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The Protein Product of the Tumor Suppressor Gene, Melanoma Differentiation-Associated Gene 7, Exhibits Immunostimulatory Activity and Is Designated IL-24

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The melanoma differentiation-associated gene 7 (mda-7) has been studied primarily in the context of its tumor suppressor activity. Although mda-7 has been designated as IL-24 based on its gene location in the IL-10 locus and its mRNA expression in leukocytes, no functional evidence supporting this cytokine designation exists. To further characterize MDA-7/IL-24 expression patterns in the human immune system, MDA-7/IL-24 protein levels were examined in human PBMC. MDA-7/IL-24 was detected in PHA- and LPS-stimulated whole PBMC lysate by Western blot and in PHA-activated CD56 and CD19 subsets by immunohistochemistry. The biological function of MDA-7/IL-24, secreted from Ad-MDA7-transfected HEK 293 cells, was assessed by examining the effect of MDA-7/IL-24 on the cytokine secretion profile of PBMC. Within 48 h MDA-7/IL-24 induced secretion of high levels of IL-6, TNF-α, and IFN-γ and low levels of IL-1β, IL-12, and GM-CSF from human PBMC as measured by ELISA. The MDA-7/IL-24-mediated induction of these Th1-type cytokines was inhibited by the addition of IL-10 to the PBMC cultures, suggesting that these two related protein family members may provide antagonistic functions. Therefore, because human blood leukocytes can be stimulated to produce MDA-7/IL-24, as well as respond to MDA-7/IL-24 by expressing secondary cytokines, MDA-7/IL-24 has the expression profile and major functional attributes that justify its designation as an IL. The Journal of Immunology, 2002, 168: 6041–6046.

Interleukin-10 is a pleiotropic homodimeric cytokine produced by immune system cells as well as tumor cells, including melanoma (1, 2). Its immunosuppressive function includes potent inhibition of proinflammatory cytokine synthesis, including the inhibition of synthesis of IFN-γ, TNF-α, and IL-6 (3). Multiple cellular and viral homologs of IL-10 have been identified (4–10), and several cellular members (IL-10, IL-19, IL-20, melanoma differentiation-associated gene 7 (MDA-7)/IL-24) of the IL-10 cytokine family are encoded in a small 195-kb gene cluster on chromosome 1q32, and have structural and sequence homologies (6, 7, 11). The MDA-7 IL-10 family member has been characterized primarily by its tumor suppressor activity. MDA-7 was identified and cloned using the differentiation induction subtraction hybridization approach after treatment of the human HO-1 melanoma cell line with IFN-β and mezerein resulted in growth arrest and terminal differentiation (10). Overexpression of MDA-7 via adenoviral vector (Ad-MDA7) infection induces apoptosis of a number of tumor cell types, but not normal cells, and in model tumor systems has led to suppressed growth and reduced metastasis (12–16). Apoptotic proteins up-regulated or activated by Ad-MDA7 include p53, caspases, Bax, and Bak (15), and attempts for its use in human cancer gene therapy are underway.

Several features of MDA-7 suggest that it may be a member of the IL family; these features include chromosomal location, translational regulation, murine and rat homolog expression, and putative protein structure (11, 17–19). Similar to GM-CSF, TNF-α, and IFN-γ transcripts, all of which contain AU-rich elements in their 3′ untranslated region targeting mRNA for rapid degradation, MDA-7 has three AU-rich elements in its 3′ untranslated region (17). MDA-7 mRNA has been identified in human PBMC (11) and, although no cytokine function of human MDA-7 has been previously reported, MDA-7 has been designated as IL-24 based on the gene and protein sequence characteristics (National Center for Biotechnology Information database accession no. XM_001405) and will be referred to as MDA-7/IL-24. The murine MDA-7/IL-24 homolog IL-4-induced secreted protein (FISP) was reported as a Th2-specific cytokine (18). Transcription of FISP is induced by TCR and IL-4R engagement and subsequent protein kinase C and STAT6 activation, as demonstrated by knockout studies. Expression of FISP was characterized, but no function has yet been attributed to this putative cytokine (18). The rat MDA-7/IL-24 homolog C49a (Mob-5) is 78% homologous to the MDA-7/IL-24 gene and has been implicated in wound healing (19, 20).

