Developmental and Comparative Immunology 33 (2009) 801–805

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

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

Short communication

Characterisation of a carp cell line for analysis of apoptosis

Montserrat Cols Vidal [1], Gwyn Williams, David Hoole *

School of Life Sciences, Keele University, Keele, Staffordshire, ST5 5BG, United Kingdom

ARTICLE INFO

Article history:
Received 23 January 2009
Accepted 14 February 2009
Available online 9 March 2009

Keywords:
Apoptosis
CLC
Cyprinus carpio
Macrophages
In vitro studies
Immunity
UV-light
siRNA

SUMMARY

Teleost fish in general, and common carp in particular, are excellent genetic models for bridging the gap in knowledge between invertebrate models such as *C. elegans* and *D. melanogaster*, on one hand, and higher vertebrates on the other hand, although, until now, there have been few well characterised fish cell lines shown to be suitable for studies on apoptosis. The present study describes the suitability of a permanent, nonleukemic, nonvirally infected carp cell line for apoptotic studies. A traditional approach using known apoptotic inducers such as UV-light combined with RNA interference, the latest ready-to-use technology widely used in higher vertebrates, was tested in the carp leucocyte cell line (CLC). This study was designed as a first step towards a better knowledge of fish macrophages and their fate after different types of apoptotic insults.

1. Introduction

Permanent fish cell lines have been mainly developed for virus propagation, however, when derived from cells of the immune system, they may also provide important tools in the study of fish immunology. Of the permanent fish cell lines that have been described in ref. [1], only a few are of haemopoietic origin [2–6]. In contrast, human and rodent cell lines have been invaluable in assisting in the characterisation of a range of immunological parameters and important cellular processes such as apoptosis in mammals. The capacity of studies on fish cell lines to facilitate the dissection of the details of apoptosis has been largely unexplored, despite their invaluable use in virology, toxicology and ecotoxicology [7–9]. The occurrence of apoptosis in fish cell lines has only been partially characterised in response to a few viruses [10–12]. The permanent cell line, carp leucocyte cell line (CLC) was first established in 1981 from peripheral blood mononuclear cells obtained from a normal, nonleukemic, nonvirally infected common carp. CLC was first characterised by Faisal and Ahne [13] as having an epithelioid-like morphology and exhibiting functional properties similar to monocytes and macrophages in that they adhered to plastic and were able to phagocytose iron particles. Weyts et al. [14] have also carried out a further characterisation of the CLC cell line and have revealed that the cell reacted with a monoclonal antibody (WCI15) raised against head kidney macrophages, which suggests the presence of shared membrane determinants with carp macrophages. In contrast, CLC showed no reaction with monoclonal antibody WCI12, indicating that the cell line did not express surface Fc receptors. The latter is in concordance with Koumans-van Diepen et al. [15] who noted that carp head kidney macrophages also do not bind Ig. CLC also exhibited respiratory burst activity when stimulated by PMA and LPS, which again was comparable behaviour to that of head kidney macrophages [15]. In addition, the CLC produced IL-1 like factors in culture supernatants, which were able to stimulate proliferation of carp peripheral blood leucocytes (PBL). Ultrastructural observations have revealed that desmosomes occur between CLC cells [14], which may indicate that CLC cells originate from a cell with the capacity to form a network in tissue. Indeed, Ellis et al. [16] also reported a similar phenomenon in teleost macrophages in kidney, spleen and heart, which formed a network of elongated cells. In summary, the above observations suggest that CLC is a suitable *in vitro* model to study the immune responses of fish and, in particular, to elucidate the induction and genetic control of apoptosis within the teleost immune system. In the present study, the CLC cell line was utilised as a tool to validate both its capacity and suitability for detailed studies of apoptosis in the immune system of fish.

* Corresponding author. Tel.: +44 1782 733673; fax: +44 1782 733516.
E-mail address: d.hoole@biol.keele.ac.uk (D. Hoole).
[1] Present address: Weill Medical College of Cornell University, Department of Pathology and Laboratory Medicine, 510 East 70th Street, New York, NY 10021, United States.

Abbreviations: AO, acridine orange; Apaf-1, apoptosis protease activating factor-1; CLC, carp leucocyte cell line; siRNA, small interfering RNA; UV, ultraviolet; z-VAD.fmk, benzyloxycarbonyl-Val-Ala-Asp(O-me).fluoromethylketone.

0145-305X/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.dci.2009.02.007

2. Materials and methods

2.1. Media and culture conditions

CLC cell line was maintained in RPMI-1640 (Sigma, UK) supplemented with 2 mM L-glutamine (Sigma, UK), 5% FBS (Fetal Clone I, Hyclone, not heat inactivated), 2.5% pooled carp serum (heat inactivated, 30 min at 56 °C in a water bath), penicillin-G (100 IU ml^{-1}, Sigma) and streptomycin (50 mg ml^{-1}, Sigma) (herein designated modified RPMI). Cells were kept at a constant temperature of 27 °C and 5% CO_2. All experiments used cells in logarithmic growth phase.

