Deglycosylation of Serum Vitamin D₃-binding Protein Leads to Immunosuppression in Cancer Patients

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ABSTRACT

Serum vitamin D₃-binding protein (Gc protein) can be converted by β-galactosidase of B cells and sialidase of T cells to a potent macrophage activating factor, a protein with N-acetylglucosamine as the remaining sugar moiety. Thus, Gc protein is the precursor of the macrophage activating factor (MAF). Treatment of Gc protein with immobilized β-galactosidase and sialidase generates an extremely high titered MAF, GcMAF. When peripheral blood monocytes/macrophages of 52 patients bearing various types of cancer were incubated with 100 pg/ml of GcMAF, the monocytes/macrophages of all patients were efficiently activated. However, the MAF precursor activity of patient plasma Gc protein was found to be severely reduced in about 25% of this patient population. About 45% of the patients had moderately reduced MAF precursor activities. Loss of the precursor activity was found to be due to deglycosylation of plasma Gc protein by α-N-acetylgalactosaminidase detected in the patient’s bloodstream. The source of the enzyme appeared to be cancerous cells. Radiation therapy decreased plasma α-N-acetylgalactosaminidase activity with concomitant increase of precursor activity. This implies that radiation therapy decreases the number of cancerous cells capable of secreting α-N-acetylgalactosaminidase. Both α-N-acetylgalactosaminidase activity and MAF precursor activity of Gc protein in patient bloodstream can serve as diagnostic and prognostic indices.

INTRODUCTION

Cancer patients often suffer from deficiency of immunity and increased susceptibility to infection (1, 2). Susceptibility of cancer patients to infection, with concomitant depression of antibody production, becomes more evident as the disease advances (3). Development of immunity and defense against infection is initiated by macrophage activation that is induced by inflammation resulting from the infection. Membranous lipid metabolites of inflamed normal and cancerous tissues, lysophospholipids and alkylglycerols, are potent macrophage-stimulating agents (4–7). Administration of 5–20-μg doses of lyso-Pc⁷ to mice efficiently activates macrophages to phagocytize target antigens or cells via the Fc-receptor (4–7) and to generate superoxide (8). In vitro treatment of mouse peritoneal macrophages with lyso-Pc did not activate macrophages (2–5). However, treatment of mouse peritoneal cells (mixture of macrophages and lymphocytes) with 1 μg lyso-Pc/ml in a serum-supplemented medium for 30 min followed by 3 h cultivation of macrophages without lymphocytes markedly enhanced the phagocytic (2–5) and superoxide-generating (9, 10) capacities of macrophages, implying a rapid signal transmission from lyso-Pc-treated lymphocytes (B and T cells) to macrophages during the 30-min treatment period. This macrophage activation requires participation of B and T lymphocytes and serum vitamin D₃-binding protein (Gc protein; Refs. 11–14). Efficient activation of macrophages can be achieved by cultivation of lyso-Pc-treated peritoneal cells in a medium containing purified human Gc protein (11–13). Lyso-Pc-stimulated B cells modify Gc protein to yield a proactivating factor that can be converted by T cells to the MAF (12, 13). This rapid generation process of the MAF was found to be a consequence of stepwise hydrolysis of Gc glycoprotein by β-galactosidase of lyso-Pc-stimulated B cells and sialidase of T cells (13, 14), as illustrated in Fig. 1a. Thus, Gc protein is a precursor for the MAF (13, 14). Treatment of Gc protein with immobilized commercial β-galactosidase and sialidase efficiently generated an extremely high titered MAF (14, 15). Administration of a minute amount (4–10 pg/mouse; 30 ng/human) of the GcMAF resulted in a greatly enhanced (3–7-fold) phagocytic capacity of macrophages (13–15).