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4 Abbreviations used in this paper: MDA-7, melanoma differentiation-associated gene-7; FISP, IL-4-induced secreted protein.
Therefore, homologs of the MDA-7/IL-24 are expressed and secreted in various species, further supporting the cytokine-like nature of this molecule.

We now present data that define human MDA-7/IL-24 as a functional cytokine; we demonstrate its expression in human PBMC and its ability to induce Th1 type cytokine secretion from human PBMC. MDA-7/IL-24 stimulation of PBMC resulted in cytokine production that was antagonized by IL-10, suggesting that the balance of these two similar molecules may provide a previously unrecognized homeostasis of the immune system. To our knowledge, this is the first report of a tumor suppressor for which the protein product can also be secreted and function as a cytokine.

Materials and Methods

Activation of PBMC

PBMC were isolated from the peripheral blood of normal healthy donors by centrifugation over Histopaque (Sigma-Aldrich, St. Louis, MO). Cells were cultured in AIMV (see Figs. 1, 4, and 5) or in RPMI 1640-based medium (see Fig. 2) supplemented with 1-glutamine, HEPES, penicillin, streptomycin, and 10% human AB serum (Pfifferz, Brown Deer, WI). Both PHA-P and LPS were purchased from Sigma-Aldrich. Four hours before harvest of cells for the Western blot and immunohistochemistry, brefeldin A (Sigma-Aldrich) was added at a final concentration of 10 μg/ml.

For PBMC subclass studies, cells were stimulated with PHA-P (5 ng/ml) for 72 h and separated into CD3+, CD19−, and CD56−-enriched populations by positive selection using a MiniMax magnetic cell sorting system (Miltenyi Biotec, Sunnyvale, CA). Peripheral blood monocytes were isolated by adherence to chamber slides (Nalge Nunc International, Rochester, NY). To acquire monocytes, total PBMC were incubated in these chambers at a concentration of 1 × 10⁶ cells/ml without or with LPS (10 μg/ml) for 72 h, and nonadherent cells were washed away. The purity of these subpopulations was determined by staining with FITC- or PE-conjugated mAbs against CD3, CD19, CD56, and CD14 (BD Immunochemistry Systems, Mountain View, CA). The cells were analyzed cytfluorometrically using a FACScan with CellQuest software (BD Immunochemistry Systems). The CD3-enriched subpopulation contained 97% CD3+, the CD19 subpopulation contained 71% CD19+, and the CD56-enriched population contained 91% CD56+. The contaminants in the CD19− and CD56−-enriched populations were CD3+ and CD56−. Human IL-10 was purchased from R&D Systems (Minneapolis, MN).

Immunohistochemical staining for MDA-7/IL-24

Immunostaining of human PBMC or subclasses was performed using a mouse mAb against human MDA-7/IL-24 (Introgen Therapeutics, Houston, TX), using an avidin-biotinylated-peroxidase complex method optimized by us previously for melanocytes and melanoma cells (21). Specificity of the anti-MDA-7/IL-24 Ab was confirmed by total blocking with recombinant MDA-7/IL-24 produced in Escherichia coli, as previously published (21).

ELISAs. The ELISA reaction to detect human MDA-7/IL-24 was conducted in 96-well plates using standard techniques and an Ab pair selected for sensitivity. Briefly, plates were coated with a mAb (I) against MDA-7/IL-24 (prepared and provided by Introgen Therapeutics) overnight at 4°C in a standard sodium carbonate coating buffer. The plate was blocked for 2 h at room temperature with blocking buffer composed of PBS containing 1% BSA. Alternatively, Protein samples or recombinant MDA-7/IL-24 produced and purified from E. coli were diluted in diuluent buffer (blocking buffer with Tween 20 added at a final concentration of 1%) and incubated with the Ab for 2 h at room temperature in the presence of 1% nonfat dry milk in diuluent. After extensive washing with 0.1% Tween 20 in PBS, a biotinylated mAb (II) against MDA-7/IL-24 was added to the plate and incubated for 1 h at room temperature. After washing, HRP-streptavidin (Southern Biotechnology Associates, Birmingham, AL) was added to the plate for 30 min at room temperature. The reaction was developed with the addition of TMB microwell peroxidase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) and stopped with 1 N H₂SO₄ after 10 min. The ODs were recorded in a microtiter plate reader at a 450-nm wavelength (Dynatech, Chantilly, VA). ELISA for other cytokines were performed with commercially available kits according to the manufacturers’ instructions as designated in the figures. Although multiple experiments were performed, each was performed in duplicate as indicated by the manufacturer, so statistical errors for each value is not possible.

