

Gc Protein-Derived Macrophage Activating Factor (GcMAF) and Autism: Do Clinical Results Require a Novel Interpretation?

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Abstract: In this Editorial I reinterpret the results observed with the Gc protein-derived Macrophage Activating Factor (GcMAF) at the light of two recent papers published on this topic. According to the results and the hypothesis emerging from these papers, the biological and clinical effects thus far attributed to GcMAF are indeed to be ascribed to a glycosaminoglycan, chondroitin sulfate, that binds both the precursor and the active form of GcMAF. Such an interpretation has the advantage of solving all the contradictions and inconsistencies that have recently characterized the field of GcMAF-based immunotherapy. This novel interpretation is particularly apt at explaining the results observed *in vitro* and *in vivo* concerning the administration of GcMAF to autistic subjects.

Keywords: Autism, Macrophages, Chondroitin Sulfate, Immunotherapy, Nagalase, Inflammation

Introduction

The Gc protein-derived Macrophage Activating Factor (GcMAF) has been the object of intense investigation since its first description (Yamamoto and Kumashiro, 1993) because of its potential therapeutic use in a number of different conditions associated with dysfunctions of the immune system. Interestingly, such an intense investigation has been paralleled by an equally intense controversy (Ugarte *et al.*, 2014).

According to the hypothesis first proposed by Yamamoto and Kumashiro (1993), GcMAF derives from a plasma protein, the Gc protein (also known as vitamin D-binding protein), following the enzymatic removal of two sugars attached to threonine 420 during immune responses. The enzymes beta-galactosidase, released by activated B lymphocytes and sialidase, released by activated T lymphocytes, would remove galactose and sialic acid, thus exposing N-acetylgalactosamine (GalNAc) as the remaining sugar moiety attached to threonine 420 of the Gc protein. GalNAc would then be the active site of GcMAF.

According to the hypothesis by Yamamoto *et al.*, (1995), patients infected with HIV, cancer patients (Yamamoto *et al.*, 1996), or patients with *Lupus* (Yamamoto *et al.*, 1997), had elevated plasma levels of an enzyme, alpha-N-acetylgalactosaminidase (nagalase). This enzyme that would remove GalNAc from the Gc

protein, thus preventing its conversion to the active GcMAF. Thus, according to this hypothesis, elevated nagalase levels would correspond to decreased production of endogenous GcMAF and, hence, to immunodeficiency (Yamamoto *et al.*, 1996; 2008).

Based on this hypothesis, Yamamoto *et al.* proposed to produce GcMAF in the laboratory by enzymatically treating Gc protein extracted from human blood and to administer this GcMAF to patients with elevated nagalase in order to overcome the supposed deficiency of endogenous GcMAF. Such an administration would have restored the functionality of the immune system in HIV and cancer patients thus leading to “eradication” of HIV and cancer.

However, the papers where the results achieved by administering GcMAF produced in the laboratory to HIV patients or patients affected by breast or colon cancer, have been retracted and the entire rationale for such an approach has been severely criticized (Retraction, 2014a; 2014b; 2014c; Ugarte *et al.*, 2014).

As a matter of fact, clinical observations and experimental findings demonstrate that the hypothesis on which GcMAF administration was based shows significant inconsistencies:

1. Subjects who cannot produce even a single molecule of GcMAF show no signs of immunodeficiency or predisposition to cancer; on the contrary, they have

reduced risk of cancer. This observation refers to subjects who harbor the Gc2 allele only (Gc2 homozygotes) of the Gc protein. These individuals are unable to glycosylate the Gc protein on threonine 420 due to its substitution by lysine. Thus, there is no GalNAc in position 420. In other words, Gc2 homozygotes are unable to produce one single molecule of GcMAF. However, despite this fact, the risk of cancer in these individuals is decreased rather than increased as one would have expected given the absence of bona fide GcMAF (Abbas *et al.*, 2008)

2. Subjects with elevated nagalase, such as autistic children, do not show signs of immunodeficiency (Bradstreet *et al.*, 2012)
3. Subjects with cancer do not show decreased levels of endogenous GcMAF; on the contrary the level of their endogenous GcMAF not only is not different from healthy subjects, but it is significantly higher than the trace amounts of the exogenously administered GcMAF that should “eradicate” their cancer (Rehder *et al.*, 2009)
4. Nagalase degrades GcMAF by removing GalNAc, thus rendering it inactive (Mohamad *et al.*, 2002; Borges and Rehder, 2016). Therefore, in patients with elevated nagalase, the trace amounts of exogenously administered GcMAF would be immediately degraded
5. The procedure to produce GcMAF in the laboratory using the enzyme beta-galactosidase from *E. Coli* (Kuchiike *et al.*, 2013; Uto *et al.*, 2015) is not consistent with experimental data. Thus, this enzyme does not cleave galactose from the Gc protein (Borges and Rehder, 2016). Therefore, it is questionable whether using such a procedure even a single molecule of GcMAF is produced.

