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The Angiogenic Growth Factor and Biomarker Midkine Is a Tumor-Shared Antigen

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The angiogenic factor Midkine (MDK) is overexpressed in various human malignant tumors, although its expression is low or undetectable in normal adult tissues. Its expression in tumors and its detection in plasma have been associated with poor disease outcome, whereas its blockade was found to contribute to tumor regression. By weekly stimulation of T lymphocytes harvested in HLA-A2 healthy donors, we derived CD8 T cell lines specific for several MDK peptides. The T cell response was mostly dominated by two nonamer peptides localized in the signal peptide and in the C-terminal part of the protein, as assessed by IFN-γ ELISPOT and HLA-A2 tetramer labeling. Peptide-specific T cell lines recognized cells transfected with an MDK-encoded plasmid and tumor cell lines naturally expressing the MDK protein, but not untransfected cells. T cell presentation of the two MDK epitopes was found to be TAP dependent. Experiments performed in HLA-A2 transgenic mice demonstrated the capacity of the two identified CD8 T cell epitopes to elicit a cytotoxic response. Altogether, our data show that the secreted MDK protein is a candidate vaccine for multiple cancers. The Journal of Immunology, 2010, 185: 418–423.

The number of well-characterized tumor Ags has steadily increased in recent years. More than 200 class I-restricted cytotoxic T lymphocyte peptide epitopes have been characterized from multiple proteins (1). Tumor Ags differ widely in their expression pattern in tumor and healthy cells, in their contribution to tumor survival, and their immunogenic properties. One major group of tumor Ags is represented by cancer-germline Ags encoded by genes that are totally silent in most normal tissues and are overexpressed in a variety of tumor types (2). Members of this group include genes encoding MAGE, BAGE, or LAGE. Another important type of Ag is composed of differentiation Ags that are found in both the tumors and the normal cells from which they derived. The most studied differentiation Ags are probably the Melan-A/MART-1 and gp100 proteins, which are present in both melanocytes and melanoma. Although both types of Ags appeared to be able to elicit a tumor-specific immune response, they are dispensable for the tumor and hence facilitate tumor escape from immune attack through loss of Ags (3, 4). Because of their almost strict tumor-restricted expression, cancer-germline Ags also suffer from interindividual expression discrepancies in cancer patients (5, 6), whereas differentiation Ags are only found in a defined type of tumor. As a result, a few identified tumor Ags exhibit a wide expression in multiple cancer patients and contribute to survival and growth of cancer cells. These cancer targets mainly comprise telomerase reverse transcriptase (7), which is required for the control of telomere length in the tumor, and survivin (8), and Bcl-2 (9), which provide resistance to apoptosis. T cell epitopes of these proteins (8–10) have been already delineated, but their efficiency in eliciting a tumor-eradicating immune response is not formerly demonstrated.

Midkine (MDK) is a heparin-binding growth factor found as the product of a retinoic acid-responsive gene (11). This secreted protein has been found to be overexpressed in various human malignancies, such as bladder, prostate, breast, lung, liver, and colon tumors, whereas its expression is typically low or undetectable in normal adult tissues (12–14). Its natural role is mainly to participate in neuronal development during embryogenesis (15). It has also been implicated in ischemic renal injury (16) and autoimmune responses (17). In tumor cells, MDK has not only an angiogenic role (18), but also a mitogenic (19) and antiapoptotic function (20). Its blockade contributes to tumor regression in a mouse model (21), demonstrating its importance in tumor expansion. Interestingly, its expression in the tumor (12, 22) and its concentration in plasma (23) were found to be related to poor disease outcome. Its expression in tumors can be detected early (13), and it was therefore considered as a relevant biomarker of disease progression. Its expression in tumors can be determined by blood (23, 24) and urinary analysis (25). Because of its functions, its wide expression in tumors, and its biomarker status, we have investigated whether MDK can induce a cellular cytotoxic response in humans. Both in vitro primary stimulation experiments performed with HLA-A2 healthy donors and immunization trials performed in HLA-A2 transgenic mice revealed the capacity of MDK to elicit tumor-specific CTL response.

