Antitumor effects of vaccination with dendritic cells transfected with modified receptor for hyaluronan-mediated motility mRNA in a mouse glioma model

TAKAYUKI AMANO, M.D.,1 KOJI KAJIWARA, M.D.,1 KOICHI YOSHIKAWA, M.D.,1 JUN MORIOKA, M.D.,1 SADAIRO NOMURA, M.D.,1 HIROSUKE FUJISAWA, M.D.,1 SHOICHI KATO, M.D.,1 MASAMI FUJI, M.D.,1 MIKIKO FUKUI, M.D.,2 YUJI HINODA, M.D.,2 AND MICHIYASU SUZUKI, M.D.1

Departments of 1Neurosurgery and 2Clinical Laboratory Science, Yamaguchi University School of Medicine, Ube, Yamaguchi, Japan

Object. The receptor for hyaluronan-mediated motility (RHAMM) is frequently overexpressed in brain tumors and was recently identified as an immunogenic antigen by using serological screening of cDNA expression libraries. In this study, which was conducted using a mouse glioma model, the authors tested the hypothesis that vaccination with dendritic cells transfected with RHAMM mRNA induces strong immunological antitumor effects.

Methods. The authors constructed a plasmid for transduction of the mRNAs transcribed in vitro into dendritic cells, which were then used to transport the intracellular protein RHAMM efficiently into major histocompatibility complex class II compartments by adding a late endosomal–lysosomal sorting signal to the RHAMM gene. The dendritic cells transfected with this RHAMM mRNA were injected intraperitoneally into the mouse glioma model 3 and 10 days after tumor cell implantation. The antitumor effects of the vaccine were estimated by the survival rate, histological analysis, and immunohistochemical findings for immune cells.

Mice in the group treated by vaccination therapy with dendritic cells transfected with RHAMM mRNA survived significantly longer than those in the control groups. Immunohistochemical analysis revealed that greater numbers of T lymphocytes containing T cells activated by CD4, CD8, and CD25 were found in the group vaccinated with dendritic cells transfected with RHAMM mRNA.

Conclusions. These results demonstrate the therapeutic potential of vaccination with dendritic cells transfected with RHAMM mRNA for the treatment of malignant glioma.

Key Words • glioma • dendritic cell • receptor for hyaluronan-mediated motility • mRNA • vaccination therapy • mouse

Malignant gliomas can be divided into World Health Organization Grade III (anaplastic glioma) and Grade IV (glioblastoma multiforme).1 The yearly incidence of these tumors is 2.5 per 100,000. The median survival duration of patients with malignant glioma is approximately 50 to 60 weeks, even with the most current treatments such as gross resection followed by radiation and/or chemotherapy.2,3,12,22,25,36,40–43 The central nervous system is an immunologically privileged site protected by the blood–brain barrier. Nevertheless, immune effector cells induced peripherally can be recruited into the central nervous system.1,14,16,37 Malignant glioma cells are known to be poor antigen presenters to the immune system, in part because of downregulation of the B7 costimulatory molecules required for direct tumor cell activation of T cells.25,46 To induce an antitumor immune response against malignant glioma, so-called professional antigen-presenting cells may be needed to internalize, process, and present malignant glioma antigen efficiently to T cells.3,31 In particular, immunotherapy using dendritic cells, which are known as professional antigen-presenting cells, has been attempted in several studies.3,20,38 Various forms of tumor antigens have been applied to improve the induction of specific antitumor immune responses; these include apoptotic tumor cells, tumor cell lysates, proteins, peptides, and nucleic acids.3,7,16,28,32,35,36,43 Among these, mRNA seems to be the most pertinent
Vaccine therapy with dendritic cells transfected with RHAMM mRNA

form for evaluating whether a new candidate gene identified using serological screening of an expression library can be useful as an antigen for dendritic cell therapy, because mRNA can easily be synthesized in vitro, transferred into dendritic cells, and subjected to molecular engineering. Furthermore, mRNA is superior to cDNA in transfection efficiency and safety in vivo and is also superior to synthetic peptides, because there is no need to determine whether the peptides have been presented to the immune system.