Rapid development of the potent MAF during inflammation via modification of serum Gc protein by stimulated lymphocytes as compared with de novo synthesis of lymphokines seems to be an emergency shunt for the host defense response in infectious and inflammatory diseases and appears to be the major inflammation-primed macrophage activation cascade. Because the inflammation-primed macrophage activation is the first step of the immune developmental process, immune responsive cells (lymphocytes and phagocytes) and Gc protein in immunosuppressed cancer patients need to be characterized. In this communication, we characterize peripheral blood monocytes/macrophages, lymphocytes, and Gc protein of 52 cancer patients undergoing curative and palliative radiation therapy and discuss the mechanism of immunosuppression in cancer patients.

MATERIALS AND METHODS

Chemicals and Reagents. Lyso-Pc, p-nitrophenyl N-acetyl-α-d-galactosaminide, and p-nitrophenyl N-acetyl-α-g-glucosaminide were purchased from Sigma Chemical Co. (St. Louis, MO). Lymphoprep (similar to Ficoll) was obtained from Polysciences, Inc. (Warrington, PA). Human Gc protein (Gc1, a mixture of Gc1f and Gc1s) was purified by vitamin D₃ affinity chromatography (16). Gc1 carries a dibranched trisaccharide with galactose and sialic acid terminals (13–15) and was used throughout this study. GcMAF was prepared by treatment of purified Gc protein with immobilized β-galactosidase and sialidase (14, 15). Lyso-Pc solution, purified Gc protein, and culture media were routinely tested for lipopolysaccharide contamination by using the Limulus amebocyte lysate assay (5).

Peripheral Blood Samples from Cancer Patients. All patient blood samples were collected in tubes containing 0.2% EDTA to prevent coagulation. A 5-ml blood sample and 5 ml saline (0.9% NaCl) were mixed, gently laid on 3 ml Lymphoprep contained in a 15-ml centrifuge tube, and centrifuged at 1000 x g for 15 min. The dense white mononuclear layer containing monocytes/macrophages (phagocytes) and lymphocytes (B and T cells) was collected using Pasteur pipettes, washed twice with 10 mm PBS containing 0.15 M NaCl, suspended in 0.1% egg albumin-supplemented RPMI 1640 (EA medium), and placed in 16-mm wells. Incubation for 45 min in a 5% CO₂ incubator at 37°C allowed adherence of phagocytes to the plastic substrate. Lymphocytes (nonadherent cells) were removed. Phagocytes (2 x 10⁶ to 5 x 10⁶ cells/well) of individual patients were cultured in EA medium supplemented with 100 pg GcMAF/ml for 3 h at 37°C and assayed for superoxide generation of the phagocytes.

Received 1/22/96; accepted 4/16/96.

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1 This investigation was supported in part by United States Public Health Service Grant ROI AI-32140 from the National Institute for Allergy and Infectious Diseases.

2 To whom requests for reprints should be addressed, at the Laboratory of Cancer Immunology and Molecular Biology, Albert Einstein Cancer Center, Korman Research Pavilion B-31, 5501 Old York Road, Philadelphia, PA 19141.

3 The abbreviations used are: lyso-Pc; lysophosphatidylcholine; Gc protein, human vitamin D₃-binding protein (group-specific component); MAF, macrophage activating factor; GcMAF, enzymatically generated macrophage activating factor.

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Mammalian Tissue. The 1-ml plasma samples of patients and healthy humans were prepared by centrifugation at 10,000 g for 15 min, then precipitated with 70% saturated ammonium sulfate. The ammonium sulfate precipitates of plasma and tumor homogenates were dissolved in 50-mM citrate phosphate buffer (pH 6.0) and dialyzed against the same buffer at 4°C overnight. The dialysates were made up to 1 ml and assayed for the enzymes found in healthy human and patient plasma and lung tumor homogenates. The resultant products were added to lyso-Pc-treated mouse peritoneal cells in EA medium was determined, and 1-ml aliquots of the cells were placed in the 16-mm-diameter wells of tissue culture plates. The cells were incubated at 37°C in a humidified CO₂ incubator for 30 min to allow the macrophages to adhere to plastic substrata. Lyso-Pc (1 μg/ml) was added to the peritoneal cells in the wells and mixed gently. After 30 min incubation at 37°C, macrophages and lymphocytes were washed separately with PBS to remove residual lyso-Pc. The mixture of lyso-Pc-treated lymphocytes and macrophages was used for assay of the precursor activity of Gc protein or α-N-acetylgalactosaminidase-treated Gc protein.

