Overview of Macrophage Activating Factor and the Nagalase Assay – Potential for Control of Micrometastatic or Early Primary Cancer

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Abstract

Macrophage activating factor (GcMAF) functions physiologically to boost the superoxide-generating, phagocytic, and cancerocidal capacities of macrophages; it is generated from vitamin D binding protein by lymphocyte beta-galactosidase and sialidase activities up-regulated by inflammation. Many and possibly most cancers secrete an N-acetylgalactosaminidase – a.k.a. nagalase – that prevents the synthesis of and also degrades GcMAF, protecting the cancer from macrophage-orchestrated destruction. Yamamoto, who discovered these phenomena, has presented evidence that elevated serum nagalase is a virtually universal feature of cancer, and that nagalase can be used to monitor cancer status when cancer is in a microscopic metastatic form. He further proposes that intramuscular injections of pre-formed GcMAF can aid cancer control by compensating for the immunosuppressive activity of nagalase and boosting macrophage activity within tumors. In clinical trials enrolling breast, prostate, and colorectal cancer patients who had achieved remission after therapy, but who were presumed to harbor microscopic metastatic disease owing to elevated serum nagalase levels, he found that weekly administration of GcMAF was associated with a gradual monotonic decline in serum nagalase; the normal range was achieved within 24-48 weeks, and these patients were reported to be alive and clinically cancer free a number of years later. Hence, it is proposed that GcMAF administration can be employed to "mop up" residual cancer cells after effective extirpative therapy; concurrent monitoring of nagalase can provide evidence that the GcMAF is working effectively, and insure that it is administered for a sufficient time to effect a cure. If this model is correct, adjuvant use of GcMAF has the potential to make surgery, chemotherapy, and radiotherapy notably more curative than they are at present. GcMAF may also have the potential to slow the spread of more advanced cancers, and it has strong anti-angiogenic activity if injected daily. Conceivably, the routine monitoring of serum nagalase could aid early detection of cancer. Although these prospects are exciting, much further research is needed to verify the utility of nagalase as a cancer marker and to confirm the efficacy of GcMAF as adjuvant therapy, in a range of cancers. The use of GcMAF in conjunction with agents that promote a pro-inflammatory M1 phenotype in tumor-associated macrophages merits evaluation as an immunotherapeutic strategy. Although GcMAF is not currently available as an investigational drug, patients can order and self-administer it, and their doctors can monitor their progress.

Macrophage Activating Factor Boosts the Cytotoxic Capacity of Macrophages

Yamamoto and colleagues have presented evidence that, within inflamed tissues, vitamin D-binding globulin (a.k.a. Gc protein, a member of the albumin family secreted by the liver) becomes structurally altered, giving rise to a "macrophage activating factor" – GcMAF – that rapidly and potently boosts the superoxide-generating and phagocytic capacity of macrophages.[1, 2] *In vitro*, exposure of macrophages to 100 pg/ml GcMAF is reported to boost their generation of superoxide by about 9-fold, with a 2-fold increase seen at 0.1 pg/ml. The increase in antibody-dependent phagocytic capacity, reflects, at least in

part, increased translocation of FcgR1 and FcgR2 receptors to the cell surface.[3] Macrophages pre-treated with GcMAF have an increased capacity to kill cancer cells with which they are co-incubated.[4] When cheek pouch cancer is induced in hamsters with DMBA, the ability of GcMAF-treated macrophages to kill hamster cheek pouch cancer cells in vitro is greatly augmented if serum from tumor-bearing hamsters (but not healthy hamsters) is added; this suggests that serum factors induced in the cancer-bearing animals (perhaps cancer-targeting antibodies?) can work in concert with activated macrophages to mediate cancer cell killing.[4] GcMAF also appears to increase the antigen-presenting activity of macrophages or dendritic cells, as administration of this agent to mice (20-100 pg) potentiated the subsequent yield of antibodies after injection of sheep red blood cells; moreover, GcMAF-primed mice injected with heat-killed Ehrlich ascites cells were immune to tumor formation when live Ehrlich ascites cells were later implanted.[5]