Materials and Methods

Peptides and proteins

MDK nonamer peptides were synthesized using standard 9-fluorenylmethoxycarbonyl chemistry on an Advanced ChemTech Apex synthesizer (Advanced ChemTech, Louisville, KY) and cleaved from the resin by 95%
trifluoroacetic acid. If necessary, peptides were purified by reversed-phase HPLC on a C18 Vydac column (Interchim, Montluçon Cedex, France). Their purity was >90%, as indicated by analytical HPLC. Sequences of the 13–21 and 114–122 peptides were ALLALTSA V and AQCQETIRV, respectively.

Construction of MDK expression plasmid

Total RNA was extracted from A549 cells and reverse transcribed into cDNA using reverse transcriptase and oligo(dT) (ImProm-II Reverse Transcription System, Promega, Charbonnières-les-bains, France). MDK cDNA was amplified by PCR using the upstream primer 5'-GTGGTTGGAAATT-CACCACCATGCAGCACCGAGGCTTCCTC-3' and the downstream primer 5'-AAACTCGAGCCAGGCTTGGCGTCTAGTC-3'. MDK cDNAs were digested with EcoRI and XhoI, then ligated into pcDNA3.1 expression vector predigestion. The corresponding construct (pMDK) was purified on plasmid kit columns under endotoxin-free conditions (Qiagen EndoFree Plasmid Maxi Kit, Qiagen, Courtaboeuf, France).

Cell culture and transfection

A549 human lung carcinoma, CIR human B lymphoblast, HepG2 human hepatocellular carcinoma, and DLD1 human colorectal adenocarcinoma were from the American Type Culture Collection (Manassas, VA). The pcDNA3.1-MDK (pMDK) (20 μg) was introduced into C1R-A2 cells (3 × 10^6 cells) by electroporation (0.9 kV, 250 μF, 0.4-cm gap) with a Gene Pulser II (Bio-Rad, Marnes-la-coquette, France). The treated cells were suspended in 40 ml prewarmed RPMI 1640 medium with 10% fetal serum and incubated under 5% CO2/air at 37˚C. Forty-eight hours later, transfection efficacy was assessed by flow cytometry as endogenous MDK expression in matured dendritic cells (DCs) and HepG2 and DLD1. Briefly, cells were permeabilized with 75 μl fixation/permeabilization solution (BD Biosciences, Le pont de Claix, France) for 20 min at 4˚C and washed twice with BD Perm/Wash buffer plus 2% FCS (BD Biosciences). They were stained for 30 min on ice with anti–MDK-PE Ab (BD Biosciences) or anti-isotype control. Cell staining buffer plus 2% FCS (BD Biosciences). They were stained for 30 min on ice with anti–MDK-PE Ab (BD Biosciences) or anti-isotype control. Cell staining buffer plus 2% FCS (BD Biosciences). They were stained for 30 min on ice with anti–MDK-PE Ab (BD Biosciences) or anti-isotype control. Cell staining buffer plus 2% FCS (BD Biosciences). They were stained for 30 min on ice with anti–MDK-PE Ab (BD Biosciences) or anti-isotype control. Cell staining buffer plus 2% FCS (BD Biosciences). They were stained for 30 min on ice with anti–MDK-PE Ab (BD Biosciences) or anti-isotype control. Cell staining buffer plus 2% FCS (BD Biosciences). They were stained for 30 min on ice with anti–MDK-PE Ab (BD Biosciences) or anti-isotype control. Cell staining buffer plus 2% FCS (BD Biosciences). They were stained for 30 min on ice with anti–MDK-PE Ab (BD Biosciences) or anti-isotype control. Cell staining buffer plus 2% FCS (BD Biosciences).
cells per well). After a 4-h incubation at 37°C, emitted fluorescence was measured on a Gemini Spectramax Fluorimeter (Molecular Devices, St. Gregoire, France) (excitation at 485 nm and emission at 530 nm).

