GcMAF: our next-generation immunotherapy

Authors
Yoshihiro Uto[1], Hitoshi Hori[1], Kentaro Kubo[2], Masamitsu Ichihashi[3], Norihiro Sakamoto[3,4], Martin Mette[5], Toshio Inui[2,3,5]

[1] Department of Life System, Institute of Technology and Science, Graduate School, The University of Tokushima, 2-1 Minamijosanjima-cho, Tokushima, Japan.

[2] Saisei Mirai Cell Processing Center, Nature21 Building 3F, 3-34-8 Okubo-cho, Moriguchi, Osaka, Japan.

[3] Kobe Saisei Mirai Clinic, Kobe Commerce Industry and Trade Center Building 23F, 5-1-14, Hamabe-dori, Chuo-ku, Kobe, Japan.

[4] Division of Food and Drug Evaluation Science, Department of Community Medicine and Social Healthcare Science, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe, Japan

[5] Inui Immunotherapy Clinic, 6-14-17 Kindacho, Moriguchi, Osaka, Japan.

Saisei Mirai, based in Osaka, Japan, is a medical organization specializing in cancer immunotherapy. The name Saisei Mirai is derived from the Japanese words for regeneration and future. It is our mission to provide cancer patients with the latest, most effective treatments while also ensuring the best possible quality of life. Cancer immunotherapy is the use of the immune system to reject cancer. In particular, it involves stimulating the patient's immune system to locate and eliminate the cells that are cancerous. This is achieved with immune cells such as Natural Killer cells (NK cells), T Lymphocytes, Dendritic Cells (DC), and macrophages, among others. A variety of treatments, such as GcMAF, Hyper T/NK cells, Coley vaccine, gene therapy, cancer vaccines, and intravenous high-dose vitamin C or α-lipoic acid are routinely used to stimulate the immune system.

The immune system is the body's vital self defense that protects against disease. It can recognize a wide variety of pathogens found in the environment and even tumor cells borne within the human body. The mechanisms of protection fall into two broad categories — innate immunity and adaptive immunity. There are several types of cells in the innate immune system: phagocytic neutrophils; macrophages; dendritic cells; mast cells; and natural killer (NK) cells. The adaptive immune system is comprised of the lymphocytes, both T cells and B cells, which are able to recognize and remember specific pathogens, and their products, including antibodies.

The progression of many diseases, such as cancer and AIDS, follows the collapse of the immune system. We now know that stimulating the immune system can halt or even reverse such diseases. Immunotherapy has become an attractive new strategy in the treatment of cancer[1]. The laboratory and clinical study of cancer immunotherapy — such as dendritic cells, autologous lymphocyte-activated killer (LAK) cells, autologous NK cells, monoclonal antibodies, cancer peptide vaccines, cytokines, and biological response modifiers (BRM) — is rapidly advancing. However in a clinical setting, the results of cancer immunotherapy are mixed.

We therefore contend that cancer immunotherapy should be customized to each individual patient based on their immune status. We propose our next-generation Gc protein-derived macrophage activating factor (GcMAF) as a promising candidate for a patient-friendly cancer immunotherapy. GcMAF, also known as vitamin D binding protein-macrophage activating factor (DBP-maf), is a potent endogenous macrophage activator found naturally in the blood. Until recently, GcMAF was not used in cancer immunotherapy in spite of it being a key player of our innate immune system.

We will review first-generation GcMAF developed by Dr Yamamoto and second-generation GcMAF currently under development at Saisei Mirai (Fig. 1). We will also present a number of other therapies used in combination with GcMAF.

At Saisei Mirai, we are currently treating cancer patients with GcMAF-based cancer immunotherapy. We use GcMAF in combination with other immune system-related therapies, such as regional and whole-body hyperthermia, Coley vaccine, which stimulates fever within the body, and Hyper-T/NK-cell therapy. All of these therapies are about strengthening the immune system and take a holistic approach to fighting cancer rather than a localized approach that is common with conventional therapies such as radiation and surgery.

Coley vaccine

Coley vaccine — also known as Coley's Toxins and Coley Fluid — was one of the

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first immunotherapies to enter the clinic. Coley-vaccine therapy is a medical treatment used to induce a fever to improve the immunological competence of the patient. For hundreds of years, doctors have reported cases of tumors that disappeared, apparently spontaneously after patients developed high fevers from infection. In many cases, when fever subsided, tumors had broken down and been absorbed or sloughed off. Infection seemed to be key to these miraculous cures. It was not until 1893 when Dr William Coley began injecting a sterile mixture of *Streptococcus pyogenes* and *Serratia marcescens* bacteria into cancer patients to induce fevers, that this observation was put into clinical practice. By using a suspension of killed bacteria, Coley was able to mimic an acute infection, without the risks of an actual pathogen.

Hyperthermia

Hyperthermia heat therapy, also called thermotherapy, is the application of heat to the body in order to treat a medical condition (Fig 2). It is used to activate a patient's immunological competence and is often utilized in combination with other medical treatments at Saisei Mirai.

At Saisei Mirai a patient can select from a wide range of suitable and compassionate integrative medical treatments in consultation with doctors experienced with cancer immunotherapy. Recently, immunotherapy treatments are being administered to people living with with infections, such as HIV, hepatitis B virus (HBV) and hepatitis C virus (HCV).

Saisei Mirai has a rich history in cancer research. It has collaborated with a number of renowned research centers such as Tokushima University, Kagoshima University and Kobe University and has contributed significantly to the clinical application of new cancer therapies.

Discovery and basic study of first-generation GcMAF

GcMAF, discovered by Dr Nobuto Yamamoto in 1991, is derived from the group specific component (Gc) protein (vitamin D binding protein), a member of the albumin superfamily[2]. GcMAF is an interesting serum glycoprotein with various biological activities.

