Neutrophils Contribute to the Measles Virus-induced Antitumor Effect: Enhancement by Granulocyte Macrophage Colony-stimulating Factor Expression

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ABSTRACT

To investigate the contribution of a neutrophil response to the oncolytic effect of replicating attenuated measles virus (MV), MVs expressing murine granulocyte macrophage colony-stimulating factor (GM-CSF) were generated. The growth characteristics and kinetics of GM-CSF production of these viruses were characterized in vitro. Their biological effects were characterized in mice transgenic for the MV receptor CD46. The oncolytic efficacy of MV GM-CSF was then compared with that of a parental MV and a control, UV-irradiated MV using a human lymphoid tumor model in immunodeficient mice. Intratumoral injection of MV resulted in significant tumor regression or slowing of progression compared with injection of the control. Injection of MV GM-CSF further enhanced the oncolytic effect. In additional experiments, the cellular response to MV, MV GM-CSF, recombinant murine GM-CSF alone, or untreated tumors was quantified. The predominant response was an influx of neutrophils. Intratumoral natural killer cells and macrophages were not detected. The magnitude of the neutrophil response correlated well with tumor regression. Our studies suggest that therapy with replicating MV stimulates a strong neutrophil antitumor response, which can be cytokine-enhanced to improve oncolysis.

INTRODUCTION

Neutrophils have received less attention than other leukocyte subsets as potential mediators of an antitumor effect. However, there is abundant evidence from many murine models of cancer that neutrophils can play an important role in the antitumor response, including direct killing of tumor cells (1). In SCID2 mice, neutrophil depletion has been shown to permit much more rapid tumor growth of a number of different human tumor cell lines and, in particular, to allow the growth of lymphoid tumors in which growth was otherwise totally suppressed (2).

Replicating attenuated MV for the therapy of lymphoid and other malignancies has recently been investigated, with encouraging results (3–5). We showed that locally or systemically injected MV resulted in regression of large established lymphoma xenografts in SCID mice (3). Viral replication within the tumor was confirmed, however, viral RNA and protein expression was distributed in discrete areas within the tumor. Despite this, we frequently observed complete regression of large established lymphoma xenografts in SCID mice. We showed that locally or systemically injected MV resulted in significant tumor regression or slowing of progression compared with injection of the control. Injection of MV GM-CSF further enhanced the oncolytic effect. In additional experiments, the cellular response to MV, MV GM-CSF, recombinant murine GM-CSF alone, or untreated tumors was quantified. The predominant response was an influx of neutrophils. Intratumoral natural killer cells and macrophages were not detected. The magnitude of the neutrophil response correlated well with tumor regression. Our studies suggest that therapy with replicating MV stimulates a strong neutrophil antitumor response, which can be cytokine-enhanced to improve oncolysis.

MATERIALS AND METHODS

Plasmids. The open reading frame of murine GM-CSF including the leader sequence was PCR amplified from plasmid p0RF (Invivogen, San Diego, CA), corrected to maintain a MV genome of hexameric length (11), and tailed to include restriction sites MvnI and AatII. The PCR product and the plasmids p+ +MVLMGP-N, p+ +MVLMGP-P, and p+ +MVLMGFP-H were digested with MvuI and AatII, and the insert was ligated into the full-length MV cDNA, creating three antigenomic MV plasmids in which mGM-CSF was expressed upstream of the viral N gene and downstream of the P and H genes, respectively. Correct plasmid assembly was demonstrated by restriction analysis and dideoxysequencing.

Virus Rescue. The viruses were rescued by Lipofectamine (Invitrogen, Grand Island, NY)-mediated cotransfection of 4 µg of full-length p+ +MVmGM-CSF cDNA, along with a set of three plasmids from which the MV polymerase complex was expressed (1 µg each of plasmids encoding MV-N and MV-P and 0.5 µg of plasmid encoding MV-L) into 293 cells that had been infected for 1 h by modified Vaccinia Ankara encoding T7 polymerase (12). After 72 h, the 293 rescue cells were overlaid onto Vero cells in 10-cm dishes. Individual syncytia were picked from the dishes and transferred to fresh Vero cells in 35-mm wells. When a cytopathic effect was evident in the wells, the virus was transferred to Vero cells in T75 flasks and subsequently propagated in bulk preparations.