**In vivo immunogenicity studies**

H-2 class I knockout, HLA-A*0201-transgenic mice (HHD II) have been previously described (27). Eight- to 10-wk-old female mice were bred and maintained under specific pathogen-free conditions in our animal facility. HHD mice were injected s.c. at day 0 and day 14 with 50 μg MDK peptides 13–21 or 114–122 along with 140 μg I-Aβ MHC class II helper peptide TPPAYRPNNAIL (T13L), which corresponds to amino acids 128–140 of the hepatitis B virus core protein in IFA. For DNA immunizations, HHD mice were injected with 50 μl cardiotoxin into each regenerating tibiaus anterior muscle 5 to 6 d prior to DNA injection. Fifty microliters of pMDK (1 μg/μl in PBS) was injected directly into each pretreated muscle at days 0 and 14. Seven to 10 d after the last immunization, spleen cells of immunized mice were harvested, and Ficoll-purified lymphocytes were isolated. Specific T cells were detected ex vivo using ELISPOT assay. For CTL function analysis, splenocytes were stimulated for 5 to 6 d with peptide-pulsed (10⁶ M) HepG2 cell line (Fig. 1A), whereas its expression was extremely low in DLD1 cells (Fig. 1B). MDK was also weakly expressed in the CIR-A2 cell line, whereas CIR-A2 cells transfected with pMDK exhibited a high expression of MDK (Fig. 1C). In contrast, human DCs did not express MDK (Fig. 1D). We also observed good expression of MDK in A549 and Caski cell lines, whereas it was not expressed in monocytes, lymphocytes, or activated T lymphocytes (data not shown). We thus confirmed that MDK was overexpressed in several tumor cell lines in contrast to normal cells derived from the blood.

**Induction of MDK peptide-specific T cell lines**

Based on HLA-A2 scores provided by the prediction programs BIMAS, SYFPEITHI, and PROPRED, 14 peptides of 9 to 10 aa were initially selected for preliminary T cell priming experiments. BIMAS, SYFPEITHI, and PROPRED, 14 peptides of 9 to 10 aas were designed and characterized by IFN-γ ELISPOT. Seven peptides, namely 13–21, 12–21, 13–22, 14–22, 63–72, 113–122, and 114–122, elicited peptide-specific T cell lines and were retained for further experiments. As shown in Fig. 2A, T cell lines 314.48 and 267.29 were activated by MDK-HLA-A2+ CIR-A2 cells presenting the peptides 113–122 and 114–122 or 12–21 and 13–21, respectively. Omission of the peptides or addition of inappropriate peptides did not activate them. Dose-range experiments (Fig. 2B) showed that the optimal peptides were the 9-mers 114–122 and 13–21. They were recognized at low concentration as compared with the 10-mers 113–122 and 12–21, the midactivating dose being ~0.01 μg/ml (equivalent to 10⁻⁸ M). The peptide-specific T cells appeared therefore to possess a good affinity for the corresponding peptide–HLA-A2 complexes. As shown in Fig. 3, the T cell line 314.7, which was specific for the 114–122 peptide in ELISPOT (not shown), was nicely labeled by the corresponding HLA-A2 tetramers (Fig. 3A), although very few cells were labeled by the 13–21 specific tetramer (Fig. 3B). In contrast, the T cell line 297.58, which was specific for...
the peptide 13–21 in ELISPOT, was labeled by the 13–21 specific tetramer (Fig. 3C), but not by the 114–122 specific tetramer (Fig. 3D). For both T cell lines, ∼7% of the cells were peptide-specific, confirming their good peptide specificity. From 12 different HLA-A2+ healthy donors, we derived seven T cell lines specific for peptide 13–21 and eight T cell lines specific for peptide 114–122, as illustrated by T cell lines presented Fig. 2C. We therefore demonstrated that peptides 13–21 and 114–122 were able to prime a CD8 T cell response in HLA-A2+ healthy donors.

