Characterization of Hela cells for presentation of exogenous antigens

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Role of MHC class I HLA A2 HC and trimeric molecules on the binding of exogenous HBV core antigen was studied in Hela cells transfected with MHC class 1 HLA A2. HC and trimeric HLA-A2 molecules could be identified with specific monoclonal antibodies. HC and trimeric HLA-A2 molecules exist in varying proportion on the cell surface of Hela cells. MHC class I HC molecules on the cell surface do not bind HBV core peptide. The exchange of peptide with MHC class 1 molecules on the cell surface is inefficient with cells exhibiting a maximal binding (12%) at 1.5μM of peptide. Hence, presentation of exogenous HBV core antigen molecules occurs after endocytosis of the antigen and MHC class 1 molecules, followed by functional trimer complex formation in endolysosomal compartments.

Keywords: Core antigen, Endocytosis, Hela, Hepatitis B virus, MHC

Introduction

Antigen presenting cells, in particular dendritic cells, process exogenous antigens for presentation by MHC class 1 molecules. CD8+ cytotoxic T lymphocytes (CTL) are cross-primed during viral and bacterial infection and by many vaccination approaches. Processing and presentation to MHC class 1 restricted CD8+ CTL have been exploited in development of vaccines against intracellular infections. There are various pathways by which antigen can be presented to CD8+ CTL. Conventional TAP dependent MHC class 1 peptide loading occurs in endoplasmic reticulum (ER). In cytosol, antigens are degraded by proteosome and transported by transporter associated with antigen processing (TAP) into the ER for presentation by MHC class 1 molecules. Alternatively, antigens can be processed in endolysosomal compartments where they bind to recycling MHC class 1 molecules and are transported to the cell surface for presentation. In mouse processing of exogenous Hepatitis B surface antigen (HBsAg), VLP, occur in endolysosomal compartment that bind to recycling MHC class 1 MHC molecules and are presented to CD8+ CTL. Surface-associated MHC class I heavy chain (HC) molecules are involved in the alternative processing and presentation pathway of exogenous HBsAg.

In acute Hepatitis B infection, class-1 restricted CTL response to Hepatitis B virus (HBV) core antigens have been detected in patients who cleared the infection successfully unlike patients with chronic HBV infection. Thus, HBV core antigen plays a critical role in terminating HBV infection and is a potential vaccine candidate. TAP independent cross presentation of HBV surface antigen has been shown in mouse. The free HC, peptide empty form of Ld molecules, which does not bind β2-microglobulin while on the surface, is involved in the presentation of antigenic peptides. This surface subset of Ld molecules was recognized by the mab 64-3-7, which binds to a sequence of amino acids including residues 48Q and 50P present on the hinge connecting β-strand with α1 helix above the ligand-binding site in Ld molecules, a conserved structure across MHC class 1 molecules. This study characterizes HLA A2 HC and trimers on transfected Hela cells using epitope transferred from mouse Ld to HLA A2.

Materials and Methods

Culture Medium and Reagents

Culture medium RPMI 1640 and modified DMEM (GIBCO BRL) were supplemented with 1mM HEPES (GIBCO, BRL), 5% Bovine calf serum (GIBCO, BRL), β2-Microglobulin, hygromycin B, 2-amino actinomycin D, G418, FITC-dextrane, polystyrene latex PE labeled beads (Sigma catalogue no L-2278), and Interferon-γ were purchased from Sigma. INSERM U580 provided anti-HC HLA-A2 antibody.
Transfection and Expression of HLA-A2 Wild and Mutant Molecules in Hela Cells

5x10⁶ Hela cells were incubated on ice for 10 min with 20μg DNA [pCDNA3.1 encoding mutated HLA-A2 gene, hygromycin B resistance, Invitrogen] in 10mM HEPES, PBS buffer pH 7.5 and electroporated at 250V, 950μF. After electroporation, cells were incubated on ice for 10 min and allowed to recover for 48 h in modified 10% FBS DMEM medium at 37°C. Cells were cultured under selection with 1 mg/ml G418 or 0.4mg/ml hygromycin B for wild and mutant HLA-A2 molecules respectively. Cells were sorted on the FACS sorter after initial sorting using goat anti mouse IgG microbeads [Miltenyl Biotec, Germany] after selecting with b.b.7.2. Cells were frozen in FBS (10% DMSO) and stored in liquid N₂.

FACS Analysis of Transfected Cells

Appropriate number of cells was washed once in PBS and labeled with trimeric and free HC MHC-class 1 HLA A2 molecules using specific monoclonal antibodies b.b7.2 and 64.3.7 respectively at 4°C for 45 min. Secondary antibody used was FITC or PE conjugated anti-mouse antibody (10μg/ml). The cells were washed twice in 1% FBS, 0.01% NaN₃ PBS and analyzed by FACS. Dead cells were labeled using 2-amino actinomycin D.

