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Mechanisms involved in apoptosis of carp leukocytes upon *in vitro* and *in vivo* immunostimulation

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ABSTRACT

During inflammation leukocyte activity must be carefully regulated, as high concentrations and/or prolonged action of pro-inflammatory mediators e.g. reactive oxygen species (ROS) can be detrimental not only for pathogens but also for host tissues. Programmed cell death – apoptosis is a most effective regulatory mechanism for down regulation of leukocyte activity, but little is known about this process in fish.

We aimed to reveal the mechanisms of initiation and regulation of apoptosis in carp neutrophilic granulocytes and macrophages.

During zymosan-induced peritonitis in carp, activated inflammatory neutrophilic granulocytes and monocytes/macrophages died by apoptosis. This correlated with a strong production of ROS, but pre-treatment of the fish with NADPH oxidase inhibitor only slightly decreased late apoptosis. Interestingly *in vitro* incubation with zymosan or phorbol ester, but not lipopolisaccharide and poli I:C induced apoptosis of head kidney neutrophilic granulocytes. This coincided with loss of mitochondrial membrane potential. Moreover, in zymosan-stimulated neutrophilic granulocytes NADPH oxidase inhibitor not only reduced the production of ROS but also apoptosis. A similar effect was not observed in cells stimulated with phorbol ester, where DPI reduced ROS production, but not apoptosis. In PMA-stimulated neutrophilic granulocytes both the respiratory burst and apoptosis were reduced by protein kinase inhibitor. Furthermore, a short neutrophil stimulation either with PMA or with zymosan did induce caspase-independent apoptosis.

These results show that in carp, apoptosis is an important regulatory process during *in vitro* and *in vivo* immunostimulation. In neutrophils, protein kinase, but not NADPH oxidase, is involved in PMA-induced apoptosis while apoptosis induced by zymosan is ROS-dependent.

1. Introduction

Apoptosis, programmed cell death, is crucial for the elimination of old, infected or endangered cells, both during homeostatic and pathological circumstances. Moreover, it is important for correct embryogenesis, including the development of the immune system. During an immune response, removal of activated lymphocytes from the periphery proceeds through apoptosis. Moreover, to avoid additional tissue damage during inflammation, activated neutrophils are eliminated quickly after pathogen eradication [1,2].

In mammals, two main pathways of cellular apoptosis have been described: the external (extrinsic) and internal (intrinsic) or mitochondrial pathway. The extrinsic pathway is characterized by activation of the cytoplasmic death domain (DD) linked to the membrane death receptors of the TNF superfamily (e.g. Fas, TNFR1, TNFR2, DR3, DR4, DR5 and DR6) after binding of the ligand (e.g. TNF-α, FasL). These activated DD domains subsequently bind other homological DD domain of adaptor proteins e.g. FADD (Fas-associated death domain protein), TRADD (TNF-R1 receptor associated death domain) or RIP (receptor interacting protein), which is followed by oligomerization and subsequent binding of death receptor effector domain (DED) to DED of procaspase-8 or -9 [3]. This activates proteolytic cleavage to their active type. Initiator caspase-8 now activates effector caspases 3, 6 and 7, while caspase-9 activates caspases 3 and 7. The active effector caspases then proteolytically degrade intracellular proteins, thereby carrying out the cell death program [4,5].

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In the intrinsic route, apoptosis inducers such as UV/gamma radiation, chemical exposure or lack of growth factors provoke changes in the mitochondrial transmembrane potential, decrease of ATP synthesis and release of cytochrome *c*. The released cytochrome *c* binds to apoptosis protease activating factor-1 (Apaf-1) and procaspase-9 to create a complex known as the apoptosome. Apoptosome formation activates initiator caspase-9 to initiate the caspase cascade and activate effector caspases 3 and 7 [6].

Interestingly, NADPH oxidase-derived ROS can also induce caspase-independent apoptosis [7]. In this case ROS directly activates protein kinase transduction pathways like ERK1/2, p38 MAPK and JNK [8–11].

Apoptosis is additionally regulated by several pro- (e.g. Bax, Bak, Bcl-xS, Bad, Bik, and Hrk) and anti-apoptotic (e.g. Bcl-2, Bcl-xL, Bcl-w, Mcl-1, Bfl-1, Brag-1 and a1) proteins. Their cellular concentrations (ratio of antagonists to agonists) and the competitive dimerization between selective pairs of antagonists and agonists determine the susceptibility to apoptosis [12]. The earliest events of apoptosis are connected with externalization of phosphatidylserine and can be effectively detected upon binding to labeled annexin V. Externalization of phosphatidyloserine precedes the loss of membrane integrity. This is characteristic for the later stages of apoptosis as well as for necrotic processes and can be measured with a vital dye such as 7-Amino-Actinomycin (7AAD) or propidium iodide.

The majority of studies on the role and regulation of apoptosis during immune activation have focused on either mammals or invertebrates. Data about apoptosis regulation in the immune system of ectothermic vertebrates, such as teleost fish are still limited. Whole genome sequencing projects, including pufferfish (*Fugu rubripes*) and zebrafish (*Danio rerio*) caused great impetus to the discovery of fish genes homologous to mammalian molecules of the cell death pathways. Till now, the sequences and molecular characterization of caspase homologs in zebrafish [13–15], sea bass [16–18], Atlantic salmon [19] and carp [20] have been described. Moreover TNF receptors and their ligands are known [21,22]. In addition some members of the Bcl-2 family have been cloned, e.g. the anti-apoptotic proteins Mcl-1 of zebrafish [23] and carp [20]. Additionally, the identification and functional analysis of the zebrafish bcl-2 homolog has been reported [24]. This remarkable conservation between fish and mammals of molecules involved in induction, execution and regulation of apoptosis [12,20] now creates opportunities to achieve significant progress in their functional characterization. An initial functional analysis of potential proteins involved in apoptosis of carp leukocytes was performed for a carp leukocyte cell line (CLC) [20]. Inhibition of Mcl-1, Apaf-1 and p-53 (by silencing RNA technique) decreased UV-light-induced cell apoptosis. This effect was reversed by the pan-caspase inhibitor Z-VAD-fmk.

