Development of Spontaneous Uterine Tumors in Low Molecular Mass Polypeptide-2 Knockout Mice\textsuperscript{1}

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Abstract

The presentation of antigenic peptides by MHC class I molecules is important for tumor rejection by CTLs. Such antigenic peptides are generated as a result of the degradation of intracellular proteins by the proteasome pathway, a process that is influenced by the IFN-\(\gamma\)-inducible low molecular mass polypeptide-2 (LMP2) subunit of the proteasome complex. LMP2 knockout mice thus exhibit a defect in proteasome function. Female LMP2\textsuperscript{−/−} mice are now shown to develop uterine neoplasms, with a disease prevalence of \(\sim 36\%\) by 12 months of age. This observation indicates that proteasome function is essential for MHC class I-mediated tumor rejection by CTLs.

Introduction

The proteasomes, the proteolytic machinery of the ubiquitin/ATP-dependent pathway, have a relevant role in many processes crucial for cell physiology and cellular processes as diverse as cell cycle progression and gene expression of numerous proinflammatory cytokines, enzymes, and cellular adhesion molecules.\textsuperscript{1} Proteasome inhibitors block this gene activation and provide anti-inflammatory effects in several animal models of peripheral inflammation. The ubiquitin-proteasome pathway thus represents an important target for the development of drugs that might prove effective in the treatment of inflammatory disease.\textsuperscript{1} IFN-\(\gamma\) is a critical inducer of proteasome activation in immune-network systems.\textsuperscript{2} Recent published data verify that IFN-\(\gamma\) and lymphocytes prevent primary tumor development, therefore showing a tumor suppressor role in the immune response.\textsuperscript{3, 4} IFN-\(\gamma\) plays a central role in diverse immune events and up-regulates large numbers of responsive genes. IFN-\(\gamma\)-regulated genes including proteasome subunits, LMP2,\textsuperscript{5} LMP7, and LMP10 appear to be obligatory for T-cell target recognition of tumors.\textsuperscript{2, 5} IFN-\(\gamma\)-deficiency apparently does not hamper CTL generation.\textsuperscript{3, 4} Recent reports demonstrate the multiple functional deficiencies of components of the MHC class I antigen pathway including LMP2 and TAP-1 in tumor cells.\textsuperscript{5, 6–9} The possible role of the IFN-\(\gamma\)-responsive gene, TAP-1, in select tumor recognition is reported.\textsuperscript{4} Here we now identify the proteasome subunit LMP2, which is a single IFN-\(\gamma\)-responsive gene, as obligatory for tumor surveillance (5) and furthermore demonstrate a tissue-specific role of LMP2 in protection from spontaneous neoplasms of the uterus. IFN-\(\gamma\)-inducible proteasome function thus plays a significant role for MHC class I-mediated tumor rejection.

Materials and Methods

Mice, Cells, and Antibodies. C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Nude (\textit{nu/nu}) mice were purchased from COX7 (Massachusetts General Hospital, Boston, MA). LMP2\textsuperscript{−/−} mice were a generous donation from Dr. Luc Van Kaer (Vanderbilt University School of Medicine, Nashville, TN). T1 cells were purchased from American Type Culture Collection (Manassas, VA); T2 cells were a kind gift from Dr. P. Cresswell (Yale University School of Medicine, New Haven, CT). Fresh MEFs were obtained from C57BL/6 or LMP2\textsuperscript{−/−} embryos at 14.5 days by standard methods. All antibodies were purchased through Santa Cruz Biotechnology, Inc. (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Histology and Immunofluorescence Staining. Uteri obtained from C57BL/6 or LMP2\textsuperscript{−/−} mice were fixed in 10% buffered formalin, incubated in 4% paraformaldehyde for 8 h, and embedded in paraffin. Sections (5 \(\mu\)m) were prepared and stained with H&E for routine histological examination or were processed further for immunofluorescence staining. After removal of paraffin by 100% xylene, the sections were treated at 95°C for 5 min and incubated for 2 h at room temperature with antibodies to Ki-67 (Santa Cruz Biotechnology, Inc.). The sections were then washed in PBS, incubated with FITC-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc.), and examined with a fluorescence microscope.

Immunoblot Analysis. MEFs obtained from C57BL/6 and LMP2\textsuperscript{−/−} embryos at 14.5 days were treated for 3 days at 37°C in DMEM with or without 50 units/ml of IFN-\(\gamma\) (Sigma Chemical Co., St. Louis, MO). Alternatively, MEFs were also treated by 20 ng/ml of TNF-\(\alpha\) (R&D, Minneapolis, MN) for various times indicated in Fig. 2C. The whole cell extracts from MEFs treated by IFN-\(\gamma\) or TNF-\(\alpha\) were subjected to SDS-PAGE on 12.5% gels under nonreducing conditions. The separated proteins were transferred electrophoretically to a polyvinylidene difluoride membrane, which was then incubated for 2 h at room temperature with TBS-T [20 mm Tris-HCl (pH 7.6), 137 mm NaCl, and 0.05% (v/v) Tween 20] containing 8% BSA. The membrane was then incubated for 12 h at 4°C with TBS-T containing the appropriate primary antibodies and washed four times with TBS-T for 15 min, each time at room temperature. The membrane was incubated for 2 h at room temperature with TBS-T containing alkaline phosphatase-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc.). After washing an additional five times with TBS-T, the membrane was subjected to the alkaline phosphatase color reaction by standard method.

