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Review Article

Alpha-N-acetylgalactosaminidase in cancer: diagnostic applications and related treatment strategies

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Abstract: Alpha-N-acetylgalactosaminidase (nagalase), a lysosomal enzyme encoded by the NAGA gene, plays a critical role in the degradation of glycoconjugates, modulation of immune responses, and regulation of vitamin D metabolism. Dysregulation of nagalase is associated with several pathological conditions, including Schindler disease, psychiatric disorders, viral infections, and notably, cancer. Elevated serum levels of nagalase, particularly the Naga6 isoform, have been observed in cancer patients and individuals with enveloped viral infections, contributing to immune evasion by impairing macrophage activation through Gc protein deglycosylation. Moreover, nagalase activity has been implicated in rare blood group changes observed in some malignancies. Although ELISA-based assays offer potential for quantifying nagalase, their clinical application is hindered by assay interferences and cross-reactivity. The immunotherapeutic potential of Gc protein-derived macrophage activating factor (GcMAF), in combination with vitamin D3 and ascorbate, has shown promise in enhancing anti-tumor immunity, particularly in prostate cancer. Nevertheless, conflicting data and methodological criticisms have led to skepticism regarding its efficacy. This review comprehensively explores the biochemical variants of nagalase, its physiological and pathological roles, its diagnostic utility as a biomarker, and emerging therapeutic strategies targeting its activity, including gene silencing and monoclonal antibody development. The findings underscore the need for rigorous clinical studies to validate the diagnostic and therapeutic potential of nagalase in oncology and immunology.

Keywords: Alpha-N-acetylgalactosaminidase, malignancy, macrophage activating factor, immunotherapy, cancer

Introduction

Lysosomes are particular organelles that are responsible for the degradation and recycling of proteins, glycolipids, and other components found both within and outside of the cell [1]. Alpha-N-acetylgalactosaminidase or nagalase, encoded by the NAGA gene, is a lysosomal enzyme found in various human tissues and organ systems. It also occurs in a diverse range of organisms, including terrestrial mammals, avians, invertebrates, and certain marine species, but notably, it is absent in the plant kingdom [2, 3]. Nagalase is important for breaking down glycoconjugates, especially by removing specific sugar molecules from glycopeptides and glycolipids. Functioning optimally within the acidic pH range of 4.5 to 5.5, this enzyme participates in several physiological processes. These processes involve breaking down vitamin D3, cleaning up cell parts, removing unwanted cells, helping with bone changes, and adjusting how the immune system works [3]. NAGA's immune modulatory activity regulates macrophage activation, neutrophil chemotaxis, and superoxide generation while inhibiting circulating G-actin. Additionally, its anti-angiogenic and anti-tumorigenic properties are significant in immune surveillance. Various studies have proven that the elevation of nagalase levels is mainly attributable to the release of active nagalase by malignant cells,

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which subsequently leads to immunosuppression [1, 3-5].

Different types of cancers and their respective severities significantly influence plasma nagalase levels. In general, more aggressive and metastatic tumors tend to release higher concentrations of nagalase into the bloodstream. This elevation is primarily attributed to the increased secretion of the Naga6 isoform by malignant cells, which facilitates immune evasion through the deglycosylation and inactivation of Gc protein-derived macrophage activating factor (GcMAF). Tumors with higher proliferation rates and greater metastatic potential - such as advanced prostate, breast, or gastrointestinal cancers - often exhibit markedly elevated nagalase levels, correlating with tumor burden and disease progression. Furthermore, the tumor microenvironment, characterized by hypoxia and altered pH, may enhance nagalase expression and activity. Thus, plasma nagalase concentration serves not only as a potential biomarker for tumor presence but also as an indirect indicator of cancer aggressiveness and immune suppression status.

Nagalase involvement in various clinical complications

Nagalase, a key player in a multitude of physiological processes, has been linked to various clinical manifestations and infections when dysregulated. Schindler disease, another disorder resulting from mutations in the NAGA gene, presents a broad spectrum of clinical complications. The severity of the disease dictates its classification into three distinct phenotypes: Type I, also known as the infantile form; Type II, or Kanzaki; disease and Type III Schindler disease exhibits an intermediate clinical severity between Types I and II. In these diseases, a wide range of symptoms can be seen, including neurodegenerative progression, developmental regression, visual impairment, seizures, severe cognitive decline, sensorineural hearing loss, and peripheral neuropathy. Dermatological symptoms such as angiokeratomas and, etc.

In Schindler disease, a reduced concentration of nagalase leads to the accumulation of specific glycoproteins within the brain's lysosomes during gestation. This may result in clinical symptoms associated with bipolar disorder or schizophrenia. While the deficiency of this enzyme is not the sole causative factor, it plays a significant role in the pathogenesis of these conditions [6, 7].

