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The HLA-DR mediated signalling increases the migration and invasion of melanoma cells, the expression and lipid raft recruitment of adhesion receptors, PD-L1 and signal transduction proteins

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ABSTRACT

The constitutive expression of Major Histocompatibility Complex (MHC) class II molecules is restricted to professional Antigen-Presenting Cells (APCs), nevertheless almost 50% of melanomas express constitutively the MHC class II molecules. Therefore, in two MHC class II constitutive expressing melanoma cell lines we studied the signalling mediated by the HLA-DR molecules in the aim to understand the consequence of class II mediated signalling on metastatic dissemination of melanoma. In particular, we reported that the HLA-DR mediated signalling play a new role in melanoma progression, increasing the migration and invasion of melanoma cells. Furthermore, we showed that the HLA-DR mediated signalling increases the expression and the lipid raft localisation of class II molecules, PD-L1 receptor, Integrin and CAM adhesion receptors, FAK, AKT and STAT3 signalling proteins. We also showed that the HLA-DR mediated signalling increases the activation of FAK, AKT, ERK, PKC and STAT3 signalling proteins and the expression of ILK, PAX, BRAF, ERK and PKC. Indeed, the results showed suggest that the HLA-DR mediated signalling provides a platform useful to frustrate an effective anti-tumour response and to increase melanoma migration and metastatic dissemination of this cancer.

1. Introduction

Melanoma, is one of the most aggressive cancers worldwide, its incidence rates increase rapidly in western population and unfortunately it is responsible of about 20% overall cancer mortality as well as of approximately 80% skin cancer related mortality [1]. Indeed, the chemotherapeutic treatment has a generally low effect on survival rates of melanoma patients, but recently targeted therapies and immunotherapies, changed significantly the therapeutic choices of metastatic melanoma patients. In particular, the discovery and the U.S. Food and Drug Administration (FDA) approval of immune checkpoint target antibodies such as the monoclonal antibodies directed against the cytotoxic T Lymphocyte Antigen 4 (CTLA-4) (ipilimumab) [2], the Programmed Cell Death 1 (PD-1) (nivolumab and pembrolizumab) [3,4] and a member of the B7 family, the Programmed Cell Death Ligand 1 (PD-L1, B7-H1) (atezolizumab) [5] receptors, inducing responses in 20–40% of patients, changed significantly the landscape of metastatic melanoma treatments. Indeed, the PD-1/PD-L1 interaction during antigen presentation of APC and cancer, inhibit the expansion and survival of antitumor T cells as well as the T-cell cytotoxic activity ultimately facilitating immune evasion [6]. Instead, the antibodies that prevent the binding of T-cell receptor PD-1 with the highly melanoma expressed PD-L1 lead to the reactivation of T-cells anti-tumour function in tumour microenvironment [3,4]. Nevertheless, the T cells reactivation could be ineffective in a tumour microenvironment heterogeneous as well as in immune resistant cancers. PD-L1 as well as the Major Histocompatibility Complex (MHC) class II molecules is expressed constitutively and after interferon-γ (IFNγ) stimulation in a wide number of cells including some cancer cells [7,8]. In particular, the MHC class II molecules are expressed in almost 50% of melanoma and although the MHC class II molecules expressed in melanoma cells may directly present tumour antigens to CD4 + T cells and trigger their effector functions [9], the constitutive MHC class II expression in melanoma is related to a bad prognosis [10–13]. The MHC class II molecules are signalling receptors whose engagement leads to the activation of several signalling pathways by tyrosine and serine/threonine kinases phosphorylation such as Src, Syk, Extracellular signal-Regulated Kinase (ERK) and Protein Kinase C (PKC) [14,15]. Furthermore, the class II signalling is transduced through Inositol lipid hydrolysis, production of cyclic AMP (cAMP) or mobilisation of intracellular $Ca2+$, ultimately leading to APCs activation or death [16–19]. Interestingly, the synergy between the class II molecules and

