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Effect of Invariant Chain on Major Histocompatibility Complex Class I Molecule Expression and Stability on Human Breast Tumor Cell Lines

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Abstract

Invariant chain (Ii) binds to the human leukocyte antigen (HLA) class II molecule and assists it in the process of peptide acquisition. In addition, Ii binds to the HLA class I molecule, although there has been little study of its effects on the HLA class I molecule. In addition to its normal expression on antigen-presenting cells, Ii expression is up regulated in a variety of tumors. By flow cytometric analysis, we found that expression of Ii resulted in an increase in the number of cell surface HLA class I molecules and in the proportion of unstable HLA class I molecules at the surface of breast tumor cell lines. These data suggest that the expression of Ii by tumor cells may quantitatively and qualitatively alter the presentation of antigens on those cells.

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Keywords

antigen presentation; breast cancer; antigens/peptides/epitopes; invariant chain; MHC class I molecule

Introduction

Ii is a transmembrane glycoprotein that participates in the assembly of MHC class II molecule-peptide complexes in antigen-presenting cells [7]. In addition to its constitutive expression in antigen-presenting cells, Ii expression is up regulated in tumors arising from several different tissues. For example, in an evaluation of colorectal carcinomas, Ii was found in 81.9% of the tumors [18]. Furthermore, in one study Ii was identified in a majority of cases (53 of 60) of renal cell cancer by immunohistochemistry [29], and a separate study reported its detection in renal cell tumors by both microarray analysis and immunohistochemistry [40]. Several breast tumor cell lines have also been noted to express Ii [2]. Heightened lymphocytic infiltration was observed in vivo in breast and renal cell carcinomas that expressed Ii [15,29]. Ii expression in tumors is not always linked to the co-expression of MHC class II molecules, as it is frequently expressed even in the absence of the MHC class II molecule [13,15,17,18].

In addition to binding to MHC class II molecules, Ii also associates with MHC class I molecules [5,24,25,34,38]. Complexes of Ii and MHC class I molecules accumulate in the cis-Golgi [38] and the endosomes [34]. Ii interacts specifically with the folded, β_2 -microglobulin-(β_2 m)-associated conformation of the MHC class I molecule, but it can be competitively displaced from the MHC molecule by the addition of peptide [5,25,38]. This latter observation suggests that Ii is sensitive to epitope binding by the MHC molecule, either due to Ii responsiveness to MHC conformational change upon peptide binding [5] or to direct Ii occupation of the MHC antigen binding groove and replacement by peptide. The finding that Ii is sensitive to MHC peptide binding suggests that it may potentially influence selection of peptide ligands. In this study, we questioned whether the expression of Ii in breast cancer cell lines affected MHC class I molecule cell surface expression on those lines. Our results indicate that the level of cellular Ii influences the number and stability of HLA class I molecules expressed at the surface of human breast cancer cell lines. These findings suggest that Ii alters the peptide repertoire presented by breast tumor cells to T lymphocytes.

Materials and methods

Cell lines

The T47D, MCF-7, and MDA-MB435S human breast cancer cell lines [3,14,30] were gracious gifts from Drs. Shantaram Joshi, Kenneth Cowan, and Vinod Labhasetwar (respectively), University of Nebraska Medical Center, Omaha, NE. The human pancreatic tumor cell lines S2-013, FG, Capan-1, Capan-2, and Hs766T [8,10,20,35,37] were provided by Dr. Michael A. Hollingsworth (University of Nebraska Medical Center). S2-013 is a cloned subline derived from the SUIT-2 human pancreatic tumor cell line, which was generated from a liver metastasis [11]. The Huh7 and DU145 prostate tumor cell lines [19,33] and the HepG2 hepatocellular carcinoma cell line [1] were donated by Dr. Richard MacDonald (University of Nebraska Medical Center).

For S2-013, FG, Capan-1, Capan-2, Hs766T, and T47D, the basal medium used was RPMI-1640. For MCF-7, MDA-MB435S, Huh7, DU145, and HepG2, the basal medium used was DMEM. For all cell lines, the basal medium was supplemented with 10% (volume/volume) fetal bovine serum, 1 mM sodium pyruvate, 2 mM L-glutamine, penicillin (100 units/ml), streptomycin (100 μ g/ml), 1X non-essential amino acids, and 1X HEPES buffer. The basal

media and all culture media additives were purchased from Invitrogen (Carlsbad, CA), with the exception of the fetal bovine serum (which was obtained from Atlanta Biologicals, Norcross, GA), and all cells were grown at 37°C in 5% CO₂.

