Inhibitory effect of vitamin D-binding protein-derived macrophage activating factor on DMBA-induced hamster cheek pouch carcinogenesis and its derived carcinoma cell line

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Abstract. This study investigated the inhibitory effect of vitamin D-binding protein-derived macrophage-activating factor (GcMAF) on carcinogenesis and tumor growth, using a 9,10-dimethyl-1,2-benzanthracene (DMBA)-induced hamster cheek pouch carcinogenesis model, as well as the cytoidal effect of activated macrophages against HCPC-1, a cell line established from DMBA-induced cheek pouch carcinoma. DMBA application induced squamous cell carcinoma in all 15 hamsters of the control group at approximately 10 weeks, and all 15 hamsters died of tumor burden within 20 weeks. By contrast, 2 out of the 14 hamsters with GcMAF administration did not develop tumors and the remaining 12 hamsters showed a significant delay of tumor development for approximately 3.5 weeks. The growth of tumors formed was significantly suppressed and none of the hamsters died within the 20 weeks during which they were observed. When GcMAF administration was stopped at the 13th week of the experiment in 4 out of the 14 hamsters in the GcMAF-treated group, tumor growth was promoted, but none of the mice died within the 20-week period. On the other hand, when GcMAF administration was commenced after the 13th week in 5 out of the 15 hamsters in the control group, tumor growth was slightly suppressed and all 15 hamsters died of tumor burden. However, the mean survival time was significantly extended. GcMAF treatment activated peritoneal macrophages in vitro and in vivo, and these activated macrophages exhibited a marked cytoidal effect on HCPC-1 cells. Furthermore, the cytoidal effect of activated macrophages was enhanced by the addition of tumor-bearing hamster serum. These findings indicated that GcMAF possesses an inhibitory effect on tumor development and growth in a DMBA-induced hamster cheek pouch carcinogenesis model.

Introduction

Oral cancer is one of the ten most common cancers in the world, accounting for approximately 2% of all cancer types and 1% of all cancer-related deaths (1). Squamous cell carcinoma (SCC) is the most common malignant tumor of the oral cavity, accounting for over 90% of the malignant neoplasms in this region (2). Despite recent advances in the diagnosis and treatment modalities of surgery, radiotherapy and chemotherapy for oral cancer, the 5-year survival rate has improved only marginally (2). This result indicates the limitations of these treatment modalities. Therefore, additional treatment strategies, such as immunotherapy, are required to improve the 5-year survival rate.

Cancer patients often suffer from immunodeficiency and increased susceptibility to infection, resulting in death (3). Macrophage activation in phagocytosis and subsequent antigen presentation are involved in immune development, and the capacity of macrophages to be activated is indicative of host immune potential (4). Serum vitamin D3-binding protein (Gc protein) is the precursor for the principal macrophage activating factor (MAF). The MAF precursor activity of the serum Gc protein of various cancer patients, including oral cancer patients, was lost or reduced since the Gc protein is deglycosylated by serum α-N-acetyl galactosaminidase (Nagalase) secreted from cancer cells (5). Deglycosylated Gc protein cannot be converted to MAF, leading to immunosuppression. Administration of the Gc protein-derived MAF (GcMAF) that was generated enzymatically in vitro from the Gc protein in human serum, bypasses the impaired macrophage activation cascade and efficiently activates macrophages. Highly activated macrophages have been reported to have a tumoricidal potential (6-9). Pilot studies have reported the efficacy of GcMAF-based immunotherapy of metastatic cancer in animal and humans (10-13).

The aim of the present study was to investigate whether or not GcMAF has an inhibitory effect on oral carcinogenesis.
and tumor growth, using a 9,10-dimethyl-1,2-benzanthracene (DMBA)-induced hamster cheek pouch carcinogenesis model. The cytotoxic effect of GcMAF on its derived squamous carcinoma cell line, HCPC-1, was also been examined, as well as the possible combination of immunotherapy with GcMAF for oral cancer.

Materials and methods

Preparation of GcMAF. Human serum was heat-inactivated at 60°C for 1 h and Gc protein fraction was precipitated by mixing with 30% saturated ammonium (14). The precipitate was dissolved in phosphate-buffered saline (PBS) (pH 7.4) containing 0.5% Triton X-100 and 0.3% tri-n-butyl phosphate, and was maintained overnight at room temperature to resolve the lipid containing microbial contaminants. The sample was precipitated by 30% saturated ammonium sulfate, dissolved in 50 mM citrate buffer at pH 4.0 and maintained overnight. Gc protein was purified using 25-hydroxyvitamin D3-affinity chromatography (15). Stepwise digestion of purified Gc protein with immobilized β-galactosidase and sialidase yielded the most potent macrophage activating factor (GcMAF) (16,17).

