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Short communication

Identification of the mRNA encoding interleukin-6 and its receptor, interleukin-6 receptor α, in five marsupial species

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ABSTRACT

Expressed coding sequences for interleukin-6 (IL-6) and interleukin-6 receptor α (IL-6R) were examined in five marsupial species. Full length expressed coding sequences for IL-6 and IL-6R were identified and characterized in the gray short-tailed opossum (*Monodelphis domestica*). For IL-6, ~225 bp fragments of the mRNA sequence were identified in the red-tailed phascogale (*Phascogale calura*), kultarr (*Antechinomys laniger*), and stripe-faced dunnart (*Sminthopsis macroura*), while ~563 bp fragments of mRNA encoding IL-6R were identified in the red-tailed phascogale, kultarr, stripe-face dunnart and fat-tailed dunnart (*Sminthopsis crassicaudata*). Relative expression levels of IL-6 and IL-6R were examined in the heart, muscle, lung, liver, spleen and kidney of adult red-tailed phascogales, and IL-6 gene expression was found to be significantly higher in the lung and spleen than the other tissues examined, while the expression of IL-6R was significantly higher in the liver, lung and spleen. These results now serve as a reference point for examining the role and levels of IL-6 and IL-6R in the health and disease of these marsupial species. The pro-tumorigenic nature of IL-6 is of particular interest, and the identification of these IL-6 and IL-6R coding sequences provides a platform for further work to evaluate the potential role of IL-6 in marsupial cancers.

1. Introduction

Cytokines play a key role in the control and regulation of many components of the vertebrate immune system. It is the interaction of cytokines and immune effectors that mediate the cell-to-cell communication that in turn produces the complex immune response characteristic of higher vertebrates (Beck and Dormer, 2004). The functions and role of cytokines in the immune response have been broadly studied in common model eutherian mammals. One such pleiotropic cytokine is IL-6 which is produced by many cell types, including T cells, B cells, endothelial cells, monocytes, and fibroblasts (Kishimoto, 1989, 2003). Interleukin-6 (IL-6) exerts a broad effect on both immune and non-immune cell types, and can act in an autocrine or paracrine manner. The effects of IL-6 have been shown to be context-dependent, and can be pro- or anti-inflammatory (Hunter and Jones, 2015).

The functional IL-6 receptor (IL-6R) complex comprises a type 1 cytokine α-receptor subunit, to which IL-6 binds, and a ubiquitously expressed signal-transducing β-receptor subunit (gp130) (Hunter and Jones, 2015). IL-6R is used by a few cytokines in the IL-6 family, including IL-6, CNTF and IL-33 (Rose-John et al., 2015). IL-6R can be present in either a membrane-bound form (mIL-6R) or soluble form (sIL-6R). IL-6 can activate cells via a classical signalling pathway, using mIL-6R or via a *trans*-signalling pathway, using sIL-6R (reviewed in Neurath and Finotto, 2011). The IL-6/sIL-6R complex enables the activation of cells that do not otherwise express IL-6R (Rose-John, 2006), and therefore widens the scope of IL-6 signalling (Drucker et al., 2010).

IL-6 can act as a growth factor for some types of cancer, and has therefore been classified as pro-tumorigenic (Rojas et al., 2011; Mihara et al., 2012). Further the activity of IL-6 is known to increase as the expression of IL-6R increases (Li et al., 2011), thus when investigating the role or effects of IL-6 in a particular context the levels of IL-6R can be just as important as a standalone increase or decrease in the level of IL-6 (Jones et al., 2001). In cancerous

Abbreviations: CNRQ, calibrated normalized relative quantity; CNTF, ciliary neurotrophic factor; FT dunnart, fat-tailed dunnart; G-CSF, granulocyte colony-stimulating factor; IL, interleukin; MGF, myelomonocytic growth factor; mIL-6R, membrane-bound interleukin-6 receptor α; mRNA, messenger ribonucleic acid; PBMC, peripheral blood mononuclear cell; SF dunnart, stripe-faced dunnart; sIL-6R, soluble interleukin-6 receptor α; WSU, Western Sydney University.

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epithelial cells the over-expression of IL-6R enhanced IL-6-induced activation and increased cell growth and tumorigenicity (Zhang et al., 2013). In some cancers, IL-6R has proved to be a more reliable marker of disease progression or status than IL-6 (Isobe et al., 2015).

Marsupials are a unique group of mammals whose reproduction is typified by a short gestation followed by long lactation. Unlike eutherian mammals marsupial young are born in an extremely altricial state and thus captive marsupials are often important models in studies of developmental and comparative immunology. Adult marsupials have similar histologically mature immune tissues to those of eutherian mammals (Borthwick et al., 2014). Genome data is available for some model species, including the gray short-tailed opossum (*Monodelphis domestica*) (Mikkelsen et al., 2007), tammar wallaby (*Macropus eugenii*) (Renfree et al., 2011), Tasmanian devil (*Sarcophilus harrisii*) (Murchison et al., 2012) and koala (*Phascolarctos cinereus*) (Hobbs et al., 2014). To date, *in silico* studies of these genomes have identified some immunological genes, including cytokines that are conserved in marsupials, and suggest that the marsupial immune system has the same level of complexity to that of eutherian mammals (Wong et al., 2006). Identification of the expressed form of IL-6 has been reported for the Tasmanian devil (Morris and Belov, 2013) and tammar wallaby (Alsemgeest et al., 2013), and the expression profile of IL-6 has been examined in cultured koala peripheral blood mononuclear cells (PBMCs) (Maher et al., 2014). However, there have been no reports of expressed IL-6R in any marsupial species to date.

