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Multiple suppression pathways of canonical Wnt signalling control thymic epithelial senescence

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ABSTRACT

Members of the Wnt family of secreted glyco-lipo-proteins affect intrathymic T-cell development and are abundantly secreted by thymic epithelial cells (TECs) that create the specific microenvironment for thymocytes to develop into mature T-cells. During ageing, Wnt expression declines allowing adipoid involution of the thymic epithelium leading to reduced naïve T-cell output. The protein kinase C (PKC) family of serine-threonine kinases is involved in numerous intracellular biochemical processes, including Wnt signal transduction. In the present study, PKCδ expression is shown to increase with age and to co-localise with Wnt receptors Frizzled (Fz)-4 and -6. It is also demonstrated that connective tissue growth factor (CTGF) is a Wnt-4 target gene and is potentially involved in a negative feed-back loop of Wnt signal regulation. Down-regulation of Wnt-4 expression and activation of multiple repressor pathways suppressing β-catenin dependent signalling in TECs contribute to the initiation of thymic senescence.

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1. Introduction

During ageing of the immune system the gradual loss of naïve T-cells is associated with the rate of thymic adipose involution that correlates with significant destruction of the epithelial network. As impaired T-cell production leads to weakened immune responses, understanding the mechanism of thymic involution has high physiological and medical importance.

In our recent studies of thymic involution Wnt-4 secretion was significantly reduced in TECs while LAP2α expression concomitantly increased triggering epithelial–mesenchymal transition (EMT) and then pre-adipocyte-differentiation (Kvell et al., 2010).

As Wnt-4 is the primary regulator of FoxN1 expression and consequently TEC identity, understanding Wnt-4 signalling carries particularly high importance (Balciunaite et al., 2002). The difficulty of signalling studies, however, stems from the general complexity of Wnt pathways (Kuhl and Pandur, 2009). Wnt-4, for example, has been described as activator of both β-catenin dependent canonical (Lyons et al., 2004) and JNK/PKC dependent non-canonical (Cai et al., 2002; Du et al., 1995) signalling pathways that interact at multiple levels. Apart from specific, there are also shared signalling elements in Wnt pathways including the main cell surface receptors Frizzleds (Fz) (Schulte and Bryja, 2007) as well as intracellular signalling molecules including G proteins (Malbon et al., 2001), Dishevelleds (Dvl) (Kuhl et al., 2001; Schulte and Bryja, 2007) and PKCs α (Kuhl et al., 2001), ζ (Ossipova et al., 2003), and δ (Kinoshita et al., 2003). PKCδ appears particularly important as this serine-threonin kinase can phosphorylate and therefore activate Dvls (Kinoshita et al., 2003) to relay ligand induced signals towards down-stream elements of Wnt cascades.

From the ten known mammalian Fz receptors, Fz-4 (Lyons et al., 2004) and Fz-6 (Lyons et al., 2004) have been confirmed to bind Wnt-4. Interestingly, while Fz-4 is an activator of the β-catenin dependent canonical pathway, signals from Fz-6 inhibit β-catenin dependent target gene transcription (Golan et al., 2004) indicating that regulation of Wnt-4 signalling might also begin at receptor level in the thymus.

As thymic involution is a complex physiological process and appears to be initiated by suppression of Wnt signals, understanding of receptor associated regulatory mechanisms can lead to target molecule recognition in the quest for re-juvenate the ageing thymus. To investigate the hypothesis, TECs of young and ageing adult Balb/c mice as well as a thymic epithelial cell line, TEP1 were used in the studies. Our experiments demonstrate that expression of Wnt receptors increase with age and that Frizzleds co-localize

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with signalling molecules PKCδ and Dvl. Examination of Wnt-4 target gene expression provides evidence for the existence of additional negative regulatory loops suppressing β-catenin dependent signalling that aids repression of thymic epithelial maintenance and provides opportunities for EMT and consequent adipocyte type differentiation.

2. Materials and methods

2.1. Cell culture

Tep1 (thymic epithelial) (Tanaka et al., 1993), 293 and Phoenix (PHX) human kidney epithelial cell lines were cultured in DMEM (Sigma–Aldrich) supplemented with 10% FCS and 100 μg of penicillin and streptomycin (Lonza Walkersville, Inc.).

