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Research Paper

The Tim-3-galectin-9 Secretory Pathway is Involved in the Immune Escape of Human Acute Myeloid Leukemia Cells

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ABSTRACT

Acute myeloid leukemia (AML) is a severe and often fatal systemic malignancy. Malignant cells are capable of escaping host immune surveillance by inactivating cytotoxic lymphoid cells. In this work we discovered a fundamental molecular pathway, which includes ligand-dependent activation of ectopically expressed latrophilin 1 and possibly other G-protein coupled receptors leading to increased translation and exocytosis of the immune receptor Tim-3 and its ligand galectin-9. This occurs in a protein kinase C and mTOR (mammalian target of rapamycin)-dependent manner. Tim-3 participates in galectin-9 secretion and is also released in a free soluble form. Galectin-9 impairs the anti-cancer activity of cytotoxic lymphoid cells including natural killer (NK) cells. Soluble Tim-3 prevents secretion of interleukin-2 (IL-2) required for the activation of cytotoxic lymphoid cells. These results were validated in ex vivo experiments using primary samples from AML patients. This pathway provides reliable targets for both highly specific diagnosis and immune therapy of AML.

1. Introduction

Acute myeloid leukemia (AML) is a blood/bone marrow cancer originating from self-renewing malignant immature myeloid precursors, which rapidly becomes a systemic malignancy. It is often a fatal disease because malignant cells are capable of suppressing anti-cancer immunity by impairing the functional activity of natural killer (NK) cells and cytotoxic T cells (Golden-Mason et al., 2013; Wang et al., 2007; Khaznadar et al., 2014). Recent evidence clearly demonstrated an involvement of the T cell immunoglobulin and mucin domain 3 (Tim-3) - galectin-9 pathway in this immune escape mechanism (Golden-Mason et al., 2013; Kikushige et al., 2015; Gonçalves Silva et al., 2016). Galectin-9 is a β-galactoside-binding lectin, which has a tandem structure and contains two carbohydrate recognition domains (CRDs) fused together by a peptide (Delacour et al., 2009). Galectin-9 has a specific receptor on AML cells known as Tim-3 which also could act as its possible trafficker (galectin-9 as all other galectins lacks a signal sequence required for transport into the endoplasmic reticulum (ER) and thus requires a trafficking protein for its secretion (Hughes, 1999; Delacour et al., 2009)). However, the mechanisms underlying the activation of biosynthesis of the components of the Tim-3-galectin-9 autocrine loop, galectin-9 secretion and its effects on cytotoxic lymphocytes (NK cells and T cells) remain poorly understood.

Recently, we discovered that human AML cells – but not healthy leukocytes – express physiologically active latrophilin 1 (LPHN1; Sumbayev et al., 2016). LPHN1, an adhesion G-protein-coupled receptor, is highly expressed in neuronal axon terminals and in many secretory cells (Davletov et al., 1998; Silva and Ushkaryov, 2010). In all cells expressing this receptor, LPHN1 activation by its most potent agonist, α-latrotoxin (LTX) from black widow spider venom (Ushkaryov, 2002), triggers intracellular Ca^{2+} signaling and exocytosis of neurotransmitters and hormones (Volynski et al., 2003). Similarly, ligand-

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induced activation of LPHN1 in AML cells facilitates exocytosis of cytokines and growth factors (Sumbayev et al., 2016). Production of LPHN1 in AML cells is controlled by the mammalian target of rapamycin (mTOR) (Sumbayev et al., 2016), a highly conserved serine/threonine kinase that acts as a central regulator of growth and metabolism in healthy and malignant human myeloid cells (Yasinska et al., 2014). To function in cell-cell interactions and cell signaling, LPHN1 can interact with at least two endogenous ligands, Lasso/teneurin-2 (Silva et al., 2011) and fibronectin leucine rich transmembrane protein 3 (FLRT3) (Boucard et al., 2014), although only FLRT3 seems to be expressed in peripheral tissues. In addition to triggering exocytosis by increasing cytosolic Ca^{2+}, LPHN1 can enhance the sensitivity of the release machinery by activating protein kinase C (Liu et al., 2005), which is also thought to be involved in galectin-9 secretion (Chabot et al., 2002). Based on these observations, we hypothesized that activation of LPHN1 by its ligands can induce secretion of galectin-9, thus protecting AML cells against NK and cytotoxic T cells. This hypothesis has been studied experimentally in the present study.

Here we report that the Tim-3-galectin-9 autocrine loop is activated in AML cells through protein kinase C (PKC)/mTOR pathways. These pathways trigger translation of both Tim-3 and galectin-9 and induce high levels of galectin-9 secretion as well as the release of soluble Tim-3. Importantly, this effect was also verified in the AML patients studied. Galectin-9 was found to impair AML cell killing by primary human NK cells. Soluble Tim-3 reduced the ability of T cells to secrete IL-2, a cytokine, which is required for the activation of both NK cells and cytotoxic T cells (Dhupkar and Gordon, 2017). Blood plasmas of AML patients contained significantly lower amounts of IL-2 compared to those of healthy donors. We confirmed that PKC activation occurred in AML cells in a LPHN1-dependent manner. The LPHN1 agonist LTX and natural ligand FLRT3 upregulated the Tim-3-galectin-9 autocrine loop in a PKC-dependent manner. Based on our findings, we conclude that LPHN1/PKC/mTOR/Tim-3-galectin-9 is a biosynthetic and secretory pathway which is operated by human AML cells resulting in a decrease of immune surveillance and promotion of disease progression.