The human Ii p33 cDNA, a kind gift from Dr. Eric Long (NIH, Bethesda, MD), was subcloned into the RSV.5neo expression vector [16]. The Ii-RSV.5neo construct was stably transfected into T47D, MCF-7, and MDA-MB435S cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and selected with 600 µg/ml G418 (Invitrogen, Carlsbad, CA).

Antibodies, immunoprecipitations, Western blotting, and flow cytometry

Anti-CD74 monoclonal antibody (against Ii) was purchased from BD Biosciences-Pharmingen (San Diego, CA) and the hybridoma secreting anti-Ii monoclonal antibody PIN1.1 was kindly donated by Dr. Peter Cresswell (Yale University, New Haven, CT). The anti-MHC class I molecule monoclonal antibody H58A was purchased from VMRD, Inc. (Pullman, WA). H58A binds to MHC class I molecules from many species (including human, mouse, and rabbit). The hybridomas secreting monoclonal antibody 30-5-7 [21], W6/32 (which recognizes the β_2 m-associated, folded HLA class I molecule [4,22]), and HC10 (which recognizes open HLA class I molecules [31,32]) were donated by Dr. Ted Hansen (Washington University, St. Louis, MO). The 30-5-7 monoclonal antibody is specific for H2-L^d [21] and was used as a isotype control for flow cytometry. The hybridoma secreting monoclonal antibody BB7.2 [23], which recognizes HLA-A2, was obtained from the American Type Culture Collection (Manassas, VA). The monoclonal antibody specific for α -tubulin was purchased from Sigma-Aldrich (St. Louis, MO).

Immunoprecipitations and Western blots were performed by procedures similar to previously described methods [36], with 4→20% acrylamide Tris-glycine gels (Invitrogen) used for electrophoresis. Flow cytometry procedures used were similar to those described in [25], with phycoerythrin-conjugated, Fc-specific F(ab')₂ portion of goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA). The cell analysis was performed on a FACSCalibur flow cytometer with Cell Quest software (BD Biosciences, San Jose, CA). For assessment of the cell surface HLA class I molecule turnover rate, treatments with 5 µg/ml brefeldin A (Sigma-Aldrich, St. Louis, MO) with subsequent flow cytometry assays were performed [39]. Statistical analyses to compare experimental results to control results were done with SPSS software (SPSS Inc., Chicago, IL).

Results

Ii was found to be endogenously expressed by the MDA-MB435S breast tumor line

To begin to understand how HLA class I molecules might be affected by Ii in tumor cells, we examined the expression of Ii by several human tumor cell lines. An Ii transfectant of T47D was also included in the flow cytometric analysis as a positive control (Fig. 1A and B). Among the tumor cell lines that we examined, the highest level of Ii was detected on MDA-MB435S breast tumor cells (Fig. 1A and C). Little or no endogenous Ii was detected on the other tumor cell lines tested (Fig. 1A). The MCF-7 breast carcinoma cell line has been reported to express Ii with a glycosylation defect [12] but in our experiments we detected no Ii in this cell line by Western blotting, although we could detect Ii expression in MDA-MB435S cells by the same Western blot (Fig. 1D). These experiments identified the MDA-MB435S cell line as a breast tumor cell line that endogenously expresses intracellular and cell surface Ii.