2.2. Primary thymic epithelial cells

Balb/c mouse thymi (1 and 9 months) were the source of primary cell material. $CD45^-$ $EpCAM1^+$ TECs were isolated using magnetic cell sorting (Miltenyi Biotech) (purity was regularly above 90% – data not shown). Purified $EpCAM1^+$ TECs were cultured in medium alone, or in medium supplemented with Wnt4.

2.3. Antibodies

For western blot analysis rabbit polyclonal anti-PKCδ (C-17), anti-Dvl(C-19) (Santa Cruz), anti-Fz-6 and anti-Fz-4 (R&D systems Inc.) antibodies were used as primary and HRP-conjugated anti-rabbit and anti-goat (Santa Cruz) were used as secondary antibodies. For fluorescent microscopy studies primary Abs were anti-PKCδ (658-676) pAb (Calbiochem) (1:100), anti-PKCδ (C-17) (Santa Cruz) (1:100), anti-Fz-4, anti-Fz-6 (R&D systems Inc.) (1:100) and FITC labelled anti-EpCAM1 (clone G8.8) (American Type Cell Culture Collection)(1:50) and anti-Ly51-PE (BD Pharmingen) (1:50) antibodies. Secondary antibodies were NorthernLights donkey anti-goat IgG-NL493 and NorthernLights donkey anti-rat IgG-NL557 and anti-rat and anti-rabbit IgG-NL663 (all from R&D Systems Inc.). (Dilution factor for all secondary antibodies was 1:200).

2.4. Histology using fluorescent antibodies

Frozen thymic sections (9 μm thick) were fixed in cold acetone for 10 min, then dried for 15 min and rehydrated and blocked using 5% bovine serum albumin (BSA in PBS for 20 min) before staining with the appropriate antibodies. The primary antibody was applied at appropriate dilution in 100 μl on all sections for 30 min followed by 3 washing steps with PBS for 5 min each. Secondary Ab was applied for 30 min followed by 3 × 5 min wash with PBS as above. PBS-glycerol 1:1 mix was applied before covering with slide covers. The sections were analysed by an Olympus BX-61 Fluorescent microscope or by Olympus Fluoview 300 confocal microscope using the Olympus Fluoview FV1000S-IX81 software.

Staining controls were the following: primary Ab with no secondary Ab, no primary just secondary Ab and irrelevant primary Ab for isotype control in combination with secondary Ab. All the stainings were repeated for a minimum of three times.

2.5. Subcloning of Wnt-4 and full length PKCδ

Wnt-4 was purchased and subcloned from a commercially available vector (Origene), while the full length PKCδ was a kind gift of Jae-Won Soh, Tnha University, Korea. Both Wnt-4 and PKCδ sequences were subcloned into the MIGRI retroviral vector (gift from W.S. Pear, Department of Pathology and Laboratory Medicine, University of Pennsylvania, PA). Retrovirus was produced by transfecting the plasmid DNA into the Phoenix packaging cell line (American Type Cell Culture Collection) using Lipofectamine 2000 (Invitrogen).

2.6. Transient transfection of siRNA PKCδ

siRNA specific for PKCδ was supplied by Santa Cruz. Tep1 cells were grown to 80% confluency and then siRNA and control siRNA was delivered using Lipofectamine according to manufacturer's instruction.

2.7. Cell sorting

Tep1 cells were infected with recombinant retroviruses encoding GFP, Wnt-4-GFP or wild type- PKCδ-GFP then sorted based on GFP expression by FACSVantage Cell Sorter (BD). GFP positive cells were cultured further under conditions described in Section 2.1.