The immobilized enzymes were removed by centrifugation. The final product, GcMAF, was filtered through a low protein-binding filter, Millex-HV (Millipore Corp., Bedford, MA, USA) for sterilization.

Animals, carcinogen treatment and GcMAF administration. A total of 29 male golden Syrian hamsters, at 5 weeks of age, were purchased from Nihon Animal Inc. (Osaka, Japan). The animals were divided into two groups: 14 hamsters with GcMAF administration by intramuscular injection with 100 pg/hamster to the thigh twice a week from the beginning of DMBA application, while 15 hamsters without GcMAF administration served as controls (Fig. 1). The GcMAF dose of 100 pg/hamster was employed according to the study reported previously in mice bearing Ehrlich ascites tumor (10). These hamsters were treated under ether anesthesia by painting a cheek pouch three times a week with 1% solution of DMBA (Wako Pure Chemical Industries, Ltd., Osaka, Japan) dissolved in acetone, as previously described (18,19). DMBA application was continued until the 13th week. The diameter of tumors formed was measured using calipers and the body weight of the hamsters was simultaneously measured once a week.

From the 13th week, when tumors had formed on the cheek pouches of all of the hamsters, GcMAF administration was started by intramuscular injection with 100 pg/hamster twice a week in 5 out of the 15 hamsters in the control group (late administration of GcMAF) and stopped in 4 out of the 14 hamsters in the GcMAF-treated group (early administration of GcMAF).

Superoxide generation assay. Peritoneal cells were collected by peritoneal lavage with cold PBS, washed three times in cold PBS and plated in a 16-mm multiwell plate in DMEM (Gibco-BRL, Grand Island, NY, USA) supplemented with 1% fetal bovine serum (PBS; Hyclone Laboratories, South Logan, UT, USA). The cells were incubated at 37°C for 30 min to facilitate the adherence of macrophages to plastic substrate and then washed with PBS gently to remove non-adherent cells. Various concentrations of GcMAF were added and the cells were incubated at 37°C for 3 h. To measure the activation of macrophages, the culture medium was replaced with 1 ml of PBS containing 20 µg of cytochrome C (Sigma-Aldrich Co., St. Louis, MO, USA) and incubated for 10 min. Approximately 30 min after the addition of 10 µl PBS containing 0.5 µg of phorbol 12-myristyl acetate (PMA; Sigma-Aldrich Co.), the superoxide-generating activity of macrophages was determined by measuring the absorbance at 550 nm.
For the \textit{in vivo} activation assay of macrophages, GcMAF (100 pg/hamster) was injected into the thigh of hamsters intramuscularly. The peritoneal cells were harvested 48 to 96 h after injection and assayed for superoxide generation as described above.

\textbf{Culture of hamster HCPC-1 cells and treatment with GcMAF-activated macrophages.} HCPC-1 cells were isolated and established from the 7,12-dimethylbenz(\(\alpha\))anthracene-induced epidermoid carcinoma of golden Syrian hamster cheek pouch. The cells were kindly provided by Dr G. Shklar (Harvard School of Dental Medicine, Boston, MA, USA) (20). To examine the cytocidal effect of GcMAF-activated macrophages on HCPC-1 cells, the cells were plated at a density of \(10^5\) cells/well in DMEM supplemented with 10% FBS in a multiwell plate and incubated at 37\(^\circ\)C for 24 h. Non-activated macrophages or macrophages activated with GcMAF \textit{in vitro} or \textit{in vivo} were then added at the effector to target ratio (E:T ratio) of 5:1 and further incubated at 37\(^\circ\)C for 48 h. Non-activated macrophages or macrophages activated with GcMAF \textit{in vitro} or \textit{in vivo} were then added at the effector to target ratio (E:T ratio) of 5:1 and further incubated at 37\(^\circ\)C for 48 h. Peritoneal macrophages were activated either by intramuscular injections with 100 pg GcMAF twice (1 and 4 days prior to collection) \textit{in vivo} or by the addition of 100 pg GcMAF to the medium and incubation at 37\(^\circ\)C for 1 h \textit{in vitro}. Viable HCPC-1 cells were counted at constant intervals by the nigrosin exclusion test in a hemocytometer.

The effect of the addition of heat-inactivated DMBA-induced tumor-bearing hamster serum on macrophage-directed cytotoxicity with GcMAF in HCPC-1 cells was also studied, since it was reported that the tumoricidal activity of activated macrophages was markedly enhanced by the addition of tumor-bearing patient serum in the human retinoblastoma cell line W24 (6).

