Tumor Cell Lines Expressing the Proteasome Subunit Isoform LMP7E1 Exhibit Immunoproteasome Deficiency

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Abstract

The immune system can recognize antigenic peptides derived from tumors by their presentation on MHC class I complexes to CTLs. Immunoproteasomes (i20S) can substantially enhance the MHC class I peptide repertoire, making down-regulation of i20S an important strategy of tumor cells in manipulating immune surveillance. Here, we report that human cancer cells express the nonfunctional immunosubunit-variant LMP7E1, in addition to, or instead of LMP7E2, in response to IFN-γ. This preferential expression of LMP7E1 and the consequent down-regulation of LMP7E2 results in i20S deficiency. The molecular explanation for this phenomenon is the incapacity of LMP7E1 to interact efficiently with the proteasome maturation protein, which regularly recruits LMP7E2 into nascent i20S precursor complexes. In contrast to previous reports, i20S formation in these cancer cells cannot be restored by IFN-γ treatment. However, expression of LMP7E2 in these cells restores the i20S-deficient phenotype. Thus, our data describe a novel mechanism that contributes to the process of oncogenesis. (Cancer Res 2006; 66(2): 649-52)

Introduction

Presentation of antigenic peptides by MHC class I molecules to CTLs is a crucial prerequisite for successful immune recognition and the elimination of transformed cells. Peptide ligands of MHC class I molecules are generated by a proteolytic cascade in which the proteasome plays a central role. IFN-γ-mediated induction of special proteasomes [i.e., the immunoproteasomes (i20S)], can improve MHC class I antigen presentation by producing certain peptides more efficiently (1). i20S have an altered proteolytic 20S core complex compared with constitutive proteasomes (c20S), where the three active sites β1, β2, and β5 are replaced by their immunosubunit counterparts LMP2 (β1i), MECL-1 (β2i), and LMP7 (β5i). In nonlymphatic cells, the immunosubunits can be induced by IFN-γ, whereby they are constitutively expressed in cells of the immune system. Concomitant with i20S, other components of the antigen presentation machinery (APM), like the transporter associated with antigen processing (TAP) or the proteasome activator 28 (PA28), are up-regulated by IFN-γ (1).

An altered or decreased cell surface expression of loaded MHC class I molecules contributes importantly to the process of oncogenesis and is thought to be one of the main mechanisms of transformed cells to escape immune surveillance. The effect of i20S on the processing of tumor-associated antigens is controversially discussed. Although tumor-derived antigenic peptides are generated more efficiently by i20S, some were reported to be destroyed by i20S, and some can be equally produced by both proteasome types (2–4). Nevertheless, the presence of a functional i20S seems to be of utmost importance for recognition of tumor cells by the immune system (2, 5–10). Various malignant tumor cells are characterized by the loss or down-regulation of components of the APM, in particular, the immunosubunit LMP7. Together with down-regulation of LMP expression, an impairment of other APM components has often been described, suggesting that these genes are controlled by common regulators whose function is altered in tumor cells (1, 7, 8, 11, 12). However, in most cases, treatment with IFN-γ restores the expression of down-regulated APM components, thereby restoring MHC class I antigen presentation (5–10).

Because active proteasomal β-subunits are expressed as inactive propeptides with NH2-terminal propeptides, the formation of both 20S species essentially requires a multistep biogenesis program starting from de novo syntheses of subunits. Final activation of the β subunits by autocatalytic removal of the propeptides is dependent on the correct integration of all components into the nascent complex (1). In fact, eukaryotic proteasome assembly and maturation is mediated by accessory proteins like the proteasome maturation protein (POMP; refs. 1, 13). We have recently shown that POMP is not only essential for c20S formation but also for i20S biogenesis. POMP recruits both β5 subunits into the nascent complex by differential interaction with either β5 or, preferably, with β5i/LMP7 (14). Vice versa, LMP7 is also crucial for the biogenesis of i20S (15, 16). The coincident induction of POMP and LMP7 by IFN-γ as well as their molecular interplay accelerate i20S biogenesis compared with c20S biogenesis and result in faster turnover of POMP (14).