On the other hand, nagalase's role in vitamin D3 metabolism intersects with the function of Gc-globulin, a vitamin D transporter. Together, nagalase and Gc-globulin work in tandem to maintain immune regulation and vitamin D homeostasis. The proper level of vitamin D is crucial for optimal immune function, and any disruption in its bioavailability can lead to immune suppression [8]. For instance, in severe cases of COVID-19, a dysregulation of Gc-globulin has been observed, which is associated with immunosuppression and an exacerbation of inflammatory responses [3, 9].

Research in the late 20th century by Japanese scientist Nobuto Yamamoto suggested that certain enzymes are produced by cancerous cells and cells infected by enveloped viruses, including HIV-infected cells. These enzymes impact macrophages, the body's first line of defense against cancer and viral infections [10]. Macrophages express Gc protein receptors on their surface, which play a crucial role in disease detection. The Gc protein, identified in blood by Hershfield, exhibits approximately 120 isoforms, with three polymorphic variants predominating in the human population [3]. Nagalase, secreted from cancer cells or virus-infected cells, deglycosylates the Gc proteins, thereby inhibiting or disrupting macrophage activation [11]. This results in immunodeficiency in individuals with advanced neoplasms and HIV infection [12]. The elevated level of nagalase observed in cancer patients is primarily due to the release of active nagalase by malignant cells, contributing to immunosuppression [13, 14].

Nagalase and cancer

Cancer is one of the most common causes of disability and mortality throughout the globe, with roughly 19.3 million new diagnoses expected to be made across the globe in the year 2020, based on the most recent data that is currently available. Metastasis is a mechanism that includes a series of alterations that makes cancer a highly complicated disease. In general, this sickness accumulates in an identified tissue or organ, and later on, it expands to

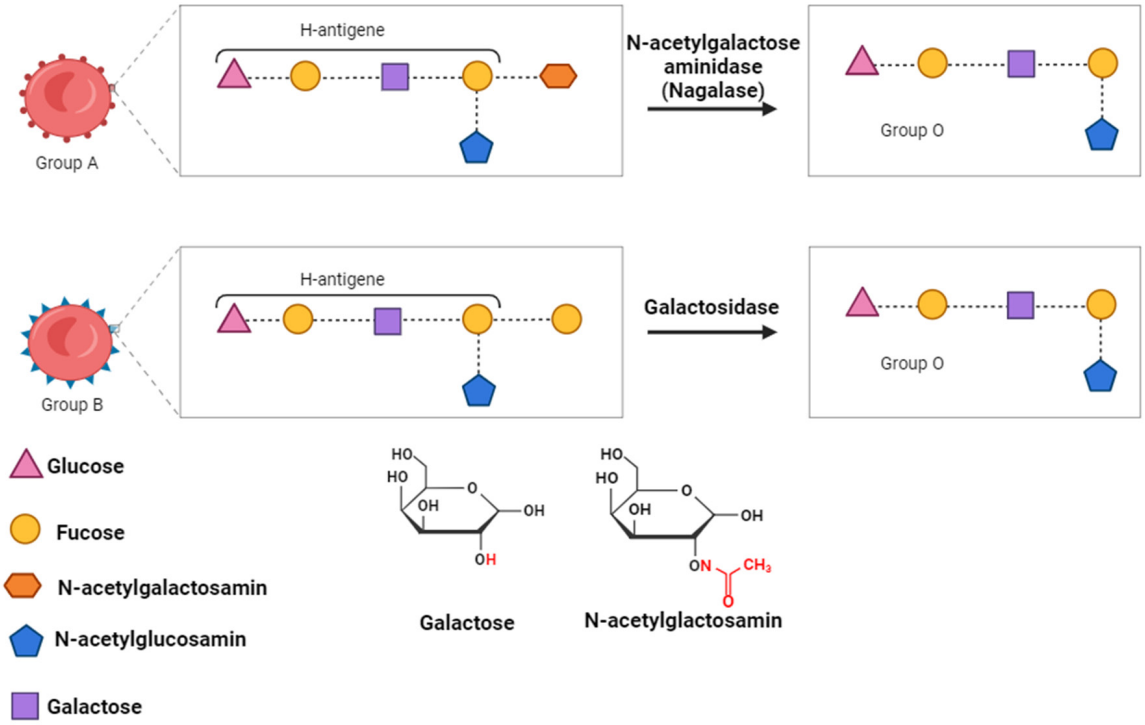

Figure 1. α-N-acetylgalactosaminidase (nagalase) and galactosidase are enzymes essential for the synthesis of surface antigens on erythrocytes that determine blood group phenotypes. If these enzymes remove the N-acetylgalactosamine and galactose residues. Created by biorender.com.

other distant regions. Metastasis is a process that occurs. Cancer patients have access to a variety of treatment options, including surgical removal of the tumor, chemotherapy, radiation therapy, immunotherapy, targeted medicines, and hormone therapies [15, 16]. The majority of patients, on the other hand, have a combination of surgical procedures and either chemotherapy or radiation therapy. The majority of chemotherapeutic drugs have a mechanism of action that is based on interference with cell proliferation. This interference is used to prevent cancer cells from randomly dividing without proper regulation. Nevertheless, normal cells are also in a phase of continual multiplication, which means that an antineoplastic will not be able to differentiate between a healthy cell and a malignant cell, which will result in very severe adverse reactions during therapy [1, 5].