The RHAMM was detected using serological screening of an expression library of tumor tissues or cultured tumor cells. The RHAMM was subsequently implicated in tumor progression and metastasis formation as well as signal transduction. In addition to the centrosome, the RHAMM distributes into multiple compartments, including the cell surface, cytoskeleton, mitochondria, and nucleus. In particular, it should be noted that the cell surface RHAMM–hyaluronan interactions regulate signaling through Ras and Ser.

The RHAMM antibodies, dominant-negative protein forms, soluble recombinant RHAMM protein, and antisense RHAMM cDNA inhibit proliferation, motility, and Ras-mediated transformation of immortalized fibroblasts in vitro. It has therefore been suggested that RHAMM may make a good tumor antigen candidate for immunotherapy.

The activation of CD4+ helper T cells is essential for the optimal induction of cytotoxic T cells in dendritic cell therapy for cancer. It is known that exogenous antigens are taken up by dendritic cells and processed in the endosomal pathway, where the resultant peptides are associated with MHC class II molecules, leading to the induction of the CD4+ helper T-cell response, whereas endogenous cytosolic and nuclear proteins are processed in a different manner (that is, by degradation by proteasomes and transport of peptides into the endoplasmic reticulum, where they bind to MHC class I).

The purpose of this study was to demonstrate the antitumor effects of mRNAs transduced into dendritic cells to transport the intracellular protein RHAMM efficiently into MHC class II compartments by adding a late endosomal–lysosomal sorting signal to the RHAMM gene–transfected dendritic cells.

Materials and Methods

Animal Model

Adult male C57BL/6 mice (purchased from Japan SLC Co., Ltd.) were maintained in a specific pathogen–free environment. Mice weighing between 25 and 35 g were used in this study. Free access to sterilized food and water was provided. This experiment was reviewed by the Committee on the Ethics of Animal Experiments at the Yamaguchi University School of Medicine and conducted under the Guidelines for Animal Experiments at the Yamaguchi University School of Medicine and according to the Law (no. 105) and Notification (no. 6) of the Japanese government.

Cell Lines

The KR158B cell is a subclone of the murine glioma cell line KR158 involving the mutation of two tumor-suppressor genes, Nf1 and Trp53. The KR158B glioma cells were maintained in complete media (RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin).

Plasmid Cassette for RNA Transcription

Plasmid constructs have been described previously. Briefly, for in vitro transfection, the plasmids were cloned with pSP64 vector (Promega Corp.). A TRP-2 signal sequence fragment and transmembrane–cytoplasmic domain were amplified from TRP-2 cDNA by using PCR (Ex Taq polymerase; Takara Bio, Inc.). The PCR products were cloned as a HindIII–PstI fragment and a BamHI–SmaI fragment into pSP64 (Fig. 1) to allow in vitro transcription under the control of an SP6 promoter to transport the RHAMM protein efficiently to MHC class II compartments for eventual cross-presentation by both class I and II on dendritic cells in a cognate manner.

In Vitro Transcription of mRNA

The total RNA of the KR158B glioma cells was corrected for reverse transcriptase PCR by using TRIzol (Invitrogen) according to the manufacturer’s protocol. The following primers were used: RHAMM forward primer, 5'-AAGTCGACAGTCTTTCCATTGAAGGC-GGCCCCTGAA-3'; and RHAMM reverse primer, 5'-AATCTAGAGCAGCTTGGGTTTGCC-3'. The products were cloned as a SalI–XhoI mouse RHAMM fragment into a pSP64 cassette (Fig. 2). In vitro transcription was performed in a 100-μl reaction mix at 37°C for 2 hours by using an mMESSAGe SP6 kit (Ambion, Inc.) to generate 5'm7GpppG-capped, in vitro–transcribed mRNA, as described previously.