Here, we describe the expression of the nonfunctional variant LMP7E1 in human cancer cells. LMP7E1, which is not expressed in primary cells, cannot be recruited by POMP into nascent i20S complexes. Consequently, cells preferentially expressing LMP7E1 are devoid of i20S, a phenotype that is not reversible by IFN-γ. Thus, we suggest expression of LMP7E1 in cancer cells is an additional strategy of oncogenesis.

Materials and Methods

Cell culture. Human cell lines were cultivated under standard conditions in RPMI 1640 (colon carcinoma: RKO, DLD-1, and Caco-2; cervical carcinoma: HeLa; melanoma: Mel15 and Mel 18) or Basal Iscove's medium (colon carcinoma: SW-480 and primary CRL-2429 cells: foreskin fibroblasts), with 10% (Caco-2: 20%) FCS, 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. Primary human melanocytes (NHM M2 cell kit, PromoCell, Heidelberg, Germany) or epithelial cells (HNEpC cell kit, PromoCell) were grown according to manufacturer's instructions.

For induction of i20S, cells were incubated 24 hours with 150 units/mL human IFN-γ. RKO cells were transiently transfected with expression

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plasmids for LMP7E1 or LMP7E2 according to manufacturer’s instruction
(LipofectAMINE 2000, Invitrogen, Inc., San Diego, CA). Stable transfectants
for LMP7E2 were subcloned and selected by Neomycin (Geneticin,
Invitrogen). Pulse-chase experiments and immunoprecipitation were done
with an antisera recognizing 20S precursor complexes and quantified as
described (14).

Immunoprecipitation and immunoblotting. Immunoprecipitation
was done with a β7 antibody. Immunoblotting was done with antibodies
for LMP7, LMP2, α4, and POMP (all laboratory stock) by glyceraldehyde-
3-phosphate dehydrogenase (GAPDH, bottom) served as loading controls. Northern blot
analysis of LMP7 in the presence or absence of IFN-γ (second) and ethidium bromide-stained 28S rRNA as loading control (third).

Northern blotting and semiquantitative reverse transcription-PCR.
For Northern blots, 3 μg of total RNA were loaded, vacuum blotted, and
hybridized overnight with digoxigenin-labeled riboprobes of LMP7 and
POMP (14). Staining of 28S rRNA served as a loading control.

Equal amounts of total RNA were supplied to the reverse transcription
with M-MuLV Reverse Transcriptase (Amersham Europe, Freiburg,
Germany) using (dT)18-oligonucleotides. The PCR amplified specifically
with M-MuLV Reverse Transcriptase (Amersham Europe, Freiburg,
hybridized overnight with digoxygenin-labeled riboprobes of LMP7 and
A

Figure 1. Human carcinoma cell lines are defective in IFN-γ−induced LMP7 expression. A, Northern blot analysis of POMP mRNA levels upon IFN-γ stimulation (+) in human cell lines (top) with ethidium bromide−stained 28S rRNA as loading control (middle). POMP is stabilized in RKO and Caco cells as shown by Western blot analysis (bottom). B, Western blot analysis of the proforms (p, pI) and the matured forms (m) of LMP2 (top) and LMP7 (fourth) in the presence (+) or absence (−) of IFN-γ. α4 (fifth) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, bottom) served as loading controls. Northern blot analysis of LMP7 in the presence or absence of IFN-γ (second) and ethidium bromide-stained 28S rRNA as loading control (third).

Results and Discussion

RKO and Caco-2 cells are defective in IFN-γ−induced LMP7 expression. We have recently shown that POMP; although induced, becomes unstable in response to IFN-γ, due to the nearly 4-fold faster biogenesis of i20S (14). Induction of LMP7 and POMP in HeLa cells led to accelerated i20S formation characterized by complete maturation of LMP2 and an apparent decrease in POMP levels (ref. 14, Fig. 1A and B). In contrast, POMP levels in the carcinoma cell lines RKO and Caco-2 were induced by IFN-γ (Fig. 1A). Although both cell lines respond to IFN-γ stimulation with a significant induction of the LMP7 mRNA, this did not result in a strong increase in LMP7 protein as in HeLa cells (Fig. 1B) or in other cell lines (14). Nevertheless, expression of LMP7 was induced by cytokine stimulation, showing that IFN-γ signal transduction was not affected. The loss of LMP7 expression indicated a reduced i20S formation, reflected by the signal for the unprocessed LMP2 proprotein.