Blood group alterations in cancer patients: unraveling the role of nagalase

An interesting implication of nagalases is their involvement in blood group shifts observed sporadically in cancer patients. Most cases don't have reliable information because of unusual blood cell activity, which can happen in diseases like chronic myeloid leukemia and acute lymphoblastic leukemia [17-20], but there are also reports of this in solid tumors like oral and breast cancers [21-23]. The primary transition occurs from blood group A to O, with less frequent shifts from RH positive to negative. Despite this, the underlying mechanisms remain poorly understood. However, some scientists have postulated that nagalase, an enzyme capable of modifying glycan structures on erythrocyte surface antigens - specifically targeting N-acetylgalactosamine in blood group may play a pivotal role in these alterations (see **Figure 1**) [24]. More research is needed to find out if higher levels of nagalase are related to changes in blood groups in cancer situations [22, 23].

Nagalase types: a critical point

Yamamoto's research has previously linked specific isoforms of the Gc protein receptor in macrophages to certain neoplasms and viral

infections. Besides the lysosomal α-N-acetylgalactosaminidase, which works best at a pH of 4, tests have found three more nagalase isoforms in the blood. This suggests the potential existence of alternative nagalase isoforms in the serum that function across a range of pH conditions. These isoforms, found in both healthy individuals and cancer patients, have optimal pH values of 5.2, 5.8, and 8. These enzymatic variants, similar to nagalase but distinct in their optimal pH for functionality, may not be detectable under standard assay conditions. However, they could be identified through the adjustment of assay conditions, such as changes in pH [3, 25].

A unique kinetic property distinguishes the Naga 4 and Naga 6 enzymes, which are active at pH 4 and pH 5.8, respectively. Additionally, the speed at which Naga4 works depends on how much serum is present, while Naga6 shows a big drop in activity when the serum pH is high. Yamamoto's research, using a uniform monoclonal antibody for detection, indicates the presence of Naga6 in various cancer types and in healthy individuals, suggesting its role as a normal human protein. Naga6 is hypothesized to be a modified exo-glycohydrolase variant of Naga4. Changes in RNA splicing mechanisms within neoplastic cells could produce Naga4 isoforms with amino acid substitution mutations. Furthermore, aberrant glycosylation, a significant characteristic of cancer, could lead to the development of the Naga6 phenotype. Simon Albracht suggests that the difference between the two forms might be caused by changes in N-glycosylation at six specific sites, which could happen with or without mutations in the amino acid sequence due to different RNA splicing [3].

Nagalase assay challenges and utilities

The Nagalase Assay, as utilized by *Yamamoto*, employs an indirect sandwich ELISA technique to determine nagalase concentrations in blood samples. This method measures the protein levels of the enzyme per milliliter of serum or plasma. Across his publications, the standardized value for nagalase activity was set at 0.25 nmol/mg/min [3, 26]. However, ELISA has some limitations: it cannot be directly applied to plasma or serum samples. This issue arises from the reduction in the antibody's binding affinity to the antigen Naga6 due to the presence of highly charged, low molecular weight substances within the serum. These substances interfere with the antibody-antigen interaction. The epitope recognized by the monoclonal antibody used in ELISA may be a conjugate of protein and carbohydrate moieties. Therefore, the inhibitory substance could be a predominant carbohydrate-rich component of the serum, such as proteoglycans, which include chondroitin 6-sulfates, Cretan sulfate, heparin, dermatan sulfate, or hyaluronate. To overcome this, the identified inhibitor must be extracted from the plasma or serum via ammonium sulfate fractionation before conducting the ELISA assay. The resulting precipitate, with an ammonium sulfate saturation ranging from 30% to 70%, is re-dissolved and subjected to dialysis to remove the inhibitors. It was observed that the activity of Naga6 remained unaltered in serum post-ammonium sulfate treatment [3]. Another challenge with ELISA is the cross-reactivity of the monoclonal antibody with both neoplastic and healthy sources. The synthesized monoclonal antibody, isolated from lung cancer tissue, exhibits cross-reactivity with nagalase molecules derived from both neoplastic and healthy sources. As a result, a specific protein is present in the serum of individuals diagnosed with cancer, as well as in the serum of healthy subjects [27].