Ii was found to increase the expression of unstable HLA class I molecules on T47D+Ii cells

To directly address the question of the effect of Ii on cell surface HLA class I molecule expression and stability, we analyzed the T47D and T47D+Ii cell lines. By Western blotting,

we confirmed that the total level of HLA class I heavy chain was very similar in the T47D and T47D+Ii cell lines (Fig. 2A). By a combination of immunoprecipitation and Western blotting, we found that Ii bound to HLA class I molecules in the T47D+Ii cells (Fig. 2B). To compare the quantities of HLA class I molecules expressed at the surface of T47D and T47D+Ii breast tumor cells, HLA class I molecule expression at the surfaces of both cell lines was determined by flow cytometry (Fig. 2C). Our results demonstrated that transfection of Ii into T47D cells increased the expression of cell surface HLA class I molecules (Fig. 2C, compare T47D and T47D+Ii at 37°C). Since the stability of cell surface HLA class I molecules is determined by the affinity of their bound peptides [9], we also included in the assay T47D and T47D+Ii cells that had been incubated overnight at 25°C to determine whether assembly of the HLA class I molecules in the presence of Ii altered the presented peptide repertoire. As displayed in Figure 2C, 25°C incubation increased the number of surface HLA class I molecules on T47D+Ii to a greater extent than on T47D. Very similar results were obtained in the same type of assay at 25°C and 37°C with a second T47D+Ii transfectant and with a separate anti-MHC class I monoclonal antibody, H58A. Overall, these data suggest that the HLA class I molecules in T47D+Ii have bound to more low affinity peptides than those in T47D.

A separate type of assay was performed to confirm that the HLA class I molecules in T47D+Ii were acquiring a high percentage of low affinity peptides. T47D and T47D+Ii cells were treated with brefeldin A to stop the progression of new HLA class I molecules from the endoplasmic reticulum to the cell surface [39], and the rate of surface turnover of HLA class I molecules was monitored. The HLA class I molecules on T47D+Ii demonstrated a more rapid turnover rate than those on T47D, as shown by experiments using two time courses: 0, 1, 2, 3, and 4 hours, or 0, 3, and 6 hours (Fig. 2D and E). A total of 4 samples were included in the assays (2 per time course). The difference in the percentage of remaining W6/32⁺ HLA class I molecules on T47D+Ii versus T47D at the 3 hour time point (the time point shared in both time courses) was analyzed by the Student's t test in SPSS and shown to be statistically significant ($p=0.002$). For the shared 3 hour time point, the means of the percentages were 60.2% for T47D+Ii and 79% for T47D, and the SEM values for the percentages were very low (1.94 and 2.63, respectively), indicating reproducibility.

Thus, by two types of assays, we found the HLA class I molecules on the surface of T47D+Ii to be unusually unstable. These findings suggest that tumor expression of Ii may lead to changes in the spectrum of presented peptides. Furthermore, since the brefeldin A assay was performed with the cells incubated at 37°C, the greater instability of the HLA class I molecules at the surface of T47D+Ii (compared to T47D) observed in this assay confirmed that the instability was evident at physiological temperature.

MDA-MB435S breast tumor cells endogenously expressing Ii were demonstrated to have temperature-inducible HLA class I molecules at the cell surface

As mentioned above, results from flow cytometry analysis after overnight incubation of T47D and T47D+Ii at 37°C or 25°C suggested that Ii transfection resulted in increased HLA class I instability (Fig. 2C). We reasoned that if endogenous Ii in breast tumor cells also facilitated the surface expression of unstable HLA class I molecules, we should be able to detect an increase in cell surface HLA class I on cells having endogenous Ii following incubation at 25°C. MDA-MB435S (Ii⁺) and MCF-7 (Ii⁻) cells were incubated at 37°C or 25°C and were tested the following day for HLA class I expression by flow cytometry with monoclonal antibody W6/32. The mean fluorescence intensity for MDA-MB435S HLA class I molecules was over 100 channels higher after 25°C than after 37°C incubation (Fig. 3, bars labeled Assay 1). In contrast, the mean fluorescence intensity for the HLA class I molecules expressed on MCF-7 cells was not increased at all (Fig. 3, bars labeled Assay 1). The assay was performed a second time to confirm the results with MDA-MB435S, and surface expression of HLA class I

molecules was again observed to increase by over 100 channels following incubation of the cells at 25°C (Fig. 3, bars labeled Assay 2). Thus, HLA class I molecules were more temperature-inducible on a cell line expressing endogenous Ii than on a cell line expressing no Ii.

