Molecular Immunology 46 (2009) 1409–1415

Contents lists available at ScienceDirect

Molecular Immunology

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

Collagen regulates the activation of professional phagocytes of the teleost fish gilthead seabream

Patricia Castillo-Briceño, María P. Sepulcre, Elena Chaves-Pozo, José Meseguer, Alfonsa García-Ayala*, Victoriano Mulero*

Department of Cell Biology and Histology, Faculty of Biology, University of Murcia, 30100 Murcia, Spain

ARTICLE INFO

Article history:
Received 18 November 2008
Received in revised form 16 December 2008
Accepted 16 December 2008
Available online 29 January 2009

Keywords:
Collagen
Innate immune system
Inflammatory response
Phagocytes
Fish

ABSTRACT

The innate immune system mediates the initial inflammatory response that follows infection or injury. Although the innate immune response of fish to infection has been relatively well characterized during recent years at both cellular and molecular levels, no studies have examined the role of extracellular matrix (ECM) in the regulation of innate immunity and inflammation. We report here that collagen and gelatin *in vitro* were able to prime the respiratory burst of phagocytes from the bony fish gilthead seabream. In addition, collagen and gelatin induced a specific set of immune-related and ECM remodelling enzymes that substantially differed from that induced by pathogen-associated molecular patterns. Notably, both collagen and gelatin induced the expression of interleukin-1β, chemokine (C–C motif) ligand 4 and matrix metalopeptidases (MMP) 9 and 13 in acidophilic granulocytes and macrophages but were unable to significantly increase the expression of other pro-inflammatory genes. Furthermore, it was found that the MMP2/MMP9 inhibitor V had a dose-dependent inhibitory effect on seabream phagocyte activation by either collagen or gelatin. In contrast, pre-treatment of collagen and gelatin by collagenase resulted in a higher stimulatory capacity compared to non-digested proteins. Collectively, these results indicate that collagen fragments produced by the action of different host proteases, and probably released by infectious agents, are sensed by fish phagocytes. Therefore, we propose that, besides to the well-established response to infection, the innate immune system of fish is able to respond to tissue injury.

1. Introduction

The innate immune system mediates the initial inflammatory response that follows infection or injury (Mollen et al., 2006). It has been proposed that both chemical and mechanical changes in the cellular microenvironment may be responsible for driving specific cellular responses (Jiang et al., 2007; Peyton et al., 2007). Thus, the extracellular matrix (ECM) not only provides structural support and adhesive substrates for the body tissues, but it also plays a significant role in regulating cell and tissue function (Schnaper and Kleinman, 1993).

ECM components and their mechanical properties induce changes in cell shape and movement, bind growth factors, and facilitate cell–cell and cell–ECM interactions. The ECM regulatory effects involve the modulation of signalling pathways that control cell growth, differentiation, proliferation, apoptosis, survival and morphogenesis (Lukashev and Werb, 1998; Peyton et al., 2007). The differentiation induced by ECM results from multiple stimuli such as: tensile forces on the cell, cytokine- or growth factor-mediated stimulation, and interaction with bioactive domains of matrix glycoproteins, such as collagen, laminin, fibronectin and hyaluronan (Schnaper and Kleinman, 1993; Vernon and Gooden, 2002; Ågren and Werthén, 2007; Leitinger and Hohenester, 2007).

The collagen fibrillar form is an abundant structural ECM protein that was very early recognized as a potential regulator of the inflammatory response in mammals (Pacifici et al., 1991). Collagen mediates its biological activities through interactions with different receptors, such as tyrosine kinases of the discoidin domain receptor (DDR) family (Shrivastava et al., 1997; Vogel et al., 1997), α1β1 and α2β1 integrins (Zaman, 2007), glycoprotein VI (Vogel, 2001), leukocyte-associated immunoglobulin-like receptor-1 (LAIR) (Lebbink et al., 2006), and members of the mannose receptor family (Leitinger and Hohenester, 2007). Notably, some of these receptors are expressed by mammalian macrophages and dendritic cells and their engagement by collagen may result in either the activation (Pacifici et al., 1991; Matsuyama et al., 2004; Lee et al., 2007) or the inhibition (Lebbink et al., 2006) of these cells.

