Developmental and Comparative Immunology 51 (2015) 148–159

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

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

Zebrafish Nk-lysins: First insights about their cellular and functional diversification

P. Pereiro [1], M. Varela [1], P. Diaz-Rosales, A. Romero, S. Dios, A. Figueras, B. Novoa *

Instituto de Investigaciones Marinas (IIM), CSIC, Vigo, Spain

ARTICLE INFO

Article history:
Received 16 December 2014
Revised 19 March 2015
Accepted 19 March 2015
Available online 23 March 2015

Keywords:
Nk-lysin
Granulysin
Zebrafish
Rag1
Ontogeny
SVCV

ABSTRACT

Nk-lysins are antimicrobial proteins produced by cytotoxic T lymphocytes and natural killer cells with a broad antimicrobial spectrum (including bacteria, fungi and parasites). Nevertheless, the implication of these proteins in the protection against viral infections is still poorly understood. In this work, four different Nk-lysin genes (*nkla*, *nklb*, *nklc* and *nkld*) were identified in the zebrafish genome. That means that zebrafish is the species with the higher repertoire of Nk-lysin genes described so far. The differential expression pattern of the Nk-lysins in several tissues, during ontogeny, among the different kidney cell populations, as well as between $Rag1^{-/-}$ and $Rag1^{+/+}$ individuals, could suggest a certain specialization of different cell types in the production of different Nk-lysin. Moreover, only two of these genes (*nkla* and *nkld*) were significantly up-regulated after viral infection, and this observation could be also a consequence of a functional diversification of the zebrafish Nk-lysins.

1. Introduction

Nk-lysins (or granulysin in humans) are antimicrobial cationic proteins produced by cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells and stored in cytolytic granules. These cells are important effectors of the immune system and play a relevant role in defense against a variety of targets (virus or bacteria-infected cells as well as tumor cells). An array of molecules, such as perforin, granulysin/Nk-lysin and numerous serine proteases (granzymes), is stored in cytoplasmic granules and released upon target cell recognition via Major histocompatibility complex (MHC) class I in order to induce apoptosis in infected cells or tumors (Lowin et al., 1994; Nagata and Golstein, 1995; Trapani and Smyth, 2002). MHC class I proteins present antigens to CTLs in the form of peptides on the surface of tumor or virus and bacteria-infected cells (Townsend et al., 1986) that are needed for the immune regulation of the NK-cells activity (Ljunggren and Kärre, 1990). The interaction of T-cell receptor (TCR) on CTLs with tumor or viral peptide–MHC class I complexes activates a signaling cascade that leads the granule polarization and release of granule toxins by exocytosis (Trapani and Smyth, 2002). In addition to their cytotoxic function, a direct and MHC class I-independent antimicrobial activity of these cells has been observed (Levitz et al., 1995; Oykhman and Mody, 2010).

Perforin is a pore-forming member of the membrane-attack-complex/perforin (MACPF) protein family with the ability to form transmembrane channels and cause osmotic lysis (necrosis) of the target cells (Trapani and Smyth, 2002). Moreover, perforin allows the entry of other cytotoxic components (mainly pro-apoptotic proteases known as granzymes) into the target cell for inducing apoptosis (Cullen et al., 2010; Trapani and Smyth, 2002). Although Nk-lysin was relegated to a secondary role during several years, its broad and direct antimicrobial spectrum (including bacteria, fungi and parasites) (Andersson et al., 1995; Endsley et al., 2004; Hirono et al., 2007; Jacobs et al., 2003; Wang et al., 2000) and the ability to lyse intracellular *Mycobacterium tuberculosis* following permeation of the cellular membrane by perforin (Stenger et al., 1998) have contributed to increase the interest in this molecule. This peptide could be required in the elimination of some intracellular pathogens, since perforin is able to kill the infected cells but not the intracellular *M. tuberculosis*. Nk-lysin is a member of the saposin-like protein (SAPLIP) family and therefore, possesses membrane-binding activity and capability to altering the membrane integrity (Ruysschaert et al., 1998). The presence of this molecule in NK cells and of related peptides from the SAPLIP family in cytoplasmic granules of *Entamoeba histolytica* (amoebapores) suggests that these proteins are ancient but highly conserved during the evolution as an important antimicrobial host defense mechanism (Stenger et al., 1999).

