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Antitumor activity of mannan-binding protein *in vivo* as revealed by a virus expression system: Mannan-binding protein-dependent cell-mediated cytotoxicity

YONG MA*, KAZUHIDE UEMURA*, SHOGO OKA*, YASUNORI KOZUTSUMI*, NOBUKO KAWASAKI†, AND TOSHISUKE KAWASAKI*‡

*Department of Biological Chemistry and Core Research for Evolutional Science and Technology Project, Japan Science and Technology Corporation, Faculty of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan; and †College of Medical Technology, Kyoto University; Sakyo-ku, Kyoto 606-8507, Japan

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ABSTRACT **Mannan-binding protein (MBP), a Ca^{2+}-dependent mammalian lectin specific for mannose and *N*-acetylglucosamine, is an important serum component associated with innate immunity. MBP activates complement and functions as a direct opsonin on binding to mannooligosaccharide-bearing pathogens. We have found that MBP recognizes and binds specifically to oligosaccharide ligands expressed on the surfaces of a human colorectal carcinoma. Interestingly, the recombinant vaccinia virus carrying human MBP gene was demonstrated to possess a potent growth-inhibiting activity against human colorectal carcinoma cells transplanted in KSN nude mice when administered by intratumoral or subcutaneous injection. Moreover, a significant prolongation of life span of tumor-bearing mice resulted from the treatment. This effect appears to be a consequence of local production of MBP. Unexpectedly, the mutant MBP, which had essentially no complement-activating activity, was nearly as active as wild-type MBP. These results indicated that MBP has a previously undescribed cytotoxic activity, which we propose to term MBP-dependent cell-mediated cytotoxicity(MDCC). In addition, this study provides a model for the development of an effective and specific host defense factor for cancer gene therapy.**

Mannan-binding protein (MBP)(1, 2), also known as mannose-binding protein (3) or mannan-binding lectin (4), is a Ca^{2+}-dependent serum lectin specific for mannose and *N*-acetylglucosamine (5). MBP is an important component of innate immunity (6) and has been shown to have complement-dependent bactericidal activity; for example, *Escherichia coli* strains *K12* and *B*, which have exposed *N*-acetylglucosamine and L-glycero-D-mannoheptose, respectively, are killed by MBP with the help of complement (7). MBP serves as a direct opsonin and mediates binding and uptake of bacteria that express a mannose-rich O-polysaccharide by monocytes and neutrophils (8). MBP functions as a β-inhibitor of the influenza virus (9) and protects cells from HIV infection by binding to gp120, a high mannose-type oligosaccharide-containing envelope glycoprotein on HIV (10). In addition, the α-mannosidase inhibitor 1-deoxymannojirimycin-treated baby hamster kidney (BHK) cells, which have high mannose-type oligosaccharide exposed on their surfaces, can be killed by MBP with the help of complement (11). MBP activates complement through interactions with complement subcomponents C1r/C1s (6, 12, 13) or two novel C1r/C1s-like serine proteases, MBP-associated serine proteases (MASP-1 and MASP-2) (4, 14). The MBP-mediated complement activation is named the MBP pathway.

Human MBP is a homooligomer composed of 32-kDa subunits. Each subunit has an NH_2-terminal region containing cysteines involved in interchain disulfide bond formation, a collagen-like domain containing hydroxyproline and hydroxylysine, a neck region, and a carbohydrate-recognition domain with an amino acid sequence highly homologous to other Ca^{2+}-dependent lectins (3). Three subunits form a structural unit, and an intact MBP consists of 2–6 structural units. The carbohydrate-recognition domain is specific for mannooligosaccharide structures on pathogenic organisms, whereas the collagen-like domain is believed to be responsible for interactions with other effector proteins involved in host defense (15, 16). Clinical studies have demonstrated a marked correlation between low serum levels of MBP and immune opsonic deficiency (17). Low serum concentrations of MBP are associated with three independent mutations in codons 52 (18), 54 (19), and 57 (20) of exon 1, resulting in amino acid replacement of Arg-52 to Cys , Gly-54 to Asp, and Gly-57 to Glu, respectively, all of which occurred in the collagen-like domain. These replacements appear to inhibit oligomerization of the structural unit of the molecule and consequently abolish the ability to initiate complement activation without impairing the original lectin-binding specificity to oligosaccharide ligands (21, 22). Normal mammalian cells such as circulating blood cells are usually covered with complex oligosaccharides terminated with sialic acids and do not bind MBP. On the other hand, malignant transformations or viral infections modify the oligosaccharide structures on cell surfaces, and some tumor tissues have been shown to bind to MBP (10).