Western blotting. Activated PBMC were washed once in 1× PBS, resuspended in modified RIPA buffer (TBS (pH 7.6), 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM sodium fluoride, 0.2 mM aprotenin, and 1 mM leupeptin), and rocked at 4°C for 20 min. Lysates were cleared by a 30-min centrifugation at 16,000 × g at 4°C. Protein concentrations were determined with the DC Protein Assay (Bio-Rad, Hercules, CA) and samples were boiled for 5 min in an equal volume of sample buffer (62.5 mM Tris-HCl (pH 6.8), 20% glycerol, 2% SDS, and 5% 2-ME). Samples were separated by SDS-PAGE on a 12% gel and transferred to nitrocellulose. The membrane was blocked for 30 min with blocking buffer (5% milk in 1× PBS, 0.1% Tween 20 (PBST)) and incubated in a rabbit polyclonal MDA-7/IL-24 Ab (Introgen Therapeutics) in blocking buffer. Subsequently the membranes were washed twice in PBST and incubated at 1/2,000 with HRP-conjugated goat anti-rabbit secondary Ab. Blots were developed with ECL reagent (Amersham Pharmacia Biotech, Piscataway, NJ). Membranes were incubated in stripping buffer (62.5 mM Tris-HCl (pH 6.7), 2% SDS, and 100 mM 2-ME) for 30 min at 60°C, washed three times with PBST for 10 min each, and reprobed with anti-actin Ab (1/1,000).

Purification of human MDA-7/IL-24

The full-length cDNA of MDA-7/IL-24 was cloned into the pCEP4 FLAG vector (Invitrogen, San Diego, CA) containing the CMV promoter. The plasmid was transfected into HEK 293 cells and stable subclones were isolated using hygromycin (0.4 μg/ml). Purification of MDA-7/IL-24 was performed using the HEK 293 cell supernatants collected from viable cells in log phase growth. The crude supernatant was determined by ELISA to contain ~30 ng/ml MDA-7/IL-24. Supernatant containing the secreted MDA-7/IL-24 was supplemented with protease inhibitors (4 μg/ml each of aprotinin, pepstatin, 1 μg/ml pepstatin, and 0.05% sodium azide) and concentrated 10-fold with an Amicon stirred cell (Amicon, Beverly, MA) on a YM10 membrane. No detectable actin was in these supernatants, supporting the assumption that it was not derived from dead cells. Ten-milliliter aliquots of concentrated supernatant were separated over an S2000 Superdex prep grade column (Amersham Pharmacia, Piscataway, NJ) in 1× PBS (pH 7.4), and fractions identified to contain MDA-7/IL-24 by Western blot and ELISA were pooled. After buffer exchange on an Amicon stirred cell to 50 mM MES (pH 6), a second purification step was performed using a Bio-Rad S column. Column conditions consisted of a 0- to 600 mM NaCl gradient and a 50-mM NaCl gradient at a 5-ml/min flow rate. The entire purification was conducted at 4°C and MDA-7/IL-24 was identified using ELISA and Western blotting procedures. The final samples contained at least 300 ng/ml MDA-7 as determined by ELISA, and the specific activity was enriched at least 28-fold over the starting supernatant material based on the elimination of extraneous protein. Individual lots of partially purified MDA-7/IL-24 were tested for endotoxin using the QCL 1000 quantitative chromogenic LAL kit (BioWhittaker, Walkersville, MD). Levels of endotoxin ranged from 0 to 10 EU/ml.