**Substrate Specificity of α-N-Acetylgalactosaminidase.** To determine the macromolecular substrate specificity of α-N-acetylgalactosaminidase activity for the enzymes found in healthy human and patient plasma and lung tumor tissue, 1 ng of purified Gc protein was incubated with an equal amount (activity, 4 nmol/min, determined by chromogenic substrate) of each enzyme preparation for 60 min. The resultant products were added to lyso-Pc-treated mouse peritoneal cells (5–7), cultured for 3 h at 37°C, and assayed for macrophage activation as determined by superoxide-generating capacity (9, 10).

If Gc protein is enzymatically deglycosylated, it loses precursor activity and cannot be converted to MAF. The decreased precursor activity of the Gc protein is expressed by decreased superoxide generating capacity. In contrast, if the enzyme is unable to deglycosylate Gc protein, unaffected Gc protein can be efficiently converted to MAF.

**RESULTS**

**Immunological Responsiveness of Patient Peripheral Blood Monocytes/Macrophages, Lymphocytes, and Gc Protein.** For characterization of monocytes/macrophages (phagocytes), lymphocytes (B and T cells), and Gc protein in cancer patients, peripheral blood samples were collected from patients with various types of cancer who were undergoing radiation therapy. When peripheral blood phagocytes of patients were incubated with 100 pg/ml of GcMAF at 37°C for 3 h, the phagocytes of all cancer patients were efficiently activated and produced more than 5.0 nmol of superoxide/min/10⁶ phagocytes, as did those of healthy humans (Table 1). When a mixture of lymphocytes and phagocytes of individual patients was treated with 1 μg lyso-Pc/ml for 30 min and cultured in a medium supplemented with Gc protein (1 ng/ml) for 3 h, the phagocytes of all cancer patients produced more than 4.0 nmol of superoxide/min/10⁶ phagocytes (Table 1). This observation indicates that the B and T lymphocytes of all cancer patients are capable of generating MAF. However, when the mixture of lyso-Pc-treated lymphocytes and phagocytes of individual patients was cultured in a medium supplemented only with their own plasma (0.1%) for 3 h, the phagocytes were not activated in approximately 25% of the patient population and typically produced less than 1.0 nmol of superoxide/min/10⁶ phagocytes (Table 1). Evidently, the MAF precursor activity of the plasma Gc protein of certain cancer patients was deficient. With this analysis, about 45% of the patients had moderately reduced MAF precursor activities, which produced 1.5–3.9 nmol of superoxide/min/10⁶
phagocytes. The remaining (30%) cancer patients had high MAF precursor activity, similar to that of healthy humans. The results of 52 cancer patients are exemplified by the first 20 patients shown in Table 1. Although phagocytes and lymphocytes of all the cancer patients were capable of development of phagocyte activation, about 70% of the patient population had lost or reduced MAF precursor activity of their plasma Gc protein, as can be seen in the last column of Table 1. This result was reproduced when a mixture of lymphocytes and phagocytes of a healthy human was treated with 1 μg lys-PC and cultured in medium supplemented with 0.1% patient plasma.