In this study, by using a recombinant vaccinia virus (RVV) expression system that produces high levels of native MBP (23), we have demonstrated that MBP is cytotoxic to a human colorectal carcinoma *in vivo* through a mechanism that we term MBP-dependent cell-mediated cytotoxicity.

MATERIALS AND METHODS

Mice. Seven-week old female KSN athymic nude mice (24) were supplied by Japan SLC (Hamamatsu, Japan). They were maintained under specific-pathogen-free conditions.

Cell Line. The human colorectal carcinoma cell line, SW1116 (ATCC CCL-233), was obtained from American Type

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Abbreviations: MBP, mannan-binding protein; WT-MBP, wild-type MBP; G54D-MBP, G54D mutant MBP; RVV, recombinant vaccinia virus; WT-RVV, vaccinia virus carrying wild-type MBP gene; G54D-RVV, vaccinia virus carrying G54D mutant MBP gene; WR-VV, Western reserve strain of vaccinia virus; FITC, fluorescein isothiocyanate.

‡To whom reprint requests should be addressed. e-mail: kawasaki@pharm.kyoto-u.ac.jp.

Culture Collection. The cells were grown in Leibovitz's L-15 medium (GIBCO) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (HyClone), from which endogenous MBP had been depleted by passage through a Sepharose 4B–mannan column as described (12). The human hepatoma cell line, HLF (JCRB 0405), was provided by the Japanese Cancer Research Resources Bank (Tokyo, Japan).

Recombinant Vaccinia Viruses. A human wild-type MBP cDNA was isolated as described (23). The mutant (G54D mutant) MBP cDNA was prepared by using oligonucleotide-directed mutagenesis with a Mutan-Express Km kit (Takara Shuzo, Kyoto). Construction, selection, amplification, and isolation of two kinds of recombinant vaccinia viruses encoding human wild-type MBP (WT-MBP) or G54D mutant MBP (G54D-MBP) cDNA were carried out as described (23). The viral titer was determined by using plaque-forming assays as described and expressed in plaque-forming units (pfu).

Antibodies. The monoclonal anti-human MBP (YM304), which recognizes the CRD portion, was prepared in our laboratory. Horseradish peroxidase (HRP)-conjugated YM304 was prepared as described by the manufacturer (Genosys, The Woodlands, TX). Fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG was purchased from Promega.

Binding of MBPs to SW1116 Cells. Cultured SW1116 cells were trypsinized, reseeded onto glass slides, and grown in Leibovitz's L-15 medium supplemented with MBP-free 10% FBS at 37°C for 2 days, and then washed with Tris-buffered saline (pH 7.8) containing 10 mM $CaCl_2$ (TBS-Ca) and 1% BSA. The cells were incubated with 5.0 μg per 50 μl of WT-MBP or G54D-MBP in the presence of 10 mM Ca^{2+}, 20 mM mannose/10 mM Ca^{2+}, 20 mM GalNAc/10 mM Ca^{2+}, 20 mM GlcNAc/10 mM Ca^{2+}, or 10 mM EDTA for 20 min, and then washed three times with TBS-Ca^{2+}. MBP-treated cells were fixed in 3% paraformaldehyde in PBS (pH 7.2) for 15 min. After being rinsed three times with PBS, cells were incubated for 1 hr with a 1:500 dilution of YM304 in PBS and then a 1:100 dilution of FITC-conjugated anti-mouse IgG for 1 hr and then analyzed with an Olympus (Tokyo) BX50–34-FLAD1 fluorescence microscope. The MBPs used in this experiment were obtained as follows. HLF cells were infected with the RVVs containing human WT-MBP or G54D-MBP cDNAs. WT-MBP and G54D-MBP were purified, respectively, from the media collected 48 hr postinfection by affinity chromatography on a Sepharose 4B-mannan column as described (23).