The tumor-bearing hamster serum, \(~\)20 weeks after DMBA application, was collected from 5 hamsters and mixed together just before the experiment. The tumor-bearing or normal hamster serum was added to the culture medium at the final concentration of 5\% after heat-inactivation at 56\(^\circ\)C for 30 min.

\textbf{Table I. Effect of GcMAF administration on DMBA-induced hamster cheek pouch carcinogenesis.}

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tumor prevalence (%)</th>
<th>Onset of tumor formation (weeks)</th>
<th>Tumor diameter (mm) 13th week</th>
<th>Tumor diameter (mm) 16th week</th>
<th>Tumor death (%)</th>
<th>Mean survival time (weeks)</th>
</tr>
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<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GcMAF-no administration (n=10)</td>
<td>10/10 (100)</td>
<td>9.9±0.9</td>
<td>5.3±3.9</td>
<td>17.9±5.0</td>
<td>3.38</td>
<td>10/10 (100)</td>
</tr>
<tr>
<td>GcMAF-late administration (n=5)</td>
<td>5/5 (100)</td>
<td>3.0±1.5</td>
<td>8.0±2.9</td>
<td>2.67</td>
<td>5/5 (100)</td>
<td>17.4±0.5(b)</td>
</tr>
<tr>
<td>GcMAF-early administration (n=4)</td>
<td>4/4 (100)</td>
<td>13.4±0.8</td>
<td>2.3±1.8</td>
<td>7.7±2.7</td>
<td>3.35</td>
<td>0/4 (0)</td>
</tr>
<tr>
<td>GcMAF-administration (n=10)</td>
<td>8/10 (80)</td>
<td>1.5±1.2</td>
<td>2.8±2.5</td>
<td>1.87(a)</td>
<td>0/8 (0)</td>
<td>(&gt;20)(c)</td>
</tr>
</tbody>
</table>

Data are the average ± SD. \(p<0.05, b p<0.01, c p<0.005\).

\textbf{Statistical analysis.} Statistical analysis of the data was performed by using the Student's t-test. P<0.05 was considered to be statistically significant.

\textbf{Results}

\textbf{Suppression of carcinogenesis and tumor growth by GcMAF administration.} SCC was produced from the 9th to the 11th week after DMBA application in all 15 hamsters of the control group without GcMAF administration, and all died of tumor burden within 20 weeks. Out of the 14 hamsters, 2 animals with GcMAF administration did not develop tumors, while the remaining 12 hamsters showed a significant delay of tumor development for \(~3.5\) weeks in addition to suppressed tumor growth. These 12 hamsters survived until the 20th week of the experimental period (Table I and Fig. 2). The body weight loss associated with tumor burden was significantly higher in the control group than that in the GcMAF-treated group (Fig. 3). When GcMAF administration was started from the 13th to the 20th week in 5 tumor-bearing hamsters in the control group, tumor growth was slightly suppressed and all of the hamsters died of tumor burden; however, body weight loss was significantly inhibited and the mean survival time was extended. In contrast, when GcMAF administration was stopped in the 13th week in 4 tumor-bearing hamsters in the GcMAF-treated group, tumor growth and body weight loss were promoted, but none of the hamsters died within the 20-week period (Table I, Figs. 2 and 3).

\textbf{Macrophage activation with GcMAF assayed by superoxide generation.} When hamster peritoneal macrophages were treated with various concentrations of GcMAF \textit{in vitro}, superoxide generation was increased 9-fold in a dose-dependent manner, indicating that the macrophage activation needed to cause efficient tumoricidal effect (Table II). Intramuscular administration of GcMAF to hamsters also showed an \(~3\)-fold increase of superoxide generation in macrophages as compared to the control at 48 and 96 h post injection.
Figure 2. Tumor growth curves in treatment with or without GcMAF. Hamsters were divided into two groups with or without GcMAF until the 13th week and then subdivided into four groups as shown in Fig. 1. Tumor diameters were measured until the 20th week. ◼, no administration of GcMAF (n=10); ●, late administration of GcMAF (n=5); ◆, early administration of GcMAF (n=4); ▲, administration of GcMAF (n=10). Data are the average ± SD. *p<0.05; **p<0.01; †p<0.005.

Figure 3. Changes of the body weight of tumor-bearing hamsters with or without GcMAF administration. The body weight of hamsters was measured once a week in each group. ◼, no administration of GcMAF (n=10); ●, late administration of GcMAF (n=5); ◆, early administration of GcMAF (n=4); ▲, administration of GcMAF (n=10). Data are the average ± SD. *p<0.05; **p<0.01; †p<0.005.