Yamamoto proposes that the transformation of Gc protein to GcMAF in vivo comes about owing to an inflammation-triggered increase in the expression of beta-galactosidase and sialidase on the surfaces of B and T lymphocytes, respectively; GcMAF can be produced in vitro by exposing Gc protein to immobilized beta-galactosidase and sialidase.[2] A linear trisaccharide comprised of N-acetylgalactosamine, galactosamine, and sialic acid is attached to Thr420 of the most common allelic variants of Gc protein;[6, 7] treatment with sialidase and beta-galactosidase results in a protein in which N-acetylgalactosamine alone is attached to Thr 420, and this is the configuration which has been thought to confer GcMAF activity. However, this model is impossible to square with the fact that the not uncommon Gc2 allele of GcMAF lacks a threonine at the 420 position, and yet is reported to give rise to GcMAF activity.[8] More recent research has established that, in both the Gc2 allelic variant and other common forms of Gc protein, Thr418 often carries a disaccharide composed of N-acetylgalactosamine and galactosamine.[9] A model consistent with available evidence is that formation of GcMAF requires removal of galactose from the Thr418 disaccharide, and sialic acid (and possibly galactose) from the Thr420 trisaccharide; this would explain why beta-galactosidase alone suffices to generate GcMAF from Gc2 – whereas beta-galactosidase must be complemented by sialidase to achieve GcMAF activity from other allelic variants of Gc protein.[6, 8, 9]

How Does Nagalase Impair GcMAF Activity?

Yamamoto's group discovered that circulating pool of Gc protein in cancer patients tends to give rise to subnormal GcMAF activity when treated with sialidase/galactosidase *ex vivo*, and that the degree of this deficit tends to correlate negatively with tumor mass and serum levels of alpha-N-acetylgalactosaminidase activity ("nagalase" for short), which almost invariably is elevated in cancer patients.[10, 11] Yamamoto has proposed that this elevation of nagalase activity is responsible for the decrease in GcMAF precursor activity observed in the serum of cancer patients, and further proposes that serum nagalase can serve as a universal cancer marker. He maintains that the secretion of nagalase by cancer cells serves to protect the cancer from immune rejection by impeding generation of GcMAF activity and hence opposing optimal macrophage activation.

Yamamoto assumed that nagalase destroys the ability of Gc protein to act as a precursor for GcMAF by cleaving off the entire trisaccharide at Thr420 via an endo-type activity.[10] However, other investigators report that the Gc protein pool in cancer patients has as much trisaccharide at Thr420 as in healthy controls; hence, there must be some other explanation for cancer's ability to decrease GcMAF precursor

activity in cancer patients.[6] This finding, however, poses no difficulty if the true form of GcMAF carries a single N-acetylgalactosamine at Thr418, as postulated above.

There is also controversy regarding the nature of the nagalase activity produced by cancer cells. A lysosomal N-acetylgalactosaminidase activity found in liver lysosomes has been well characterized, and genetic lack of it causes a disorder known as Schindler disease.[12] However, some investigators report that the nagalase activity secreted by cancer cells has a different pH dependence than the lysosomal enzyme, retaining significant activity at neutral pH.[13] Moreover, whereas the lysosomal enzyme has exo-type activity, there are reports that nagalase has both exo- and endo- activity.[14] Another report suggests that nagalase is an exo enzyme.[13] At this point, it is unclear whether tumor-secreted nagalase is related to the lysosomal N-acetylgalactosaminidase; it might stem from an entirely separate gene (so far uncharacterized), or perhaps might arise from the same gene, but express different properties owing to variant splicing or post-translational modifications.

Treatment of GcMAF with nagalase *in vitro* abolishes its capacity to stimulate macrophages.[13, 14] Arguably, the exo activity of nagalase could deactivate GcMAF by removing N-acetylgalactosamine from Thr418 – whereas its endo activity, by removing the N-acetylgalactosamine-galactosamine disaccharide from Thr418, would degrade the ability of the plasma pool of Gc protein to give rise to GcMAF – as repeatedly observed by Yamamoto in cancer patients.[10] If this model is accurate, a testable implication is that Thr418 should be underglycosylated in cancer patients with elevated nagalase.

Nagalase – A Near-Universal Cancer Marker?

Even though further clarification is required regarding the structure and generation of GcMAF, Yamamoto's body of work has given rise to two key propositions that may have extraordinary importance if sustained by future research: namely, that serum nagalase can serve as a virtually universal marker for cancer, and that parenteral administration of pre-formed GcMAF can exert a profound immunostimulant effect in cancer patients, enabling the effective control and eradication of many cancers when they are in a micrometastatic or very early nascent form.