***In Vivo* Assay of Antitumor Activity of MBPs.** KSN athymic nude mice were divided into four groups with at least five mice per group: vaccinia virus carrying the wild-type MBP gene (WT-RVV), vaccinia virus carrying G54D mutant MBP gene (G54D-RVV), Western reserve strain of vaccinia virus (WR-VV), and saline control groups. The mice in each treatment group were injected subcutaneously with 1×10^7 SW1116 cells. Approximately 21 days later, when all mice had developed palpable tumors, each mouse was injected in a blinded, randomized fashion either intratumorally or subcutaneously with 5×10^6 pfu of WT-RVV, G54D-RVV, WR-VV, or saline solution. Three booster injections of the same agent were given on days 35, 49, and 63 (i.e., after 5, 7, and 9 weeks). Serial tumor measurements were made every 3–4 days in three dimensions with Vernier calipers. Differences in tumor growth were statistically analyzed with the Kruskal–Wallis and the Wilcoxon tests. Significance was defined as $P < 0.005$. For the survival study experiment, the mice were monitored for 92 days (13 weeks). The death of mice in each group was noted daily. An autopsy was performed on each dead mouse to confirm the cause of death.

Immunohistochemistry of the Recombinant Virus-Treated Tumors. The tumors taken from each treatment group were embedded in OCT compound (Miles) and frozen. Sections (12 μm thick) were made by cryostat (Finetec, Tokyo), placed on poly-L-lysine (Sigma)-coated slides, and then fixed in ice-cold acetone for 30 sec. Endogenous peroxidase activity was blocked by incubation with 1% periodate for 10 min. Nonspecific binding sites were blocked by using 5% normal mouse serum and 3% BSA in PBS for 30 min. The sections were incubated with HRP-conjugated YM304 diluted 1:100 with PBS for 1 hr. Antibody binding was histochemically detected by using a HistoMark Orange Kit (Kirkegaard & Perry Laboratories) supplemented with 10 mM NaN_3 to block endogenous peroxidase activity. After color development, sections were postfixed with 2.5% glutaraldehyde in PBS for 30 min, counterstained in Contrast Green Solution for 3 min, mounted with a Clearmount mounting solution (Zymed), and then observed and photographed with an Olympus Vanox AHBS3 light microscope. Cytochemical controls were prepared by reaction with 1% BSA in PBS instead of HRP-conjugated YM304.

Recombinant Human MBPs Produced by SW1116 Cells. SW1116 cells were maintained in Leibovitz's L-15 medium supplemented with endogenous MBP-free 10% FBS. Carcinoma cells were grown at 37°C to a concentration of 5×10^5 cells per ml in 20 ml of medium in 250-ml Falcon flasks. WT-RVV or G54D-RVV was then added to the cells at a multiplicity of infection close to 5. The media were harvested 48 hr postinfection, and the recombinant WT- and G54D-MBPs secreted into the media were purified by affinity chromatography on a Sepharose 4B-mannan column as described (2).

Passive Hemolysis Complement Activation Assay. Sheep erythrocytes were coated with yeast mannan sensitized with WT-MBP or G54D-MBP produced by SW1116 cells, and the sensitized cells were incubated with guinea pig complement as described (12). The degree of specific lysis was calculated based on the absorption at 541 nm of an equivalent amount of cells totally lysed in water and expressed as a percentage. Correction was made with the blank value obtained in the absence of the complement, which was usually less than 2% of the total lysed cell value.

PAGE. SDS/PAGE was performed according to the method of Laemmli (25) in a 3–10% gradient polyacrylamide gel under reducing or nonreducing conditions, and proteins were stained with Coomassie Brilliant Blue R250.