Although the innate immune response of fish has been relatively well characterized during recent years at both cellular and molecular levels, no studies worked into the role of ECM in the regulation of innate immunity and inflammation. We have previously shown that gelatin is capable of activating acidophilic granulocytes from

* Corresponding authors. Tel.: +34 968 367581; fax: +34 968 363963.
E-mail addresses: agayala@um.es (A. García-Ayala), vmulero@um.es (V. Mulero).

0161-5890/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.molimm.2008.12.005

the gilthead seabream (*Sparus aurata* L.) (Chaves-Pozo et al., 2008). As these cells, together with macrophages, have been described as the professional phagocytes of this species, and their main activities have been found to be regulated by different pathogen-associated molecular patterns (PAMPs) (Sepulcre et al., 2007), we have studied the main functional activities and the gene expression profiles of these cells in response to collagen-stimulation and compared it with PAMP-stimulation.

2. Materials and methods

2.1. Fish

Healthy specimens (800 g mean weight) of gilthead seabream (*S. aurata* L.) (Actinopterygii, Perciformes, Sparidae), in their second reproductive cycle, were obtained from CULMAMUR, S.L. (Águilas, Spain). The fish were kept at the Spanish Oceanographic Institute (Mazarrón, Murcia) in 14 m^3 running seawater aquaria (dissolved oxygen 6 ppm, flow rate 20% aquarium volume/h) with natural temperature and photoperiod, and fed twice a day with a commercial pellet diet (Trouvit, Spain) at a feeding rate of 1.5% of fish biomass. Fish were fasted for 24 h before sampling. The experiments described comply with the Guidelines of the European Union Council (86/609/EU) and the Bioethical Committee of the University of Murcia (Spain) for the use of laboratory animals.

2.2. Cell isolation

Head kidneys were removed from fish specimens, dissociated through a 100 μm nylon mesh and adjusted to 10^7 viable cells/ml in sRPMI [RPMI-160 culture medium (Gibco) adjusted to gilthead seabream serum osmolarity (353.33 mosmol) with 0.35% NaCl] (Sepulcre et al., 2002) supplemented with 10% fetal calf serum (FCS, Gibco), 100 IU/ml penicillin and 100 μg/ml streptomycin (Biochrom).

Acidophilic granulocytes were obtained by magnetic-activated cell sorting (MACS) as described previously (Roca et al., 2006). Briefly, head kidney cell suspensions were incubated with a 1:10 dilution of a monoclonal antibody (mAb) specific to gilthead seabream acidophilic granulocytes (G7) (Sepulcre et al., 2002), washed twice with phosphate-buffered saline (PBS) containing 2 mM ethylenediaminetetraacetic acid (EDTA) (Sigma) and 5% FCS, and then incubated with 100–200 μl per 10^8 cells micro-magnetic-bead-conjugated anti-mouse IgG antibody (Miltenyi Biotec). After washing, $G7^+$ and $G7^-$ cell fractions were collected by MACS following the manufacturer's instructions and their purity was analysed by flow cytometry (Roca et al., 2006). Head kidney macrophage monolayers were then obtained after overnight culture of $G7^-$ fractions in FCS-free medium and their identity was confirmed by the expression of macrophage colony stimulating factor receptor (M-CSFR) (Roca et al., 2006).