In 1996, Yamamoto and colleagues published a study in which they assayed serum nagalase in 20 patients with a wide range of cancers, as well as in 5 healthy subjects.[10] Whereas the serum nagalase activity in the healthy subjects averaged 0.23 (nmoles/mg/min), it ranged in the cancer patients from a low of 0.64 to a high of 5.21. In several patients who were receiving radiation therapy, the serum nagalase activity declined progressively, coming close to the normal range in some. Conversely, the ability of Gc protein derived from the patients to give rise to GcMAF activity in vitro (assessed by stimulation of superoxide production by macrophages) tended to be low in cancer patients, and vary inversely with serum nagalase; this precursor activity increased progressively during radiation therapy.

A year later, Yamamoto published a study confirming these findings in 36 patients with oral cancer; whereas serum nagalase averaged 0.29 in twelve healthy controls, this activity was above 1 in all except one of the cancer patients (0.74), and was above 6 in four of them.[11] Once again, serum precursor activity for GcMAF generation tended to be subnormal and correlated inversely with serum nagalase in the cancer patients. Surgical excision of primary tumors or metastasized nodes was followed by a rapid decline in serum nagalase, coming close to the normal range in 3 patients.

Yamamoto's assessment of serum nagalase in patients with micrometastatic breast, colorectal, and prostate cancers appears to provide further confirmation for the utility of nagalase as a cancer marker; these findings are discussed below.[15-17]

To date, two independent clinical groups have published assessments of serum nagalase in cancer patients. Researchers at the University of Madras measured serum nagalase in 85 healthy controls, and in 210 patients with squamous cell carcinoma of the uterine cervix, both before and after a course of radiotherapy.[18] In the controls, nagalase averaged 0.26 – very similar to Yamamoto's control values. Prior to treatment, nagalase averaged 0.72, 1.54, 3.78, and 5.05 in patients with Stage 1, 2, 3, and 4 cancers, respectively. After radiotherapy, these values averaged 0.39, 0.64, 1.38, and 3.14, respectively. These findings appear wholly confirmative of Yamamoto's proposition that serum nagalase can serve as a cancer marker and can be used to assess the effectiveness of therapy. More recently, Greek researchers have examined nagalase in patients with pre-surgical melanoma.[19] Nagalase levels were found to be significantly elevated relative to controls in patients with stage 3 disease, but not in patients with earlier lesions. Nagalase correlated directly with tumor thickness (Breslow index); in patients with stage 2 or 3 disease, it declined significantly after surgery. Hence, whereas much further clinical research is needed to confirm that nagalase is the nearly universal cancer marker that Yamamoto proposes it to be, the published clinical evidence so far is consistent with this proposition – although it may not always be elevated in early cancer.

Several studies in rodent models likewise confirm an elevation of serum nagalase in tumor-bearing animals. Moreover, they suggest that this elevation of nagalase tends to be proportional to tumor mass. However, whereas one such study – in nude mice implanted with human squamous cell carcinoma[11] – found that nagalase correlated directly with tumor mass, a subsequent study in mice carrying Ehrlich ascites tumors found that serum nagalase varied directly with the *log* of tumor mass.[20] This latter finding might explain why patients with advanced cancer can display serum nagalase that isn't orders of magnitude higher than those with micrometastatic disease (as discussed below).

If, as appears likely, serum nagalase is elevated in a very high proportion of patients with cancer, what might account for this? So far, the molecular biology behind these elevations remains virtually unexplored – as noted, the gene responsible for tumor-associated nagalase hasn't yet been characterized. However, a study focusing on the molecular biology of Ehrlich ascites cancer is of intriguing relevance. Segura and colleagues, noting that increased expression of glutaminase is a common feature of cancer thought to contribute to its malignant behavior, used anti-sense technology to impede the expression of glutaminase in Ehrlich ascites cells.[21] In this altered cell line (0.28-AS-2), the expression of two proteins thought to influence the susceptibility of cancer cells to immune rejection, mucin-1 and nagalase, was notably decreased. (Nagalase expression was assessed by its enzymatic activity.) Whereas wild-type Ehrlich ascites cells grew prolifically in immunocompetant mice, the altered cells could not produce tumors in these mice, and a far higher number of activated macrophages were found in the ascitic cavity of the mice injected with the 0.28-As-2 cells. However, these cells readily formed tumors in immunodeficient nude mice, killing them within 20 days. These findings suggest that glutaminase knockdown, likely in part by blocking nagalase expression, abrogates mechanisms which Ehrlich ascites cells employ to evade immune rejection – mechanisms which are superfluous when cancer is implanted in immunodeficient mice. It remains unclear why glutaminase expression or activity might influence nagalase expression, and no subsequent research has examined this intriguing lead. Glutaminase is now