Viruses and Virus Production. MVs were inoculated onto 106 Vero (African green monkey) cells in T75 tissue-culture flasks at a MOI of 0.01 in

2 ml of OptiMEM (Life Technologies, Inc., Rockville, MD) at 37°C for 2 h. The viral inoculum was removed and replaced by normal medium. The cultures were then observed until maximal syncytial formation had occurred, at which point the cells were harvested in 2 ml of OptiMEM and the virus was released by two cycles of freeze-thawing. Virus was titrated on Vero cells, and the 50% tissue-culture infectious dose was calculated according to the method of Kärber (13).

Cells. Vero cells (ATCC CCL-81; American Type Culture Collection, Manassas, VA) were grown in DMEM supplemented with 5% FCS. Raji cells (ATCC CCL-86) were grown in RPMI supplemented with 10% FCS (Life Technologies, Inc.). The 293 cells were grown in DMEM supplemented with 10% FCS.

Animals. Four-week-old CB17 SCID mice (Harlan Sprague Dawley, Indianapolis, IN) were housed in a barrier facility and cared for according to standards set by the Institutional Animal Care and Use Committee. Mice received s.c. injections in the flank region with 10^6 viable Raji tumor cells. For MV therapy, after the tumors reached a volume of approximately 0.4 cm^3, they were injected daily with MV or MvGM-CSF/FP in a total volume of 100 μl for 10 days. As controls, tumors were injected daily with the same volume of UV-inactivated virus. Tumor measurements were made daily in two diameters, and the tumor volume was calculated according to the formula V = a*b/2 where a is the shortest and b the longest diameter. Mice whose tumors reached a volume of 2.5 cm^3 or had begun to invade surrounding tissues were euthanized. The experimental protocol was approved by the Mayo Clinic Institutional Animal Care and Use Committee.

mGM-CSF ELISA. mGM-CSF was quantified by ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

Immunohistochemistry. Residual tumors were carefully removed, and a portion was fixed in 10% neutral buffered formalin. After paraffin embedding, the tissue was cut in 0.5-μm sections. CD18 staining was performed on paraffin-embedded tissue sections after deparaffinization and antigen retrieval by steaming in citrate (pH 6.0). Tissue sections were incubated with biotinylated antimouse CD18 (C71/16) or the isotype control at a concentration of 10 μg/ml for 1 h, followed by horseradish peroxidase-streptavidin conjugate. The labeled antigen signal was amplified using Tyramide Signal Amplification ( Molecular Probes, Eugene, OR), followed by another incubation with HRP-streptavidin. The peroxidase label was developed using the chromogen 3-amino-9-ethylcarbazole (DAKO Corp., Carpinteria, CA).

Flow Cytometric Analysis of Tumor Cells. Tumors were dissected into small pieces and suspended in a digestion mixture consisting of 0.4 mg/ml collagenase, 0.4 mg/ml DNase, 0.2 mg/ml CaCl2, and 0.2 mg/ml MgCl2 in PBS for 45 min at 37°C, with occasional agitation. The tumor digest was filtered through one layer of gauze, then centrifuged for 5 min at 1000 rpm. The pellets were resuspended in a volume of 2.5 cm^3 or had begun to invade surrounding tissues were euthanized. The experimental protocol was approved by the Mayo Clinic Institutional Animal Care and Use Committee.

A Replicating Attenuated MV-producing mGM-CSF Is Not Toxic in Mice Expressing a Human MV Receptor, CD46. Next, we characterized the effects of in vivo systemic administration of the mGM-CSF-expressing viruses, using a murine model permissive for MV replication. CD46-Infar" mice are transgenic for human CD46, with a knockout mutation of the αβ IFN receptor. Attenuated MV replicates and disseminates systemically in these mice after intranasal and i.p. administration (14–16) and is ultimately self-limited. We wished to determine whether, after systemic administration, GM-CSF would be detectable in the circulation, whether any effects on the peripheral blood cells would be observed, and whether infection with

RESULTS

Recombinant MV-expressing mGM-CSF at Different Levels. Three MV constructs expressing mGM-CSF from ATU at various positions within the MV genome were generated, as depicted in Fig. 1A. The growth kinetics of the mGM-CSF-expressing viruses did not differ significantly from that of the parent virus, MvVNSe, except for a slight delay in replication of the virus expressing mGM-CSF before the N gene (data not shown). Fig. 1, B and C, shows that, after infection of 10^6 cells at a MOI of 0.1, mGM-CSF in nanograms per milliliter quantities was detected in the cell supernatant of both Vero and Raji cells. Thus, mGM-CSF can be expressed from ATU at various positions in the genome of an attenuated MV without compromising viral replication.