**Detection of N-Acetylgalactosaminidases in Patient Plasma.** When the Gc protein content of patient plasma was analyzed by Western blot, no quantitative change was detectable as compared with that of a healthy human. Deficiency of the precursor activity of patient plasma Gc protein led us to suggest deglycosylation of the Gc protein (Fig. 1b). We thus postulated the presence of endo-N-acetylgalactosaminidases in the blood of some cancer patients. To test this idea, cancer patient and healthy human plasma were precipitated with 70% saturated ammonium sulfate. The precipitates were dissolved and dialyzed in 50 mM citrate buffer (pH 6.0) and assayed for N-acetylgalactosaminidases. As shown in Table 2, all cancer patients and healthy humans carried similar activity levels (about 20.6 ± 3.1 nmol/min of β-N-acetylgalactosaminidase. However, healthy humans had very low α-N-acetylgalactosaminidase activities ranging from 0.2 to 0.4 nmol/min. In contrast, patients who had a severely reduced precursor activity of plasma Gc protein demonstrated α-N-acetylgalactosaminidase activities ranging from 2.1 to 5.2 nmol/min in their plasma. Patients having moderately reduced precursor activity of plasma Gc protein had α-N-acetylgalactosaminidase activities ranging from 1.10 to 1.89 nmol/min in their plasma. Patients having high precursor activity of plasma Gc protein had low α-N-acetylgalactosaminidase activities ranging from 0.64 to 0.99 nmol/min in their plasma. Thus, the precursor activity of plasma Gc protein showed an inverse correlation with α-N-acetylgalactosaminidase activity in the patient plasma but no correlation with β-N-acetylgalactosaminidase activity, as can be seen in Table 2. Fig. 2a illustrates typical representation of such results with these two enzymes. Because both cancer patients and healthy humans had about the same range of plasma β-N-acetylgalactosaminidase activity, this enzyme seems unlikely to be involved in deglycosylation of Gc protein. In fact Gc protein is known to be O-glycosylated (19, 20), suggesting that Gc protein would be particularly susceptible to deglycosylation by α-N-acetylgalactosaminidase.

**Substrate Specificity of the α-N-Acetylgalactosaminidase.** Gc protein was incubated with an equal amount (activity, 4 nmol/min determined with chromogenic substrate) of the enzyme of cancer patient and healthy human plasma for 60 min and bioassayed for precursor activity (determined by superoxide generating capacity of mouse macrophages). Patient enzyme-treated Gc protein showed a 68% reduced precursor activity as compared with that of native Gc protein. It seems likely that the enzyme activity in healthy human plasma is the activity of either another isozyme or α-galactosidase. The latter enzyme is able to share the same chromogenic substrate with α-N-acetylgalactosaminidase because these enzymes carry 46.9% amino acid sequence homology and their genes are derived from the same progenitor (17, 18). The macromolecular substrate, Gc protein, seems to distinguish these enzymes. Therefore, plasma α-N-acetylgalactosaminidase was detected in all the cancer patients we studied.

**Demonstration of α-N-Acetylgalactosaminidase in Tumor Tissue.** Fresh postoperative tumor tissues from another source were homogenized in 15 mM Tris-HCl (pH 7.0). The homogenates were treated with 70% saturated ammonium sulfate, and the precipitates were dissolved and dialyzed in 50 mM citrate phosphate buffer (pH 6.0) at 4°C. Both β-N-acetylgalactosaminidase and α-N-acetylgalactosaminidase were detected in tumor tissue homogenates as represented by enzyme activities (at 41.5 and 32.1 nmol/mg/min, respectively) of a lung tumor tissue in Fig. 2a. Furthermore, the latter