A novel approach is the quantification of nagalase concentrations in serum samples. This quantification enables the prognosis of an incipient neoplasm attempting to evade the immune system, which is accompanied by an elevation in Naga6 levels. This assay is useful for individuals afflicted with malignancies and those with infections caused by enveloped viruses, including but not limited to HIV-1, influenza, rubella, measles, and potentially SARS-CoV-2. However, special consideration is needed for the quantified ELISA assay to be applicable, i.e., the patients should not have started any form of therapeutic intervention, or it should have been at least 4 weeks since their last treatment. Using the ELISA method, Yamamoto's research demonstrated that the surgical excision of tumors, which impedes the process of metastasis, leads to a prompt reduction in the levels of nagalase in the serum. Therefore, the serum nagalase concentration is reflective of the cumulative rate of tumor gen-

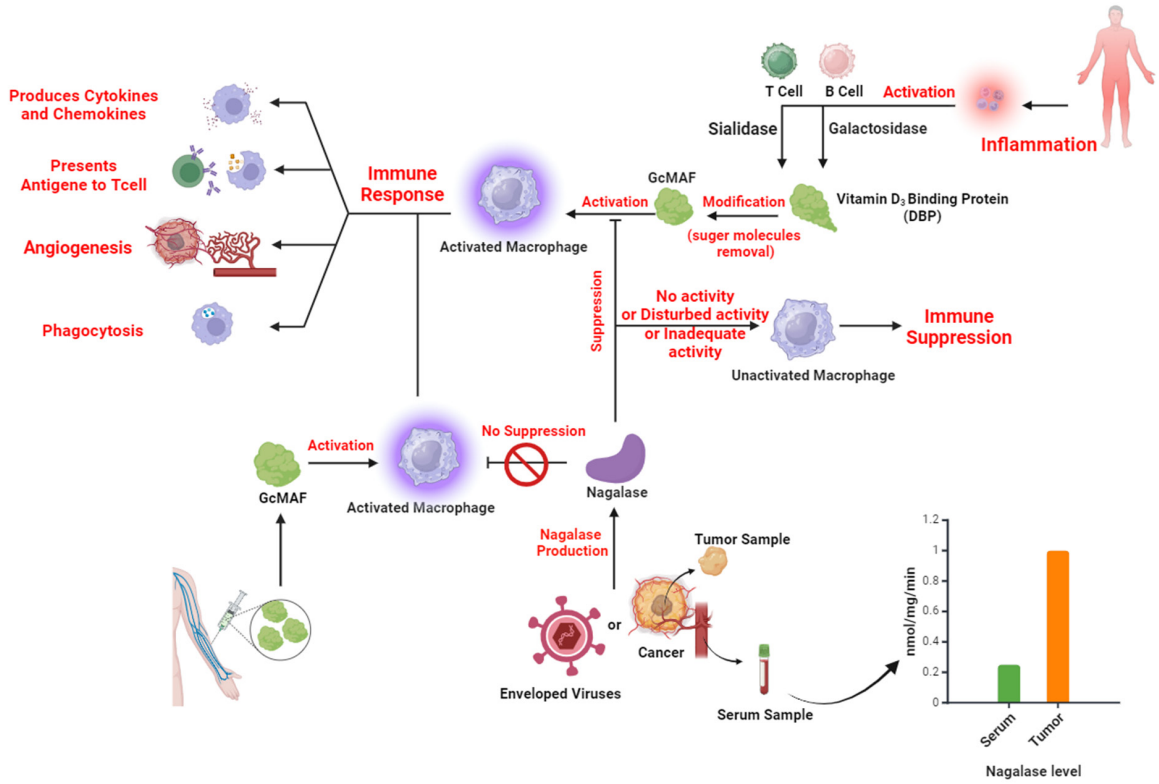

Figure 2. In the vicinity of a tumor with a 1 cm diameter, nagalase concentration is about four times higher than in serum. This elevated concentration enables the tumor to destroy GcMAF produced by the immune system in the tumor microenvironment (TME). Created by biorender.com.

eration, metastatic spread, and subsequent removal [3].

The lysosomal form performs best in an acidic environment (pH<5), whereas the extracellular form works at plasma pH values of about 7.4. Differences in active site, carboxylic or aminic-ends, glycosylated side branches, or cofactor/chaperone addition affect plasma pH function. Plasma nagalase levels depend on cancer type, severity, metastatic ability, primary or recurring tumor status, and cancer treatment. Healthy persons have nagalase levels between 0.32 and 0.95 nM/min/mg substrate [28-31].

