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Proteome dynamics in neutrophils of adult zebrafish upon chemically-induced inflammation

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ABSTRACT

Neutrophils are the most abundant polymorphonuclear leukocytes, presenting the first line of defence against infection or tissue damage. To characterize the molecular changes on the protein level in neutrophils during sterile inflammation we established the chemically-induced inflammation (ChIn) assay in adult zebrafish and investigated the proteome dynamics within neutrophils of adult zebrafish upon inflammation. Through label-free proteomics we identified 48 proteins that were differentially regulated during inflammation. Gene ontology analysis revealed that these proteins were associated with cell cycle, nitric oxide signalling, regulation of cytoskeleton rearrangement and intermediate filaments as well as immune-related processes such as antigen presentation, leucocyte chemotaxis and IL-6 signalling. Comparison of protein expression dynamics with transcript expression dynamics suggests the existence of regulatory mechanisms confined to the protein level for some genes. This is the first proteome analysis of adult zebrafish neutrophils upon chemically-induced inflammation providing a valuable reference for future studies using zebrafish inflammation models.

1. Introduction

Inflammation is an essential organismal defence mechanism triggered by chemical, mechanical, metabolic or pathogenic stimuli with neutrophilic granulocytes representing the first line of defence against tissue injury or bacterial infection [1,2]. Failure to mount an inflammatory response allows excessive tissue damage and pathogen invasion. On the other hand, failure to resolve an immune response also causes severe tissue damage and may lead to chronic inflammation, triggering diseases such as atherosclerosis, asthma, inflammatory bowel disease and various autoimmune diseases [3–5]. Hence, it is essential to understand the regulation of this process in order to address the pathological conditions associated with inflammation.

Recent studies on inflammation and leucocyte biology successfully used zebrafish as a suitable animal model for deciphering the regulatory processes of inflammation [6–8]. Temporal segregation of immune system development in zebrafish larvae allows the study of innate immunity independent of the adaptive immune system, which is functionally mature only three weeks post fertilization [9]. Zebrafish neutrophilic granulocytes show morphological, biochemical and functional similarity to mammalian neutrophils [10,11]. Given the relative ease in breeding them, their transparency during early development and the availability of multiple lines with fluorescently-tagged immune cells, the zebrafish model has been applied for *in vivo* screening of immune-modulatory compounds [12–14]. An interesting assay employs chemically-induced inflammation (ChIn), using copper sulphate to trigger robust sterile inflammation. Copper sulphate induces cell death of the sensory hair cells in the lateral line system of zebrafish larvae, which eventually regenerate within 24 h after removal of the damaging agent [15,16]. Cell death of hair cells induces rapid granulocyte recruitment to the affected tissue, a process that can be quantified using appropriate transgenic lines [14]. The ChIn assay hence, enables automated screening procedures towards identification of immune-modulatory activities of candidate compounds using zebrafish larvae. However, it is currently unknown whether the ChIn assay could also be applied in adult zebrafish. Here we established the applicability of chemically-induced inflammation in adult zebrafish and investigated the protein dynamics within neutrophils upon inflammation using mass spectrometry [17–19].

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We identified 48 proteins, including 37 up-regulated proteins and 11 down-regulated proteins exhibiting differential expression in neutrophils of adult zebrafish upon inflammation. Furthermore, we validated these changes in protein levels on the RNA level and performed *in silico* analysis to determine localization, biological process, molecular function and biological pathway association of identified proteins.

2. Materials and methods

2.1. Fish husbandry

Wild type (AB) and transgenic (*Tg(lyz:DsRED2)nz50*) zebrafish were maintained and raised under recommended conditions [20,21]. Zebrafish husbandry and experimental procedures were performed in accordance with the German animal protection standards (Animal Protection Law, BGBl. I, 1934 (2010) and were approved by the local government of Baden-Württemberg, Regierungspräsidium Karlsruhe, Germany (Licence number: Proteome analyses of adult zebrafish: Az.: 35–9185.81/G-170/12 and general licence for fish maintenance and breeding: Az.: 35–9185.64).

2.2. Inflammation induction in adult fish

Healthy transgenic zebrafish (4–8 months old) were treated with 25 μM $CuSO_4$ for 1 h. Following incubation, inflammation was confirmed visually by consideration of leucocyte influx to the gills.