Dendritic Cell Generation From Bone Marrow

The animals’ femurs and tibias were removed. The marrow was flushed with RPMI 1640 by using a syringe with a 26-gauge needle and the marrow was then filtered through a 70-μm cell strainer. The bone marrow cells were adjusted to 2 × 10^7 cells/ml in complete media and plated on 100-mm dishes. They were cultured for up to 7 days in the presence of 1000 U/ml of granulocyte-macrophage colony-stimulating factor and 500 U/ml of interleukin-4 at 37°C with 5% CO2. On Day 4 of the culture, the same amount of cytokines was added to the cells. To isolate the dendritic cell population, the cells were collected and suspended in 5 ml of complete media. The same volume of 14.5% (weight/volume) metrizamide in complete media was added, and the suspension was centrifuged at 1200 G for 20 minutes at room temperature. After centrifugation, the cells in the interface were collected and washed three times with RPMI 1640, and these were used for subsequent RNA transfection. Before and after RNA transfection, the expression of the surface molecules on the dendritic cells was analyzed using flow cytometry (Epics XL; Beckman Coulter Co.).

Transfection of mRNA Into Dendritic Cells Before Vaccination

The dendritic cells were resuspended in RPMI 1640, 10^6 dendritic cells, and 10 μg of mRNA was mixed under control RNA lipofection with TransMessenger Transfection Reagent (Qiagen), and incubated for 3 hours at 37°C. After transfection the cells were separated into two groups, 50 μm chloroquine (Sigma-Aldrich) was added to one group, and these cells were incubated for 72 hours at 37°C to block the pathway of the MHC class II presentation. After the chloroquine treatment, the expression of the surface molecules on the dendritic cells was analyzed using flow cytometry.

Implantation of Tumor Cells Into the Marine Brain

The mice were anesthetized with an intraperitoneal injection of ketamine and xylazine. The skin was incised, and a 1.5-mm bur hole was made 2 mm to the right of the bridge. The injection device consisted of a stereotactic frame with a 27-gauge needle connected via a catheter to a 10-μl Hamilton syringe. A total of 2 × 10^6 cells in 2 μl PBS was injected into the brain within 5 minutes (the needle was held in place for 5 minutes, after which it was retracted). Closure of the wound was performed with bone wax and sutures.

The injected mice were divided into two groups. One group was allowed to live without a time limit to evaluate the survival time. The survival data were tested statistically by log-rank analysis, as described previously. Differences with a probability value of less
than 0.05 were considered significant. The mice in the other group were killed 20 days after cell implantation for histological and immunohistochemical examination.

**Vaccination With Dendritic Cells**

The mice were classified into four groups. Three and 10 days after tumor cell implantation, the first group was injected intraperitoneally with $3 \times 10^5$ RHAMM mRNA–transfected dendritic cells, the second group was injected intraperitoneally with $3 \times 10^5$ dendritic cells treated with chloroquine after the RHAMM mRNA transfection, and the third group was injected intraperitoneally with $3 \times 10^5$ PBS-pulsed dendritic cells. The fourth group received no treatment after tumor cell implantation.

