps (<0.5 mm) or percoll isolated leukocytes were maintained in MGFL-15. Survival in culture was assessed using trypan blue and propidium iodide (particularly for tissue clumps).

2.4. Fluorescence microscopy and cytochemistry

Cytochemistry was done on cytospins (Shandon Instruments) of either freshly collected peritoneal macrophages and neutrophils or