Veterinary Immunology and Immunopathology 154 (2013) 62–67

Contents lists available at SciVerse ScienceDirect

Veterinary Immunology and Immunopathology

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

Short communication

# Cloning and characterization of bottlenose dolphin (*Tursiops truncatus*) interleukin-10

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ARTICLE INFO

*Article history:*
Received 5 February 2013
Received in revised form 15 April 2013
Accepted 17 April 2013

*Keywords:*
Dolphin
Interleukin-10

ABSTRACT

The function of cytokines in cetaceans has so far only been determined for the proinflammatory cytokines. In this study, we cloned bottlenose dolphin (*Tursiops truncatus*) interleukin-10 (IL-10) cDNA from concanavalin A (Con A)-stimulated peripheral blood mononuclear cells (PBMC), and investigated the mRNA expression levels in various tissues and the bioactivity of recombinant dolphin (rd) IL-10. The gene encodes a polypeptide of 178 amino acids which encompasses the mature protein sequence of 158 amino acids. Quantitative expression analysis of dolphin IL-10 revealed that the highest mRNA levels are found in the spleen. To assess its function, rdIL-10 was produced in human embryonic kidney 293 cells and its bioactivity was demonstrated through IL-10-induced inhibition of proinflammatory cytokine mRNA expression IFN-γ, TNF-α, and IL-2 of Con A-stimulated PBMC. These results indicated that the structure and function of bottlenose dolphin IL-10 is similar to that of other animals. This is the first report of the characterization of an anti-inflammatory cytokine in cetaceans.



## 1. Introduction

Many anatomical systems in cetaceans are highly modified for their aquatic lifestyle, with dramatic changes in the ear region, skin, limbs and cranium when compared to terrestrial mammals (Uhen, 2007). In this process of adaptation, cetaceans are likely to have developed some unique immunological features, and in fact, cetaceans lost absent in melanoma 2 (Aim2) gene linked to innate detection of nucleic acids (Brunette et al., 2012; King et al., 2001). Cytokines, which are essential for a functioning immune system, are broadly divided into two groups, pro- and anti-inflammatory cytokines. So far, molecular cloning studies on cetacean cytokines have been carried out to investigate IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-13, IL-18, TGF-β and TNF-α, and the deduced amino acid sequences were similar to those of terrestrial mammals (Beineke et al., 2010; King et al., 2001). However, only the function of a few proinflammatory cytokines, such as IL-2 and TNF-α, has so far been confirmed (Itou et al., 2002; Ness et al., 1998). In order to understand regulation of the immune system of cetaceans, it is essential to study the functions of anti-inflammatory cytokines.

In Pacific white-sided dolphin, bottlenose dolphin and beluga whale mononuclear leukocytes stimulated with concanavalin A (Con A), mRNA expression of the anti-inflammatory cytokine IL-10 was highly variable between species (Sitt et al., 2008). Expression levels were increased in the Pacific white-sided dolphin, unchanged in the bottlenose dolphin, and decreased in the beluga whale. There are differences in immune regulation in these species compared to unstimulated mononuclear leukocytes. In humans and mice, IL-10 is produced by activated T cells, B cells, monocytes/macrophages, mast cells and keratinocytes as

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http://dx.doi.org/10.1016/j.vetimm.2013.04.009

a 17–21 kDa cytokine containing two disulfide bridges which functions in the form of a homodimer (de Waal Malefyt et al., 1992). IL-10 is a central anti-inflammatory cytokine that was first identified in supernatants of Con A-stimulated T cells based on its ability to inhibit the synthesis of proinflammatory cytokines such as IFN-γ (Fiorentino et al., 1989). The importance of IL-10-mediated immune regulation is exemplified by lethal, uncontrolled to IL-12 and IFN-γ production in IL-10-knock-out mice infected with the protozoan pathogen *Toxoplasma gondii*, a strong driver of the inflammatory immune response (Gazzinelli et al., 1996).

In this study, we cloned IL-10 cDNA isolated from Con A-stimulated peripheral blood mononuclear cells (PBMC) of bottlenose dolphins. We investigated the mRNA expression in various tissues and the capacity of rdIL-10 to inhibit proinflammatory cytokine mRNA expression. This is the first report of the quantitative analysis of mRNA expression and functional characterization of IL-10 in cetaceans.