It is worth noticing that, despite these obvious inconsistencies, GcMAF has been independently studied for about two decades by several research groups that reported consistent results *in vitro* and *in vivo* (for a recent review, see Ruggiero *et al.*, 2016). My former research group, among others, has witnessed the effects of GcMAF derived from human blood on human mononuclear cells and on angiogenesis (Pacini *et al.*, 2010; 2012a), on human breast cancer cells (Pacini *et al.*, 2012b; Thyer *et al.*, 2013a) and on human neurons and glial cells (Morucci *et al.*, 2015; Branca *et al.*, 2015). We have also observed the clinical effects of GcMAF administration in the context of an integrated immunotherapeutic protocol for cancer (Ruggiero *et al.*, 2014) and members of my former research group have described the effects of integrated GcMAF immunotherapy in diseases as diverse as cancer (Thyer *et al.*, 2013b), chronic fatigue syndrome, multiple

sclerosis, Lyme disease, amyotrophic lateral sclerosis, syphilis and autism (Thyer *et al.*, 2013c).

These observations of ours have been independently corroborated, among others, by researchers from Japan who have reported encouraging results obtained using a similar immunotherapeutic approach in cancer (Inui *et al.*, 2013; 2014; Inui *et al.*, 2016a), multiple sclerosis (Inui *et al.*, 2016b), serious infections and chronic fatigue syndrome (Inui *et al.*, 2015).

In an effort to reconcile the results observed by us and many others with the inconsistencies described above, we recently hypothesized that the biological and clinical effects attributed to GcMAF are indeed to be ascribed to a glycosaminoglycan, chondroitin sulfate, that binds to Gc protein and GcMAF in plasma and other bodily fluids as well as on the surface of the cells of the immune system (Ruggiero *et al.*, 2016). According to this hypothesis, the glycosylation status of the Gc protein is irrelevant in determining the biological and clinical effects that we and others have observed with GcMAF and the active GalNAc would be the one that is a constituent part of chondroitin sulfate.

This novel hypothesis is particularly apt at explaining the results obtained with GcMAF in autism spectrum disorders as they were firstly described by the late Dr. Bradstreet (Bradstreet *et al.*, 2012) and later confirmed by members of my former research group (Thyer *et al.*, 2013c).

In the study by Bradstreet *et al.* (2012), it was observed that, in a cohort of forty autistic children, all subjects had elevated levels of nagalase. After an average 14 weeks of weekly subcutaneous injections with human blood-derived GcMAF, all autistic subjects, but one, showed a decrease of nagalase levels and a significant percentage of them showed improvement of the clinical symptoms of autism evaluated according to Clinical Global Impression of Improvement scale. These results are now difficult to interpret at the light of the recent observation that elevated nagalase should have degraded the exogenously administered GcMAF as it was demonstrated by Borges and Rehder (2016). The source of elevated nagalase could not be accounted for and the conundrum of the lack of signs of immunodeficiency in spite of elevated nagalase could not be explained.

These inconsistencies between the biochemistry of GcMAF and the clinical results appear all the more striking considering that GcMAF has been described by the late Dr. Bradstreet as “one of the most powerful tools I have ever used for autism” during his last public speech at the AutismOne Conference in May 2015 (the speech of Dr. Bradstreet is publicly available at <https://www.youtube.com/watch?v=6I2Wr9ihvV0> and his last words on GcMAF are at minute 52:26).

If, however, we accept the hypothesis that the biological effects of GcMAF are either due to, or mediated by chondroitin sulfate, then all the inconsistencies cease to exist (Ruggiero *et al.*, 2016). Thus, chondroitin sulfate is constituted by GalNAc, the supposed active site of GcMAF, but it is not affected by nagalase since this enzyme is not endowed with endoglycosidic function (Borges and Rehder, 2016).

Interestingly, chondroitin sulfate exhibits most, if not all, the biological and clinical properties attributed to GcMAF, including the anti-inflammatory and immune modulating effects recently reported by Theoharides *et al.* (2016) when referring to the use of GcMAF in autism. Thus, it is well known that glycosaminoglycans such as chondroitin sulfate are associated with central nervous system development, maintenance and disorders. The relevance of chondroitin sulfate in the pathogenesis of autism and other neurological and psychiatric disorders is corroborated by the evidence that, as a component of the extracellular matrix that interacts with the perineuronal nets, chondroitin sulfate plays a fundamental role in regulating synaptic functions and plasticity (Pantazopoulos and Berretta, 2016).

