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DOα⁻β⁺ expression in favor of HLA-DR engagement in exosomes

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ABSTRACT

The expression of DOβ and not DOα, in addition to the high intracellular DR, low DM levels and absence of surface DR expression in K562 and HL-60 cells introduce alternative regulatory pathways in DR trafficking and consequently the antigen presentation process. The present study attempted to define the naturally occurring DOα negative state and explain the role of DOβ in the intracellular DR accumulation in K562 and HL-60 cells. Despite the absence of DOα, the DOβ chain was detected in the endosomal compartments. The lack of DOα was found to be partially responsible for the absence of DR from the cell membrane since stable K562-DOα transfectants allowed expression of membrane DR. This expression could be significantly increased upon DM induction by IFN-γ, indicating that DM was another limiting factor for the migration of DR to the cell surface of K562 and HL-60 cells. Furthermore, intracellular DR co-localized with the exosome specific marker CD9, while culture supernatants were shown to contain exosome-engaged and exosome free DR activity as evaluated by SDS-page followed by western blot, ELISA and transmission electron microscopy analysis. These findings indicated that in DOα⁻β⁺ cells, DR molecules were programmed to secretion rather than surface expression. The presented results provide novel regulatory processes as to DR trafficking, avoiding expression to the cell surface.

Introduction

Non-classical HLA-DO (DO) is believed to negatively regulate the antigen presentation process in B cells, trophoblasts, thymic epithelial cells and possibly other not yet defined cell types, providing an additional mechanism for the maintenance of a tolerogenic state to the organism (Denzin et al. 1997). DO expression has also been detected in many cases of primary acute myeloid leukemia, where the DO:DM ratio correlated with the CLIP:DR ratio (Chamuleau et al. 2004). Previous studies in the leukemic K562 and HL-60 cell lines have shown that although these cells do not express surface HLA-DR (DR), they contain intracellular pools of these molecules and express high levels of DOβ and CD74, but not DOα chains (Papadimitriou et al. 2008). It has thus been postulated that DO is part of the escape mechanism from immune surveillance in leukemic cells.

DO is known to be a lysosomal-resistant protein with low polymorphism, expression limited to specific cell types, regulating immune response by controlling the availability of DM (Denzin et al. 2005; vam Ham et al. 1997; vam Ham et al., 2000; Chen et al. 2002). On the other hand, DM is catalyzing the release of CLIP and loading of antigenic peptide to DR, is present in all antigen presenting cells (APCs), is lysosome resistant, shows limited polymorphism and does not bind antigenic peptides (Morris et al. 1994; Kropshofer et al. 1996). The activity of DO is pH-dependent and has been postulated to exert its inhibitory activity to DM in the early compartments of the endocytic pathway by limiting the pH range in which DM is active (vam Ham et al. 2000). Recently, DO has been postulated to impair the incorporation of DM into exosomes, promoting thus DR excretion through exosomes (Xiu et al. 2011). The restricted cellular distribution of DO, implies different regulatory control mechanisms as compared to classical MHC class II (MHCII) antigens.

Although DOα has been shown to co-regulate with other MHCII molecules (similar enhancing elements in the promoter sequences and inducibility by interferon-γ (IFN-γ), DOβ is subjected to different regulatory mechanisms and is not stimulated by IFN-γ (Tonnelle et al. 1985; Wake and Flavell 1985), while its activity seems to depend on a di-leucine cytoplasmic motif, which is a sorting signal regulating trafficking and endocytosis (Xiu et al. 2011). However, it has been shown that mutation of the di-leucine

Abbreviations: K-DOα, stable transfectants of K562 cells with DOα; K-DOβ, stable transfectants of K562 cells with DOβ; mbDR, membrane HLA-DR; Intradr, intracellular HLA-DR; DR, HLA-DR; DM, HLA-DM; DO, HLA-DO; IFN-γ, interferon-γ; MHCII, major histocompatibility class II.

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sequence in DOβ did not alter its lysosomal sorting when associated with DM molecules (Brunet et al. 2000).

The differential role of DOα and DOβ seems to play an important role in DR trafficking. H2-Oa – deficient mice generated by homologous recombination have been shown to express physiological levels of classical MHCII molecules on APCs, while in the absence of H2-M these were not loaded with antigenic peptide (Liljedahl et al. 1998). Other studies, using various HeLa transfectants suggested that association of DO with DM is required for efficient exit of DO from the ER (Samaan et al. 1999), while additional studies report that although DOβ contains functional sorting motifs, the tight compartmentalization of HLA-DO observed inside B lymphocytes is controlled by the DOα chain and DM (Brunet et al. 2000). The present study was designed to exploit the role of DOα in the negative expression of surface DR and correlate its expression to DR excretion. Using the K562 and HL-60 cell lines which naturally lack DOα, express high levels of DOβ and low levels of DM, it could be possible to exploit the mechanisms disallowing DR migration to the cell membrane, which could be thereafter linked to escape from immune surveillance.