Table II. Superoxide generation in macrophages with GcMAF treatment in vitro and in vivo.

<table>
<thead>
<tr>
<th>GcMAF treatment</th>
<th>Generation of superoxide (nmol/min/10^6 cells)</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>In vitro</strong> experiment (treatment with GcMAF at 37°C for 3 h)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GcMAF no administration</td>
<td>0.094±0.02</td>
<td>1.0</td>
</tr>
<tr>
<td>GcMAF (0.1 pg/ml)</td>
<td>0.209±0.04</td>
<td>2.2</td>
</tr>
<tr>
<td>GcMAF (1 pg/ml)</td>
<td>0.335±0.04</td>
<td>3.6</td>
</tr>
<tr>
<td>GcMAF (10 pg/ml)</td>
<td>0.586±0.05</td>
<td>6.2</td>
</tr>
<tr>
<td>GcMAF (100 pg/ml)</td>
<td>0.848±0.06</td>
<td>9.0</td>
</tr>
<tr>
<td><strong>In vivo</strong> experiment (intramuscular injection of 100 pg of GcMAF/hamster)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GcMAF no administration</td>
<td>0.094±0.02</td>
<td>1.0</td>
</tr>
<tr>
<td>48 h post injection</td>
<td>0.293±0.03</td>
<td>3.1</td>
</tr>
<tr>
<td>96 h post injection</td>
<td>0.324±0.05</td>
<td>3.4</td>
</tr>
</tbody>
</table>

Peritoneal macrophages were treated with various concentrations of GcMAF in vitro at 37°C for 3 h and superoxide generated was assayed as described in Materials and methods. In the in vivo experiment, GcMAF 100 pg/hamster was intramuscularly injected into the thigh of each hamster and superoxide generation was assayed for peritoneal macrophages harvested 48 and 96 h after injection (n=6).

Table III. Cytocidal effect of peritoneal macrophages activated with GcMAF in vitro on HCPC-1 cells.

<table>
<thead>
<tr>
<th>Macrophages (Mφ) treated</th>
<th>No. of viable HCPC-1 cells (x10^4)</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100.3±10.0</td>
<td>1.00</td>
</tr>
<tr>
<td>Non-activated Mφ</td>
<td>69.0±4.5</td>
<td>0.69^a</td>
</tr>
<tr>
<td>Activated Mφ with 1 pg of GcMAF</td>
<td>66.5±3.4</td>
<td>0.66^a</td>
</tr>
<tr>
<td>Activated Mφ with 10 pg of GcMAF</td>
<td>62.3±4.6</td>
<td>0.62^a</td>
</tr>
<tr>
<td>Activated Mφ with 100 pg of GcMAF</td>
<td>54.8±5.6</td>
<td>0.55^a</td>
</tr>
<tr>
<td>Activated Mφ with 200 pg of GcMAF</td>
<td>54.3±2.6</td>
<td>0.54^a</td>
</tr>
</tbody>
</table>

The non-activated macrophages (Mφ) or macrophages activated with GcMAF were added to the HCPC-1 culture at an E:T ratio of 5:1 and further incubated at 37°C for 48 h. Peritoneal macrophages were activated by the addition of various concentrations of GcMAF to the medium and incubated at 37°C for 1 h in vitro. Viable HCPC-1 cells were counted by the nigrosin exclusion test in a hemocytometer. Data are the average ± SD (n=4). *p<0.005.
Cytocidal effect of GcMAF-activated macrophages on HCPC-1 cells. The cytocidal effect of GcMAF-activated macrophages was examined by treatment with an E:T ratio of 5:1 at 37°C for 48 h on HCPC-1 cells. As shown in Table III, the number of viable cells was decreased by 69% even with the addition of non-activated macrophages, but decreased to ~55% in the case of macrophages activated with 100 and 200 pg of GcMAF. A similar or stronger effect was obtained by treatment with macrophages activated in vivo as well as in vitro (Table IV). To investigate the effect of tumor-bearing hamster serum on macrophage-directed cytotoxicity in HCPC-1 cells, tumor-bearing hamster serum was added at a final concentration of 5% to the culture medium. As shown in Table V, the tumor-bearing hamster serum was more cytotoxic than normal serum, and the number of viable cells was decreased by 60%. This was a similar level to that obtained through the addition of non-activated macrophages. However, treatment with GcMAF-activated macrophages reduced the viable cells by 46% and the combined treatment with GcMAF-activated macrophages and tumor-bearing hamster serum markedly reduced the viable cells by 11%. HCPC-1 cells attacked by macrophages became round and were eradicated (Fig. 4).