Analysis of the protein structure as well as the hydrophobicity was performed by analyzing the amino acid sequence using the ProtParam tool, ExPASy server (us.expasy.org). Hydrophobicity was assessed using the Kyte and Doolittle scale provided by this site, applying the linear weight variation model.

Results

Human PBMC can be induced to express MDA-7/IL-24 protein

Fresh normal donor human PBMC were either unactivated or treated with the polyclonal stimuli of PHA or LPS, and were examined for intracellular MDA-7/IL-24 expression by immunoblotting and immunohistochemistry. As shown in Fig. 1, untreated PBMC do not express detectable levels of MDA-7/IL-24. However, after treatment with PHA or LPS for 72 h, MDA-7/IL-24 with an observed molecular mass of 23 kDa was evident. Two of four other donors tested had a detectable level of MDA-7/IL-24 upon PHA and LPS stimulation, and one of the three donors had a very low amount of MDA-7/IL-24 in cultured unactivated PBMC (data not shown). The levels of MDA-7/IL-24 may be at the threshold of sensitivity of our Ab and blotting procedure; therefore, MDA-7/IL-24 may not be detected in the donors that are just below the threshold. These results correlate with a recent report of the detection of MDA-7/IL-24 mRNA in the thymus, spleen, and...
PBL (11) as well as MDA-7/IL-24 secretion from Con A-treated PBMC (22).

MDA-7 is expressed by non-CD3 subsets

To determine which subclasses of PHA-stimulated PBMC express MDA-7/IL-24, subtypes were analyzed by immunohistochemistry. Positively selected CD3+ T cells (six of six experiments, Fig. 2a) and monocytes (three of three experiments, Fig. 2d) were routinely negative for MDA-7/IL-24, but 15–20% of the CD19+ (three of three experiments, Fig. 2b) and 50–80% of the CD56+ (three of three experiments, Fig. 2c) subpopulations resulting from the same starting PBMC and separation procedures were unequivocally positive. Membrane staining of MDA-7/IL-24 was most evident in the CD56+ cells, and a granular location in both B cells and NK cells was observed.

MDA-7 can be a secreted protein

One of the characteristics of a cytokine is the ability to be secreted. Usually, a short stretch of hydrophobic amino acids at the amino terminus signals and targets the protein to a secretory pathway. As shown in Fig. 3a, the MDA-7/IL-24 contains a leader sequence consisting of 49 amino acids; this is depicted in more detail in the hydrophobicity plot (Fig. 3c). The predicted cleavage site was determined by the von Heijne SignalP predictions program (23); however, this cleavage site in MDA-7/IL-24 has not been confirmed experimentally. To demonstrate secretion of MDA-7/IL-24 from mammalian cells, stable transfectants of HEK 293 cells containing the human full-length MDA-7/IL-24 cDNA were generated. Supernatants were analyzed for the presence of MDA-7/IL-24 by Western blot (Fig. 3b) and four bands of MDA-7/IL-24 were detected in the culture supernatants of MDA-7/IL-24 transfected but not untransfected HEK 293 cells. Based on the amino acid sequence, the full-length MDA-7/IL-24 is expected to have a molecular mass of 23,824 Da, and the final secreted peptide is 18,419 Da.

MDA-7 induction of secondary cytokines is inhibited by IL-10

Another hallmark of the cytokine family is belonging to a cascade of molecules involved in cellular activation or inhibition. To address the biological function of MDA-7/IL-24 as a cytokine, its induction of secondary cytokine secretion by PBMC was examined. Preliminary experiments using recombinant MDA-7/IL-24, expressed in E. coli and Saccharomyces cerevisiae, showed that MDA-7 could induce robust production of IL-6, TNF-α, and IFN-γ, very low levels of GM-CSF and IL-10, and no IL-2, IL-4, and IL-5 (data not shown). However, very high doses (quantities measured in micrograms per milliliter) of bacterial MDA-7/IL-24 were required to stimulate a response, possibly due to improper folding or lack of glycosylation of the recombinant protein. Therefore, secreted MDA-7/IL-24 was purified from supernatants of HEK 293 cells (MDA-7/IL-24 expressing stable transfectants) and used to test the effect of MDA-7/IL-24 on human PBMC.