**Restriction, function, and tumor specificity of peptide-specific T cell lines**

HLA-A2 restriction of five T cell lines was confirmed using HLA-A2+-transfected and untransfected APCs. Representative experiments with the T cell lines 314.48 and 297.58 are presented in Fig. 4A. In contrast to the HLA-A2+ CIR-A2 cells, HLA class I-deficient CIR cells were unable to present the appropriate peptide to T cell lines specific for either 13–21 or 114–122. Moreover, transfection of pMDK into MDK− CIR-A2 cells was as efficient as peptides 13–21 or 114–122 in stimulating the T cells, demonstrating that both epitopes were appropriately processed and presented by the APCs. Because transfection led to a higher production of endogenous MDK, we also assessed whether HLA-A2 tumor cells (HepG2) naturally expressing MDK could be recognized by the peptide-specific T cell lines. As shown for four different T cell lines specific for 114–122 (Fig. 4B, left panels) and 13–21 (Fig. 4B, right panels), very strong stimulation of the T cell lines occurred in the presence of the HLA-A2+ MDK+ HepG2 cell line, whereas CIR-A2 and DLD1 were almost unable to activate them. The 616.11 T cell line was also tested with success for recognition of other HLA-A2+ MDK+ tumor cells (Caski) (Fig. 4B). For two T cell lines (Fig. 2C), we showed that tumor recognition occurred in an HLA class I-restricted manner. Activation of the 13–21 specific T cell lines 648.24 and 654.7 was inhibited by the HLA class I-specific mAb (W6/32) but not by the isotype control Ab. We investigated the TAP dependence of the tumor recognition (Fig. 5B). CIR-A2 cells loaded with the 114–122 and 13–21 peptides were more efficient than unloaded CIR-A2 to inhibit the T cell activation. Altogether, MDK peptides 13–21 and 114–122 elicit HLA-A2–restricted T cells in different donors. The T cells were cytotoxic and specifically recognized MDK+ tumor cells and MDK-transfected cell lines.

**Induction of MDK-specific T cells in HHD mice**

We also investigated the immunogenic properties of pMDK and the identified CD8 T cell epitopes in HHD mice. Mice were immunized with pMDK (Fig. 6A) or the individual peptides (Fig. 6B), and the ex vivo T cell response specific to the two HLA-A2–restricted peptides was evaluated by IFN-γ ELISPOT. All of the individual mice immunized with pMDK exhibited a T cell response to the peptides 114–122 and 13–21. Immunization with individual peptides led to good peptide-specific T cell responses transfection of the T2 cells with pMDK did not restore T cell stimulation. This plasmid was, however, used with success to activate the same T cell lines (Fig. 4A) by transfection of CIR-A2. Therefore, presentation of both 13–21 and 114–122 appeared to be TAP dependent. Finally, we evaluated the cytotoxicity of T cell lines by their capacity to release perforin (Fig. 5A) or to lyse labeled cells (Fig. 5B). T cell lines 365.55 and 365.36, which were specific for peptide 114–122 and 13–21, respectively, gave rise to a large number of perforin spots when they were in contact with CIR-A2 loaded with the appropriate peptide or with HepG2 (Fig. 5A). They remained inactivated in contact with unloaded CIR-A2 cell lines. Cytotoxic activites of MDK-specific T cell lines.

**FIGURE 5.** Cytotoxic activites of MDK-specific T cell lines. A. HLA-A2–transfected C1R cells (CIR-A2) were pulsed for 4 h with peptide 13–21 or 114–122. Target cells (HepG2 or CIR-A2) were incubated (3 × 10⁶ cells per well) with 114–122 (365.55) or 13–21 (365.36) specific T cell lines. T cell cytotoxicity was revealed by perforin ELISPOT. B. Calcineloaded HepG2 cells (5000 cells per well) were incubated with the MDK 114–122 specific T cell line 581.5 (20,000 cells per well) with CIR-A2 loaded with the peptide (black) or unloaded (blank). Specific cell lysis was calculated using values obtained with killed calcineloaded HepG2 as maximum values and unkillled calcineloaded HepG2 as background. Data were reported as inhibition percentage of the recognition in absence of competitive cells.