Cancer cell lines express the LMP7E1 transcript. Despite an
IFN-γ−induced expression of the LMP7 message, the above data revealed virtually no LMP7 protein in RKO cells and a strongly reduced LMP7 level in Caco-2 cells (Fig. 1). The human LMP7 gene encodes two variants: the nonfunctional LMP7E1 isoform and LMP7E2, which is incorporated into nascent i20S (17, 18). Therefore, we investigated the LMP7 transcripts in more detail by performing reverse transcription-PCR (RT-PCR) analyses with specific primers for either LMP7E1 or LMP7E2 mRNA. As expected, LMP7E2 was induced in response to IFN-γ in all cells with increased levels of LMP7 protein (see Fig. 1). Surprisingly, LMP7E1 was expressed in all cancer cell lines tested to different extents and in dependence on IFN-γ. Importantly, in primary cells as fibroblasts, epithelial cells, or melanocytes, no LMP7E1 transcript was detectable, indicating that expression of LMP7E1 is a specific feature of cancer cells (Fig. 2, top). Remarkably, Caco-2 and RKO, as well as the formerly described LMP7− and PA28-deficient melanoma Mel18 cells (11), displayed low

Figure 2. Human cancer cells express the nonfunctional variant LMP7E1. Analysis of LMP7 transcript variants E1 (top) and E2 (middle) of unstimulated (−) and IFN−γ−stimulated (+) human cell lines by RT-PCR using variant-specific primers. Actin expression was unaffected by IFN-γ and served as an internal standard (bottom). All PCR reactions have been carried out on the same cDNA per cell at 54°C annealing temperature, 1 minute extension time, and 28 cycles to amplify fragments of 1014 bp (LMP7E1), 942 bp (LMP7E2), and 1014 bp (actin). Primary cell lines (foreskin fibroblasts, CRL-2249, melanocytes, NHEM; or epithelial cells, HNEpC; first three); cancer cell lines as indicated.
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and in total lysates (left) and in immunoprecipitated 20S complexes (right). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and GAPDH were used as loading controls. Glyceraldehyde-3-phosphate dehydrogenase levels represent 15% input.

LMP7E2 can restore the i20S-deficient phenotype in RKO cells. To further test whether the loss of LMP7E2 expression is indeed the molecular reason for defective i20S formation in RKO cells, we transiently transfected RKO cells with plasmids expressing either LMP7E1 or LMP7E2. Transfection with LMP7E1 resulted only in marginal synthesis of the LMP7E1 protein and had no significant effect on cellular POMP levels (Fig. 4A). Thus, an efficient recruitment of LMP7 by POMP into i20S complexes not only accelerates i20S formation but may also protect not yet incorporated LMP7 from further turnover. However, RKO cells transfected with LMP7E2 and stimulated with IFN-γ expressed high levels of the immunosubunit, which was efficiently incorporated into i20S as indicated by the amount of matured LMP7. This result was also supported by significantly decreased POMP levels as soon as LMP7E2 was expressed (Fig. 4A).

The above result was also confirmed with RKO cells stably expressing LMP7E2. Quantification of the POMP turnover kinetics upon IFN-γ stimulation showed a dramatic decrease of precipitated POMP within precursors compared with untransfected RKO cells (Fig. 4B). Thus, expression of LMP7E2 restored the dynamics of i20S formation.

Immunoblot analyses of these transfected RKO cells showed a high constitutive expression of LMP7 independent of the IFN-γ signal. LMP2, however, was clearly induced by the cytokine (Fig. 4C, left). A quantitative processing of LMP2 could only be detected in the IFN-γ-stimulated transfectant (Fig. 4C, left, compare with Fig. 3B). For further support, we did immunoprecipitation analyses of 20S complexes from cellular extracts of these cells. Indeed, the expression of LMP7E2 in RKO cells resulted in a reconstitution of i20S (Fig. 4C, right). Thus, the accelerated formation of mature and active i20S upon IFN-γ stimulation is exclusively dependent on the molecular interplay of LMP7E2 and POMP. This fundamental role of the immunosubunit LMP7E2 was established for the first time on cancer cell lines defective in LMP7E2 expression.