Endo- or exo-glycohydrolase activity of nagalase

In the context of GcMAF and Naga6, it has been hypothesized that the immune response is selectively inactive within the neoplastic environment and its immediate vicinity. Yamamoto suggested that neoplasms evade immunological detection through the secretion of nagalase, an enzyme with endo glycohydrolase activity, which degrades the GcMAF precursor within the bloodstream. This degradation process does not occur when GcMAF is administered exogenously [10]. However, Borges et al., using mass spectrometric analysis of cancer patient serum, have refuted this mechanism of GcMAF precursor degradation. They propose that the "precursor" is synthesized by local B and T lymphocytes and is subsequently inactivated by elevated levels of nagalase produced within and around the tumor, indicative of exo-glycohydrolase activity [3]. It is estimated that the concentration of nagalase, or any analogous GcMAF inactivating agent, is approximately four-fold greater in the vicinity of a 1 cm diameter tumor in the 5.5 L blood of an adult person compared to their serum concentration (**Figure 2**). This increase is believed to lead to the degradation of locally produced GcMAF. Nevertheless, it is theorized that macrophages situated distally from the tumor, once activated by injected GcMAF, remain impervious to this inactivation mechanism. Further elucidation of the differential activities of endo- and exo-glycohy-

drolase, potentially employing the residue T418 as a substrate, may provide valuable insights into this complex interaction [3].

Vitamin D binding protein: how to communicate with nagalase

As mentioned earlier, vitamin D and its related products play an important role in fighting various diseases. VDR genotypes and cancer prognosis may vary by tumor size [32]. There is emerging evidence linking vitamin D status to health [30, 31]. Many chronic illnesses and poor prognoses are linked to low vitamin D [29]. Apoptosis, cell proliferation, adhesion, migration, metastases, and angiogenesis may be regulated by 1,25-dihydroxy vitamin D, or calcitriol, which binds a type II nuclear receptor (VDR). Lower 25OHD levels were detected in advanced cancers and bigger tumors [30, 31]. Single nucleotide polymorphisms (SNPs) of vitamin D receptor (VDR) and vitamin D binding protein (GC gene) may interfere with vitamin D activity, affecting breast cancer features and outcomes [33, 34]. These polymorphisms may alter gene expression by controlling mRNA stability, but they do not modify the protein's amino acid sequence [4, 35-37].

But as the most important aspect, macrophages are crucial to eliminating cancer. The fate of a monocyte, whether it becomes an M1 or M2 macrophage, is influenced by a variety of surrounding biomolecules and cytokines. The M1 phenotype in monocytes is induced in vitro by a combination of lipopolysaccharide (LPS) and interferon-gamma (IFNγ), while the M2 phenotype is promoted by interleukin-4 (IL-4) or interleukin-13 (IL-13). Furthermore, macrophage colony-stimulating factors (CSFs) have been implicated in the polarization of monocytes towards M1 (CSF-2) or M2 (CSF-1) macrophages. The source of these differentiation signals - whether they are secreted by the cancer cells themselves, by recycled immune cells, or both - is an ongoing subject of investigation. It is crucial to acknowledge that the characteristics of monocyte-derived macrophages observed in vitro may vary considerably across different experiments. Moreover, it is recognized that data derived from in vitro studies may not accurately reflect the interactions and phenomena that occur within the in vivo environment [10].

But on the other hand, MQ activation involves vitamin D binding protein (VDBP or Gc). Vitamin D binding protein (also known as Gc globulin, Gc protein, and D binding protein) becomes Gc-MAF produced by the liver after being modified by T and B lymphocyte-secreted sialidase and galactosidase. Gc-MAF anti-tumor actions include anti-angiogenic, adjuvant, osteoclast activation, chemotaxis (with C5-derived peptide), and G-actin scavenging. This enzyme deglycosylates Gc-MAF [4, 35]. As mentioned earlier, macrophages involved in tumor-killing activity are classified as M1-like. This contrasts with tumor-associated macrophages (TAMs) that are categorized as M2-like macrophages and are implicated in facilitating angiogenic processes. These TAMs may secrete a range of cytokines that contribute to suppressing the immune system, potentially leading to the inactivation of GcMAF synthesized by local B and T lymphocytes. It is hypothesized that neoplastic cells modulate the expression profiles of TAMs, thereby promoting their tumorigenesis and angiogenic activity [3].

It was noted that the nagalase enzyme can stop the activation process of Gc by changing its side branches. Alongside the increase in Nagalase concentration in patients with neoplasms, there is a corresponding change in an additional neoplastic biomarker. It is well-documented that nascent tumors have a tendency to attract immune cells. In the case of malignant solid tumors, a substantial portion of their mass, potentially up to 50%, may consist of infiltrating immune cells, predominantly macrophages. This infiltration is accompanied by an increase in the concentration of the precursor molecule, Globulin component Macrophage Activating Factor (GcMAF), which plays a crucial role in activating macrophages and enhancing their tumor-killing capabilities, thereby serving to hinder neoplastic progression [3].