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several transmembrane receptors and signalling proteins is enabled by the lipid raft localisation of these molecules [20]. The lipid rafts are specific microdomains of the plasma membrane enriched in freely diffusing, stable assemblies of sphingolipids (sphingomyelin and glycosphingolipids) and cholesterol implicated in selective protein–protein interactions as well as in the assembly of transient signalling platforms [21]. Notably, in cancer cells, the lipid rafts localisation and activation of a wide number of receptors such as Integrins and Cell Adhesion Molecules (CAMs) as well as of some signalling molecules is related, for example, to the regulation of cell adhesion and migration during each phase of cancer development and progression [21–23]. In particular, the aggressive metastatic trend of melanoma is associated to the lipid raft recruitment of these molecules but also to their improved expression, to the deregulation of their functions and to the adhesion receptors-mediated interaction between tumour cells and cellular blood components, that promotes the tumour cells detachment from primary tumour and facilitate the survival of melanoma cells in the vascular system. Indeed, Integrins α/β are heterodimeric transmembrane receptors that mediating the cells anchorage to extracellular matrix (ECM), interacting with cytoskeleton and through the activation of downstream signalling pathways, play a key role in cell adhesion, migration, invasion, cell survival, growth and gene expression [24]. In particular, the Integrin functions are mediated by the interaction with structural proteins such as Paxillin (PAX), Talin and Vinculin and modulate the regulation of signalling pathways such as Focal Adhesion Kinase (FAK), Integrin-Linked Kinase, Src kinases, the ERK/Mitogen-Activated Protein Kinase (MAPK) and the Phosphoinositide 3-kinase/AKT pathways [24]. In human metastatic melanoma the α2, α5, αV, β1 and β3 Integrin subunits are up regulated and between them the Integrin β3 is one of the most specific markers of vertical growth phase of melanoma [25]. Furthermore, Melanoma Cell Adhesion Molecule (MCAM, MUC18 or CD146) a marker of melanoma metastatic progression and Intercellular Adhesion Molecule-1 (ICAM-1) are transmembrane receptors often over expressed in advanced primary and metastatic human melanoma cells [26]. In particular, MCAM mediates homotypic melanoma adhesion and, as well as ICAM, the heterotypic adhesion between melanoma, leukocytes and endothelial cells, promoting the formation of cellular clumps and allowing melanoma to survive in lymphatic and vascular systems. Furthermore, MCAM and ICAM adhesion receptors mediate the interaction of melanoma cells with vessel walls thus supporting the melanoma cells intravasation and extravasation in distant organs [24,27]. In this contest, the aim of our work was to understand the consequences on melanoma metastatic progression, migration and immune escape of HLA-DR mediated signalling. Notably, we previously reported the increased lipid rafts recruitment of HLA-DRα in melanoma cells after HLA-DR engagement [28] and we and others reported that the signalling activated by MHC class II molecules is associated in different tumour cell lines, to the lipid rafts localisation of these molecules [20,28]. Furthermore, the HLA-DR mediated signalling could inhibit, in melanoma cells, the Fas mediated apoptosis [14]. Therefore, in this paper we reported, in response to a sustained continuous HLA-DR stimulation, the increased expression of HLA-DRα, MCAM, ICAM, Integrin β1, Integrin β3, Integrin α5, Integrin αV and PD-L1 receptors. We also showed the increased expression of FAK, PAX, BRAF, ERK, ILK, AKT, PKC and Signal Transducers and Activators of Transcription 3 (STAT3) signalling proteins, the increased activation of FAK, PAX, ERK, AKT, PKC and STAT3 as well as the lipid rafts recruitment of HLA-DRα, MCAM, ICAM, Integrin β1, Integrin β3, PD-L1, FAK, AKT and STAT3 in response to a sustained continuous HLA-DR stimulation. Furthermore, we identified the HLA-DR mediated signals depending on lipid rafts integrity through the treatment of melanoma cells with the methyl-β-cyclodextrin (MβCD) that disrupt the lipid raft domains through cholesterol depletion. Finally, we reported that the HLA-DR mediated signalling increases the melanoma cell migration and invasion, thus suggesting a new role played by the HLA-DR molecules on metastatic dissemination and on immune evasion of melanoma.