2.8. Reverse transcription polymerase chain reaction (RT-PCR) and quantitative RT-PCR (Q-RT-PCR)

RT-PCR was conducted as described previously (Kvell et al., 2010). Q-RT-PCR was performed using SYBR Green Q-RT-PCR reagents and random hexamer primers (Applied Biosystems) as recommended by the manufacturer using an ABI Prism 7900HT sequence detection system. Threshold cycles (C_T) for three replicate reactions were determined using Sequence Detection System software (version 2.2.2), and relative transcript abundance was calculated following normalization with a β-actin PCR amplicon. Quantitation of Q-RT-PCR products were based on a standard curve generated from untreated TEP1 cell line gene expression. PCR primer sequences are listed in Table 1.

2.9. PKCδ activation assay

Tep1 cells were lysed in RIPA buffer supplemented with protease and phosphatase inhibitors (Sigma–Aldrich) and immunoprecipitated with rabbit anti-PKCδ (658-676) pAb (Calbiochem) and protein G resin (Sigma–Aldrich) overnight at 4 °C. Kinase assay was performed using an HTScan PKCδ Kinase–assay Kit (Cell Signaling Technology Inc.) with biotinylated substrate peptide in the presence of diluted PKCδ. Active PKCδ kinase GST fusion protein was supplied to the kit as positive control. PKCδ specific activity was quantified in a colorimetric ELISA Assay using 96-well streptavidin-coated plates (Institute of Isotopes, Budapest, and Soft Flow Hungary Ltd., Hungary). Phosphorylation level of biotynilated substrates from each kinase reaction mix were measured using a rabbit anti phosphoSer/Thr-antibody (1:1000) (provided with the kit) detected by a HRP-labelled anti-rabbit (1:1000) (Santa Cruz) in the presence of TMB substrate. Optical density (absorbance) was read in an iEMS Reader MF V2.9 (Thermo Scientific, Waltham, MA) spectrophotometer using a bi-chromatic measurement system at 450 nm and 620 nm as reference.

2.10. Purification of proteins from cell membrane and cytosol

Tep1 cells (1×10^6/condition) were treated with Wnt-4 and control supernatants for 30 min then cells were pelletted and cytosolic and membrane proteins were isolated as described previously (McMillan et al., 2003). Proteins of cytosolic and membrane fractions were separated in 10%SDS PAGE, blotted, blocked in 3% fat-free milk and probed for PKCδ protein. To ensure equal loading protein levels were visualised by Ponceau Red staining, when proteins were entering the separating gel.

2.11. Immunoprecipitation and Western blotting

Cell lysates were immunoprecipitated using anti-Fz-4 and anti-Fz-6 antibodies (R&D systems Inc.) and protein G resin (Sigma–Aldrich) in the presence of protease and phosphatase inhibitors (Sigma–Aldrich). Proteins were resolved in 10% SDS–PAGE, blotted onto nitrocellulose membranes, then blocked in buffer containing 3% fat-free dried milk and probed for the proteins of interest with primary then in the appropriate HRP-conjugated secondary antibodies. Proteins were visualized by enhanced chemiluminescence (Pierce) according to the manufacturer's instructions in a FUJI LAS4000 image station.

Table 1
PCR primers.

Gene	Accession nos.	Forward primer	Reverse primer
β-actin	NM_007393	TGG CGC TTT TGA CTC AGG A	GGG AGG GTG AGG GAC TTC C
Wnt-4 cloning primers	NM_030761	gaagatcttc ATGAGTCCCCGCTCGTGC	ccgctcgagcgg TCATCGGCACGTGTGCAA
Wnt-4 PCR primers	NM_030761	CTC AAA GGC CTG ATC CAG AG	TCA CAG CCA CAC TTC TCC AG
CTGF	NM_010217	GGCCTCTTCTGCGATTTCG	CCATCTTTGGCAGTGCACACT
PKC-δ PCR primers	NM_011103	AGGCCGTGTTATCCAGATTG	CGGTTCATGGTTGGAAACTT
Frizzled 4	NM_008055	TCTGCTTCATCTCCACCACCTT	GCGCTCAGGGTAAGAAAACCT
Frizzled 6	NM_008056	GCGGCGTTTGCTTCGTT	CACAGAGGCAGAAGGACGAAGT
Frizzled 8	NM_008058	TTCCGAATCCGTTCAGTCATC	GCGGATCATGAGTTTTTCTAGCTT

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