LMP7E1 Expression in Cancer Cells

The nonfunctional LMP7E1 variant prevents i20S formation. The proform of the immunosubunit LMP7E2 directly binds to the maturation factor POMP, which recruits LMP7 into the nascent complex and essentially mediates the accelerated biogenesis of i20S (14). To examine this molecular interaction for the LMP7E1 variant, we analyzed its interaction with POMP after in vitro coexpression in pull-down assays (Fig. 3A). In contrast to LMP7E2, the proform of LMP7E1 could not be pulled down with histidine-tagged POMP (Fig. 3A, left). Thus, the LMP7E1 protein is not only barely expressed in vivo (15) but also not capable of interacting efficiently with POMP. Thus, the functional incapacity of LMP7E1 to interact with POMP is the molecular explanation for the missing integration of this LMP7 species into i20S complexes.

The above results imply a failure of i20S biogenesis in cells expressing predominantly LMP7E1. Therefore, we investigated i20S formation in LMP7E2 deficient RKO and Mel18 compared with HeLa cells by immunoprecipitation and Western blot. The inability to incorporate LMP7E1 into i20S precursor complexes in RKO and Mel18 cells further resulted in a lack of i20S formation in response to IFN-γ (Fig. 3B), although LMP7 transcription is apparently induced as evidenced by Northern blots (Fig. 1B). Thus, these tumor cells exhibit a LMP7-deficient phenotype as shown for several other i20S-deficient tumor cells. Importantly, however, this phenotype cannot be restored by IFN-γ treatment. Taken together, these data suggest that expression of the nonfunctional variant LMP7E1 prevents i20S formation upon IFN-γ stimulation.

or barely detectable amounts of LMP7E2 mRNA even after IFN-γ stimulation (Fig. 2, middle). On the other hand, IFN-γ enhanced the expression of LMP7E1 in these cells (Fig. 2, top). This result can explain the apparent contradiction between induction of LMP7 mRNA in Northern blots (Fig. 1A) and the concomitant deficiency of LMP7 protein in these cells (Fig. 1B). Down-regulation of immunosubunits has been described as an important mechanism of tumor cells to evade immune surveillance (7, 8, 12). These data are therefore of particular importance for transcription analyses of tumors using microarrays, Northern blots, or RT-PCR, which usually do not differentiate between LMP7E1 or LMP7E2 transcripts.

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In conclusion, our results show that the observed impairment of i20S in human RKO, Caco-2, and Mel18 cells is due to the preferential expression of the immunosubunit isoform LMP7E1. The human LMP7 gene PSMB8 is transcribed into two mRNAs due to two putative promoters, which use different initial exons (17, 19, 20). The resulting protein isoforms differ only in their prosequences. However, LMP7E1, which has no counterpart in mouse or rat, is not capable of interacting efficiently with POMP (Fig. 3A). Thus, LMP7E1 is not incorporated into nascent i20S (Fig. 3B; refs. 15, 16, 18, 19). Interestingly, an alternative human LMP2 variant of unknown function has also been described, which results from differential splicing (accession no. NM_148954). The detection of LMP7E1 mRNA in all cancer cell lines tested is an unexpected and interesting result and indicates a novel regulatory function of this isoform. We propose that the interdependence of the expression of both alternative LMP7 transcripts, LMP7E1 and LMP7E2, guarantees a fine tuning of i20S biogenesis. A critical balance between the alternative transcripts could determine the efficiency of i20S formation. Although the promoters for LMP7E1 and LMP7E2 are purely defined, their positions suggest that the expression of LMP7E1 competes with that of LMP7E2. Transcription from LMP7E2 promoter is only favored once IFN-γ-induced factors like IFN regulatory factor-1 are allowed to bind (20). Probably this can only occur when the LMP7E1 expression is low. Because we failed to find a mutation within this putative promoter sequences of the PSMB8 locus in RKO cells, a dysregulation of the respective transcriptional activators is most likely the reason for the impaired LMP7 expression.

Taken together, our data provide evidence for an additional mechanism, which results in the failure to express sufficient amounts of i20S. Ultimately, this ensures the immune escape of malignant tumor cells due to less efficient presentation of peptides by MHC class I to CTLs.

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