### Table 1 Characterization of monocytes/macrophages, lymphocytes, and Gc protein of individual cancer patients

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Cancer type</th>
<th>Assayed on phagocytes</th>
<th>Assayed on lymphocytes</th>
<th>Specific activity (nmol/mg/min)</th>
<th>Precursor activity (nmol/min/10^6 phagocytes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Prostate ca.</td>
<td>0.10</td>
<td>0.10</td>
<td>2.34</td>
<td>0.36</td>
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<tr>
<td>2</td>
<td>Lung ca.</td>
<td>0.14</td>
<td>0.14</td>
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<tr>
<td>3</td>
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<tr>
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<td>6</td>
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<tr>
<td>7</td>
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<td>0.35</td>
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<td>8</td>
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<td>9</td>
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<td>1.90</td>
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<td>0.50</td>
<td>4.00</td>
<td>2.10</td>
</tr>
<tr>
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<td>0.55</td>
<td>4.25</td>
<td>2.30</td>
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<tr>
<td>12</td>
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<td>0.60</td>
<td>4.50</td>
<td>2.50</td>
</tr>
<tr>
<td>13</td>
<td>Prostate squamous cell ca.</td>
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<td>0.65</td>
<td>4.75</td>
<td>2.70</td>
</tr>
<tr>
<td>14</td>
<td>Cervix squamous cell ca.</td>
<td>0.70</td>
<td>0.70</td>
<td>5.00</td>
<td>2.90</td>
</tr>
<tr>
<td>15</td>
<td>Prostate ca.</td>
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<td>0.75</td>
<td>5.25</td>
<td>3.10</td>
</tr>
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<td>16</td>
<td>Prostate ca.</td>
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<td>0.80</td>
<td>5.50</td>
<td>3.30</td>
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<tr>
<td>17</td>
<td>Prostate ca.</td>
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<td>0.85</td>
<td>5.75</td>
<td>3.50</td>
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<td>0.90</td>
<td>6.00</td>
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<tr>
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<td>0.95</td>
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<td>3.90</td>
</tr>
<tr>
<td>20</td>
<td>Prostate ca.</td>
<td>1.00</td>
<td>1.00</td>
<td>6.50</td>
<td>4.10</td>
</tr>
<tr>
<td>C Healthy human</td>
<td>0.76</td>
<td>0.76</td>
<td>0.76</td>
<td>4.62</td>
<td>2.83</td>
</tr>
</tbody>
</table>

* All values indicate mean of duplicate assays.

**a** Phagocytes (monocytes/macrophages) were lys-PC-untreated.

**b** Mixture of lyso-PC-treated lymphocytes and phagocytes.

**c**. Average of 5 healthy humans.

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* DEGLYCOSYLATED Gc PROTEIN AND IMMUNOSUPPRESSION

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or patient 2 or lung tumor tissue, incubated with lyso-Pc-treated mouse peritoneal cells for...enzyme activities in plasma of a healthy human and patient enzyme was able to inactivate the MAF precursor activity of Gc protein. When Gc protein content in patient plasma was analyzed by Western blot, no quantitative difference in plasma Gc protein concentration of patients and healthy humans was observed. Thus, the lost or reduced MAF precursor activity of Gc protein in certain cancer patients led us to propose deglycosylation of Gc protein by α-N-acetylgalactosaminidase from cancer patients (Fig. 1b). Deglycosylated Gc protein cannot be converted to MAF and thus lacks the precursor activity. Therefore, macrophage activation cannot develop in certain cancer patients. Because macrophage activation is the first step in the inflammation-primed immune development cascade, such cancer patients become immunosuppressed. This may explain at least in part why cancer patients die from overwhelming infection.

An impairment of immune function of the hosts will enhance opportunities for survival and proliferation of neoplastic cells (21). HIV-infected/AIDS patients also carry α-N-acetylgalactosaminidase in their bloodstream (22). Because this enzyme deglycosylates plasma Gc protein leading to immunosuppression in the hosts, AIDs patients are at extremely high risk for development of various forms of malignant tumors (23).

In tumor invasion, cancer cells produce extracellular-matrix degrading enzymes, various proteases and exo- and endoglycosidases (24-26). Secretion of endoglycosidases from cancerous cells into the peripheral blood can result in deglycosylation of Gc protein in plasma. Indeed, deglycosylation of plasma Gc protein appeared to be due to the presence of α-N-acetylgalactosaminidase in patient bloodstream (see Fig. 1b). Consequently, the precursor activity of plasma Gc protein decreased. Thus, the gradual recovery of the precursor activity of patient plasma Gc protein during radiation therapy can be explained by reduction in the number of cancerous cells capable of secreting α-N-acetylgalactosaminidase. In a separate study, we observed that surgical removal of malignant lesions results in subtle decrease of plasma α-N-acetylgalactosaminidase activity, particularly if malignant cells are localized. In a mouse tumor model, we found that plasma α-N-acetylgalactosaminidase activity is directly proportional to the number of the transplanted tumor cells (27).