Furthermore, disaccharides derived from chondroitin sulfate have been implicated in the inhibition of neurodegeneration by influencing microglia activation (Ebert *et al.*, 2008) in a manner consistent with what we had observed treating microglial cells *in vitro* with GcMAF (Branca *et al.*, 2015). Consistent with these evidences, experimental and clinical data suggest that chondroitin sulfate might be a useful therapeutic agent in neurological diseases that are characterized by inflammation such as Parkinson's and Alzheimer's diseases, multiple sclerosis and amyotrophic lateral sclerosis (Vallières and du Souich, 2010) that are some of the conditions successfully treated with GcMAF (Thyer *et al.*, 2013c).

Also the molecular structure of chondroitin sulfate is consistent with the hypothesis that this glycosaminoglycan is the molecule responsible for the macrophage stimulating activity that we and others had attributed to GcMAF. Thus, researchers at the Arizona State University, demonstrated that the macrophage stimulating activity resides in a GalNAc residue exposed in the context of an alpha helix (Bogani *et al.*, 2006) and chondroitin sulfate is precisely composed of a chain of alternating GalNAc and glucuronic acid that are arranged in a right handed helical structure (Németh-Csóka *et al.*, 1975). Therefore, it can be hypothesized that the GalNAc of chondroitin sulfate and not that exposed on the Gc protein is responsible for activating macrophages and directing the immune response (Wrenshall *et al.*, 1999). This observation would explain the reason why Gc2 homozygotes, who cannot produce a single

molecule of GcMAF but have plenty of chondroitin sulfate, do not suffer of immunodeficiency and are not predisposed to cancer (Abbas *et al.*, 2008).

Furthermore, it is important to notice that the GcMAF used in the study on autism quoted above was obtained by enzymatic treatment of a plasma protein, the Gc protein, that had been isolated from human plasma by vitamin D₃-Sepharose or actin-agarose affinity chromatography with no description of further purification steps aimed at removing plasma glycosaminoglycans (Bradstreet *et al.*, 2012). It is well known that Gc protein belongs to the superfamily of albumin and it is present in fraction IV of Cohn-Oncley fractionation of human plasma. Since 2007, we had demonstrated that chondroitin sulfate is highly represented in this very Cohn-Oncley fraction (Cecchi *et al.*, 2007) and, considering that in 1999, DiMartino and Kew had demonstrated that Gc protein binds to chondroitin sulfate (DiMartino and Kew, 1999), it can be deduced that Gc protein and hence the studied GcMAF, were associated and co-extracted with chondroitin sulfate.

Another point that requires reinterpretation is the role of nagalase in autism; thus, it is difficult to envisage the source of nagalase in those patients since autism is not associated with viruses known to produce the enzyme such as influenza virus (Yamamoto and Urade, 2005) or HIV-1 (Yamamoto, 2006). If, however, we consider that autism is associated with widespread inflammation and with higher concentration of pro-inflammatory cytokines (Masi *et al.*, 2015), then it can be speculated that nagalase, being a lysosomal enzyme (Suh *et al.*, 2015), may be interpreted as a marker of chronic inflammation rather than a marker of immunodeficiency. Since chondroitin sulfate is endowed with anti-inflammatory properties and it exerts this action upstream of the inflammasome by inhibiting activation of NF-kappaB transcription factors (Stabler *et al.*, 2016), it is conceivable that the observed reduction of nagalase was due to chondroitin sulfate rather than to GcMAF.

Conclusion

The two most recent publications on GcMAF (Borges and Rehder, 2016; Ruggiero *et al.*, 2016) help reinterpreting the biological and clinical results independently observed *in vitro* and *in vivo* by a number of researchers. The hypothesis that chondroitin sulfate may be responsible for the effects thus far attributed to GcMAF, solves all the inconsistencies and contradictions that have characterized this field of immunotherapy. Furthermore, this hypothesis lays the foundation for the development of non-proteinic macrophage activating factors that are not extracted from human blood, thus avoiding all the risks associated with human blood-derived products.

Author's Contribution

Marco Ruggiero wrote and revised the manuscript.

Conflict of Interest

The Author is a retired full professor of molecular biology at the University of Firenze, Italy and he is currently a consultant for the company "dr. reinwald healthcare", a company that organizes seminars and trainings for therapists and commercializes nutritional supplements and therapy devices. None of the products or devices distributed by the company is mentioned in this article and the Author has not received financial compensation for writing this article. The Author is member of the Editorial Board of The American Journal of Immunology and is waived from the Article Processing fee for this contribution; the Author receives no remuneration for his editorial work.

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