viewed as an oncogene, and its increased expression is often a reflection of increased myc activity – so perhaps myc is the prime driver of nagalase overexpression in cancer.[22, 23]

The possibility that nagalase might be elevated in other pathologies – raising the possibility of false positives when nagalase is used as a cancer marker – requires much further study. Certain viruses, including the HIV virus, also express a nagalase-like activity, and serum nagalase activity is increased in HIV patients.[24] An elevation of serum nagalase has also been reported in systemic lupus erythematosis; the basis and source of this increase remains obscure, but Yamamoto intriguingly proposes that this increase may responsible for a decrease in phagocytic activity that contributes to the elevation of circulating immune complexes in this disorder.[25]

In any case, it is clear that nagalase may have great clinical potential as a cancer marker – perhaps a near-universal cancer marker that could be of great utility for guiding cancer therapy, particularly when cancer is present in micrometastatic or early form, and hence difficult or impossible to assess with radiological techniques. It is distressing that, 17 years after Yamamoto's first pertinent clinical publications, so few independent groups have chosen to study this issue – and so little progress has been made on defining the molecular biology of tumor-associated nagalase.

Potential of GcMAF Administration for Cancer Control

As noted, Yamamoto's other key contribution is to demonstrate, both in rodent studies and clinical trials, that parenterally administered GcMAF has intriguing potential as a cancer therapy. His work in this regard was rooted in the presumption that most cancer patients experience a deficit of GcMAF activity reflecting secretion of nagalase in the tumor microenvironment. (Whereas other researchers have shown that nagalase pre-exposure quenches GcMAF's bioactivity, presumably by removing a key N-acetylgalactosamine group, Yamamoto believed that nagalase was destroying Gc protein's precursor activity.) Since optimally activated macrophages have the potential to kill cancer cells in a variety of ways, Yamamoto further presumed that the cancer-imposed deficit of GcMAF activity could promote the survival and spread of cancer and that, conversely, parenteral administration of pre-formed GcMAF could compensate for this deficit and exert a beneficial immunostimulant effect in cancer patients.

To evaluate the therapeutic potential of GcMAF, Yamamoto and his colleagues have examined the impact of such therapy on Ehrlich ascites cancer, a mouse squamous cell carcinoma, and DMBA-induced cheek pouch carcinoma in immunocompetant rodents.[4, 20, 26] In each of these models, substantial tumor control was seen. When Ehrlich ascites cancer was implanted intraperitoneally in mice, untreated controls survived an average of 13 days. In the mice who received a single subcutaneous dose of 100 pg GcMAF on the day of transplantation, one survived past 60 days, and the other 7 had an average survival of 21 days. In the mice who received two injections of GcMAF (day 0 and day 4), 2 survived past 60 days, and the other 6 survived an average of 31 days. In mice who received three GcMAF injections (days 0, 4, and 8), 6 survived past 60 days, and the other two survived an average of 38 days. The researchers reasoned that repeated injections of GcMAF would be required for optimal results, since the lifespan of an activated macrophage was judged to be about 6 days. As they had expected, serum nagalase levels tracked lower in the mice receiving multiple injections. They further demonstrated that, in mice receiving a single injection of GcMAF on the day of implantation, a subsequent intraperitoneal injection of Freund's adjuvant was associated with apparent cure in all mice – whereas Freund's adjuvant alone had a non-significant impact on survival.[20]

In another series of studies, the interaction between photodynamic therapy and GcMAF – administered both intraperitoneally and intratumorally – was observed in mice implanted with squamous cell carcinoma SCVII.[26] Photodynamic therapy was applied when tumor diameter had reached 5-6 mm. After testing a number of regimens, they found that a photodynamic therapy sufficiently intense to cure 25% of the mice would cure all of the mice who also received i.p and intratumoral injections of GcMAF on days 0, 4, 8, and 12 after therapy. GcMAF therapy alone was not useful for controlling this poorly immunogenic cancer. This set of studies laid the groundwork for subsequent clinical evaluation of GcMAF as an adjuvant cancer therapy.