RESULTS

The Presence of MBP Ligands on Carcinoma Cell Surfaces. The presence of MBP ligands on a cultured human colorectal carcinoma cell line, SW1116, was demonstrated (Fig. 1). SW1116 cells were incubated with recombinant human MBPs followed by anti-human MBP mAb (YM304) and FITC-conjugated anti-mouse IgG. On incubation with either WT-MBP (Fig. 1*A*) or a mutant G54D-MBP (Fig. 1*B*), SW1116 cells stained strongly with YM304/FITC-conjugated anti-mouse IgG. The binding of human MBP to the SW1116 cell surfaces was mannose/*N*-acetylglucosamine-specific and calcium-dependent. Thus, the addition of 10 mM EDTA without Ca^{2+}, which completely blocks binding of MBP to sugar ligands, resulted in total staining inhibition (Fig. 1*C*). Similarly, the addition of ligand sugars [either 20 mM mannose (Fig. 1*D*) or 20 mM *N*-acetylglucosamine (Fig. 1*F*)] almost completely inhibited staining, but a nonligand sugar (20 mM *N*-acetylgalactosamine) did not significantly affect staining (Fig. 1*E*). These results clearly indicate that human colorectal carcinoma SW1116 cells express on their surface oligosaccharides that are specifically recognized and bound by human MBP.

Recombinant Vaccinia Virus Carrying Human MBP Genes Inhibit Tumor Growth *in Vivo*. The detection of MBP ligands on the surface of tumor cells prompted us to test the antitumor

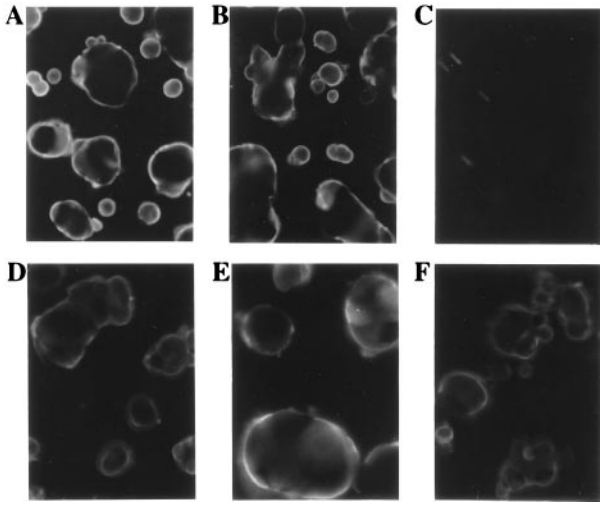

FIG. 1. Expression of the MBP ligands on human colorectal carcinoma SW1116 cell surfaces. The cultured SW1116 cells were incubated with human MBPs, followed by immunofluorescent staining with anti-human MBP mAb YM304 and with FITC-conjugated anti-mouse IgG. The cells were incubated with either WT-MBP (*A*, *C–F*) or G54D-MBP (*B*) in the presence of 10 mM Ca^{2+} (*A* and *B*), 10 mM EDTA (*C*), 20 mM mannose/10 mM Ca^{2+} (*D*), 20 mM GalNAc/10 mM Ca^{2+} (*E*), and 20 mM GlcNAc/10 mM Ca^{2+} (*F*).

effect of MBP *in vivo* with a recombinant virus expression system that produces high levels of human MBP. SW1116 cells were first inoculated subcutaneously in the right back region of KSN athymic nude mice. Palpable tumors developed in 3 weeks after tumor cell transplantation. At this time, WT-RVV, G54D-RVV, WR-VV, or saline solution was injected directly into the tumor mass or subcutaneously into adjacent nontumor sites. Fig. 2 shows pictures of mice after two doses of RVVs taken at a 2-week interval. There were marked differences in tumor size between WT-RVV-treated (Fig. 2*A*) or G54D-RVV-treated mice (Fig. 2*B*) and WR-VV-treated (Fig. 2*C*) or saline solution-treated (Fig. 2*D*) mice.

The mean tumor size (in cubic millimeters) was measured with calipers as a function of time. As shown in Fig. 3*A*, the

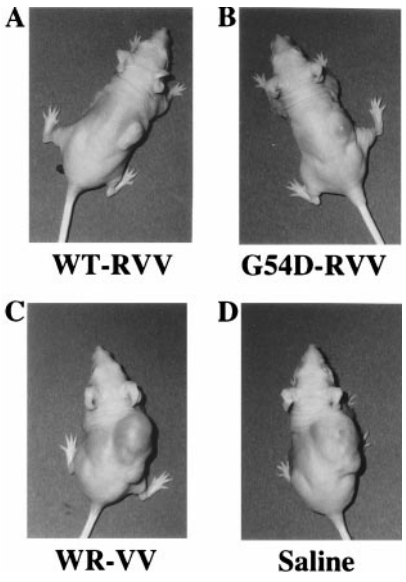

FIG. 2. Photographs of SW1116 tumor-bearing KSN nude mice treated with the recombinant vaccinia virus carrying MBP genes. The pictures were taken 48 days after tumor transplantation. The tumor-bearing mice were inoculated intratumorally with two consecutive doses of WT-RVV (*A*), G54D-RVV (*B*), WR-VV control (*C*), or saline control (*D*).