Fig. 1. mGM-CSF can be expressed from additional transcription units of MV. A, schematic representation of the full-length MV cDNA, depicting the sites at which the gene encoding murine GM-CSF was inserted. The genes are denoted by open boxes.

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the GM-CSF-expressing MVs resulted in any symptoms or pathology related to the expression of this cytokine in a replicating virus.

Therefore, CD46GeInfar^ko mice received intraperitoneal injections of 10^6 pfu of each of the three mGM-CSF-expressing viruses i.p. (n = 10 per group). White cell counts with differentials, serum mGM-CSF levels, and anti-MV antibody response were serially monitored and compared with those of control mice that received injections of MVNSe. In an additional experiment, a mouse was sacrificed at various time points during the experiment, so that relevant organs could be sectioned and examined.

The general health of the mice was closely observed. None of the mice suffered any apparent toxicity. Fig. 2A shows the levels of mGM-CSF measured in the serum at four time points over a 28-day period after infection. The highest serum level of mGM-CSF was detected in mice that received injections of MV GM-CSF N, lower levels in mice receiving injections of MV GM-CSF P, and no elevations in serum GM-CSF beyond background levels were detected in mice that received injections of MV GM-CSF H. This is consistent with expected levels of protein production generated by the MV transcription gradient. Serum mGM-CSF was not detectable at any time later than the 3-day time point, consistent with previous studies, in which the peak of virus replication in this model occurs at 3 days after infection (13).

Despite the transient elevations in serum GM-CSF levels, the leukocyte counts of MV GM-CSF-infected mice did not differ from those of MVNSe-infected control mice, nor did they differ significantly from each other, as shown in Fig. 2B. In particular, as shown in Fig. 2C, no elevation in neutrophil counts were noted.

An antibody response, measured by ELISA, was observed to each of the injected viruses at days 14 and 28 after infection. There was no significant difference in the magnitude of this response between each of the GM-CSF-expressing viruses and the MVNSe control (data not shown). H&E staining of sections of various organs, in particular, the spleens, did not differ between MV-GM-CSF-infected mice and MVNSe-injected controls (data not shown). Thus, administration of MV GM-CSF N and MV GM-CSF P results in transient elevations in serum GM-CSF levels but does not significantly affect the peripheral blood counts nor result in any overt toxicity in CD46 transgenic mice. A typical antibody response is generated against the GM-CSF-expressing MVs, and these viruses are eliminated with similar kinetics to the control MVNSe virus.

MV-producing mGM-CSF Has Enhanced Oncolytic Activity in a SCID/Raji Model of Human Lymphoma. We chose MV GM-CSF P, expressing GM-CSF at intermediate levels, to investigate the antitumor efficacy of MV GM-CSF in a model of human lymphoma in SCID mice. Raji tumors were seeded and allowed to grow to a volume of 0.2–0.4 cm^3. Groups of mice (n = 10/group) then received injections intratumorally, daily for 10 days, of MV GM-CSF P, MVNSe, or UV-irradiated MV control.

Fig. 3A shows the change in tumor volume over time. There was a
significant difference in tumor progression between the three groups. In the tumors injected with UV-inactivated MV, all of the animals required euthanization by day 15. Consistent with our previous experience, injection of MVNSe resulted in a significant slowing of tumor growth compared with control. Moreover, after the injection of MV GM-CSF, there was an additional improvement in antitumor efficacy that was especially pronounced at later time points. Histological examination of sections of residual Raji tumors revealed a significant infiltrate in tumors treated with MVNSe and MV GM-CSF P, which stained positively with an anti-CD18 antibody. This was not present in control tumors treated with UV-irradiated MV, suggesting that leukocytes were involved in the antitumor response. Photomicrographs of representative tumor sections from mice that received injections of MVNSe and UV-irradiated MV are shown in Fig. 3, B and C.