Ii was demonstrated to increase the expression of unstable MHC class I molecules on other breast tumor cell lines

With T47D, we had observed that increasing Ii expression raised the cell surface expression of HLA class I molecules (Fig. 2). We also demonstrated that this finding could be extended to other breast tumor cell lines. MCF-7 cells were stably transfected with Ii, and Ii expression was verified by Western blotting (data not shown). The MCF-7+Ii cells had increased expression of HLA class I molecules at the plasma membrane, as detected by two antibodies, H58A and W6/32 (Fig. 4A). Similar results to those shown in the figure were also obtained with a separate set of flow cytometry samples. The H58A monoclonal antibody is usable for flow cytometry and immunoprecipitation but not for Western blotting analyses of MHC class I molecules (according to the manufacturer and observations in our laboratory), which suggests that it recognizes a conformational determinant.

MDA-MB435S cells, which already express endogenous Ii (Fig. 1), were transfected with either Ii or with vector alone, and the resulting increased expression of Ii in MDA-MB435S +Ii was confirmed by Western blotting (data not shown). We found that MDA-MB435S cells also undergo an increase in HLA class I molecule expression when transfected with Ii, compared either to untransfected MDA-MB435S or vector-transfected MDA-MB435S cells (Fig. 4B). The variation of mean fluorescence intensity with the W6/32 staining in the assay results shown in Fig. 4B is very low, with SEM values <10 for all three cell lines (as shown by the error bars). Statistical analysis using the non-parametric Mann-Whitney test in the SPSS software indicated that there was a significant difference between the HLA class I molecule surface expression on MDA-MB435S+Ii compared either to MDA-MB435S+V ($p=0.002$) or MDA-MB435S ($p=0.002$). Furthermore, comparable results were also obtained in a separate flow cytometry assay.

Thus, our observation that a rise in Ii expression can induce an elevation in HLA class I expression at the plasma membrane was reproducible with three different human breast cancer cell lines: T47D, MCF-7, and MDA-MB435S.

Discussion

Our results from this study indicate that Ii can increase the surface expression of MHC class I molecules on human breast tumor cell lines. In addition, the low temperature inducibility and rapid surface turnover of the HLA class I molecules on the T47D+Ii cells, together with the low temperature inducibility of the HLA class I molecules on MDA-MB435S, suggest that the array of peptides presented is altered and biased toward low affinity peptides by Ii. Since Ii interacts with folded (and not open) MHC class I molecules, Ii present in the endoplasmic reticulum of tumor cells may bind to nascent MHC class I molecules in the process of assembly and influence their choice of peptide cargo. Alternatively, Ii may direct folded MHC class I molecules to the endosomes, where they may exchange their peptide ligands.

The HLA class I heavy chain co-immunoprecipitated with Ii from lysates of T47D+Ii (Fig. 2), suggesting that the intracellular binding of these two proteins is mechanistically related to the Ii-induced rise in cell surface HLA class I molecule expression. We did not detect HLA class I heavy chain interaction with Ii in MCF-7+Ii cell lysates, which may have been due to the lower intracellular level of HLA class I molecules in MCF-7 compared to T47D (data not shown).

Previous studies indicated that higher levels of surface HLA class I molecules increase the recognition of tumor cells by cytotoxic T lymphocytes (CTLs) [6,28]. Our data indicate that Ii expression in breast tumor cells induces a quantitative increase in surface HLA class I molecule expression, and therefore increased CTL recognition may potentially result from endogenous Ii expression in tumors. Our findings also suggest Ii may cause HLA class I heavy chain binding of an unusually high proportion of low affinity peptides. Presentation of a wider variety of peptide epitopes, even of low affinity, may increase the effectiveness of cellular immune responses to tumors expressing Ii [26,27]. Alternatively, CTL recognition may not be increased (or may even be decreased), if there is skewed presentation of a particular low affinity peptide or small set of peptides by Ii-expressing cells. Furthermore, CTL recognition may be decreased due to loss of the low affinity peptides at the cell surface. Consistent with this possibility, in one study the expression of Ii in colorectal carcinomas was not found to correlate with any reduction in the rate of cancer recurrence after surgery [18]. Further studies will determine the precise nature of the changes in peptide repertoire and the effect of Ii on T cell recognition of breast tumors in vivo. In total, these data suggest that breast tumor expression of Ii can lead to changes in the spectrum of presented peptides, in addition to changes in the number of MHC class I molecules that are present at the cell surface.

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Figure 1.