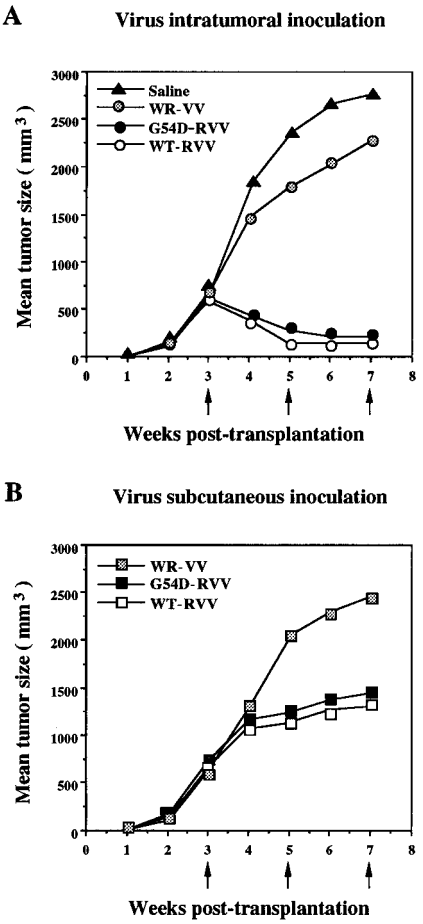

FIG. 3. Time course of SW1116 tumor growth inhibition by the recombinant vaccinia virus carrying MBP genes. Four different groups of SW1116 tumor-bearing KSN nude mice were inoculated intratumorally (*A*) or subcutaneously (*B*) with three consecutive doses of 5×10^6 pfu of WT-RVV, G54D-RVV, WR-VV control, or saline control (arrows). Tumor size was measured in three dimensions every 3–4 days with Vernier calipers. Differences in tumor size were statistically analyzed with the Kruskal–Wallis test and the Wilcoxon test.

intratumoral administration of the recombinant vaccinia virus carrying a MBP gene caused a marked reduction in the tumor size as compared with the control groups ($P < 0.005$). This effect was confirmed in duplicate experiments. WT-RVV was most effective for tumor regression, although G54D-RVV was also very effective. The virus vector itself, WR-VV, given intratumorally, had no significant antitumor effect. Tumors grew progressively in WR-VV-treated controls, and no significant toxicity or death resulted from WR-VV administration.

The effects of subcutaneous administration on tumor-growth inhibition was assessed, and the results are shown in Fig. 3*B*. The recombinant vaccinia virus carrying a human MBP gene inhibited tumor growth as compared with the WR-VV treatment group ($P < 0.005$). However, the magnitude of tumor size reduction was much lower compared with intratumoral administration of the recombinant virus. On the other hand, the difference in the antitumor activity of WT-RVV and G54D-RVV was small, consistent with the results of intratumoral administration.

The Recombinant Vaccinia Virus Treatment Prolongs Survival of Tumor-Bearing Mice. The effect of the recombinant virus treatment on the life span of tumor-bearing mice was investigated (data not shown). On the 7th week, after three consecutive intratumoral inoculations of WT-RVV, G54D-RVV, or WR-VV, all of the mice tested survived. However, on the 10th week (1 week after the final injection), only 1/3 of the

saline-treatment group, and none of the WR-VV-treated mice, survived. In contrast, all of the mice in the WT-RVV and G54D-RVV treatment groups survived. It is clear that the recombinant virus-treated groups survived significantly longer than control groups ($P < 0.05$, log-rank analysis of Kaplan–Meier survival curves). All of the dead mice contained massive tumors in their right back region, suggesting that death was caused by the tumor. Note that the WT-RVV-treated group and the G54D-RVV treatment group did not exhibit any significant differences in prolonging of life span.