The Enhanced Oncolytic Activity of MV mGM-CSF Is Correlated with Greater a Tumor Infiltration by Neutrophils. Next, we defined and quantified host cell response to the tumor. We repeated the injections of established tumors, using MV GM-CSF P, MVNSe, or recombinant murine GM-CSF alone as an additional control. After six injections, the Raji tumors were removed, the cells dissociated and subjected to flow cytometric analysis with an antibody to murine CD45, an antigen expressed on all leukocytes. Fig. 4A shows mean (±/−SE) murine CD45-positive (CD45+) cells as a percentage of total tumor cells, for tumors injected with MV GM-CSF, MVNSe, or recombinant GM-CSF (n = 10/group). Infilarating CD45+ cells were seen in all groups. However, there was a very significant difference in the magnitude of the infiltrate between each group. Whereas in tumors injected with recombinant GM-CSF alone, the mean CD45+ infiltrate less than 5% of total cells, those injected with MVNSe contained a mean of 16% CD45+ cells and those with MV GM-CSF a mean of 34% CD45+ cells. This suggested that MVNSe in itself stimulated a considerable host leukocyte response to the tumor. Furthermore, the magnitude of the infiltrate elicited by MV GM-CSF was greater than that stimulated by GM-CSF and MVNSe added, raising the possibility of a synergistic interaction between the two.

To further define this host cell response, the experiment was repeated, and the analysis was performed in the presence of antibodies against macrophages (Mac-3, M3/84), NK cells (DX 5, CD49b, Pan NK), and neutrophils (Ly6G,RB6–8C5). On this occasion, untreated tumors were used as controls. The results are shown in Fig. 4B as the mean (+/−SE) percentage of total cells detected by Ly6.1 for each group. We did not detect any macrophages or NK cells within the tumors. Murine neutrophils were detected in each of the groups. In a result that was consistent with the previous experiment, the tumors injected with MV GM-CSF P contained the greatest percentage (26%) of neutrophils; once again, there was a larger percentage of neutrophils in the MV GM-CSF P-injected tumors than in both the MVNSe (16%) and untreated tumors (4%) added together, again suggesting a possible synergistic interaction between MV and the virally expressed GM-CSF in stimulating a host response. Interestingly, the untreated tumors in this experiment contained a similar percentage of neutrophils to those tumors that, in the previous experiment, had been injected with recombinant GM-CSF. Again, this suggests synergistic local interaction.

To verify that the neutrophil response to the tumors did not result merely from increased numbers of neutrophils in the peripheral blood, we performed leukocyte counts with differentials on all mice, shown in Fig. 4, C and D. There was no difference in the mean peripheral blood counts and differential counts between mice with MVNSe-treated tumors and those with untreated mice. Mice with MV GM-CSF-treated tumors had slightly but significantly lower peripheral blood leukocyte counts than the other two groups and, in addition, a slight but significant difference in the percentage of neutrophils. This finding possibly resulted from large numbers of neutrophils having been recruited to the tumor site.

We measured serum GM-CSF levels in all of the mice at the same time point. GM-CSF was detected only in the serum of mice whose tumors were injected with MV GM-CSF P and not in controls. The mean GM-CSF level after six injections of virus was 13.2 pg/ml (range, 0–33), a level similar to that detected on day 3 after infection of the CD46G6fna-k+ mice with one dose of MV GM-CSF P.

Taken together, these data support the hypothesis that therapy with replicating MV stimulates a neutrophil antitumor response. The hypothesis is further supported by the fact that this response can be cytokine enhanced, with a concomitant improvement in the oncolytic capacity of MV.
cells did not increase it. Thus, the mechanism by which MV replication is necessary to achieve this effect, because there was a neutrophil response in this model of lymphoma is enhanced by a coccal preparation, OK-432 (18, 19). It is, therefore, of interest that MV infection of Raji tumor cells directly stimulated neutrophil chemotaxis.