Perforin-granzymes synergy is the main mechanism used by the cytotoxic cells to combat viral infections. The role of Nk-lysin in viral clearance is still poorly understood and even contradictory results were observed in some cases. The main objective of this work was to identify and characterize the zebrafish Nk-lysins, as well as to

* Corresponding author. Instituto de Investigaciones Marinas (IIM), CSIC, Vigo, Spain. Tel.: +34 986 214 463; fax: +34 986 292 762.
E-mail address: beatriznovoa@iim.csic.es (B. Novoa).
[1] These authors contributed equally to the work presented in this paper.

http://dx.doi.org/10.1016/j.dci.2015.03.009
0145-305X/

conduct some analysis in order to investigate the potential implication of these molecules in viral clearance. Moreover, the expression studies of the four zebrafish Nk-lysins in different tissues, during ontogeny, among different cell populations isolated from kidney and in Recombination activation gene 1 deficient ($Rag1^{-/-}$) mutant zebrafish, shed some light about the specialization of different cell types in the production of certain Nk-lysins.

2. Materials and methods

2.1. Sequence retrieval and characterization of zebrafish Nk-lysin genes

An exhaustive BLAST search (Altschul et al., 1997) was performed against the *Danio rerio* full genome (version Zv9) using Nk-lysin nucleotide sequences from fish (including an Nk-lysin gene from *D. rerio*) that were retrieved from the public NCBI nucleotide database (http://www.ncbi.nlm.nih.gov/nucleotide). Synteny conservation between zebrafish and several teleost species (*Astyanax mexicanus*, *Tetraodon nigroviridis*, *Takifugu rubripes*, *Gasterosteus aculeatus* and *Oryzias latipes*) as well as with the human granulysin gene was investigated using Genomicus v75.01. Four Nk-lysins genes located in tandem were identified. The full-length coding sequence of these genes was confirmed by PCR using specific primers (Supplementary data Table S1) and subsequent linking of the PCR product into pCR2.1-TOPO vector (Invitrogen) for their cloning using One Shot TOP10F' competent cells (Invitrogen) following the protocol instructions. cDNA sequencing of 4 selected clones from each Nk-lysin was conducted using an automated ABI 3730 DNA Analyzer (Applied Biosystems, Inc., Foster City, CA, USA).

The presence of signal peptide was analyzed with the SignalP 3.0 Server (http://www.cbs.dtu.dk/services/signalp-3.0/) (Emanuelsson et al., 2007) and the presence of specific domains with SMART 4.0 (http://smart.embl.de/) (Letunic et al., 2004). Molecular weight and isoelectric point were determined using the Compute pI/Mw tool from ExPASy (Gasteiger et al., 2003). The potential disulphide bonds between cysteines were analyzed using the server DiANNA 1.1 (Ferrè and Clote, 2005) and the 3D-structures of zebrafish Nk-lysins were predicted using I-TASSER server (Roy et al., 2010) selecting the model with the best C-score and viewed by PyMOL (http://www.pymol.org). The Template Modelling Score (TM-score), a measure of structural similarity between two proteins, was also considered in order to identify those structural analogs with known crystal architecture in the Protein Data Bank (PDB; http://www.rcsb.org/pdb/).

2.2. Sequence conservation and phylogenetic analysis

An alignment between *D. rerio* Nk-lysins and several Nk-lysins/Granulysins sequences from other fish and vertebrates was conducted using the ClustalW server (Thompson et al., 1994). Sequence similarity and identity scores were calculated with the software MatGAT (Campanella et al., 2003) using the BLOSUM62 matrix. Moreover, a phylogenetic analysis was conducted using several proteins belonging to the saposin-like proteins (SAPLIPs) family (Nk-lysins/Granulysin, Prosaposins, Acyloxyacyl hydrolases – AOAH –, Pulmonary surfactant protein B – SP-B – and Amoebopores). The alignment was constructed also with ClustalW and the tree was drawn using Mega 6.0 software (Tamura et al., 2013). Neighbor-joining algorithm (Saitou and Nei, 1987) was used as clustering method, the distances matrix was computed using Poisson correction method, and complete deletion of the positions containing alignment gaps and missing data was conducted. Statistical confidence of the inferred phylogenetic relationships was assessed by performing 10,000 bootstrap replicates. The GenBank accession numbers of the sequences used in this section are listed in Supplementary data Table S2.