## 2. Materials and methods

### 2.1. Samples

Three bottlenose dolphins (*Tursiops truncatus*), one adult and two neonates, who had accidentally died at Minamichita Beach Land Aquarium and Yokohama Hakkeijima Sea Paradise, respectively, were examined in this study. The adult dolphin was apparently healthy until the day before it died and the two neonates died as a result of drowning. There were no detectable abnormalities in the three dolphins at necropsy. Various tissues (muscle, thymus, spleen, bone marrow, heart, liver, kidney and lung) were collected from the dolphins. PBMC were isolated as previously described (Inoue et al., 1999; Itou et al., 2001). Briefly, dolphin peripheral blood from Shinagawa Aquarium ($n = 4$) was overlaid onto Lymphoprep (AXIS-SHIELD PoC AS, Oslo, Norway) and after centrifugation at 800 × *g* for 30 min, PBMC were isolated and resuspended to a final concentration of $1 \times 10^7$ cells/ml in RPMI 1640 (Wako, Osaka, Japan) containing 10% (v/v) fetal bovine serum (FBS; Biowest, Caille, France), 100 U/ml penicillin (MP Biomedicals, Solon, OH), 100 μg/ml streptomycin (Gibco, Grand Island, NY, USA).

### 2.2. Cloning of bottlenose dIL-10

PBMC ($1 \times 10^6$ cells/ml) in RPMI 1640 containing 10% (v/v) FBS (Biowest), 100 U/ml penicillin (MP Biomedicals), 100 μg/ml streptomycin (Gibco) and 2 μg/ml Con A (Wako Pure Chemicals Industries, Ltd., Osaka, Japan) were incubated for 6 h at 37 °C in 5% $CO_2$. Total RNA was isolated using an RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The primers used in this study are shown in Table 1. PCR primers were designed based on published human, canine and bovine IL-10 cDNA sequences (GenBank accession number: M57627, U33843 and U00799, respectively). First-strand cDNA synthesis and amplification of partial dIL-10 cDNA was performed as previously described (Segawa et al., 2010). After the decision on partial dIL-10 nucleotide sequences, 5′ and 3′ RACE cDNAs were generated using a SMART RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions. This was followed by PCR using dIL-10-specific primers in addition to the universal primer mix included in the SMART RACE kit. Nucleotide sequences of the PCR products were determined by direct sequencing using an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The nucleotide sequences of dIL-10 were determined from three dolphins. The signal peptide sequence and mature protein were predicted using SMART (http://smart.embl-heidelberg.de/). Multiple alignments of mature dIL-10 and IL-10 amino acid sequences from other animals were generated and analyzed using ClustalW (Thompson et al., 1994), and phylogenetic trees were created by the neighbor-joining (NJ) method with 1000 bootstrap replications using MEGA 4.0 software.

### 2.3. Analysis of dIL-10 mRNA expression in tissues

Total RNA was isolated from the tissues as described above and cDNA was synthesized from 1 μg total RNA using a High Capacity RNA-to-cDNA kit (Applied Biosystems) according to the manufacturer's instructions. Real-time PCR was performed with an ABI Prism 7500 (Applied Biosystems) and expression levels were calculated using the ΔCt method relative to an endogenous control gene, β-actin as described previously (Segawa et al., 2013). The β-actin appeared to be more stable among the tissues of three dolphins because the coefficient of variation (CV) was 5.6%. The relative values were normalized against the tissue with the lowest observed expression levels (muscle). The primers used in this study are listed in Table 1.

### 2.4. Production of recombinant dIL-10 (rdIL-10)

Human embryonic kidney (HEK) 293 cells were routinely grown in DMEM (Wako) containing 10% (v/v) FBS (Biowest), 100 U/ml penicillin (MP Biomedicals) and 100 μg/ml streptomycin (Gibco) at 37 °C in 5% $CO_2$. HEK 293 cells were transfected with a total of 2.5 μg of plasmid pcDNA 3.1 + dIL-10 (Invitrogen, Carlsbad, CA) or empty pcDNA 3.1 as a plasmid control (pcDNA) containing a V5 epitope tag using Lipofectamine LTX with Plus Reagent (Invitrogen) according to the manufacturer's instructions. The dIL-10-transfected cells were selected with 500 μg/ml of geneticin for 3–5 weeks to make stably transfected cells. The stably transfected cells were maintained in DMEM containing 10% (v/v) FBS (Biowest), 100 U/ml penicillin (MP Biomedicals) and 100 μg/ml streptomycin (Gibco). The presence of rdIL-10 in the supernatant was confirmed by western blot analysis. Briefly, debris was removed by centrifugation and the supernatants were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 15% polyacrylamide gels. After separation, semi-dry western blotting onto an Immobilon-P Transfer Membrane (Millipore, Bedford, MA, USA) was carried out for 1 h at room temperature. The membrane was blocked in 20 mM Tris–HCl, 150 mM NaCl, and 0.1% (v/v) Tween 20, pH 7.6 containing Block Ace (Dainihon Pharmaceutical, Osaka, Japan) for 1 h at room temperature. After blocking,