Effect of Cytokines and Peptide on Expression of HC and Trimeric HLA A2 Molecules

Hela A2w and A2m cells were incubated in media supplemented with IFN-α (500u/ml) or IFN-γ (500u/ml) at 37°C for 48 h and analyzed by FACS. To check peptide effect, cells were incubated in presence of HBV core peptide at 10μM for 18 h and analyzed by FACS for MHC HLA A2 HC and trimeric HLA A2 molecules.

Depletion of Peptide from Cell Surface Molecules

Cells (2x10⁶) were washed in PBS, spun at 1500 rpm for 5 min, supernatant was discarded and the pellet incubated with 50μl of 300mM glycine, 1% BSA (pH 2.5) for 3 min at room temperature. The reaction was neutralized with 50 vol of serum free RPMI medium, cells were spun and washed once in serum free medium and re-suspended in 300μl medium. Direct staining of 100μl reaction was done using monoclonal antibodies b.b7.2 and 64.3.7. The remaining was split into two batches and incubated with and without HBV core antigen 18-27 at a final

Phagocytosis of Latex Beads

Hela cells were plated at 0.5x10⁶/ml in 24 well plates and latex PE beads suspension (Sigma catalogue no L-2278) was added at a final concentration of 0.2mg/ml to each well. The cells were incubated at 37°C for various time periods, washed twice with cold PBS containing 1% FBS and analyzed by FACS using 2-AAD to exclude dead cells.

Endocytosis of Dextrane

Hela cells were suspended at 1x10⁶/ml in IMDM (20% FCS) medium. FITC dextrane was added to a final concentration of 1 mg/ml and incubated at 4°C and 37°C for 1 h. Cells were washed thrice with cold PBS (1% FBS) and analyzed by FACS using 2-AAD to exclude dead cells.

64.3.7 and anti trimeric HLA-A2 antibody bb7.2 stocks. Interferon-α, HBV core peptide 18-27 (FLPSDFFPSV) given by Dr Antonio Bertoletti, UCL, London. pCDNA3.1 encoding mutant HLA-A2, the HC of which was detectable using mab 64.3.7 and anti trimeric HLA-A2 antibody bb7.2; Sequence change made to transfer the mouse HLA A2 mutant epitope recognized by mab 64.3.7, to HLA A2 mutant.

Fig. 1—Peptide binding domain of HLA A2 showing region recognized by mab 64.3.7; Sequence change made to transfer the mouse Ld epitope recognized by mab 64.3.7, to HLA A2 are indicated.

Transfection with G418 (Sigma). Wild HLA A2 was cloned into pCDNA 3.1 (Invitrogen), selected after transfection with G418 (Sigma).
concentration of 100 μM for 2 h at 37°C in the presence of Brefeldin A (5 μg/ml). The cells were washed and labeled with antibodies bb7.2 and 64.3.7 for analysis by FACS.

Binding of Peptide to Cell Surface Molecules

Cells were acid treated as mentioned above, washed in RPMI and incubated with varying concentrations of peptide (0.156-5 mM) in the presence of 6 μg/ml of β2-microglobulin and 5 μg/ml BFA at room temperature for 2 h followed by analysis on FACS using bb7.2. The percent maximal binding was calculated using maximal fluorescence intensity of untreated cells as maximal binding and the MFI of treated cells with no peptide as minimum binding.

Results

HBV core antigen is known to be involved in the termination of HBV infection and is therefore a potential candidate vaccine to initiate a cell mediated immune response. In mouse processing of exogenous HbsAg, VLP occurred in endolysosomal compartment through a TAP independent processing pathway. HBsAg bind MHC class I Ld molecule and β2-microglobulin for presentation to CD8+ CTL. Surface-associated HC MHC class I molecules are involved in this alternative processing pathway requiring the presence of exogenous β2-microglobulin. MHC class I presentation of exogenous HBV core antigens are expressed on the cell surface of infected hepatocytes which are recognized by cytotoxic T cells leading to the killing of infected hepatocytes. Hence, hepatitis B core antigen is a potential candidate for a therapeutic vaccine.

Hela cells as a Model Cell System for Antigen Presentation

Hela cells were used for the following reasons: 1) Endocytosis using FITC-dextran was higher (Fig. 2a) than phagocytosis using PE labeled polystyrene latex beads (Fig. 2b) in Hela cells; and 2) It can be used to study cell surface expression of HC and trimeric HLA A2 molecules since they are HLA A2 negative. Hela was transfected with the modified HLA A2 molecule containing Ld epitope recognized by mab 64.3.7. As control, Hela was transfected with wild type HLA A2 molecules (Hela A2w). HLA A2-peptide-β2-microglobulin trimer complex was recognized by mab b.b.7.2 in both Hela A2m and Hela A2w cells (Fig. 3). Mab b.b.7.2 has the same affinity by scatchard analysis for both HLA A2m and HLA A2w trimer molecules indicating no structural change was caused by transferring the 64.3.7 epitope from mouse Ld to HLA A2 molecules.