Optimum levels of MDA-7/IL-24 to induce maximal IL-6, TNF-α, and IFN-γ secretion were determined in a dose-response curve. Maximal IL-6 secretion was achieved by only 2 ng/ml MDA-7/IL-24, while maximal TNF-α and IFN-γ secretion required 20 ng/ml MDA-7/IL-24 (data not shown). High levels of IL-6, TNF-α, and IFN-γ were secreted by PBMC in response to MDA-7/IL-24 in the representative donor shown in Fig. 4. In fact, MDA-7/IL-24 was a more potent inducer of TNF-α than LPS, a known inducer of inflammatory cytokines that was used as a positive control. All donors tested secreted IL-6, TNF-α, and IFN-γ in...
response to MDA-7/IL-24; some cytokine levels were higher and some were lower than the donor in Fig. 4. PHA, another positive control, always induced robust cytokine secretion from all donors and untransfected 293 supernatants did not induce any significant levels of cytokines (data not shown). A similar pattern of MDA-7/IL-24 stimulation of cytokine production, albeit much lower levels, was observed with IL-1β, IL-12, and GM-CSF as shown in Fig. 5. Values in a similar range of the amount of cytokines from the donor shown in Fig. 5 were detected in supernatants from three additional donors. Using polyclonal antiserum specific for MDA-7/IL-24, depletion of the MDA-7/IL-24 by >90%, as determined by ELISA, resulted in significant reduction of induction of IFN-γ secretion (data not shown), indicating that the induction of these secondary cytokines was MDA-7/IL-24 specific.

As the IL-10 family member MDA-7/IL-24 stimulates the production of proinflammatory cytokines, we hypothesized that MDA-7/IL-24 and IL-10, a potent immunosuppressive cytokine, may be antagonists. To test this hypothesis, human rIL-10 and human recombinant MDA-7/IL-24 were added to the PBMC cultures at the same time. Under the conditions used, IL-10 completely abrogated TNF-α, IFN-γ, IL-12, and GMCSF induction by MDA-7/IL-24 and partially blocked IL-6 and IL-1β induction by MDA-7/IL-24 (Figs. 4 and 5). As a positive control, IL-10 coadministration also reduced or completely blocked the production of all cytokines, except IL-6, in response to LPS. The lack of inhibition of LPS-induced IL-6 secretion by IL-10 is probably due to the extremely potent stimulatory activity of LPS and the IL-6 val-

FIGURE 4. Effect of MDA-7/IL-24 and IL-10 on IL-6, TNF-α, and IFN-γ inflammatory cytokine secretion from PBMC. Two milliliters per well (2 × 10^6 cells/ml) PBMC were plated in a 24-well plate and cultured untreated or with indicated amounts of MDA-7/IL-24 (a, 2 ng/ml; b and c, 20 ng/ml), 5 μg/ml LPS, 500 U/ml IL-10 (−17 ng/ml; R&D Systems), or a combination of IL-10 and MDA-7/IL-24. Supernatants were harvested at 48 h and analyzed for cytokine content by ELISA (Endogen, Woburn, MA) according to the manufacturer’s instructions. Data from one representative donor are reported.

FIGURE 5. MDA-7/IL-24 induction of IL-1β, IL-12, and GM-CSF secretion from PBMC is inhibited by IL-10. Human PBMC were isolated and cultured as in Fig. 4 and treated with 5 μg/ml LPS, 500 U/ml IL-10, and 5 ng/ml MDA-7/IL-24. Supernatants were harvested as in Fig. 4 and analyzed for cytokine content by ELISA according to the manufacturer’s instructions. * Value greater than 500 pg/ml IL-1β. ** Actual values are 539 pg/ml IL-12 and 893 pg/ml GM-CSF. Data from one representative donor are reported.