2.3. Cell culture and treatments

Total head kidney leukocytes and macrophages and acidophilic granulocytes fractions were stimulated for 4 or 16 h at 23 °C with 10 μg/ml lipopolysaccharide (LPS, Sigma) and 50 μg/ml phenol-extracted genomic DNA from *Vibrio anguillarum* ATCC19264 cells (VaDNA), 0.01–10 μg/ml type I collagen from calf skin (C9791, Sigma) or 0.01–10 μg/ml gelatin from bovine skin (G9391, Sigma) in supplemented sRPMI. As type I collagen is acid-soluble and needs to be dissolved in 0.1 M acetic acid, a final concentration 0.001 M acetic acid was added to all samples. In some experiments, cells were pre-treated for 30 min with 20–200 nM MMP2/MMP9 inhibitor V (Cat. No. 444285, Calbiochem) or incubated for 16 h in the presence of 40 μg/ml polymyxin B (P4932, Sigma) before being stimulated as previously described. In another series of experiments, the culture plates were coated overnight at 4 °C with the stimuli, then treated for 10 min at 37 °C with 0.25–0.50 U/ml collagenase from *Clostridium histolyticum* (C5138, Sigma) in Hanks' balanced salt solution (HBSS, Gibco) and washed with PBS to remove the enzyme before adding the cells which were incubated for 16 h.

2.4. Reactive oxygen intermediate (ROI) production assays

Respiratory burst activity for head kidney cells was measured as the luminol-dependent chemiluminescence (Mulero et al., 2001) brought on by adding 100 μM luminol (Sigma) and 1 μg/ml phorbol myristate acetate (PMA) (Sigma). Then, the chemiluminescence was recorded every 117 s for 1 h in a FLUOstart luminometer (BGM, LabTechnologies). The values reported are the average of at least three fish, expressed as curve maximum, from which the apparatus background was subtracted.

2.5. Analysis of gene expression

Total RNA was extracted from cell pellets with TRIzol Reagent (Invitrogen) following the manufacturer's instructions and treated with DNase I, 1 U/μg RNA Amplification grade (Invitrogen). The SuperScript III RNase H^- ReverseTranscriptase (Invitrogen) was used to synthesize first strand cDNA with oligo-dT_{18} primer from 1 μg of total RNA at 50 °C for 50 min. Real-time PCR was performed with an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems). Reaction mixtures were incubated for 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 1 min at 60 °C, and finally 15 s at 95 °C, 1 min at 60 °C and 15 s at 95 °C. For each mRNA, gene expression was normalized to the ribosomal protein S18 content in each sample using the comparative Ct method ($2^{-\Delta\Delta Ct}$). The primers used are shown in Table 1. In all cases, each PCR was performed with triplicate samples and repeated with at least two independent samples.

Table 1
Primer sequences used for gene expression analysis. The gene symbols followed the Zebrafish Nomenclature Guidelines (http://zfin.org/zf_info/nomen.html).

Gene	Accession number	Primer name	Nucleotide sequence (5′ → 3′)
rps18	AM490061	F	AGGGTGTTGGCAGACGTTAC
		R	CTTCTGCCTGTTGAGGAACC
il1b	AJ277166	F3	ATGCCCGAGGGGCTGGGC
		R2	CAGTTGCTGAAGGGAACAGAC
tnfa	AJ413189	FE2	TATGGGGGCATACACAACA
		RE5	TTAAAGTGTCAAACACACCAAA
cox2	AM296029	F1	GAGTACTGGAAGCCGAGCAC
		R1	GATATCACTGCCGCCTGAGT
ccl4	AM765840	F1	GCTGTGTTTGTGCTGATGCT
		R1	GCTGGCTGGTCTTTTGGTAG
tgfb1	AF424703	F	AGAGACGGGCAGTAAAGAA
		R	GCCTGAGGAGACTCTGTTGG
il6	AM749958	F1	AGGCAGGAGTTTGAAGCTGA
		R1	ATGCTGAAGTTGGTGGAAGG
il1r2	AM296027	F	AAGGACTCCAGCTCCACTGA
		R	ACGCCTTCTACATGGACCAC
mmp9	AM905938	F1	GGGGTACCCTCTGTCGATTT
		R1	CCTCCCCAGCAATATTCAGA
mmp13	AM905935	F	CGGTGATTCCTACCCATTTG
		R	TGAGCGGAAAGTGAAGGTCT
timp2a	AM905937	F	CAAAGGTGGTGGGAGAGAAA
		R	TTGACGTCCAGGGTAACTCC
timp2b	AM905936	F	ATGTCGTTATCAGGGCGAAG
		R	AGAAGTGGGAGCGGTGTAGA