GcMAF therapy has also been assessed recently in hamsters in whom cheek pouch carcinoma was induced by repeated applications of DMBA for 13 weeks, at which time tumors had formed in all the hamsters.[4] Some of the hamsters also received twice-weekly thigh injections of GcMAF. In some of the mice, these injections began when DMBA was first applied, and continued through the 20 weeks of observation. Some mice only received GcMAF for the first 13 weeks, and some only received it from week 13 to week 20. The untreated mice all died within the 20 weeks, on average surviving 15 weeks. Those who received GcMAF beginning at week 13 did slightly better – average survival was 17.4 weeks, albeit tumor size at 16 weeks (8.0 mm) was about half as large as in the untreated group (17.9 mm). In those who received GcMAF for only the first 13 weeks, all four of the animals survived the 20 weeks, but tumor size at 16 weeks was 7.7 mm. In those mice who received GcMAF continuously throughout the study, all 8 animals survived 20 weeks, and tumor size at 16 weeks was only 2.8 mm.

In aggregate, these studies suggest that continuous bi-weekly administration of GcMAF achieves best results, and the effect is optimized if tumor mass is minimal when therapy commences. These findings appear to be logical consequences of the thesis that activated macrophages are best capable of coping with small nests of tumor cells, and that GcMAF must be administered repeatedly to avoid nagalase-mediated suppression of macrophage cytotoxicity.

A complementary line of investigation was opened up by the intriguing discovery that GcMAF can exert anti-angiogenic activity via a direct anti-proliferative effect on endothelial cells.[27] This effect may be mediated through the endothelial CD36 receptor (the thrombospondin receptor), as it is blocked by monoclonal antibodies targeting this receptor.[27] GcMAF also suppresses VEGF-mediated phosphorylation of VEGFR-2, and inhibits VEGF-stimulated migration, sprouting, and tube formation[28] – effects analogous to those of the anti-angiogenic protein thrombospondin.[29] The anti-angiogenic effect of GcMAF was demonstrated first in endothelial cell cultures and in mouse corneas.[27] Subsequently, its ability to slow or block the growth of human tumors transplanted into nude mice has been demonstrated by three separate groups, including one involving Judah Folkman.[30-32] In these studies, best results were seen when GcMAF was administered daily, since the anti-proliferative effect on endothelial cells must be sustained if angiogenesis is to be blocked; 4 ng/kg/day was especially effective. Optimal regimens brought tumor growth to a virtual standstill. Since the mice employed in these studies were immunodeficient, anti-angiogenesis likely was the key mediator of the benefits observed.

Yamamoto's Clinical Trials with GcMAF

The first, and so far only, clinical studies evaluating GcMAF as a cancer therapy were published by Yamamoto and colleagues in 2008. These three reports describe the treatment of 16 patients with breast cancer, 16 with prostate cancer, and 8 with colorectal cancer.[15-17] All of these patients had had previous

treatment with standard modalities – surgery, chemotherapy, hormone therapy, and/or radiotherapy – which had eliminated all lesions detectible by CT scan; clinically, they were in complete remission. Nonetheless, since serum nagalase was elevated in all of these patients, it was judged that they had microscopic metastatic lesions likely to give rise to recurrences. (The report on the breast cancer patients, unlike those with the prostate and colorectal cancer patients, does not explicitly state that radiologically detectible lesions were absent in the patients, but it seems likely that this was an inadvertent oversight, as the studies are in other respects quite homologous.) Patients with anemia were excluded from these trials, as it was thought that concurrent treatment with erythropoietin might complicate interpretation of the results. Each of the enrolled patients then received weekly intramuscular injections of 100 ng GcMAF; Yamamoto states that, in previous pilot trials, this dose had achieved the most potent stimulatory effect on the phagocytic capacity of macrophages measured *ex vivo*, and was not associated with evident adverse effects. Status of the patients was monitored primarily by weekly assays of their serum nagalase, although in the prostate and breast cancer trials, at least some of the patients were also monitored with more standard markers (PSA, CEA, CA15-3, CA27.29).