The potential action of IL-6 in marsupials is unknown. Thus, the present study will address the paucity of knowledge by identifying IL-6 in five marsupial species where tumorigenesis is known to occur (Attwood and Woolley, 1973; Ley, 1997; Stannard and Old, 2014). Thus our aim is to isolate IL-6 and IL-6R in these animals and to examine the expression levels of these genes in the tissues of normal, healthy individuals. These values may subsequently be used to determine if higher than normal levels of IL-6 or IL-6R expression may induce or aid cancer development in these species, as has been reported in eutherian species.

2. Materials and methods

2.1. Animals and tissues

All work received prior approval from the Western Sydney University (WSU) Animal Care and Ethics Committee (approval number A9690) and the University of Sydney Animal Ethics Committee (approval number K22/10-2011/3/5618). Samples from phascogales (spleen, n = 10; liver, n = 6; lung, n = 6; muscle, n = 6; heart, n = 6; kidney, n = 6) and kultarrs (spleen, n = 2) were collected opportunistically from animals housed in the WSU School of Science and Health Small Native Mammal Teaching and Research Facility, Richmond, NSW, Australia. These animals were euthanized during routine population management procedures. A postmortem was conducted immediately for each animal to ensure there were so signs of disease or tumors. Spleen samples were also collected from stripe-faced dunnarts (n = 2) and fat-tailed dunnarts (n = 2) maintained at the University of Sydney, School of Medical Sciences, Camperdown, NSW, Australia. Spleen tissue from an adult opossum (n = 1) was kindly donated by Professor Norman Saunders (University of Melbourne, VIC, Australia).

Total RNA was extracted from collected tissues using the SV Total RNA Isolation System (Promega; Wisconsin, USA). Extracted RNA was used to synthesize cDNA using the SuperScript III First-Strand Synthesis SuperMix (Invitrogen; California, USA) using oligo (dT) primers. Opossum spleen total RNA was purified using the Oligotex mRNA Mini Kit (Qiagen; Maryland, USA), and used to synthesize RACE-ready cDNA using the SMARTer RACE cDNA Amplification Kit (Clontech; California, USA).

2.2. Primers

Consensus primers for RT-PCR were designed using Oligo6 software (Molecular Biology Insights; Colorado, USA) based on a consensus alignment of the predicted marsupial IL-6 and IL-6R sequences available in Ensembl (release 75) (Flicek et al., 2012), (see Table 1 for all primers designed and used in this study). Opossum IL-6 and IL-6R sequence fragments identified here were used to design primers for RACE PCR. RACE PCR sequences were then used to design 5′ and 3′ UTR primers for sequencing. Preliminary phascogale IL-6 and IL-6R sequences were used to design suitable qPCR primers for the gene expression studies.

2.3. PCR

RT-PCR reactions followed a typical cycle of: 95 °C for 3 min; 35 repeats of: 95 °C for 30 s, 50–60 °C for 50 s and 72 °C for 60 s, followed by a final extension step at 72 °C of 10 min. Specific annealing temperatures for each primer set are listed in Table 1. GoTaq Flexi (Promega) was used for all RT-PCR reactions. All RACE reactions were carried out using the SMARTer RACE cDNA Amplification Kit (Clontech; California, USA) and recommended cycle conditions. For qPCR, GAPDH and β-actin were chosen as reference genes based on their expression stability across phascogale tissues as examined by Ong et al. (unpublished). Gene expression was examined in the lungs, heart, liver, spleen, muscle and kidney from normal and healthy adult phascogales (n = 6). Females (n = 3) and males (n = 3) were equally represented. Reactions were carried out on a Rotor-Gene Q with a Rotor-Gene SYBR Green PCR kit (Qiagen; Hilden, Germany). All reactions followed the same cycle program of: 95 °C for 5 min, followed by 45 repeats of 95 °C for 5 s, 55 °C for 5 s, and 72 °C for 10 s. Melt curves from 55 °C to 95 °C were included in each run. Temperature was increased by 0.5 °C per step with 5 s between each.

A standard curve for each gene was constructed from a pool of all cDNA samples to be examined to assess reaction efficiency, presence of inhibitors, limit of detection (LOD) and limit of quantification (LOQ). 'No template' controls were included for each reaction, and inter-run calibrators were included in each run to correct for any potential run-to-run variation. The coefficient of determination (R^2) of all primer pairs was $\geq$0.99, and reaction efficiencies were between 90 and 102%. Amplification curves for each gene were examined using the relevant standard curve in the Rotor-Gene Q software, before the calculated quantification cycle (Cq) values were imported into qBase+ (Biogazelle; Gent, Belgium) for analysis. Expression values were normalized using the geometric mean of the reference genes. Calibrated normalized relative quantities (CNRQ) for each tissue were expressed relative to the heart tissues as a control group, as expression in this tissue was consistently low (Cq data not shown). For reference the equations for calculating CNRQ using qBase + can be found in Hellemans et al. (2007). CNRQ values were log transformed using $\log_{10}(x)$ before running an ANOVA to determine if the differences in gene expression across tissues or between groups were significant.

2.4. Cloning and sequencing

PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega; Wisconsin, USA), and cloned prior to sequencing, using the TOPO TA Cloning® Kit for Sequencing (Invitrogen; California, USA) to ligate the PCR products into a vector that