MDA-7 does not appear to function as a growth factor for human PBMC

Because some cytokines can also function as growth factors, the ability of MDA-7/IL-24 to act as a proliferative stimulus was addressed using human PBMC and the results are reported in Table 1. IL-10 was included as a negative cytokine control. PHA was used as positive control and induced a robust uptake of [³H]thymidine in all three donors tested. As expected, IL-10 did not induce increased [³H]thymidine uptake of PBMC over the course of 4 days. Our results show that MDA-7/IL-24, purified from HEK 293 supernatants, did not induce significant proliferation during 4 days of culture of the PBMC population in any of the three donors tested. Earlier studies using recombinant MDA-7/IL-24 (up to 5 μg/ml) expressed in E. coli or S. cerevisiae also did not show a proliferative response in human PBMC from three donors (data not shown).

Discussion

In addition to the tumor suppressor activity of MDA-7/IL-24 (12–16), we have demonstrated that MDA-7/IL-24 is expressed by PHA-activated human lymphocytes of the CD56 and CD19 subclasses and is a secreted protein that can induce the further secretion of inflammatory cytokines from human PBMC. Although IL-10 and MDA-7/IL-24 are accepted as members of the same family, our results indicate that they have antagonistic functions in PBMC, as IL-10 inhibited the MDA-7/IL-24-induced cytokine secretion. However, similar to IL-10, MDA-7/IL-24 does not appear to function as a growth factor for PBMC. The combination of structural data, including homology to known cytokines, chromosomal location, a predicted signal peptide, and the evidence of secretion, and functional data we have presented all support the
classification of MDA-7/IL-24 as a cytokine. To our knowledge, this is the first report of a tumor suppressor molecule for which its secreted protein product functions as an IL in the immune system.

Activation of immune cells in vitro with PHA or LPS or, in vivo with a microbial infection results in transcription, translation, and secretion of cytokines, including MDA-7/IL-24, as indicated by us in our in vitro studies using human PBMC. These cytokines can then act locally or systemically to initiate further cytokine cascades that are critical to the regulation of effective immunity. Because immune cells have been demonstrated to respond to MDA-7/IL-24 by secretion of cytokines, it can be hypothesized that these cells must express an MDA-7/IL-24 receptor. The MDA-7/IL-24 receptor has recently been identified and uses one of the IL-10R subunits (22, 24), similar to another IL-10 homolog, IL-22, which uses the IL-10Rβ chain in addition to its own unique chain (25). In fact, MDA-7/IL-24 can bind both the IL-22R1/IL-20R2 and the IL-20R1 (IL-20R1/IL-20R1) (22) and induce STAT3 phosphorylation (24).

Because IL-10 and MDA-7 share a receptor subunit, the mechanism of IL-20R1/IL-20R1 may be smaller than in the Western blot in Fig. 1, because the protein may not have been fully glycosylated yet, as secretion had not occurred. In Fig. 2, IL-10 by immunohistochemistry (2). We hypothesize that, based on the relative levels of MDA-7/IL-24 and IL-10, immune cells in proximity to the primary melanoma lesion could be either activated and secrete proinflammatory cytokines (if MDA-7/IL-24 predominates) or be suppressed (if IL-10 is present in excess). If the MDA-7/IL-24:IL-10 ratio were higher, some of these secondary cytokines could activate APCs that are presenting melanoma tumor Ags, thereby creating an antitumor immune response. Therefore, we hypothesize that loss of MDA-7/IL-24 in melanoma progression (17, 21), and thus the lack of expression of MDA-7/IL-24 in metastatic melanomas, together with high expression of the immunosuppressive cytokine IL-10 in melanoma, could contribute to the ineffective immune response to melanoma.

The interactions of MDA-7/IL-24 and IL-10 and the cytokines they induce or repress comprise a very complex network. We hypothesize that MDA-7/IL-24 loss plays a role in the tumorigenesis of melanoma and may have a dual function as tumor suppressor and cytokine. To date this molecule has been commonly called MDA-7; for future studies as a functional cytokine, the term IL-24 may be more appropriate when referring to the immune or IL-like properties associated with MDA-7.

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