Materials and methods

Reagents

Uncoupled mouse anti-HLA-DP, DQ, DR (HB145; ATCC, Rockville, Maryland, USA) or FITC-labeled mouse anti-HLA-DR (L243,Santa Cruz, CA, USA), mouse anti- HLA-DO (DoB.L1, BD Biosciences, San Diego, California, USA), anti-rab7 (C19, affinity purified goat polyvalent antibody, Santa Cruz) as well as coupled to PE mouse anti-HLA-DM (MaPDM1, BD Biosciences) and anti-CD9-PE conjugated (ImmunoTools GmbH, Friesoythe, Germany) monoclonal antibodies were used at the concentration of 1 μg/ml in immunofluorescence experiments. Anti-mouse IgG Fab fragment coupled to FITC was used as secondary antibody for HLA-DOβ detection and anti-goat IgG Fab fragment coupled to PE was used as secondary antibody for rab7 detection. Anti-mouse and anti-goat IgG Fab fragment coupled to horseradish peroxidase (Sigma–Aldrich Co., MO, USA) were used as secondary antibody for HLA-DOβ and rab7 detection in ELISA experiments. Human recombinant IFN-γ was purchased from Endogen (Cambridge, MA, USA) and used at the concentrations of 1500 pg/ml.

Immunofluorescence experiments

Immunofluorescence was performed as previously described (Papadimitriou et al. 2008). For the intracellular staining, the cells were fixed with ice-cold 4% paraformaldehyde for 5 min and permeabilized using a HBSS–saponin solution (HBSS, 0.01 M HEPES, 0.3% saponin). Fluorescence intensity was evaluated using a FACS analysis system (Calibur Flow Cytometer, Becton Dickinson). Where appropriate, the negative controls included mouse IgG (Sigma, 1 μg/ml) or control buffer (PBS-BSA). Expression levels were evaluated using the histogram subtraction tool of FCS express® software. For confocal microscopy analysis the cells were fixed using 25 μl/ml Mowiol (Sigma). All experiments were repeated at least 3 times.

Native and transfected cell lines

HL-60, K562 and Raji cells were purchased from ATCC and maintained in RPMI culture medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Gibco). Full length cDNAs of the HLA-DOβ or HLA-DOα chains were obtained by PCR using mRNA isolated from Raji cells. Initial cloning of the cDNA was performed using the pBlueScriptII KS vector and thereafter sub-cloned using the pEGFP-C2 or pDsRed-monomer-C2 vectors. Gene products were verified by sequencing. The produced HLA-DOβ was fused at the amino-terminus with the fluorescent protein EGFP, whereas HLA-DOα was fused at the amino-terminus with the fluorescent protein dsRed, facilitating thus its identification within the cellular compartments. The constructs shown in supplement Fig. 1 were used to produce single or double transfectants of K562 cells by electroporation. Stable transfectants were selected for a one-month period using the antibiotic G418 (1181131, Gibco). All types of cells were submitted to immunofluorescence experiments followed by flow cytometry analysis, whereas culture supernatants were tested for the presence of free or exosome-engaged DR activity by ELISA, transmission electron microscopy (TEM) or Western blot analysis.

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.imbio.2012.12.003.

Exosome isolation and analysis

Exosomes were collected from the media of 4 ml K562 or HL-60 cells (2×10^6 cells/ml) cultured for 24 h in serum -free medium. Culture media was placed on ice and centrifuged at $800 \times g$ for 6 min to sediment the cells and subsequently at $12{,}000 \times g$ for 20 min at 4 °C to remove the cellular debris and concentrated to a volume of 1 ml. The resulting supernatants were further centrifuged at $100{,}000 \times g$ for 2 h at 4 °C. The exosome pellet was resuspended either in 20 μl paraformaldehyde 1% in phosphate buffer 0.2 M, pH 7.4 for TEM analysis, or in loading buffer for SDS-PAGE analysis or in 200 μl of PBS for ELISA experiments. For the ELISA experiments, exosome membranes were osmotically disrupted. ELISA, SDS-PAGE and western blot analysis were performed as previously described (Ranella et al. 2005; Athanassakis and Iconomidou, 1996; Papadimitriou et al. 2008). In these experiments DR was detected using an uncoupled anti-HLA-DP-DQ-DR antibody. For TEM analysis, exosomes were placed on Formvar carbon-coated electron microscopy grids, left to dry for 2 min, washed twice with PBS-BSA 0.1% and incubated with PBS-BSA 5% for 30 min at room temperature. After washing twice with PBS-BSA 0.1% grids were incubated overnight with uncoupled anti-HLA-DP-DQ-DR at 4 °C, washed twice and developed using an anti-mouse IgG coupled to 1.2 nm nanogold particles (4 h at room temperature). Nanogold signal was enhanced using the HQ SILVER™ enhancement kit (Nanoprobes NY, USA). After extensive washing, samples were observed using a JEOL-100C electron microscope equipped with a GATAN camera.

Sub-cellular fractionation

Sub-cellular fractionation was performed following the technique described by Qiu et al. (1994). Briefly, 70×10^6 cells treated as described above were collected, re-suspended in 2 ml of homogenization buffer (HB; 10 mM Tris, 1 mM EDTA, 0.25 M sucrose, pH 7.4) and dounced gently in a Dounce Tissue Grinder (Wheaton 357542; Wheaton Industries, Millville, NJ). The homogenate was centrifuged at $900 \times g$ and after collecting the supernatant in a clean tube, the pellet was resuspended in 1 ml HB and the $900 \times g$ spin was repeated. The resulting supernatant was combined with the first one and all were centrifuged at $10{,}000 \times g$ to remove mitochondria. Two milliliters of the resulting supernatant were loaded on a 9 ml Percoll gradient (Pharmacia LKB Biotechnology Inc., Piscataway, NJ; 1.05 g/ml) at $35{,}000 \times g$ using a SW41 rotor (Beckman). After collecting 0.5 ml samples from bottom to top, the resulting 18–20 fractions were tested by ELISA for detection of DO activity and rab7. The results are expressed as percent of optical density increase over background values.