### Table 3 Effect of radiation therapy on the MAF precursor activity of patient plasma Gc protein and α-N-acetylgalactosaminidase activity in bloodstream

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Day</th>
<th>Precursor activity, 0.1% plasma-lymphocytes/phagocytes</th>
<th>α-N-acetylgalactosaminidase specific activity (nmol/mg/min)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>Day 0</td>
<td>0.56</td>
<td>3.64</td>
</tr>
<tr>
<td></td>
<td>Day 7</td>
<td>0.87</td>
<td>3.21</td>
</tr>
<tr>
<td></td>
<td>Day 14</td>
<td>1.04</td>
<td>2.68</td>
</tr>
<tr>
<td>2</td>
<td>Day 0</td>
<td>0.89</td>
<td>5.21</td>
</tr>
<tr>
<td></td>
<td>Day 7</td>
<td>1.90</td>
<td>2.28</td>
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<td>Day 14</td>
<td>4.42</td>
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<td>3</td>
<td>Day 0</td>
<td>3.96</td>
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<td>Day 7</td>
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<tr>
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<td>Day 0</td>
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<td></td>
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<td>0.47</td>
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<tr>
<td>12</td>
<td>Day 0</td>
<td>2.98</td>
<td>3.64</td>
</tr>
<tr>
<td></td>
<td>Day 7</td>
<td>1.89</td>
<td>0.74</td>
</tr>
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α Radiation doses, 1.5-3.0 Gy/day. All values indicate mean of duplicate assays.

DISCUSSION

All 52 cancer patients, but not the healthy humans, carried plasma α-N-acetylglactosaminidase. This plasma enzyme inactivated the MAF precursor activity of Gc protein. When Gc protein content in patient plasma was analyzed by Western blot, no quantitative difference in plasma Gc protein concentration of patients and healthy humans was observed. Thus, the lost or reduced MAF precursor activity of Gc protein in certain cancer patients led us to propose deglycosylation of Gc protein by α-N-acetylgalactosaminidase from cancer patients (Fig. 1b). Deglycosylated Gc protein cannot be converted to MAF and thus lacks the precursor activity. Therefore, macrophage activation cannot develop in certain cancer patients. Because macrophage activation is the first step in the inflammation-primed immune development cascade, such cancer patients become immunosuppressed. This may explain at least in part why cancer patients die from overwhelming infection.

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<td>2</td>
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Various types of tumors contained various levels of \( \alpha-N \)-acetyl-
galactosaminidase activity. It seems likely that secretory capacity of
individual tumor tissue for \( \alpha-N \)-acetylgalactosaminidase varies among
tumor types depending upon tumor size, staging, and the degree of
malignancy or invasiveness. This would result in various degrees of
the MAF precursor activity of patient plasma Gc protein. Despite
coexistence of \( \alpha-N \)-acetylglucosaminidase and Gc protein in patient
plasma, the enzyme and precursor activities were highly reproducible
(\(<5\%\) deviation) even after prolonged storage at least up to 1 month.
This was found to be due to the presence of the product inhibitor in
patient plasma. This reproducibility of the enzyme and precursor activities
allowed us to assay stored plasma and to design the time
course study of radiation therapy by assaying patient plasma collected
at different times. Therefore, both \( \alpha-N \)-acetylglucosaminidase activity
and MAF precursor activity of individual patients may serve as
diagnostic and prognostic indices. These biochemical measurements
could be readily performed in a clinical laboratory for assessment of
cancer patients and their immune status.

ACKNOWLEDGMENTS

We thank Dr. Sidney Weinhouse for his critical advice, guidance, and
interest in our work.

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Deglycosylation of Serum Vitamin D₃-binding Protein Leads to Immunosuppression in Cancer Patients

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