**Immunocytochemical Examination for RHAMM and Cellular Immune Response**

Twenty days after implantation of the tumor cells, some of the mice were killed for histological and immunohistochemical examination. Their brains were cut with a cryostat through the area of tumor implantation at a thickness of 10 μm, and the sections were either mounted on glass slides for routine histological staining with H & E or were prepared for immunohistochemical investigation. The sections used for immunohistochemical examination were fixed in cold ethanol treated with hydrogen peroxide and blocked with 1.5% rabbit serum before being incubated overnight at room temperature with the rat monoclonal antibodies TIB122 (anti-CD45), KT3 (anti-CD3), YTA3.1 (anti-CD4), YTS169.4 (anti-CD8), FA/11 (macrosialin), and TIB222 (anti-CD25). After being washed with PBS, the sections were incubated with horseradish peroxidase–conjugated rabbit anti–rat immunoglobulin G (DAKO) that had been adsorbed with murine serum. The sections used for immunocytochemical testing for mouse RHAMM were also fixed with cold ethanol, treated with hydrogen peroxide, and blocked with 1.5% goat serum before being incubated overnight at room temperature with the rabbit polyclonal antibody for the mouse, rat, and human RHAMM (Santa Cruz Biotechnology, Inc.). After washing with PBS, the sections were incubated with biotinylated goat anti–rabbit immunoglobulin G for 30 minutes. After another wash with PBS, the sections were incubated with the VECTASTAIN avidin–biotin complex reagent (Vector Laboratories, Inc.) containing avidin DH and biotinylated horseradish peroxidase H reagent, which were especially prepared to form ideal complexes for immunoperoxidase staining. Horseradish peroxidase was detected with diaminobenzidine. The sections were counterstained with crystal violet or hematoxylin, dehydrated, and mounted.

**Quantification of Infiltrating Cells in the Tumor Tissue**

The number of CD8 and CD4 T cells seen in the RHAMM-treated group was semiquantified. The infiltration pattern of T cells in the tumors was heterogeneous, and therefore the number of these T cells was counted in the infiltrating area. The degree of the T-cell infiltration was graded as follows on the basis of one visual field at $\times 200$ magnification: –, none evident; +, rare cells (1–20) found; ++, scattered cells (21–40) found; ++++, larger number of cells (>40) found.

**Results**

**Detection of RHAMM mRNA Expression in KR158B Cells**

To clarify the RHAMM expression in the KR158B glioma cell line, we examined the RHAMM mRNA expression by using conventional reverse transcriptase PCR. The results showed that RHAMM mRNA was expressed in the KR158B glioma cell line (data not shown).

**Dendritic Cell Phenotypes Before and After RHAMM mRNA Transfection**

The dendritic cell phenotypes before RNA transfection showed CD11c+; CD40+, CD86+, H-2Kb+, I-Ab+, and DEC205+. The results of the flow cytometry revealed no
Vaccine therapy with dendritic cells transfected with RHAMM mRNA

change in the dendritic cell phenotypes after RNA transfection. After chloroquine treatment, the expression of the surface molecules on the RHAMM mRNA–transfected dendritic cells was analyzed using flow cytometry. The dendritic cell phenotypes after chloroquine treatment also were the same as before treatment.

Antitumor Effects of Dendritic Cells Transfected With RHAMM mRNA

As shown in Fig. 3, the mice immunized with dendritic cells transfected with RHAMM mRNA survived significantly longer (mean survival 61.8 days) than the ones vaccinated with nontransfected dendritic cells (mean survival 31.6 days; \( p < 0.001 \), log-rank analysis of survival curves) and the ones without immunization (mean survival 25.6 days, 20 mice in each group; \( p < 0.001 \), log-rank analysis of survival curves).

In addition, the mice immunized with dendritic cells transfected with RHAMM mRNA survived significantly longer than the ones immunized with chloroquine-treated dendritic cells transfected with RHAMM mRNA (mean survival 45.5 days, 15 mice; \( p < 0.001 \)).

All mice from the group that was not immunized with nontransfected dendritic cells died by Day 50, and all the mice from the group vaccinated with chloroquine-treated dendritic cells transfected with modified RHAMM mRNA died by Day 55. However, 50% of the mice vaccinated with dendritic cells transfected with modified RHAMM mRNA were still alive on Day 55. Even at Day 80, 15% of the mice in this group were healthy and neurologically normal.

Immunocytochemical Analysis

The intracranial tumor volumes in the mice vaccinated with dendritic cells transfected with modified RHAMM mRNA were smaller than those in the other groups (Fig. 4).

In our glioma model, RHAMM was consistently overexpressed on the surface of the tumor cells, and its distribution was relatively homogeneous (Fig. 5A). By contrast, RHAMM immunostaining was not detected in normal brain tissue or structures (Fig. 5B).