Expression of WT- and G54D-MBPs in Tumor Tissues. Histochemical studies indicated that the genetically engineered human MBP was actually produced locally in tumor tissues. Thus, tumor specimens inoculated intratumorally with WT- and G54D-RVVs were stained heavily with HRP-conjugated anti-human MBP mAb YM304, as shown in Fig. 4 *A* and *B*. In contrast, the implanted tumor cells treated intratumorally with saline (Fig. 4*C*) or WR-VV (data not shown) were not stained. Human MBPs accumulated within the cells and also were secreted into the surrounding tumor space. This confirms that MBP genes in the recombinant virus produced MBP on injection into SW1116-bearing KSN mice.

Biochemical Characterization of WT- and G54D-MBPs Expressed *in Vitro*. On infection of human WT- and G54D-RVVs to SW1116 cells *in vitro*, both WT- and G54D-MBPs accumulated in the culture media almost linearly over time up to 48 hr (data not shown). The yield of WT- and G54D-MBPs 48 hr postinfection were 3.65 mg/liter and 3.12 mg/liter, respectively, comparable to values seen in hepatoma cell lines (20). SDS/PAGE under reducing conditions indicated that both WT- and G54D-MBPs migrated as a 32-kDa protein (Fig. 5*A*, lanes 1 and 2), identical to native human MBP (1). On the other hand, SDS/PAGE under nonreducing conditions indicated that recombinant MBPs migrated as a mixture of several components, corresponding to monomers, dimers, and trimers of the subunit and to higher oligomers of the structural unit, respectively (Fig. 5*A*, lanes 3 and 4). However, the ratios of higher oligomers in the recombinant MBPs obtained from SW1116 cells were much lower than those obtained from hepatoma cell lines (23). In fact, tetramers and pentamers of the structural units are the major constituents of the recombinant MBP produced by HLF cells (23). Because formation of higher oligomers by disulfide bonds in the structural units are important for complement activation by MBP, MBPs produced by SW1116 cells should have much lower complement-activating activity than those obtained from the hepatoma cells, which was the case. About 1,200 ng of WT-MBP resulted in about 44% hemolytic activity, whereas 1,800 ng of G54D-MBP resulted in a maximum of 5% lysis (Fig. 5*B*). It was noted in earlier experiments that <100 ng of WT-MBP isolated from HLF cells was required to give 100% hemolytic activity under the same conditions (23). These results suggest strongly that the antitumor activity of MBP does not depend on the formation of higher oligomers and complement activation but involves some sort of cell-mediated activities in immunity.

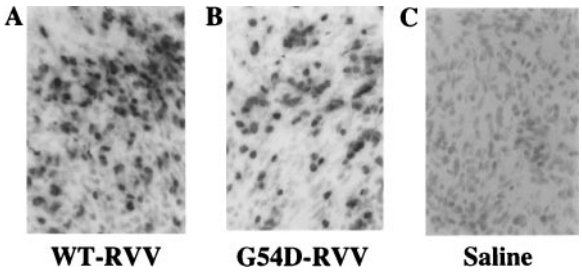

FIG. 4. Immunohistochemistry of SW1116 tumors from tumor-bearing mice treated with the recombinant vaccinia virus carrying MBP genes. Tumor specimens inoculated intratumorally with three consecutive doses of the recombinant virus were stained with HRP-conjugated anti-human MBP mAb YM304. WT-RVV (*A*), G54D-RVV (*B*), and saline control (*C*).