Expression of HC and Trimeric HLA A2 Molecules

Ld epitope was transferred to HLA A2 DNA by mutagenesis (provided by INSERM U580) and transfected into Hela cells (Hela A2m), a HLA A2 negative cell line, was recognized by mab 64.3.7. As control, Hela was transfected with wild type HLA A2 molecules (Hela A2w). HLA A2-peptide-β2-microglobulin trimer complex was recognized by mab b.b.7.2 in both Hela A2m and Hela A2w cells (Fig. 3). Mab b.b.7.2 has the same affinity by scatchard analysis for both HLA A2m and HLA A2w trimer molecules indicating no structural change was caused by transferring the 64.3.7 epitope from mouse Ld to HLA A2 molecules. In Hela A2m cells, trimeric and HC HLA A2 molecules were expressed in varying proportion on the cell surface with MFIs by FACs of 181 and 4.23 respectively (Fig. 3 a, b). The expression of HC and functional trimeric class 1 molecules is known to vary between cell types.

Cell Surface HC HLA-A2 Molecules Bind Peptide Inefficiently

Stripping Hela A2m cell surface molecules of peptide by incubating cells under acidic conditions confirmed the loss of trimeric HLA A2 molecules by the complete loss of mab b.b.7.2 binding. While, there was an increase in HC HLA A2 molecules recognized
Fig. 3—FACs analysis using mab b.b7.2 and 64.3.7 of: a) Hela A2m; and c) Hela A2w; Secondary antibody used was FITC or PE conjugated anti-mouse antibody.

by mab 64.3.7, wild HC HLA A2 cell surface molecules on Hela A2w were not recognized by mab 64.3.7 (Fig. 4a). Binding of peptide-to-peptide depleted cell surface, HLA-A2 molecules in Hela A2m cells was not observed. There was no peptide binding on incubating peptide with Hela A2m cells at various temperatures; RT, 37°C. Peptide binding to peptide depleted cell surface Hela A2w molecules occurred to a maximal binding (12.5%) at 1.5μM HBV core concentrations (Fig. 4b).

Effect of Cytokines, Serum and Peptide on Expression of HC and Trimeric HLA-A2 Molecules

Hela A2m cells in the presence of IFN-α showed an increase in trimeric (63%) and HC HLA A2 (111%) molecules (Fig. 5a). Similarly in the presence of IFN-γ, there was an increase in trimeric (20%) and HC HLA A2 (100%) molecules (Fig. 5a). In presence of peptide with serum (Fig. 5a), there was an increase in trimeric (50%) and HC HLA A2 molecules (45%). In absence of serum, there was an increase in trimeric (36%) and HC HLA A2 (20%) molecules in Hela A2m cells (Fig. 5a). In Hela A2w cells, an increase of 18% and 66% trimeric molecules in the presence of IFN-γ and peptide serum respectively was observed (Fig. 5b). In the presence of IFN-γ and absence of serum, there was no effect on the expression of trimeric HLA A2 molecules in Hela wild cells (Fig. 5b).

Discussion

Hepatitis B is a viral infection of the liver infecting 350 million people with 1 million deaths annually. The available vaccine, HbsAg, provides protection against development of chronic Hepatitis B infection16. An obstacle to the development of an efficacious vaccine against chronic Hepatitis B is the weakened immune response against Hepatitis antigens by T cells of infected patients. Development of an efficacious vaccine requires induction of specific CD8+ cytotoxic T cells. Chronic Hepatitis B is not treatable using HBsAg but is treatable with interferons and lamivudine, which are not feasible in developing countries due to the high cost17.
Pathways of MHC Class 1 Molecules Presentation

Cytosol antigens are degraded by proteosome and transported through TAP into ER for loading onto MHC class 1 molecules\(^9\). Antigens can be processed in endolysosomal compartments where they bind to recycling MHC class 1 molecules\(^18\). Binding of peptides produced by alternative pathway to MHC class 1 molecules occur in specialized cytosolic organelles (phagolysosomes) formed by fusion of ER membrane with nascent phagosomes\(^19\).