Significantly, GcMAF exerts direct anti-angiogenic effects on a wide variety of endothelial cells across different species, thereby influencing their behavior. This agent effectively counteracts chemotactic stimuli, notably FGF-2, Ang2, and VEGF-A, which are known promoters of tumorigenesis via angiogenesis. Furthermore, GcMAF seems to spare the MAPK signaling cascade within endothelial cells, a pathway often implicated in angiogenic responses. Recent findings suggest that Gc protein-derived

macrophage activating factor (GcMAF) may play a role in suppressing endothelial cell-mediated angiogenesis [3, 38].

Simultaneously, GcMAF may enhance the activity of angiogenic macrophages, potentially exacerbating tumor progression [10]. While commercial preparations of GcMAF are known for their high purity, with minimal risk of contamination by vitamin D3 derivatives, it is plausible that such contaminants could contribute to the observed anti-angiogenic activity. For instance, the CD36 receptor emerges as a significant therapeutic target, given its involvement in the anti-angiogenic mechanisms of both GcMAF and Thrombospondin-1 (TSP-1). Therefore, CD36 represents a promising direction for anti-angiogenic therapeutic interventions aimed at reducing solid tumor growth, thereby forming a critical component of anti-angiogenic strategies in oncological treatment paradigms [39].

Immunotherapeutic potential of GcMAF: dosing concerns

Yamamoto has suggested a treatment plan that uses a series of therapies purified Gc protein, alpha-galactosidase, and sialidase. This approach produces the highly potent macrophage-activating factor, known as GcMAF. This factor has been recognized as the most effective immunotherapeutic to date and has not shown any adverse effects in human subjects [10]. Furthermore, GcMAF-based immunotherapy has proven to be effective against SARS-CoV-2 [3].

Macrophages activated by GcMAF develop a unique receptor profile capable of identifying abnormalities on the surface of malignant cells. These macrophages possess strong defensive capabilities, and GcMAF demonstrates resistance to degradation by Nagalase. Therefore, GcMAF stands as a highly specific and extraordinary macrophage activator [10]. Measuring the amount of GcMAF protein shows that even a very small dose of GcMAF can quickly boost the activity of macrophages throughout the body. It has been demonstrated that a dosage of 100 ng of GcMAF, with a molecular weight of 51.2 kDa, distributed within the average adult blood volume of 5.5 liters, achieves a picomolar concentration of 0.355 pM. This concentration is sufficient to activate approximately 1.5 × 10^10 Gc proteins, leading to significant macrophage activation. The intramuscular injection of such a dose resulted in a 40-fold increase within four days, and intravenous injection led to an amplification exceeding 100-fold in two days. However, Yamamoto's findings suggest that administering 500 ng does not significantly differ in effect from a 100 ng dose (**Figure 3**). On the other hand, experiments have shown that as the concentration of GcMAF increases, its ability to activate macrophages actually decreases, eventually leading to a strong blocking effect [10].

Therefore, the graph showing how well GcMAF works at different concentrations looks like a bell shape. The GcMAF molecule has two binding sites: the first one works well at low concentrations and boosts GcMAF activity, while the second one gets filled at higher concentrations with less strength, which reduces its ability to activate and interferes with the first site [3, 26].

Interplay of vitamin D3 and C with GcMAF immunotherapy

Similar to GcMAF, MAF proteins display unparalleled bioactivity. These peptides include Gc protein domains that lack affinity for actin, vitamin D, or fatty acids. Clinical observations have shown that higher levels of plasma Naga6 are linked to lower levels of ascorbate and 25-hydroxyvitamin D3 in both cancer and non-cancer patients. Optimal immune function requires sufficient plasma concentrations of these vitamins.

Albracht suggested in 2021 that ascorbate and 25-hydroxyvitamin D3 work together to improve how immune cells function, and current research is looking into how they depend on each other for the immune system to work well. As a result, during GcMAF-based immunotherapy, it is recommended to maintain plasma ascorbate levels at approximately 70 to 80 μM (corresponding to an intake of 200 to 500 mg/day) and 25-hydroxyvitamin D3 levels at 80 to 200 nM (equivalent to a daily intake of 50 to 70 μg) [3, 40-42].