Astoundingly, in all of the patients reported on in these studies, nagalase levels decreased monotonically during GcMAF administration. In the patients with breast and prostate cancers, nagalase had fallen to the normal range after a maximum of 24 weeks. The decline of nagalase in colorectal cancer patients tended to be more gradual, but nagalase was at or near the normal range after 48 weeks in all of them. The initial decline in nagalase tended to be steeper during the first weeks of therapy, with the slope flattening a bit thereafter; Yamamoto suggest that this reflects the fact that sensitivity of cancer cells to killing by GcMAF-activated macrophages is non-homogeneous, such that less differentiated cells, more readily perceived as aberrant, are killed more rapidly. In four patients with breast cancer, and in two prostatectomized patients with prostate cancer, the decline in serum nagalase is shown to parallel declines in the other more established cancer markers measured. However, in prostate cancer patients who had not received a prostatectomy (who presumably had been treated with androgen antagonists), PSA levels failed to decline notably; Yamamoto attributes this to persistent inflammation of the prostate triggered by the cancer. It is not clear why alternative cancer markers were reported in only a handful of patients, rather than the whole cohorts.

At the time of publication, Yamamoto states that all of the treated patients are clinically cancer free – 7 years later in the patients with prostate and colorectal cancer, 4 + years in the patients with breast cancer. If one accepts the proposition that serum nagalase is a reliable cancer marker – a big "if" given that relatively few groups have so far reported on this issue (albeit all who have agree with this conclusion) – these studies strongly suggest that, at least in breast, prostate, and colorectal cancer, patients who continue to harbor microscopic metastatic lesions following definitive therapy (and hence would be at high subsequent risk for recurrence) can frequently be cured by a prolonged weekly course of GcMAF injections. If this conclusion is correct, it implies that inclusion of GcMAF as an adjuvant therapy for patients who remain nagalase positive has the potential to make definitive extirpative therapies – surgery, chemotherapy, hormone therapy, radiotherapy – much more curative than they currently are.

A weakness or ambiguity of these reports is that they do not clarify whether Yamamoto was reporting on *all* of the patients with presumed microscopic disease which Yamamoto's group treated during the period of the studies. For example, the prostate cancer report states that "a group of 16 nonanemic prostate cancer patients was included in this study." Analogous statements are made in the breast cancer and

colorectal cancer reports. Were these patients cherry-picked from a larger cohort of treated patients – or did literally all of the patients who met the inclusion criterion respond with a normalization of nagalase and apparent cure? It would be good for the authors to clarify this point. However, even if adjuvant GcMAF therapy of microscopic disease is only effective in a significant fraction of patients, these observations are evidently of great importance if confirmable.

The Need for Confirmatory Research

Five years after the publication of these quite provocative studies, no further clinical trials with GcMAF have emerged. Apparently, pharmaceutical companies are attempting to develop more-readily-patentable synthetic analogs of GcMAF as drugs; there appears to be no current effort to register natural GcMAF for therapeutic use. Several biotech companies have recently stepped into this void to sell GcMAF, packaged in sterile vials suitable for use in injections, via mail order internationally, without explicit drug claims; a number of cancer patients, and a few "integrative" doctors, are availing themselves of this resource. It seems likely that the majority of cancer patients currently using GcMAF have advanced lesions that are unlikely to respond definitively to immunotherapies; GcMAF is still a little-known option, and the patients who seek it out are likely to be searching for options after standard therapies have failed them. Yamamoto's clinical reports have no evident implications for those with advanced cancer – albeit the possibility that GcMAF therapy might sometimes help to slow the growth and spread of progressive cancers, as suggested by some of the rodent studies, certainly merits consideration. The anecdotal experience of cancer patients currently using GcMAF may cast some light on this issue.