Infiltration of inflammatory cells into the intracranial tumors was detected in all groups. The distributions and populations of the infiltrating cells in the brain were determined immunocytochemically. The findings were as follows: CD45+ leukocytes were detected in and around the tumors in all groups (Fig. 6A and B). In the control (no treatment) group, infiltrating cells were mainly macrophages (Fig. 6C), and few T lymphocytes were detected (Fig. 6D). In the group vaccinated with chloroquine-treated dendritic cells, only a few T lymphocytes were detected. In contrast, the group vaccinated with dendritic cells transfected with modified RHAMM mRNA showed greater numbers of CD3+ lymphocytes (Fig. 6E), and these lym-
phocytes contained not only CD8+ (Fig. 6F) but also CD4+ (Fig. 6G) T cells. Furthermore, these lymphocytes contained T cells activated by CD25+ (Fig. 6H).

The degree of T-cell infiltration in the glioma is summarized in Table 1. In the group treated with the RHAMM-transfected dendritic cells, the degree of CD4 T cells was +++ and that of CD8 T-cells was +++ or at least ++.

By contrast, in the group that received no treatment, no CD4 or CD8 cells were detected.

**Discussion**

The RHAMM was recently identified as an immunogenic antigen by serological screening of cDNA expression libraries. It was reported that RHAMM mediates glioma cell migration and proliferation. Using immunohistochemical staining, we have checked the degree and distribution of RHAMM expression in the glioma specimens. We also checked RHAMM expression in human malignant gliomas and found that in these tumors RHAMM was overexpressed, but the degree and distribution varied from case to case. The RHAMM expression was heterogeneous in all human malignant gliomas. However, RHAMM was not detected in the normal human brain tissue (data not shown). Moreover, we reported that RHAMM mRNA showed a very restricted expression in normal mouse tissues, but it showed an overexpression in the KR158B glioma cell line. Using immunohistochemical staining, we also examined
Vaccine therapy with dendritic cells transfected with RHAMM mRNA

normal mouse brains for RHAMM expression, and detected none. Considering that RHAMM has a mitotic spindle-stabilizing function and exhibits oncogenic activity, it is reasonable to think that its expression would be limited to organs, tissues, or cells that have increased proliferation potential. Finally, we examined the normal brain tissues of mice immunized with RHAMM mRNA–transfected dendritic cells by using histological methods, and we could detect no toxicity in these tissues. Given this fact, we considered it suitable for immunotherapy to be targeted against RHAMM in tumor cells.

It is necessary for antigen peptides to be presented, not only on MHC class I, but also on MHC class II to induce a strong immunoresponse. In this study, to transport the RHAMM protein efficiently to both the MHC class I and II compartments (cross-presentation), modified mRNA joining the leader sequence and cytoplasmic domain containing a late endosomal–lysosomal sorting signal of TRP-2 to the N and C termini of RHAMM, respectively, was constructed and transfected to dendritic cells. This strategy has previously been demonstrated to be useful for the efficient induction of specific immune responses. In that previous study, we reported that vaccination with dendritic cells transfected with modified RHAMM mRNA induced strong antitumor effects in vitro. In the present study, we estimated the antitumor effects of vaccine therapy performed in vivo with dendritic cells transfected with modified RHAMM mRNA by calculating the survival ratio of mouse glioma models in which KR158B glioma cells had been implanted in the brain. It would also be interesting to look at animals without tumors that are immunized with the transfected dendritic cells followed by tumor implantation to see if there would be a memory response to this specific tumor antigen. We are proceeding with this investigation.