GcMAF immunotherapy in prostate cancer

GcMAF immunotherapy has shown potential benefits for cancer patients who are unresponsive to standard treatment procedures. However, due to the immune system's limited capacity for tumor eradication, it is crucial to

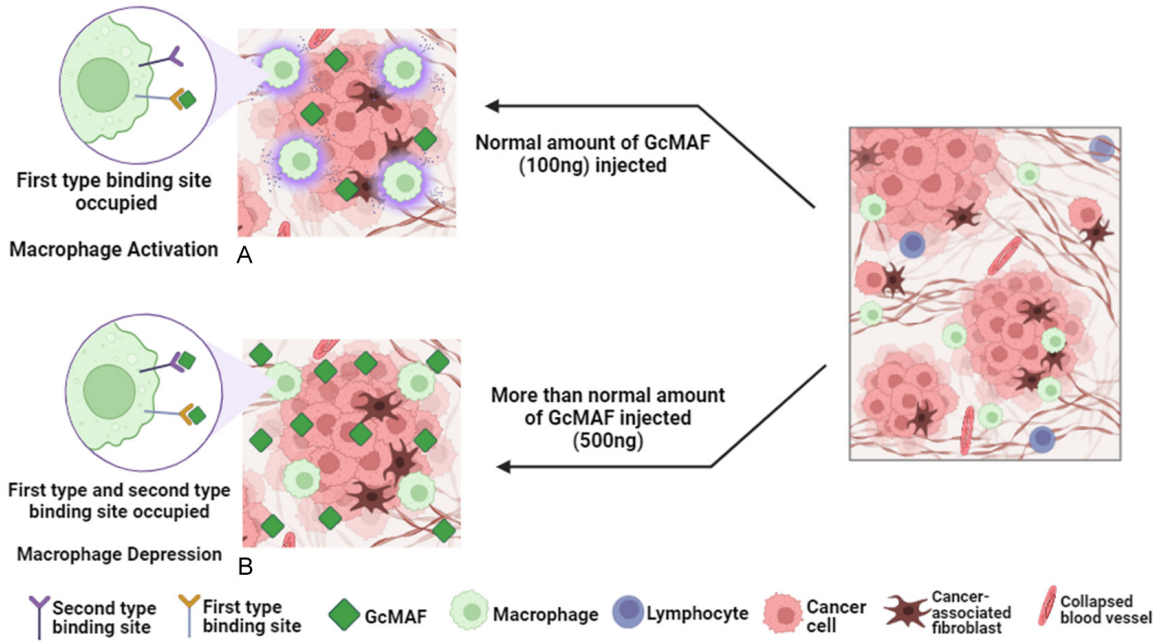

Figure 3. Effect of different nagalase concentrations on macrophage function. A. Normal Dose Injection: At a normal injection concentration, GcMAF molecules bind to high-affinity receptors (type I) on macrophages, leading to their activation. B. High Dose Injection: A much higher GcMAF concentration can result in binding to both high-affinity (type I) and lower-affinity receptors (type II). While some macrophages may still be activated through high-affinity receptors, binding to lower-affinity receptors might not trigger activation or might even interfere with activation by high-affinity receptors. This could potentially impair the immune response. Created by biorender.com.

remove the tumor through surgical or radiological interventions before initiating immunotherapy. A recovery period of at least four weeks post-chemotherapy is necessary for adequate immune restoration. Quick action is needed if new cancer growth is found, which can be shown by high serum Naga6 levels or other tests. Empirical data suggests a correlation between the reduction of Nagalase activity and tumor markers following treatment, affirming Nagalase as an indicator of tumor burden [3]. Although Prostate-Specific Antigen (PSA) is the prevalent biomarker in the case of prostate cancer, Naga6 can be a valuable additional biomarker. Primary PSA synthesis occurs in the prostate epithelium, but PSA is also detectable in several other tissues at significantly lower concentrations. Notably, seminal fluid exhibits PSA concentrations ranging from 0.5 to 2 mg/ml, significantly surpassing the serum levels observed in healthy male individuals [43, 44].

However, the specificity of PSA as a biomarker for prostate cancer diagnostics has been questioned. Elevated serum PSA levels may result from benign causes such as mechanical pressure, prostatitis, or benign prostatic hyperplasia. This study has sparked a discussion on the reliability of PSA as a sole indicator of malignancy, given the detection of PSA levels within the range of 8-12 ng/ml in individuals without oncological manifestations [3]. In a series of investigations, Yamamoto et al. monitored the serum concentrations of Nagalase and PSA in patients undergoing GcMAF therapy, administered at a dosage of 100 ng/week. Following prostatectomy, a simultaneous decline in both Nagalase and PSA was observed, with Nagalase levels normalizing within a 24-hour window, seemingly reflecting the excision of metastatic deposits. Conversely, in the absence of prostatectomy, despite a reduction in nagalase, PSA levels persisted, suggesting residual tumoral activity or tissue damage. Yamamoto observed that patients who had prostate surgery did not experience any return of cancer for up to seven years after stopping GcMAF therapy [3, 41, 45].

Diverse methods for GcMAF production

Research by Albrecht has shown that the effectiveness of the macrophage activating factor in

GcMAF depends on having N-acetylgalactosamine (GalNAc) attached to the threonine at position 418 (T418) in the Gc protein. As a result, the recombinant production of Gc protein in Escherichia coli (E. coli), for example, cannot be converted into active GcMAF due to the bacterium's inability to glycosylate proteins [3]. However, advancements in molecular glycoengineering have enabled E. coli to synthesize glycosylated proteins, including GcMAF. This technique, known as bacterial glycoengineering, involves transferring the glycosylation pathways (the mechanisms for adding sugars to proteins) from other organisms, typically eukaryotes, into E. coli.