It is clear that two research initiatives are urgently needed: the utility of nagalase as a cancer marker should be evaluated aggressively in a wide range of cancers, correlating it, when feasible, with other cancer markers that are more broadly accepted; and surgical oncologists should evaluate weekly injections of GcMAF as an adjuvant strategy in post-surgical patients who are radiologically cancer free but remain nagalase positive, in conjunction with whatever adjuvant chemotherapy or radiotherapy that is considered indicated. Unless and until GcMAF is formally registered as an investigational drug, legal consideration may make clinical trials with this agent problematic; nonetheless, this issue is of such potential importance that avenues for evading these legal restrictions should be explored – particularly in light of the fact that no serious side effects of GcMAF therapy have been reported to date. Notably, nothing currently prevents cancer patients, on their own initiative and at their own expense, from acquiring GcMAF for personal use. Currently, GcMAF acquired from a European source costs about $600 per month to use if 100 ng GcMAF is injected weekly.

As noted, rodent studies suggest that daily administration of GcMAF may have important potential as an anti-angiogenic agent; this strategy might prove to be of some utility in advanced cancers unlikely to be controllable by immunotherapy per se. To date, no clinical trials have evaluated this strategy; the current expense of GcMAF would discourage most cancer patients from trying this. Moreover, if GcMAF does have the potential to evoke significant adverse effects, such effects might be more likely with a daily administration regimen. Nonetheless, this strategy merits clinical evaluation. A proviso is that anti-angiogenic therapies to date, while they often temporarily slow cancer spread, have had little impact on overall survival, probably because the hypoxic tumor environment induced by suppression of angiogenesis tends to select for highly aggressive cancer variants.[33-35]

M1/M2 Macrophage Polarization in Tumors – How Does GcMAF Fit into the Picture?

Macrophages are usually the most prominent immune cell within tumors, reflecting the fact that a high proportion of cancer make cytokines (e.g. CSF-1, CCL2, VEGF, angiopoietin-2) that attract macrophages.[36] A peculiarity of the research literature on GcMAF is that it seems to be completely divorced from the broader burgeoning research literature examining the impact of macrophages on cancer spread. Notably, there are hundreds of studies which have focused on the role of tumor-associated macrophages; ironically, at least in advanced cancers, these macrophages are prone to promote tumor progression. This reflects the fact that macrophages are capable of assuming several phenotypes.[36, 37] So-called "classically activated" or "M1" macrophages have tumoricidal capacity; this killing of cancer cells is achieved both indirectly – via activation of natural killer cells and cytotoxic T lymphocytes – and directly, by production of such agents as TNF, TRAIL, Fas ligand, nitric oxide and oxygen radicals, and by antibody-assisted phagocytosis. How M1 macrophages recognize cancer cells to target them is still somewhat mysterious, but perceived differences in cell membrane lipids and protein glycation are likely to play a role.[38] M1 macrophages are characterized by production of IL-12, IL-23, TNF, IL-1, superoxide and nitric oxide, and they have high capacity for antigen presentation.[36] This M1 phenotype is promoted by exposure to microbial products that activate various pattern recognition receptors, and by interferon-gamma secreted by T helper cells or natural killer cells. Activation of a range of transcription factors, including IRF3, IFRF5, IRF7, and NF-kappaB are downstream mediators of this phenotype.[39, 40]

However, macrophages can also assume an "alternative", or "M2" phenotype, which is far less cytotoxic, actively suppresses recruitment of cytotoxic T lymphocytes and natural killer cells, and promotes tumor spread by evoking angiogenesis and boosting matrix proteolytic activity.[36] And, not surprisingly, progressing cancers typically master the knack of converting tumor-associated macrophages to M2 behavior; how they achieve this is in need of further clarification. M2 macrophages typically produce the immunosuppressive cytokine IL-10, as well as TGF-beta and pro-angiogenic factors; they have limited capacity for antigen presentation or superoxide production.[41] Intracellular mediators of this behavior include Stat3, IRF4, and p50 homodimers that functionally antagonize NF-kappaB activity.[42-44]