Among the types of dendritic cell vaccination therapies performed for the KR158B mouse glioma model, the group vaccinated with dendritic cells transfected with modified RHAMM mRNA survived significantly longer than the group vaccinated with nontransfected dendritic cells or the control group. This might reflect the fact that the presence of the RHAMM peptide would be effectively immunogenic. Also, dendritic cells transfected with mRNA coding tumor antigen have the potential to be used in a vaccination therapy for malignant tumors. However, the durability of the RHAMM-transfected dendritic cells is unclear, and it is difficult to get confirmation on this issue. Barratt-Boyes et al. reported that injected dendritic cells migrate spontaneously and rapidly to draining lymph nodes, where they remain for at least 5 days. The injected cells become integrated with T cells in the parafollicular and paracortical zones and retain a high level of expression of CD86, CD40, and MHC class II molecules, reflecting a phenotype of potent antigen-presenting cells. With regard to the only twofold prolongation of survival for the mice in this study that were treated with dendritic cells transfected with RHAMM, we consider that RHAMM is one of the tumor antigens and that the immunogenicity might not be strong enough to induce complete tumor cell cytotoxicity in vivo. In addition, the number of times dendritic cell therapy is performed might be too few to obtain a complete response.

The group treated by vaccination with dendritic cells transfected with modified RHAMM mRNA also survived significantly longer than the group vaccinated with dendritic cells transfected with modified RHAMM mRNA after treatment with chloroquine to inhibit lysosomal degradation. It is known that chloroquine raises the pH in the endosomal and lysosomal compartments, thus inhibiting protein hydrolysis by cathepsins. As a result, chloroquine inhibits lysosomal degradation and the antigen peptide–MHC class II complex presentation. The antitumor effects of vaccination with dendritic cells transfected with the mRNA coding tumor antigen were suppressed by the addition of chloroquine to the dendritic cells. This suggested that the efficient cross-presentation of antigen peptides is essential for the induction of strong antitumor effects.

The tumor volume in the mice treated by vaccination with dendritic cells transfected with modified RHAMM mRNA tended to be smaller than those of the other groups. It was anticipated that vaccination with dendritic cells transfected with modified RHAMM mRNA would suppress tumor growth. Immunohistochemical analysis revealed that the group treated in this fashion showed greater numbers of CD3+ lymphocytes in the tumor and that these lymphocytes contained not only CD8+ but also CD4+ T cells. Moreover, these infiltrating cells contained T cells activated by CD25+. In contrast, in the group treated by vaccination with chloroquine-treated dendritic cells as well as in the nontreated group, infiltrating cells were mainly macrophages. Only a few or no T lymphocytes were detected. Based on these results, we suggest that this vaccine therapy induced specific immune responses to the tumor as well as strong antitumor effects by activated and recruited T cells. This effect was suppressed by the chloroquine treatment of dendritic cells transfected with modified RHAMM mRNA. Based on our findings, we suggest that vaccination with dendritic cells transported the RHAMM protein efficiently into both MHC classes I and II, induced cross-presentation, and activated not only CD8+ but also CD4+ T cells.

We previously reported on the in vitro antitumor analysis of spleen cells from mice immunized with dendritic cells transfected with mouse RHAMM mRNA against KR158B cells. The KR158B cells were shown to be
weakly positive for both MHC classes I and II. Although the expression of MHC classes I and II on the surface of the KR158B cells was low, vaccine therapy in which dendritic cells transfected with modified RHAMM mRNA were used induced strong antitumor effects in vitro. In this study, we revealed similar antitumor effects in vivo by using a mouse brain tumor model.

Conclusions

In this study we demonstrated the therapeutic potential of vaccination with dendritic cells transfected with RHAMM mRNA for the treatment of malignant glioma. Moreover, with the method presented here, we will be able to perform vaccinations easily with dendritic cells transfected with other tumor antigens’ mRNA by cloning other tumor antigen fragments into the pSPl64 plasmid cassette. Nevertheless, further analyses of the effects induced by vaccine therapy with dendritic cells containing tumor antigens are necessary before the treatment’s potential for clinical application can be fulfilled.

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Vaccine therapy with dendritic cells transfected with RHAMM mRNA


Address reprint requests to: Koji Kajiwara, M.D., Department of Neurosurgery, Yamaguchi University School of Medicine, 1-1-1, Minami-Kogushi, Ube, Yamaguchi 755-8505, Japan. email: koji@yamaguchi-u.ac.jp.