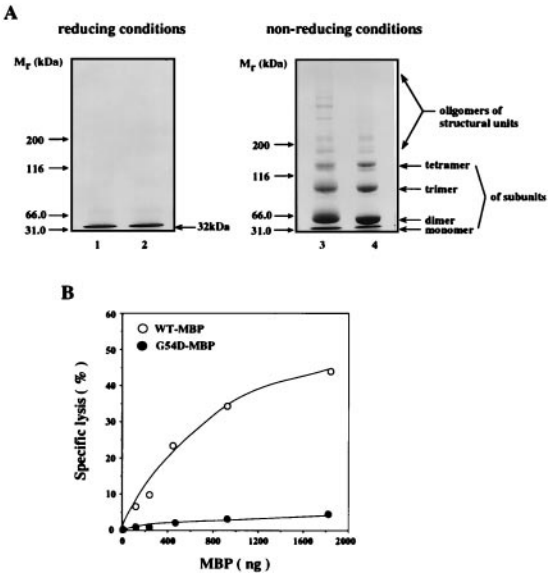

FIG. 5. (*A*) SDS/PAGE of WT- and G54D-MBPs expressed in SW1116 cells. Samples were electrophoresed on a 3–10% polyacrylamide gel according to the method of Laemmli (21) under reducing (*Left*) and nonreducing (*Right*) conditions. Proteins were stained with Coomassie Brilliant Blue R250. The positions of the molecular markers (kDa) are indicated on the left, and the arrows on the right indicate the positions of MBP monomers or oligomers of the subunit and the structural units. WT-MBP, lanes 1 and 3; G54D-MBP, lanes 2 and 4. (*B*) Dose dependence of the complement activation assay of wild-type and mutant G54D-MBPs expressed in SW1116 cells by passive hemolysis. Mannan-coated sheep erythrocytes were sensitized with 0–2,000 ng of recombinant wild-type or mutant G54D-MBPs and lysed with the guinea pig complement (2CH50).

DISCUSSION

We have demonstrated that MBP recognizes and binds specifically to mannose or *N*-acetylglucosamine-terminated oligosaccharide ligands found on the surfaces of a human colorectal carcinoma SW1116 (Fig. 1). More importantly, *in vivo* human MBP gene delivery by the recombinant vaccinia virus administered intratumorally or subcutaneously resulted in a marked inhibition of tumor growth and significant prolonging of the life span of tumor-bearing mice. This effect appears to be a consequence of local production of MBP. No significant toxicity or lethality resulted from intratumoral or subcutaneous treatment.

Initially, we expected involvement of the complement system in the MBP-mediated tumor-killing activity. However, the differences between WT-RVV (which produces MBP with complement-activating activity) and G54D-RVV (which produces MBP with no complement-activating activity) treatment groups for tumor regression were not significant, suggesting that the effect is not the result of MBP-mediated complement activation. In addition, the *in vitro* treatment of SW1116 cells with MBP followed by incubation with complement produced no significant cellular toxicity (data not shown), strengthening this hypothesis and suggesting the involvement of cell-mediated activities.

Although the mechanism of MBP-mediated tumor-growth inhibition has not yet been clearly defined, we propose calling this activity MBP-dependent cell-mediated cytotoxicity (MDCC) on the basis of an apparent similarity with the antibody-dependent cell-mediated cytotoxicity (ADCC) in adaptive immunity (26). Interestingly, MBP and the comple-

ment subcomponent C1q have strikingly similar three-dimensional structures resembling flower bouquets and including a collagen-like region contiguous with a globular domain (13). MBP and C1q also share functional similarity. C1q binds to the Fc region of antibodies in immune complexes and initiates the classical complement pathway. MBP binds specifically to mannooligosaccharide-bearing pathogens and subsequently activates complement through the MBP pathway (3). Besides activating complement, C1q triggers a variety of cell-mediated biological responses such as stimulation of oxidative metabolism (27) and enhancement of antibody-dependent cell cytotoxicity (28). In addition, both MBP and C1q enhance 126-kDa C1qR-mediated phagocytosis and stimulate CR1-mediated phagocytosis by both monocytes and macrophages (29). Thus, the collagenous stalks of both C1q and MBP may share common receptors. These lines of evidence strongly suggest that some cellular receptor(s) are involved in the MDCC reaction. To investigate the molecular mechanisms of the MDCC reaction, we have recently established an *in vitro* experimental system. By using this system, identifications of the cellular receptor(s) bound to MBP, mediator molecules responsible for tumor killing, and effector cells associated with the MBP binding and the mediator production can be studied.

Finally, cancer gene therapy is emerging as a promising new approach in treating cancer. *In vivo* delivery and expression of recombinant genes for therapy can be achieved by using viral recombinants without great cost or technical difficulty. This study indicates the potential usefulness of the recombinant-virus gene delivery system to target measurable amounts of MBP to cancer cells.

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