In view of the importance of phagocytes (neutrophilic granulocytes and macrophages) to accomplish an effective inflammatory response, we now aim to obtain insight into the mechanisms of initiation/regulation of apoptosis in carp phagocytic leukocytes.

2. Materials and methods

2.1. Animals

Common carp (500–600 g, *Cyprinus carpio* L.) from the Institute of Ichthyobiology and Aquaculture, Polish Academy of Science, Golysz, Poland, were kept at 20 °C in recirculating tap water at the aquatic facility of the Institute of Zoology, Jagiellonian University in Krakow. Fish were fed dry food pellets (Trouvit, Nutreco) at a daily maintenance rate of 1% of their estimated body weight. The tanks were equally positioned to avoid unnecessary additional interference and stress. All injections and samplings were carefully performed by the same person and at the same time of the day to avoid differences in handling. Tricaine methane sulphonate (TMS; Sigma–Aldrich, St. Louis, MO; 0.2 g/l) buffered with $NaHCO_3$ (POCH, Gliwice, Poland; 0.4 g/l) was used for animals anaesthetize.

All experiments were conducted according to license no. 23/2012 from the local ethical committee.

2.2. Cell isolation, stimulation and in vitro culture

Head kidney cell suspensions were obtained by passing the tissue through a 50 μm nylon mesh with carp RPMI (cRPMI), (RPMI 1640, Invitrogen, Carlsbad, CA, adjusted to carp osmolarity of 270 mOsm kg^{-1} with distilled water) containing 10 IU ml^{-1} heparin (Leo Pharmaceutical Products Ltd., Weesp, the Netherlands) and washed once. This cell suspension was layered on a discontinuous Percoll (Amersham Biosciences, Piscataway, NJ) gradient (1.020, 1.070, and 1.083 g cm^{-3}) to retrieve neurophilic granulocytes [25] and centrifuged for 30 min at 800 × g with the brake disengaged.

Cell fractions at 1.083 g cm^{-3} (64% neutrophilic granulocytes and 10% of macrophages [25]) were collected, washed, and seeded at 5×10^6 cells/ml in cell culture plates at 27 °C, 5% CO_2 in complete cRPMI++ (cRPMI supplemented with 0.5% (v/v) pooled carp serum, 1% L-glutamine (Merck, Whitehouse Station, NJ), 1% (v/v) penicillin G (Sigma–Aldrich, St. Louis, MO), and 1% (v/v) streptomycin sulphate (Sigma–Aldrich, St. Louis, MO)).

Neutrophils were preincubated with diphenyleneiodonium chloride (DPI; Sigma–Aldrich, St. Louis, MO; 50 μM/ml; for 1 h), pan-caspase inhibitor VI (Z-VAD-fmk; Merck, Darmstadt, Germany, 50 μM/ml; for 1 h), staurosporine (1 μg/ml for 1 h) or superoxide dismutase together with catalase (SOD; Sigma–Aldrich, St. Louis, MO; 200U and CAT; Sigma–Aldrich, St. Louis, MO; 200U for 1 h) and subsequently stimulated for 1.5 h or 48 h with phorbol 12-myristate 13-acetate (PMA; Sigma–Aldrich, St. Louis, MO; 0.1 and 1 μg/ml) or for 1.5 h with zymosan (Sigma–Aldrich, St. Louis, MO; 0.5 mg/ml), polyinosinic:polycytidylic acid (poli I:C; 25 μg/ml) or with lipopolisaccharide (LPS; Sigma–Aldrich, St. Louis, MO; 50 μg/ml).

Some cells were preincubated for 1 h with caspases inhibitors: caspase-3 inhibitor, caspase-8 inhibitor or caspase-9 inhibitor (Calbiochem, San Diego, CA; 25 μM/ml) before PMA or zymosan-treatment.

2.3. Zymosan-induced peritonitis

Fish were kept untreated (intact fish, time 0) or they were i.p. injected with freshly prepared zymosan A (Z; Sigma–Aldrich, St. Louis, MO; 2 mg/ml, 1 ml/50 g b.w.) in sterile phosphate-buffered saline PBS (270 mOsM) [26]. Some fish, for three consecutive days before zymosan-treatment, were i.p. injected with diphenyleneiodonium chloride (DPI; Sigma–Aldrich, St. Louis, MO; 1 mg/kg b.w. in DMSO) or DMSO only (VDPI; DMSO; Sigma–Aldrich, St. Louis, MO). Just after the last DPI or VDPI injection animals received zymosan. At selected time points post injection (4, 24, 48 or 96 h), animals were sacrificed and peritoneal cavities were lavaged with 5 ml of ice-cold PBS. Total numbers of peritoneal leukocytes (PTL) were counted in a hemocytometer after Türk staining [27]. To determine the cell composition of the peritoneal leukocytes, peritoneal fluid samples (24 h after injection) were analyzed with a FACScalibur flow cytometer (BD Biosciences). 5000 threshold events per sample were analyzed for their forward scatter (FSC) (for cell size) and sideward scatter (SSC) (cell complexity) profiles. Data were analyzed using WinMDI 2.9 software (Joe Trotter, http://facs.scripps.edu).