Discussion

Inflammation of cancer tissues induced by the intratumoral administration of Bacillus Calmette-Guerin (BCG) or other bacterial cells has been widely established to result in the regression of local as well as metastasized tumors, suggesting the development of specific immunity against the tumor (21,22). Since cancer tissues comprise alkylphospholipids, inflamed cancer tissues release lysoalkylphospholipids and alkylglycerols (23-26). Lysoalkylphospholipids and alkylglycerols are both potent macrophage-activating agents, and inflammation-derived macrophage activation is the principal macrophage activation process requiring serum vitamin D3-binding protein (Gc Protein) (16,27-31). Hydrolysis of the Gc protein with the inducible membrane β-galactosidase of inflammation-primed B cells as well as the membranous Neu-1 sialidase of T cells generates GcMAF. GcMAF can be generated enzymatically in vitro by the stepwise treatment of highly purified Gc protein with immobilized β-galactosidase and sialidase (10,17,28). Pilot studies have reported GcMAF antitumor effects on a variety of cancers, such as colorectal, breast and prostate cancer (11-13). Moreover, 83% of patients...
with oral SCC were found to have decreased precursor activity of serum Gc protein and, by contrast, increased Nagalase activity, which efficiently deglycosylated Gc protein (5). Therefore, exogenous administration of GcMAF is considered to inhibit oral carcinogenesis and tumor growth. This study aimed to investigate the antitumor effect of GcMAF on oral cancer by using a well-established DMBA-induced cheek pouch carcinogenesis model and its derived carcinoma cell line.

Consequently, administration of GcMAF from the beginning of DMBA application to cheek pouches resulted in decreased tumor prevalence and a significant delay in tumor formation. Tumor growth was retarded significantly and no death due to tumor burden was noted until the 20th week of observation.

On the other hand, all hamsters without GcMAF administration developed tumors by the 11th week and died by the 20th week. This antitumor effect of GcMAF was further evidenced by starting or stopping GcMAF administration halfway through the experiment. On the other hand, tumor growth was slightly suppressed when GcMAF administration was commenced from the halfway point, but the mean survival time was prolonged significantly. On the other hand, stopping GcMAF administration at the halfway point promoted tumor growth, but maintained no tumor death and the mean survival time was extended (Fig. 2 and Table I). In this case, since the mean tumor diameter between two subgroups of 10 hamsters without GcMAF administration and 5 hamsters with late administration of GcMAF differed by more than 2-fold, the results in tumor growth and mean survival time may be biased. However, when the two subgroups of early and late administration of GcMAF were compared, early administration of GcMAF was found to be more effective in the elongation of life span than late administration. In addition, GcMAF administration has no observed side effects and has prevented body weight loss in tumor-bearing animals (Fig. 3). To evaluate the macrophage activation with GcMAF, the generation of superoxide from peritoneal macrophages was measured. Superoxide was efficiently generated in a dose-dependent manner by in vitro treatment with GcMAF. Similarly, in vivo treatment with GcMAF generated superoxide to the same extent at 48 and 96 h post injection (Table II).

Highly activated macrophages with GcMAF have already been reported to have a strong tumoricidal activity (6-9). In order to investigate the antitumor effect of GcMAF on DMBA-induced hamster cheek pouch carcinogenesis, tumoricidal activity of GcMAF-activated macrophages was examined using HCPC-1, which is a squamous carcinoma cell line derived from DMBA-induced cheek pouch carcinoma. Consequently, macrophages activated in vitro and in vivo with GcMAF demonstrated a significant tumoricidal activity on HCPC-1 cells, as compared to non-activated macrophages. This activity was dose-dependent with a plateau in 100 pg administration of GcMAF (Tables III and IV). Since it was reported that the tumoricidal activity of macrophages photodynamically activated with hematoporphyrin derivative was markedly enhanced on human retinoblastoma cells by the addition of retinoblastoma patient serum in culture (6), the effect of the addition of tumor-bearing hamster serum was tested on tumoricidal activity for HCPC-1 cells. As expected, a marked tumoricidal activity for HCPC-1 cells is not as efficacious in oral cancer as compared to other types of cancer. DMBA-induced cheek pouch carcinoma is also well-differentiated SCC. These facts suggest that GcMAF therapy is not as efficacious in oral cancer as compared to other types.
of cancer. In the present study, however, GcMAF exhibited a marked retardation in tumor development and growth was demonstrated by, along with increased survival time, without any noteworthy side effects. GcMAF treatment may therefore have therapeutic potential for oral cancer, as supported by this animal model.

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References