**FIGURE 6.** Induction of MDK-specific CTL responses in HHD transgenic mice. Groups of four HHD transgenic mice (M1, M2, M3, and M4) were immunized with MDK DNA (A) or with the individual peptides 114–122 (B, left panel) and 13–21 (B, right panel). At day 10, splenocytes were harvested and incubated (1 × 10⁵ cells per well) with 2.5 μg/ml MDK peptide 13–21 (gray) or 114–122 (black) or with culture medium (white). T cell activation was evaluated by IFN-γ ELISPOT assay. Data were reported for each mouse. C. Splenocytes of four HHD transgenic mice immunized with MDK DNA were stimulated with the individual peptides 114–122 (left panel) and 13–21 (right panel) for 1 wk. A standard 4-h [³⁵S] release assay was then performed using RMAS-HHD cells pulsed for 4 h with the individual MDK peptides (black squares) or an irrelevant peptide (gray squares) as targets at the indicated E:T ratios (100 represents 1 × 10⁵ effector cells per well). Results are expressed as the mean specific lysis obtained with four mice.
in all of the mice. Finally, cytotoxic activity of the peptide-specific T cells was demonstrated by the [3¹Cr] release assay (Fig. 6C). Our data obtained in vitro on human cells or in vivo in HLA-A2 transgenic mice show that MDK constitutes a novel tumor-shared Ag.

Discussion

We considered the first evidence that the protein MDK can be considered as a tumor-shared Ag. Human CD8⁺ T cell response specific for MDK-expressing tumor cells was induced in vitro using PBMCs from healthy donors, and immunized HHD transgenic mice developed a cytotoxic response specific for MDK. 

Because of its wide expression in cancer tissues and its contribution to tumorigenesis, MDK appears as an attractive candidate for a cancer vaccine.

MDK was initially isolated as a protein induced in the early stage of differentiation of embryonal carcinoma cells (11). Further investigations demonstrated its wide expression in tumors (12–14) and its multiple functions in favor of tumor expansion (28). These studies supported therapeutic strategies based on MDK neutralization using antisense DNA (21) or Abs (29) to block the growth of tumor cells. A gene suicide-based strategy using MDK promoter has been also proposed (30). We therefore considered the opportunity to target MDK by the cellular response to obtain a T cell response specific for a wide range of tumors. Although a basal level of MDK was seen in healthy individuals, we did not fear some deleterious effect in eliciting an MDK-specific cytotoxic response. Although required for neuronal embryogenesis, its role in adults rarely appears beneficial, as MDK seems to participate in ischemic renal injury (16) and autoimmune responses (17). Accordingly, MDK-deficient mice do not exhibit serious developmental abnormalities (16, 31), and mice vaccinated with peptides and DNA in our studies did not show any signs of autoimmunity. Finally, its crucial role in tumor growth is expected to limit tumor escape from immune attack through its selective loss. Its angiogenic function is extremely attractive to limit metastasis, as proposed for the angiogenic cell growth factor-1, for which a mutated form has been found to be the natural target of CTL T cells in patients with multiple myeloma (32).