2. Materials and Methods

2.1. Materials

RPMI-1640 medium, fetal bovine serum and supplements and basic laboratory chemicals were purchased from Sigma (Suffolk, UK). Maxisorp™ microtitre plates were provided either by Nunc (Roskilde, Denmark) and Oxley Hughes Ltd. (London, UK). Mouse monoclonal antibodies directed against mTOR and β-actin, as well as rabbit polyclonal antibodies against phospho-S2448 mTOR, galectin-9, HRP-labelled rabbit anti-mouse secondary antibody were purchased from Abcam (Cambridge, UK). Mouse monoclonal antibody against FLRT3 was obtained from Santa Cruz Biotechnology (Heidelberg, Germany). The polyclonal rabbit anti-peptide antibody (PAL1) against LPHN1 was described previously (Davydov et al., 2009). LTX was purified as previously described (Ashton et al., 2000). Goat anti-mouse and goat anti-rabbit fluorescence dye-labelled antibodies were obtained from LI-COR (Lincoln, Nebraska USA). ELISA-based assay kits for the detection of galectin-9, Tim-3 and IL-2 were purchased from Bio-Techne (R&D Systems, Abingdon, UK). Anti-Tim-3 mouse monoclonal antibody, its single chain variant as well as human Ig-like V-type domain of Tim-3 (amino acid residues 22–124), expressed and purified from *E. coli* (Prokhorov et al., 2015) were used in our work. Secondary antibodies for confocal laser microscopy and imaging flow cytometry (goat anti-mouse and goat anti-rabbit Alexa 488, Alexa 555 and Alexa 647) were from Invitrogen (Carlsbad, USA). All other chemicals purchased were of the highest grade of purity.

2.2. Cell Lines and Primary Human Cells

THP-1 human myeloid leukemia monocytes, K562 chronic myelogenous leukemia cells and Jurkat T cells were obtained from the European Collection of Cell Cultures (Salisbury, UK). Renal clear cell carcinoma RCC-FG1 cells were obtained from CLS Cell Lines Service (Eppelheim, Germany). Cells were cultured in RPMI 1640 media supplemented with 10% fetal bovine serum, penicillin (50 IU/ml) and streptomycin sulfate (50 μg/ml). LAD2 mast cells were kindly provided by A. Kirshenbaum and D. Metcalfe (NIH, USA). Cells were cultured in StemPro-34 serum-free media in the presence of 100 ng/ml SCF (Kirshenbaum et al., 2003).

Primary human AML mononuclear blasts (AML-PB001F, newly diagnosed/untreated) were purchased from AllCells (Alameda, CA, USA) and handled in accordance with the manufacturer's instructions. Primary human NK cells were purified from buffy coat blood (prepared from healthy donors) obtained from the National Health Blood and Transfusion Service (NHSBT, UK) following ethical approval (REC reference: 16-SS-033). Primary CD34-positive HSCs were obtained from Lonza (Basel, Switzerland).

Femur bones of six-week-old C57 BL16 mice (25 ± 2.5 g, kindly provided by Dr. Gurprit Lall, School of Pharmacy, University of Kent) were used for the experiments following approval by the Institutional Animal Welfare and Ethics Review Body. Animals were handled by authorized personnel in accordance with the Declaration of Helsinki protocols. Bone marrow was isolated from femur bone heads as described before (Swamydas and Lionakis, 2013) and whole extracts (1 mg protein/ml) were then obtained.

2.3. Primary Human Plasma Samples

Blood plasma of healthy donors was obtained from buffy coat blood (originated from healthy donors undergoing routine blood donation) which was purchased from the National Health Blood and Transfusion Service (NHSBT, UK) following ethical approval (REC reference: 16-SS-033). Primary human AML plasma samples were obtained from the sample bank of University Medical Centre Hamburg-Eppendorf (Ethik-Kommission der Ärztekammer Hamburg, reference: PV3469).

2.4. Western Blot Analysis

Tim-3, galectin-9, FLRT3, LPHN1 and Gαq were analyzed by Western blot and compared to β-actin in order to verify equal protein loading, as previously described (Yasinska et al., 2014). Briefly, cells were lysed using lysis buffer (50 mM Tris–HCl, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet-40, 1 mM PMSF, pH 8.0). After centrifugation, the protein content in the supernatants was analyzed. Finally, samples were added to the same volume of 2× sample buffer (125 mM Tris–HCl, 2% sodium dodecyl sulfate (SDS), 10% glycerine, 1 mM dithiothreitol (DTT), 0.002% bromophenol blue, pH 6.9) and boiled for 5 min. Proteins were resolved using SDS–polyacrylamide gels followed by blotting onto nitrocellulose membranes. Molecular weights were calibrated in proportion to the running distance of rainbow markers. For all primary antibodies (see Materials section) a 1:1000 dilution was used, except those against LPHN1 and FLRT3 (where a 1:500 dilution was used). β-actin staining was used to confirm equal protein loading as described previously (Yasinska et al., 2014). LI-COR goat secondary antibodies (dilution 1:2000), conjugated with fluorescent dyes, were used in accordance with manufacturer's protocol to visualize target proteins (using a LI-COR Odyssey imaging system). Western blot data were quantitatively analyzed using Odyssey software and values were subsequently normalized against those of β-actin.

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