Chemotaxis of Human Neutrophils to MV-infected Raji Cells. We set out to determine whether neutrophils were specifically attracted to MV-infected Raji cells. Chemotaxis assays were performed in triplicate, using isolated human peripheral blood neutrophils from five different individuals. Fig. 5 shows the mean fluorescence of the migrated neutrophils (+/− SE). Medium alone was used as a negative control, and FMLP was used as a positive control. In all individuals, neutrophils migrated toward Raji cells but MV infection of the cells did not increase the migration. Thus, we did not find evidence that MV infection of Raji tumor cells directly stimulated neutrophil chemotaxis.

DISCUSSION

We have shown that MV-mediated regression of large established human Raji tumors in SCID mice is accompanied by an intense infiltrate of neutrophils, not seen in control tumors injected with control UV-irradiated MV, the growth of which progresses unchecked. The local expression of GM-CSF attracts more neutrophils to the tumors and augments the oncolytic activity of MV, further suggesting that neutrophils may play an important role in mediating the antitumor effect in this model. The augmentation of the oncolytic effect ascribed to MV GM-CSF was more pronounced in the last 2 weeks of the experiment (Fig. 3A), again suggesting a cellular host-response effect as opposed to direct viral oncolysis.

An antitumor cytotoxicity of neutrophils has been described previously. In vitro and in vivo cytotoxicity of neutrophils has been demonstrated against various murine tumor cells, in particular, after activation of the neutrophils with substances derived from microorganisms [e.g., a fungal derivative, β1–3-α-glucan (17), or a streptococcal preparation, OK-432 (18, 19)]. It is, therefore, of interest that a neutrophil response in this model of lymphoma is enhanced by a replicating virus that has oncolytic activity. Our studies indicate that virus replication is necessary to achieve this effect, because there was no augmentation of antitumor activity when the control, UV-irradiated MV was used.

We showed, in vitro, that chemotaxis of human neutrophils was stimulated by the Raji tumor cells themselves, but MV infection of the cells did not increase it. Thus, the mechanism by which MV replication within the Raji tumors in vivo stimulates neutrophil infiltration remains unclear. It has been demonstrated that “tumor take” after injection of murine adenocarcinoma can be controlled by neutrophils and that the tumor rejection was associated with neutrophil chemotaxis in vivo after G-CSF-producing tumor cells were injected (20). However, a recent study in which CD95 (Fas) ligand-expressing tumors were rejected by antitumor T cell receptor transgenic, perforin knockout mice strongly suggested that neutrophils were responsible for the tumor rejection, albeit that no chemotactic activity of CD95 ligand was demonstrated, nor could antitumor cytotoxicity of the neutrophils be shown in vitro (21).

A number of mechanisms are proposed in the literature for direct killing of tumor cells by neutrophils, including release of hydrogen peroxide (22), the nitric oxide pathway (23), and neutrophil-mediated inhibition of glutamine uptake by tumor cells (24). It is of interest that glutamine seems to play a particularly important role as an energy source for cells of lymphoid origin (25). Determination of the mechanistic aspects of enhanced tumor cell killing by neutrophils are of interest to us in future studies.

It is important to recall that our studies have been performed in a SCID mouse model, allowing for the study of the role of neutrophils in the cytolytic response in relative isolation. However, the lack of T cells as antitumor immune effector cells may have exaggerated the role of neutrophils in this model system. Nonetheless, our work clearly demonstrates that cytolysis and tumor regression after infection with an oncolytic virus is, at least in part, attributable to a neutrophil inflammatory response. This conclusion is supported by the fact that oncolytic response is enhanced by viral expression of GM-CSF that correlates with a considerable increase in neutrophil number within the tumors. We have also demonstrated that attenuated replicating MVs expressing GM-CSF are not toxic in CD46 transgenic mice. Thus, in the presence of a fully intact immune system, other activities of GM-CSF, shown to be the most potent cytokine to induce durable antitumor immunity when secreted by tumor cells (26), may provide additional antitumor effects.

Our studies in CD46 transgenic mice, used as a preclinical model for toxicity studies, demonstrate that replicating attenuated MVs expressing murine GM-CSF are safe after systemic administration. This virus has provided further insights into the mechanisms of MV-mediated oncolysis. MV expressing human GM-CSF may be more effective than parental MV as a replicating vector for the therapy of human cancer.

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REFERENCES

The role of Neutrophils in MV-Mediated Oncolysis

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