The transfer is accomplished by transfecting the relevant genetic information encoding these pathways into E. coli. While this method may be less efficient and more complex compared to eukaryotic systems inherently equipped with the glycosylation machinery, it provides a cost-effective alternative [2, 46-48].

In addition to prokaryotes, eukaryotic host cells are also used to express the cloned glycosylated GcMAF. These include insect cells, Pichia pastoris yeast, and mammalian kidney cells (HEK), all of which can produce a protein that can be converted into active GcMAF. Notably, ExpiCHO-S cells demonstrate enhanced expression of Gc2 and have the inherent ability to directly produce glycosylated active GcMAF [49]. Each host system possesses unique expression characteristics that align with specific research or therapeutic goals. For instance, human embryonic kidney (HEK) cells are often chosen when a glycosylation profile similar to that of humans is required. On the other hand, Pichia pastoris is preferred for its simplicity and scalability of production. In situations that require both human-like post-translational modifications and large-scale production, insect cells are the ideal choice [49, 50].

Moreover, Gc protein has been successfully extracted from bovine colostrum, the initial milk produced by cows. It is suggested that MAF derived from bovine colostrum could act as an immunostimulant and an antimicrobial agent. This variant of the protein, once converted to GcMAF, can activate human macrophages. Although it might not be good for injections, it could be taken by mouth to help boost the immune system by affecting macrophages in areas like the tonsils or gut-related lymph tissue. When administered as an aerosol, GcMAF can stimulate resident macrophages within the pulmonary lymphoid tissue. This method of adjusting the immune system, especially using GcMAF from cow's first milk, is thought to help treat SARS-CoV-2 infection [3, 45, 48].

Debate over GcMAF immunotherapy

Yamamoto's findings have faced skepticism from the scientific community, which has deemed them "overly optimistic" and "illogical", leading to the retraction of several publications. The criticisms primarily revolve around the small sample size and the reliance on nagalase as a measure for cancer staging, rather than conventional imaging and diagnostic assays. Also, the claimed activation of macrophages by GcMAF doesn't have strong proof, which raises doubts about whether the treatments are really effective and suggests that any positive effects might be more about the patients' mindset. A recent trial conducted by a Dutch clinician, who involved GcMAF immunotherapy in a group of 33 cancer patients, produced results similar to those reported by Yamamoto. However, these findings have been viewed with skepticism due to the limited statistical population. An oncologist has commented on the medical community's lack of confirmation for GcMAF's application, pointing out the inability to scientifically corroborate these results. Despite this, Albrecht maintains that there have been numerous instances where popular scientific opinion has contradicted and rejected many cases. He champions the potential superiority of GcMAF immunotherapy, especially when used in benign neoplasms and in conjunction with conventional therapies, to mitigate the humoral immune response at sites of advanced tumor involvement [3].

Approaches for the suppression of nagalase

Gene silencing represents a significant category within gene therapy and has demonstrated increasing significance in recent decades. Nonetheless, the emergence of unforeseen adverse reactions has prevented it from achieving global approval as a treatment. Gene silencing can be accomplished using siRNA, shRNA, or CRISPR-Cas9; however, these techniques remain confined to research laboratories and have not yet been integrated into clinical thera-

py [51]. It is anticipated that employing gene silencing techniques will lead to a decreased expression of nagalase, thereby diminishing the invasive potential of cancer cells. The most likely approach utilizing this approach would involve employing siRNA/shRNA directed at the NAGA gene, its enhancer, or a proposed sequence that facilitates the secretion of nagalase [29, 35].

Creating a monoclonal antibody that targets plasma nagalase could greatly help by improving how the immune system recognizes, presents, and removes nagalase. If these antibodies are labeled, both distant and local metastasis may be identified, as previous studies indicate that the highest levels of nagalase are found near cancer cells. Consequently, potential advantages could encompass quicker and more efficient detection and treatment of metastatic sites. Conversely, considering these findings and the preference of nagalase for acidic conditions, it may be possible to attain a loss of its activity by locally increasing the pH near these locations [35].

Conclusion

Despite the evidence and assumptions, it seems that the role of alpha-N-acetyl galactosaminidase in the occurrence, severity, prognosis and diagnosis of all types of cancers is still in an aura of uncertainty. High levels of nagalase seem to be primarily caused by the release of active nagalase from malignant cells, which results in ongoing immunosuppression and further increases the levels of the nagalase enzyme. Conducting clinical studies is necessary to validate the aforementioned cases.

Disclosure of conflict of interest

None.

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