In Vivo Tumorigenesis Assay. T1 and T2 cells were cultured in DMEM (Life Technologies, Inc., Grand Island, NY) containing 10% FCS (Life Technologies, Inc.) at 37°C. Ten-week-old wild-type C57BL/6 female mice (\textit{n} = 4) and age-matched Nude (\textit{nu/nu}) female mice (\textit{n} = 4) were injected intracutaneously on day 0 with inocula of 10\(^{5}\) T1 or T2 cells (two sites/mouse for individual injection of cell type). Tumor diameters in these animal hosts were monitored 3 and 14 days after transplantation. All animal studies were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care.

Results and Discussion

A large multicatalytic protease complex of 28 subunits called the proteasome plays a major part in cytosolic protein degradation.\textsuperscript{1, 5} The production of peptide ligands for MHC class I molecules is regulated by proteasome-mediated protein processing.\textsuperscript{5} Three proteasome subunits, LMP2, LMP7, and LMP10, are significantly induced by IFN-\(\gamma\) treatment.\textsuperscript{2} IFN-\(\gamma\)-treated cells, via the induced...
LMP subunits, induce degradation activity for hydrophobic and basic substrates, but reduce degradation activity for acidic substrate (2, 10). Moreover, proteasomes of mutant cell lines or murine hosts lacking LMP subunits show decreased rates of cleavage after hydrophobic and basic residues (5, 10–12). This produces peptides with COOH-terminal residues that are preferred anchor residues for peptide binding to surface MHC class I molecules, one process probably critical for attraction of CTL to tumor cells for efficient immune rejection (13, 14). Most spontaneously arising tumors do not solicit T-cell migration, recognition, and rejection (13). Such tumors lack either distinctive antigenic peptides presented by MHC class I molecules, the adhesion or costimulatory molecules necessary to elicit a primary T-cell response (15, 16).

In mice with a targeted disruption of LMP2 proteasome subunits, ATP-dependent proteasome function is impaired (10–12). In this study, we now show that LMP2+/− mice are prone to the development of uterine neoplasms (Fig. 1). The percentage of animals with overt tumors increases with age after 6 months, with a cumulative prevalence of disease in female mice of 36% (10 of 28) by 12 months of age and no apparent plateau at this late observation time (Fig. 2A). Leiomyosarcoma, which is the most common tumor of the uterus, was observed in the LMP2+/− female mice (Fig. 1, B and D) but not in C57BL/6 mice with the same genetic background as LMP2+/− mice (Fig. 1, A and C). Histological examinations of the LMP2+/− uterine tumors revealed the common characteristic abnormalities of leiomyosarcomas (Fig. 1, F and H). The tumors lacked lymphoid infiltrates, which is a sign of immune recognition, and consisted of uniform elongated smooth muscle cells arranged into bundles. The nuclei of the tumor cells varied in size and shape; furthermore, mitosis was frequent (Fig. 1, F and H). In contrast, the uterine smooth muscle cells of C57BL/6 mice were normal in appearance (Fig. 1, E and G). Whereas relatively few Ki-67-positive cells, the proliferating cells of solid tumors, were observed in the basal cell layer of the normal uterine smooth muscle (Fig. 1, I), most of the basal cells vividly expressed Ki-67 in LMP2+/− mice. This immunohistochemical staining indicates abnormal proliferation of the LMP2+/− cells in the basal layer (Fig. 1J).

As expected, whereas MEFs derived from C57BL/6 mice exhibited a significant increase in LMP2 expression in response to IFN-γ, MEFs derived from LMP2+/− mice did not express LMP2 in the absence or presence of IFN-γ (Fig. 2B). The abundance of the other IFN-γ-inducible proteasome subunits, LMP7 and LMP10, was markedly increased by IFN-γ treatment in MEFs derived from both C57BL/6 and LMP2+/− mice (Fig. 2B). The expression of the proteasome subunit CP9, as well as that of the cyclin-dependent kinases CDK7 and CDK8 (none of which are encoded by IFN-γ-responsive genes), also did not differ between the two mouse strains. Furthermore, whereas IκBα, an inhibitor of the transcription factor NF-κB and known substrate of LMP2-containing proteasomes (13, 14), was completely degraded in C57BL/6 MEFs within 40 min of exposing to TNF-α, IκBα was phosphorylated but not degraded in LMP2+/− cells treated with TNF-α (Fig. 2C). These observations are consistent with the previous demonstrations that proteasomes purified from the spleens of LMP2+/− mice exhibit impaired peptidase activities and that antigen presentation by MHC class I molecules in these animals is defective (10).