Currently, a great deal of research effort is being devoted to defining strategies which might be capable of converting tumor-associated M2 macrophages to an M1 phenotype; experimental strategies which achieve this in tumor-bearing rodents are sometimes capable of achieving rapid and substantial tumor kill.[37, 38, 45] Although many avenues are being pursued, double-stranded RNA, or drugs which mimic this, are of particular interest, as they have been employed for over 40 years to achieve macrophage activation and tumor control in rodent studies.[46] Moreover, several phase 1/2 clinical trials provide evidence that these agents may often have at least modest activity as adjuvant cancer therapies, and can be well tolerated in defined dose schedules; the agents poly A:U and poly I:C have received the most attention in this regard.[47-52] Double-stranded RNA is "perceived" by our cells as a sign of viral infection, and endosomal TLR3 and TLR7 receptors, as well as various intracellular proteins, can interact with double-stranded RNA to generate signals that induce interferon generation while also promoting M1 behavior in macrophages.[53-55] Other agents capable of promoting M1 polarization in macrophages via activation of toll-like pattern recognition receptors include monophosphoryl A (for TLR4), iquimod (for TLR7), and CpG-oligodeoxynucleotide (for TLR9), which have all shown promising anti-tumor activity in pre-clinical studies, and are starting to receive clinical evluation.[37, 56-59]

The known impact of GcMAF on macrophages is to very markedly activate superoxide generation and boost phagocytic capacity; the intracellular signaling pathways which achieve this remain mysterious. There is currently no evidence that GcMAF can influence M1/M2 polarization in macrophages; indeed, Folkman's group failed to observe any impact of GcMAF pre-treatment on macrophage production of IL-12, a key product of M1 macrophages.[30] Others reported that GcMAF treatment failed to influence TNF or NO production by macrophages.[60] It would be interesting to determine whether GcMAF's impact on M1 macrophages differs from its impact on M2 macrophages. Would M1 macrophages become extraordinarily potent cancer-killing machines if treated with GcMAF? Does GcMAF treatment of the M2 polarized macrophages that tend to predominate in advanced tumors have any utility? And would GcMAF administration be more useful for control of advanced tumors, or more potently and uniformly effective for eliminating micrometastases, if allied with practical measures that promote M1 polarization, such as poly I:C or microbial products? It would be of great interest to address these issues in cell culture studies and rodent tumor models. GcMAF was characterized over twenty years ago, and the integration of GcMAF research into the broader stream of research focused on tumor-associated macrophages is long overdue.

Monitoring Nagalase for Early Cancer Detection?

The intriguing possibility that nagalase assessment and GcMAF treatment might enable early detection and elimination of nascent cancers, has been raised in an insightful monograph posted online by Tim Smith.[61] If indeed many incipient cancers raise serum nagalase levels, the inclusion of nagalase in standard health profiles might enable detection of cancers before they become symptomatic – and in an early phase where they might be susceptible to elimination, not only by surgery (if a source can be established), but also conceivably by a course of GcMAF treatment. Of course, it is still unclear whether very early cancers are prone to raise serum nagalase; Yamamoto's data pertain to patients with well established, often pre-treated, cancers, and recall that the evaluation of nagalase in melanoma patients failed to note an elevation of serum nagalase in patients with phase 1 or 2 disease. But, if nagalase proves to be a genuine and near-universal marker for cancer, the possibility that it might be employed to achieve early detection at a curable stage of at least *some* cancers is quite exciting.

Questions to Address in Future Research

Clearly, the interrelated research literature on GcMAF and nagalase shows very intriguing promise, but it is currently underdeveloped, and far too isolated from major research trends in cancer immunology. Questions that merit consideration in future research include:

Is nagalase the nearly universal cancer marker that it has been purported to be?

If so, why – what oncogenes drive its expression?

Is serum nagalase elevated in a high proportion of early tumors, so that it could be monitored as an "early warning system" for cancer?

Precisely how does nagalase impair the generation or maintenance of GcMAF activity in tumors?

What is the molecular biology that underlies GcMAF's ability to boost superoxide production and phagocytic activity in macrophages?

Does GcMAF work in other ways to aid the cancer-killing activity of macrophages?

Does GcMAF influence M1/M2 polarization in tumor-associated macrophages?

Does GcMAF treatment notably boost the tumoricidal activity of M1 polarized macrophages?

Is weekly GcMAF administration as effective for eliminating microscopic metastatic disease as suggested by Yamamoto's published clinical trial – at least for certain major cancers?

If so, which types of cancer respond most effectively?

Can GcMAF administration slow the growth of more advanced cancers?

Would addition of certain M1-polarizing agents to GcMAF therapy boost its efficacy and make it more useful for control of macroscopic tumors?

Would daily administration of GcMAF have utility as an anti-angiogenic strategy in cancer therapy – and would such regimens be safe?

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