Hepatitis B Virus Core Antigen – Candidate Vaccine Molecule

HBV vaccination against chronic Hepatitis B infection was because of an altered Th response due to inappropriate activation events and varied antigen presentation. A randomized placebo-controlled double-blind vaccination trial of chronically infected patients with pre-S1, pre-S2 and S antigenic components of HBV showed HbsAg specific induction of Th2 but not Th1 cytokines\(^20\). In acute Hepatitis B infection, class-1 restricted CTL response to HBV core antigens have been detected in patients who cleared infection unlike in chronic HBV infection. Thus, HBV core antigen plays a critical role in terminating HBV infection and can be used as a potential vaccine candidate\(^21\). Pre S1, a Hepatitis B surface protein, was fused with carboxyl terminal of core antigen 1-155. Core antigen induced a specific T cell response in BALB/C mice indicating that pre S1 and HBsAg may be used as a therapeutic vaccine against chronic infection\(^22\). HBcAg, which is also a strong Th1 adjuvant, can be used along with traditional HBsAg to develop a more potent and therapeutic vaccine by activating mucosal T cells by nasal immunizations\(^23\).

DNA immunization involves an expression vector encoding foreign antigen with regulatory elements needed to express them. Hepatitis B core antigen used as a DNA vaccine for immunization of Balb/c mice induced strong humoral and cellular responses\(^24\). In C57BL/6 mice, an increase in T cell activation assessed by CTL cytolysis and IFN-\(\gamma\) secretion was seen when a DNA vaccine of HBcAg minimal peptide 18-27 was fused with an ER targeting sequence\(^25\). Similarly, interleukin IL-1\(\beta\) increased the humoral and cellular responses to foreign antigens and may serve as an adjuvant. Immune responses were higher when peptide 163-171 of IL-1\(\beta\) was fused with the DNA encoding HBsAg\(^26\).

Characteristics of MHC Class 1 HC Molecules

In acute Hepatitis B infection, class 1 restricted cytotoxic CD8\(^+\) T cell response to exogenous HBV
core antigens has been detected. HBV core antigen was used to characterize cell surface MHC class 1 free HLA-A2 HC and functional trimeric molecules. MHC class 1 HC and trimeric HLA-A2 molecules exist in small proportion on the cell surface of Hela cells. While in KG-1 a dendritic cell line, and C1R a B cell line, proportion of HC to trimer molecules were equal (unpublished observation). Hence, level of cell surface MHC class 1 HC molecules varies between cell types.

In presence of cytokines IFN-α and IFN-γ, proportion of cell surface HLA-A2 free HC increases more than 10 fold in Hela cells (Fig. 5). While in a dendritic like cell line (KG-1) and C1R cells, there was no effect of cytokines on the levels of cell surface MHC class 1 HC HLA A2 / trimeric molecules (unpublished observation). This was unexpected as interferons are known to induce the upregulation of MHC class 1 molecules in various cells. It is concluded that interferons induce expression of MHC class 1 molecules in certain cell types. Hence, cells used as APCs involved in alternative presentation of exogenous antigen may be treated with interferons to enhance the level of MHC class 1 presentation for effective presentation to cytotoxic T cells.

Antigens involved in MHC Class 1 Alternative Processing Pathway

Are cell surface MHC class I molecules involved in cell surface exchange of peptide? Study demonstrates that MHC HC HLA A2 molecules on the cell surface do not bind HBV core peptide even in presence of exogenous β2-microglobulin but has to be internalized to form functional trimeric complex. In Hela cells, acid stripped cell surface molecules demonstrated a maximal binding of 12%. In a similar study on KG-1 and C1R cells, no binding of cell surface peptide was observed (unpublished observation). This could be due to the effect of chaperons like calreticulin, which localize in ER and cytosol, and are also present on the cell surface in association with MHC class 1 HC molecules. In addition, tapasin related protein (TRP), which is similar to the MHC class 1 binding protein, tapasin is known to localize to the cell surface. Both of these chaperons binding to the cell surface HC MHC class 1 molecule and may prevent the binding of both the peptide and β2-microglobulin.

Conclusions

MHC class 1 HC exists in small proportions on Hela cells cell surface, which is increased in the presence of interferons. The study demonstrates that cell surface MHC class 1 HLA A2 HC does not bind peptide, but has to be internalized for MHC-peptide complex formation. Cell surface MHC class 1 HLA A2 molecules are involved in the presentation of HBV core antigen independent of the conventional MHC class 1 pathway. Hence, presentation of exogenous antigens accessing the alternative pathway should be primed in the presence of interferons to increase the cell surface expression of MHC class 1 associated with exogenous antigen.

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Abbreviations

HBV, Hepatitis B virus; MHC, major histocompatibility antigen; HC, Class 1 MHC free heavy chain; HbcAg, Hepatitis B virus core antigen; HbsAg, Hepatitis B virus surface antigen; 2-AAD, 2-amino actinomycin D; mab, monoclonal antibody; TAP, Transporter associated protein; MFI, mean fluorescence intensity; APC, Antigen presenting cells

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