2.2. Microscopical detection of cell viability, apoptosis and proliferation in CLC

CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega, UK) was used in conversion assays following manufacturer's protocol. Viability of cells was assessed by vital dye exclusion with nigrosin (0.2%, w/v), while appearance of typical apoptotic morphology in the cells was assessed after double staining with the Caspase Detection Kit (Chemicon®, UK) and the DNA intercalating agent acridine orange (Sigma, UK) at a concentration of 10 μg/ml. The mean percentage of apoptotic cells from triplicate samples was determined by counting 200 cells per sample.

2.3. UV irradiation of cells

After seeding plates and an incubation period of 48 h at 27 °C and 5% CO_2, cells were exposed to a desired dose of U.V. radiation (UVG-54 lamp, UVP) and immediately afterwards the irradiated culture medium was replaced with fresh medium. Five replicates were made at the different irradiation doses (1560, 627, 376, 250 and 31 J/m^2) and cell viability and the levels of apoptosis assessed at time points 24, 48, 72, 96 and 144 h post exposure. In addition, these two parameters were determined in the cells prior to UV exposure and also in cells not exposed to UV treatment.

2.4. Inhibition of apoptosis with z-VAD.fmk

In order to determine whether any cell death induced after UV exposure was mediated via apoptosis due to activation of caspases, cells were cultured in the presence of the pan-caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp(O-me).fluoromethylketone (z-VAD.fmk) [17]. Cells were cultured in 35 mm Petri dishes and exposed to 376 J/m^2 with or without 50 μM of z-VAD.fmk dissolved in DMSO. In the controls, e.g. no UV exposure or no caspase inhibitor added, the solvent, DMSO was added to the culture at the same concentration as added to those cultures treated with z-VAD.fmk. Cells were incubated for 20 min following the addition of z-VAD.fmk and prior to irradiation with UV-light. Apoptosis was detected using acridine orange up to 144 h post-exposure to UV treatment.

2.5. Apoptosis induction by soluble agents

The ability of a range of chemotherapeutic agents known to induce apoptosis in mammalian cells (Supplementary Data Table 1) to induce apoptosis in the CLC cell line was determined. The concentration and solvent utilised were based on previously known optima obtained using mammalian systems and after initial experiments using CLC exposed to a wide range of concentrations of chemotherapeutic agents.

2.6. siRNA treatment of CLC

Potential siRNA target sites in the available sequences for common carp genes associated with the apoptotic process in mammals were determined using Cenix algorithm (Ambion and Cenix BioScience, Warrington, UK) program. Synthesized siRNA duplexes for apoptosis protease activating factor-1 (Apaf-1), Mcl-1 and p53 carp homologs were purchased from Ambion (UK) at a concentration of 5 nM and QIAgen's RNAiFect™ Transfection reagent (QUIgen, UK) and the manufacturer's protocol was followed in order to transfect the siRNAs into the CLC culture. GAPDH siRNA was also synthesized in order to be used as a positive control for its house-keeping gene characteristics. Ambion scrambled siRNA was included in all experiments as negative control.

Ambion *Silencer*® siRNA Labelling Kit with Cy™3 as a dye was used for labelling small interfering RNAs, following the manufacturer's recommendations.

2.7. Data analysis

Data was tested for normality and homogeneity of variances, by the Anderson-Darling test and subsequent transformations made as necessary, prior to ANOVA analysis using the statatistical software package MiniTab14. The effects of apoptosis were assessed by a two-way Analysis of Variance (ANOVA). If ANOVA was significant, Tukey's test was further used in order to determine which pair of means were significantly different.

When the means of the treatment(s) were expressed as percentages or proportions of the control (no treatment), standard errors were estimated and a 95% confidence interval for a ratio of this kind was calculated using a formula derived from [18]. In all cases, data were expressed as mean of at least three independent experiments ± standard error of the mean. $p \leq 0.05$ was accepted as the significant level.

Regression studies using MiniTab 14 were carried out in the cases of putative soluble agents and UV-light exposure in order to show correlations between doses and percentages of apoptosis.

3. Results and discussion

The aim of this study has been to determine the suitability of the CLC cell line as an *in vitro* model to elucidate the details of the apoptotic process in the fish immune cell. For this reason, a classical approach was undertaken using different types of apoptotic inducers combined with the monitoring of morphological changes characteristic of programmed cell death. Apoptosis of macrophages has been observed during infection [19,20], or after exposure to other cell stressors such as hypoxia [21]. However, it has long been established that monocytes/macrophages are resistant to apoptosis upon activation, and at the same time these cells undergo apoptosis constitutively in culture without the appropriate stimuli [22,23]. Our results indicate that the CLC cell line, derived from a monocyte/marcrophage origin, is a suitable model system to undertake detailed investigations on the apoptotic process in fish immune cells. In mammalian systems non-ionizing UV irradiation *in vitro* has been found to induce DNA damage dependent on UV dose and the tissue or cell type involved [24]. Historically, UV-radiation has also been used for immunosuppression [25], which has been linked to diseases involving inappropriate cell survival which results from apoptosis failure [26]. In our studies both UV-exposure and time after exposure had a significant effect ($F_{(5,4)} = 13.99$, $p < 0.0001$ and $F_{(5,4)} = 22.23$, $p < 0.0001$, respectively) on apoptotic levels in CLC. The highest dose of UV used (1560 J m^{-2}) had a significant effect on the levels of apoptosis, compared with the controls and 31.35 J/m^2 (Fig. 1a).