2. Material and methods

2.1. Cell lines, antibodies and reagents

A375 (ATCC-CRL-1619) [29], HT-144 (ATCC-HTB-63), M74 melanoma cell lines (kindly given by Prof. C. Alcaide-Loridan, Paris Diderot University, Paris, Institut Jacques Monod) and LAN-5 neuroblastoma cells (kindly given by Dr. M. Di Carlo, IBIM-CNR) were grown in RPMI 1640 supplemented with 10% FCS and 1% penicillin-streptomycin (10,000 U/ml and 10,000 μg/ml, respectively) in 5% CO_2 at 37 °C. The mouse monoclonal antibody direct against the C-terminal intracellular tail of the HLA-DR-α-chain (DA6.147) [30] was a kind gift of Prof. C. Alcaide-Loridan. Rabbit polyclonal antibody direct against caveolin-1 (clone pAb) and mouse monoclonal antibodies direct against the HLA-DR (clone G46-6, mouse IgG2a), pTyr-397 FAK motif, Integrin β1, Integrin α5, Integrin αV, ICAM-1, MCAM, and PAX were obtained from BD Biosciences (Lexington, KY). The rabbit polyclonal antibodies direct against Integrin β3, FAK, MCAM, STAT3, Pdcd-1 L1 and PKCα as well as the mouse monoclonal antibody direct against Raf-B, ILK and pSTAT3 (Tyr-705) were purchased from Santa Cruz (Santa Cruz, CA, USA). The rabbit polyclonal antibodies direct against Phospho-PKC pan (γThr514), Phospho-PAX (Tyr-118), AKT, pAKT (Ser-473), p44/42 MAPK and Phospho-p44/42 MAPK (Thr-202/Tyr-204) were obtained from Cell Signaling Technology. Anti-β-actin mouse monoclonal antibody and the mouse IgG2a isotype control Ig were obtained from Sigma (St Louis, MO). Infrared dye-conjugated IRDye800 anti-mouse and anti-rabbit (LI-COR Biosciences) as well as anti-mouse and anti-rabbit Alexa Fluor 680 conjugated (Molecular Probes) were purchased as secondary antibodies. All other chemicals were of analytical grade and were purchased from Sigma Chemical Co., Merck/VWR, or J. T. Baker (Phillipsburg, NJ).

2.2. Preparation of total cell extracts and isolation of raft fractions

Semi-confluent cells were stimulated with 1 μg/ml of the mouse L243 antibody direct against a conformational epitope of the HLA-DR molecules [31] for 24 h, 48 h and 72 h or with 1 μg/ml of isotype-matched control antibody for 48 h. To obtain total cell extracts, the cells were lysed on ice for 30 min in RIPA buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1% NP40, 0,5% DOC, 0,1% SDS) containing proteases inhibitors (4 mM PMSF and protease inhibitors cocktail, Sigma) and phosphatase inhibitors (cocktail 2 and 3 of phosphatase inhibitors, Sigma) and then cleared of cellular debris by centrifugation at 12,000 × *g* at 4 °C for 30 min. The protein concentration of the supernatants was determined using the Bradford protein assay (Bio-Rad laboratories GmbH, München, Germany). Otherwise, to isolate the raft fractions [28,32], the cells were lysed in MBS buffer (25 mM MES, 2 mM EDTA pH 8 and 150 mM NaCl) containing 1% Triton × 100, protease and phosphatase inhibitors for 30 min on ice. The lysates mixed with an equal volume of 85% sucrose (*w/v*) in MBS buffer, were placed at the bottom of a polycarbonate ultracentrifuge tube (Beckman Instruments, Palo Alto, CA), overlaid with 2 ml of 35% sucrose and 1 ml of 5% sucrose in MBS buffer containing 2 mM EDTA pH 8, protease and phosphatase inhibitors and were centrifuged at 100,000 × *g* for 20 h at 4 °C in a SW55Ti rotor (Beckman Instruments, Palo Alto, CA). Nine fractions of 550 μl/each were collected from the top of the discontinuous sucrose gradient.

2.3. Western blot analysis

40 μg of total cell extracts or 28 μl of raft fractions were resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and absorbed to nitrocellulose membrane (Hybond ECL, GE Healthcare, Biosciences). Blocking was performed in blocking buffer (LI-COR Biosciences) overnight at 4 °C in TBS buffer. Incubation with primary antibodies diluted in 1:1 blocking solution (LI-COR Biosciences) and