MDK is a secreted protein like other identified tumor Ags (33–36) and is therefore produced in the endoplasmic reticulum (ER) after cleavage of the signal peptide. Because presentation of the MDK peptide 114–122 appeared to be TAP dependent, three alternative pathways could be employed to present it to CD8 T cells. First, secreted proteins like calreticulin (37) could be present in the cytosol due to an aborted translocation mechanism. Alternatively, the secreted molecule is either translocated from the ER into the cytosol (38) or captured by the APCs through a receptor-mediated mechanism, as shown for matrix metalloproteinase-2 (34). Capture by the LRP receptor has indeed been implicated in the targeting of MDK to proteasome degradation (39). Interestingly, the T cell epitope MDK 13–21 we identified in HLA-A2 healthy donors and HLA-A2 transgenic mice is localized in the signal peptide (MDK 1–22). Signal peptides comprise multiple hydrophobic residues that serve to anchor the nascent polypeptide to the membrane (40). They could also serve as anchor residues for HLA class I molecules, especially for HLA-A2, which has a marked preference for hydrophobic residues (41). Accordingly, elution of naturally processed peptides revealed their natural presentation by HLA-A class I molecules (42), and CD8 T cell epitopes derived from signal peptides have been described in virus (43) and tumor Ags (44, 45). In contrast to a recently identified T cell epitope localized in the peptide signal of preprocalcitonin (35), the 13–21 MDK T cell epitope did not seem to be processed in the lumen of the ER but required translocation from the cytosol to the ER by the TAP transporter and hence should be processed in the cytosol. This is not a particular feature of MDK CD8 T cell epitopes, as presentation of other epitopes derived from the signal peptide was also TAP dependent (43).

MDK has been proposed as a biomarker for diagnosis of tumor-bearing patients. MDK was found to be expressed at early stages of carcinogenesis of colorectal (13) and prostate (46) cancers. As a secreted protein, MDK can also be quantitated in the serum (47). In healthy donors, the serum level of MK is ~0.15 ng/ml with a cutoff of 0.5 ng/ml, whereas it is significantly higher in various types of human carcinomas (48), such as hepatocellular (48) esophageal (49), gastric (50), and neuroblastoma cancers (23). MDK was detectable at early stages (48), and the plasma level has been reported to be correlated with poor outcome (23, 24). Interestingly, MDK was also detectable in urine samples of patients with cancer (25). Although significant, the increase in serum concentration observed between healthy patients and patients with cancer remains limited (48). This increase is therefore not expected to dramatically alter the CD8 T cell response observed with healthy donors, because this response seems to be affected by high concentrations of soluble Ags only (51). In contrast to many tumor Ags, MDK expression in tumors could be revealed by its detection in the blood or the urine. MDK-based vaccination using peptides, DNA, the whole protein, or viral vectors could be applied to patients who have a significant level of MDK in their body fluids.

We showed that human CD8⁺ T cell response specific for MDK-expressing tumor cells can be induced in vitro using PBMCs from healthy donors and immunized HHD transgenic mice developed a cytotoxic response specific for MDK. Preliminary experiments performed on eight different patients with cancer did not reveal any spontaneous T cell response specific for the MDK epitopes (Supplemental Fig. 1), but we did not have access to MDK expression in these donors. We also did not show any effect of peptide immunization on the growth of B16 transfected with HLA-A2 and naturally expressing the murine MDK (data not shown). These preliminary experiments were not fully conclusive as several differences exist between human and murine MDK sequences especially in the 13–21 epitope. Further studies will address the vaccine potential in HHD mice using stable transfectants of human MDK. In conclusion, because of its wide expression in cancer tissues and its contribution to tumorigenesis, MDK appears as a novel attractive candidate for a cancer vaccine.

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Disclosures

J.K. and B.M. are coauthors of a pending patent.

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Figure S1 Spontaneous T cell response to MDK peptides

PBMCs from cancer patients were incubated with MDK, EBV, or CMV peptides at 37°C and 5% CO2. IL-7 (1ng/ml) was added at day 2 and IL-2 (20 UI/ml) at day 3 and 7. IFn-γ Elispot was performed on day 10 using irradiated PBMC as antigen presenting cells. T cell response of eight cancer patients was investigated. They included one lung cancer (blank bars) three head and neck cancer (black bars) and four renal carcinoma patients (Hatched bars).