Ii was demonstrated to be expressed by MDA-MB435S. (A) The mean fluorescence intensity for Ii staining with the anti-CD74 monoclonal antibody is shown on the bar graph for several tumor cell lines. The mean fluorescence intensity obtained with the secondary antibody only control is indicated by the bar labeled "2nd Ab", and the mean fluorescence intensity obtained with an isotype control antibody (monoclonal antibody 30-5-7) is indicated by the bar labeled "Isotype". (B) Ii was stably transfected into T47D+Ii, as shown by Western blotting. Lysates of T47D and T47D+Ii were electrophoresed on a 4-20% acrylamide Tris-glycine gel, transferred to a blot, and probed with the PIN1.1 monoclonal antibody. (C) Flow cytometry data for MDA-MB435S are displayed on a histogram. The mean fluorescence intensity for anti-CD74 Ii staining is shown as a dotted line, the mean fluorescence intensity for the labeled secondary antibody only control is shown as a black, solid line, and the mean fluorescence intensity for the isotype control is shown as a gray, solid line. (The data for the secondary antibody only control and the isotype control are virtually overlapped.) (D) Ii expression was detectable in MDA-MB435S but not MCF-7 cell lysates by Western blotting with the PIN1.1 monoclonal antibody (top panel); Western blotting with an α -tubulin monoclonal antibody was included as a control.

Figure 2.

The expression of HLA class I molecules on the surface of T47D was elevated by Ii transfection, and HLA class I molecule expression on T47D+Ii was more unstable than on T47D. (A) Western blot of lysates of T47D and T47D+Ii, probed with the HC10 monoclonal antibody to identify the HLA class I heavy chain (HC). (B) Ii immunoprecipitation was performed on a lysate of T47D+Ii (or T47D as a control), and after transfer of the electrophoresed immunoprecipitates to a membrane, the blot was probed with the HC10 monoclonal antibody to identify the co-immunoprecipitated HLA class I heavy chain (HC). The two upper bands that appear in both lanes are non-specific background bands. (C) The expression of folded HLA class I molecules was more inducible by incubation at 25°C on T47D+Ii than on T47D. T47D and T47D+Ii were incubated overnight at 25°C or at 37°C and compared by flow cytometry with W6/32. Bars display the average of the mean fluorescence intensity values obtained with duplicate samples. (D,E) The HLA class I molecules at the surface of T47D+Ii have a relatively rapid turnover rate. T47D+Ii and T47D were cultured in the presence of brefeldin A, and the cells were analyzed by flow cytometry using monoclonal antibody W6/32. Results from two assays, using different time courses, are shown (0, 1, 2, 3, and 4 hours in D, and 0, 3, and 6

hours in E). The percentage of folded HLA class I molecules remaining at each time point was calculated by the formula $[(\text{mean fluorescence after brefeldin A treatment after the specified number of hours} / \text{mean fluorescence after brefeldin A for 0 hours}) \times 100]$ and the percentages are shown on the graphs.

Figure 3.

The expression of folded HLA class I molecules on MDA-MB435S was inducible by incubation at 25°C. Results are shown on the graph for two assays. Assay 1 included both MDA-MB435S and MCF-7, and Assay 2 included MDA-MB435S. The cells were incubated overnight at 25°C or at 37°C and compared by flow cytometry with W6/32.

Figure 4.

Transfection of Ii into other human breast tumor cell lines resulted in an increase in cell surface HLA class I expression. (A) Transfection of Ii into MCF-7 cells caused an overall increase in HLA class I surface expression. MCF-7 and MCF-7+Ii cells were analyzed by flow cytometry with monoclonal antibody H58A (pan-MHC class I) or W6/32 (recognizes folded, β_2m -associated HLA class I molecules). (B) Transfection of Ii into MDA-MB435S cells caused an overall increase in HLA class I molecule surface expression. MDA-MB435S, MDA-MB435S+V (vector only), or MDA-MB435S+Ii cells were analyzed by flow cytometry with W6/32. The difference between the HLA class I molecule surface expression on MDA-MB435S+Ii compared either to MDA-MB435S+V ($p=0.002$) or MDA-MB435S ($p=0.002$) was significant, as assessed by the Mann-Whitney test.