The in vivo tumorigenesis assay was performed using hybrid lymphoblast T1 and T2 cells instead of LMP2+/− tumor cells. T1 cells are a cloned hybrid between T and B lymphoblasts and express large amounts of MHC class I and class II. T2 cells are mutant-derived T1 cells; they lack a large segment of chromosome 6 that encodes MHC class II-linked genes, including the Lmp2 proteasome subunit gene. T2 cells do not present antigenic peptides with MHC class I molecules. Ten-week-old wild-type C57BL/6 female mice, the parental mice of LMP2+/− mice, and age-matched Nude (nu/nu) female mice were injected intracutaneously on day 0 with inocula of 10^7 T1 or T2 cells. Parental T1 cells and T2 cells grew progressively in immunodeficient Nude mice when injected at 10^7 cells/mouse (data not shown). Although T2 cells significantly grew in C57BL/6 mice, T1 cells formed small an intracutaneous mass that expanded for the first 2
of these mice types was clearly higher than in normal mice (4). Indeed, the incidence of chemical carcinogen-induced cancer in both LMP2−/− mice induces antigenic peptide presentation with MHC class I molecules. These mice lack IFN-γ/Stat1 and therefore recognize and kill by CTLs. Cancer cells of wild-type mice express antigenic peptides derived from novel proteins on the cell surface in association with MHC class I molecules. These cells are therefore recognized and killed by CTLs, and tumors do not progress. In contrast, the presentation of antigenic peptides is impaired in LMP2−/− cancer cells as a result of a defect in proteasome function. These LMP2−/− cancer cells are therefore not attacked by CTLs, and tumors progress.

Our research demonstrates the requirement of LMP2 for rejection of tumor cells by CTLs. Spontaneously arising tumors are rarely rejected by CTLs (14). It is probable that they lack either distinctive antigenic peptides or the adhesion and the costimulatory molecules necessary to elicit primary T-cell responses. Tumors tend to be genetically unstable and can lose their antigens by mutation so that in the event of an immune response, further escape mutants might be generated (13). Several research reports indicate functional deficiencies of components of the MHC class I antigen pathway, including LMP2 and TAP-1 in individual tumor cells (6–9). Recent data also support a reciprocal and linked derangement in the immune response to new tumor development (3, 4, 17, 18). For instance, IFN-γ-insensitive mice that lack either the IFN-γ receptor or Stat1 test the role of immune surveillance for cancer cell progression (4). These mice lack the IFN-γ signal pathway that induces antigenic peptide presentation with MHC class I molecules. Indeed, the incidence of chemical carcinogen-induced cancer in both of these mouse types was clearly higher than in normal mice (4). TAP-1 is one of the IFN-γ-inducible molecules and is also an essential factor for antigenic peptides presentation. TAP-1-transfected cancer cells derived from tumors in both of the mouse strains formed small s.c. masses that expanded for the first 5–10 days but then disappeared 2 weeks after inoculation; thus, TAP-1 function plays a significant role for tumor rejection by CTLs (4). The current studies support the idea that TAP-2 and LMP2 may be another important gene for cancer target recognition. In this study, LMP2−/− mice revealed that this proteasome subunit significantly influences antigen processing for MHC class I molecule-binding peptides, and LMP2−/− mice have reduced levels of immunological competency by CD8+ CTLs (10). The defective antigen presentation associated with TAP-1 or LMP2 deficiency may therefore promote direct escape from immune recognition by CTLs.

We have now shown that LMP2 is important for the immune response that prevents the development of spontaneous uterine neoplasms. Moreover, spontaneous hepatocellular carcinoma or severe lung tumors were also observed at low incidence in both male and female LMP2−/− mice (data not shown), but no cancer progression was detected in age-matched C57BL/6 mice, an identical background murine strain. Diverse tumors that lose the expression of only one type of MHC class I molecule are able to escape a specific CD8+ CTL response (13, 15, 16), but select IFN-γ-induced genes, at least in the murine model, confer target specificity to interrupted immune recognition (Fig. 3). When a tumor loses expression of all MHC class I molecules, it can no longer be recognized by CTLs, although it may become susceptible to natural killer cells (13). However, tumors that lose only one MHC class I molecule may be able to avoid recognition.
by specific CD8\(^+\) CTLs while remaining resistant to natural killer cells, conferring a selective advantage in vivo. The ultimate immune response may depend on where and when tumor-specific antigens form (4, 17, 18). The tumor will be eliminated naturally unless they have developed specific resistance mechanisms, such as down-regulation of MHC or the antigen-processing machinery. As predicted, clinically mediated proteasome inhibition has an anti-inflammatory effect by presumed hampered immune recognition. The ubiquitin-proteasome pathway thus represents an important target for the discovery of drugs that might prove effective in the treatment of inflammatory disease or lethal malignancy (19–22). However, the results of our present study suggest that caution is warranted with regard to possible long-term treatment with proteasome inhibitors; a potential undesirable side effect might be the development of tumors.

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References

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