2.3. Isolation of adult neutrophils

Whole kidney marrow (WKM) of treated and control eighty adult fish was extracted and subsequently placed into ice-cold 1X Leibovitz's medium (GIBCO) plus 5% FCS (PAA Labs). Single-cells suspensions were generated by aspiration, followed by gentle pipetting and filtering using a 40 μm nylon mesh filter. Cells were centrifuged at 250 rpm for 15 min at 4 °C. The supernatant was discarded, cell pellets resuspended in ice-cold 1X Leibovitz's medium plus 5% FCS, and passed again through a filter with 40 μm pore size. Cell sorting was performed under sterile conditions at room temperature. Cells were sorted based on granularity (SSC), size (FSC), and dsRED fluorescence on a FACSAria (BD Bioscience). Sorted cells were collected in 1X Leibovitz's medium plus 10% FCS. After cell sorting, cells were spun down and resuspended. Further re-analysis of sorted cells was performed by FACS, for this purpose cells were loaded in FACS aria and 10,000 events were recorded and subsequently fluorescent cells population was gated to calculate the percent of fluorescent cells. In addition, purity of cells was also validated microscopically (Figure S1). We routinely obtained 3×10^6 cells per fish. After sorting and purity assessment (>90%), the cells were spun down and snap frozen for subsequent protein or RNA extraction.

2.4. Protein extraction

Total protein from adult neutrophils was extracted by homogenization and sonication in SDS lysis buffer (4% SDS, 100 mM Tris/HCl pH 7.6). Additionally, lysates were centrifuged at 15,000 g for 15 min at room temperature. Supernatants were collected and protein content was determined using the detergent compatible Bradford method (Bio-Rad) against a bovine serum albumin standard. A total of 45 μg of equally quantified protein samples were loaded and subsequently separated by gel electrophoresis on precast 4–12% Nu-PAGE gradient gels (Invitrogen). Gels were stained with the Colloidal Blue Staining Kit (Invitrogen). Each lane from inflamed and non-inflamed zebrafish cell extracts was cut equally into 9 sections, destained, washed, subjected to reduction and alkylation in the presence of DTT and IAA (Sigma), and further dehydrated, followed by overnight digestion at 37 °C in the presence of trypsin endopeptidase enzyme (Sigma). Peptides were extracted, desalted and concentrated using homemade C18 columns.

2.5. LC-MS/MS analysis

Trypsin digested inflamed and non-inflamed samples, representing the peptide content of one gel piece were analysed by nano-reversed Phase Chromatography using an Agilent 1100 nanoflow system that was online coupled via in house packed fused silica capillary column emitters (length 15 cm; ID 75 μM; resin ReproSil-Pur C18-AQ, 3 μm) and a nanoelectrospray source (Proxeon) to an LTQ Orbitrap XL mass spectrometer (Thermo Scientific). Subsequently, peptide extraction was carried out from the C18 column by applying a linear gradient from 5 to 35% buffer B (80% acetonitrile, 0.5% acetic acid) over 150 min. The mass spectrometer was operated in the data-dependent mode for collecting collision induced MS/MS spectra from the five most intense peaks in the MS (LTQ-FT full scans from m/z 300 to m/z 1800; resolution $r = 60{,}000$; LTQ isolation and fragmentation at a target value of 10,000). The five most intense peaks from full MS scan were fragmented in a linear ion trap using CID (35% normalized collision energy) and for LTQ Orbitrap measurements (MS/MS), 15 most intense peaks were selected for fragmentation in the linear ion trap.

2.6. Data analysis

MaxQuant software was used to analyse the generated raw data against the International Protein Index sequence database (zebrafish IPI, version 3.54). The database was extended with commonly observed contaminants and concatenated with reversed versions of all sequences. The parameter settings were as follows: trypsin as digesting enzyme, a maximum of two missed cleavages, a minimum of six amino acids, carbamidomethylation at cysteine residues as fixed and oxidation at methionine residues as variable modifications. Moreover, proteins identified with a minimum of two peptides and 1% false discovery rate were considered for further analysis. Statistical analysis was carried out with Perseus (Version 1.3.8.3).

2.7. Data visualization

Differentially regulated proteins were further analysed using the STRAP software program for their cellular localization, biological process and molecular function [22,23]. Moreover, identified protein Uniprot accession numbers were mapped into Entrez gene IDs using the Uniprot database. Subsequently, IDs were uploaded to MetaCore from GeneGo Inc for process (www.genego.com), network and pathway map analysis.

2.8. Real time PCR analysis

RNA was extracted from inflamed and control neutrophils using PeqGold Trifast reagent (Peqlab) according to the manufacturer's protocol, and cDNA was prepared using QuantiTect Reverse Transcription Kit (Qiagen). qPCR was performed on an ABI StepOnePlus Thermo Cycler with CRX96 real-time system and using GoTaq qPCR master mix (Promega). PCR was performed in triplicate using the following standard program: one cycle of 95° for 15 min, and 40 cycles of 95° for 15 s plus 60° for 30 s with the following primers: *myd88* CGAACACAGGAGAGAGAAGGAGTC (forward), TCAAAGGTCTCAGGTGTCAGTCC; *il6* GATGACAGTGAAGCTCTTGGACAC (forward), CCGATTCAGTCTGACCGGAGATTG (reverse); *tnfa*

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