2.3. Animals

Adults, embryos and larvae from wild-type zebrafish were obtained from our experimental facility, where zebrafish are cultured following established protocols (Nusslein-Volhard and Dahm, 2002; Westerfield, 2000) (also see http://zfin.org/zf_info/zfbook/zfbk.html).

The sacrifices were performed via MS-222 overdose (500 mg/L^{-1}). Fish care and challenge experiments were reviewed and approved by the CSIC National Committee on Bioethics under approval number (07_09032012).

2.4. Experimental treatments

In order to analyze the constitutive expression of Nk-lysin genes in different tissues of adult zebrafish, spleen, liver, kidney, gill, caudal fin, whole head, muscle and intestine were sampled and pooled, yielding a total of 4 pools of 5 fish per organ.

With the aim of identifying the immune cells involved in the production of the different Nk-lysins, a total leukocyte suspension from kidney (using 10 adult zebrafish) was prepared by passing the tissues through a 100-μm nylon and the cells were diluted in Leibovitz's L-15 medium supplemented with Primocin (Invivogen) and 2% fetal calf serum (all components were from Life Technologies). Cells were centrifuged at 400*g* (4 °C) for 10 min and diluted in Leibovitz's L-15 medium supplemented with Primocin (Invivogen) and 10% fetal calf serum for being analyzed by flow cytometry based on forward and side scatter on a FACSCalibur flow cytometer (Beckton Dickinson) equipped for cell-sorting (Traver et al., 2003). A total of 300,000 events were sorted in 1× PBS from the regions corresponding to myeloid (R2), lymphoid (R3) and precursor (R4) population, and they were pelleted by centrifugation at 400*g* for 5 min at 4 °C and processed for gene expression. The cells from the total population (non-sorted) were also processed. The correct distribution of the cell populations was corroborated by analyzing the expression level of different cell markers: *marco* (macrophages), *mpx* (neutrophils), *cd4* and *cd8a* (T lymphocytes). Primer sequences are listed in Supplementary data Table S1. In addition, the expression level of these four genes was studied in kidney from $Rag1^{-/-}$ mutants and compared with $Rag1^{+/+}$ wild-type adult zebrafish using 4 biological replicates (4 fish/replicate).

To determine the expression levels of the different genes during zebrafish ontogeny, wild-type zebrafish larvae were sampled at the following dpfs: 1 dpf, 2 dpf, 3 dpf and at 3-day intervals from 5 to 29 dpf. Due to differences in animal size, 10–15 animals were necessary to yield biological replicates from 3 dpf to 14 dpf, whereas only 6–8 individuals from 17 to 29 dpf were used for biological replicates. A total of 3 biological replicates per sampling point were obtained.

Fish challenge experiments were also conducted in order to investigate the induction of the Nk-lysin genes upon viral infection. The rhabdovirus, spring viraemia of carp virus (SVCV isolate 56/70) was used in these experiments. The virus was propagated on EPC cells (ATCC CRL-2872) and titrated in 96-well plates. The $TCID_{50}$/mL was calculated according to Reed and Muench (1938) and the experimental challenges were performed at 23 °C. Zebrafish larvae were infected through microinjection (using a glass microneedle using Narishige MN-151 micromanipulator and Narishige IM-30 microinjector) into the duct of Cuvier as described in Varela et al. (2014a) at 3 days post-fertilization (dpf). Two nL of 3×10^6 $TCID_{50}$/mL of SVCV was microinjected per larvae and PBS microinjection was used as a control in larvae experiments. At 24 hours post-stimulation (hps), 3 biological replicates composed by 10 larvae each were collected. Moreover, adult zebrafish