It is reported that during an inflammatory response, β-galactosidase of an activated B-cell and sialidase of a T-cell hydrolyze the terminal galactose and sialic acid saccharides of Gc protein to produce GcMAF[3]. GcMAF has interesting biological activity; it activates macrophages via superoxide radical generation and phagocytic activation[4],

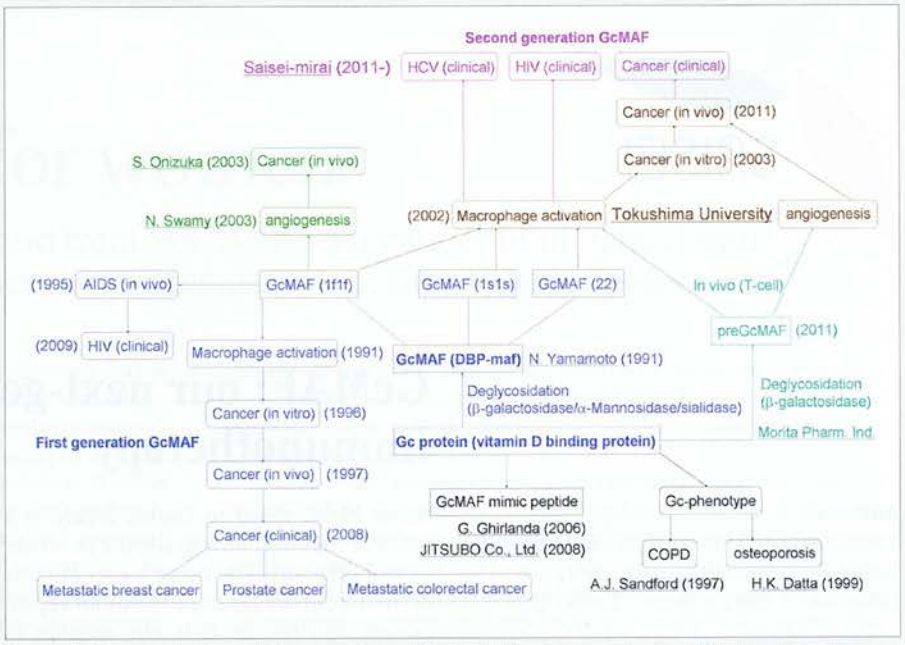

Figure 1: Research map of Gc protein and GcMAF. First-generation GcMAF developed by Dr Nobuto Yamamoto. Second-generation GcMAF proposed by Dr Yoshihiro Uto, Dr. Hitoshi Hori, DrToshio Inui and Dr Kentaro Kubo.

Figure 2: Whole body hyperthermia. This equipment can heat tumors within the patient's body with a special heat control system.

and has been demonstrated to have anti-angiogenic and anti-tumor activity *in vivo*[5]. GcMAF also directly inhibits proliferation and migration of human prostate cancer cells or human breast cancer cells independent of its macrophage activation ability[6].

Clinical study of first-generation GcMAF for cancer and HIV treatment

Clinical trials using GcMAF in patients with metastatic breast cancer[7], prostate cancer[8], and metastatic colorectal cancer[9] have been conducted. Cancer did not recur over a four to seven year period in all subjects administrated weekly doses of 100 ng of GcMAF for 7 to 19 weeks; a result that took everyone by surprise. However, there are some problems with these clinical trials: there were no clear classifications of patients' histopathological types, grades, and stages, and the curative judgment was based solely on a patient's N-acetylgalactosaminidase (Nagalase) activity, and neither tumor markers or cytokine levels were measured, and there was no control group.

There is also an interesting clinical report of HIV treatment using GcMAF[10]. The weekly administration of 100 ng of GcMAF to 15 non-anemic HIV-infected patients showed that the number of $CD4^+$ cells increased to normal levels within 6 weeks, and were maintained for the entire 7 years after GcMAF therapy, while the number of $CD8^+$ cells decreased to normal levels, and the amount of HIV-1 RNA and p24

antigen were detectable in these patients blood stream. It is believed that this positive effect resulted from GcMAF-activated macrophages phagocytosizing and destroying the HIV virus. GcMAF was active in the monocytes/macrophages isolated from AIDS patients[11].

Pharmaceutical development of first-generation GcMAF for immunotherapy

There is little or no research being conducted on the pharmaceutical development of GcMAF as a biological drug. The pharmaceutical development of GcMAF with its high homogeneity and purity from human serum is very difficult owing to its parent Gc protein having six major subtypes, namely homodimer or heterodimer of 1f, 1s, and 2, each with a different sugar moiety (Fig. 3). In order to overcome this problem, Dr Yamamoto manufactured the GcMAF mimic glycoprotein using a baculovirus expression system. Dr Giovanna Ghirlanda and colleagues developed a small molecule GcMAF constituting 23-mer glycopeptides with a similar sugar chain of GcMAF with macrophage phagocytic activation abilities comparable to GcMAF[12]. We also developed 12-mer GcMAF-mimic glycopeptides having their macrophage phagocytic activation abilities and *in-vivo* anti-angiogenic activities in the chick embryo's chorioallantoic membrane (CAM) assay in a joint study with JITSUBO Co., Ltd. We hope that practical pharmaceutical development of GcMAF for clinical use will begin soon.

Pharmaceutical development and clinical study of second-generation GcMAF for immunotherapy

The first-generation GcMAF were prepared by artificial enzymatic treatments from 1f1f-subtype Gc protein. First-generation GcMAF treatment is not personal and does not consider each patient's immune status, possibly caused by differences of Gc protein types, GcMAF level, and immune state of each subject (Fig. 4A). Accordingly, we propose second-generation GcMAF, which considers the patient's individual immune status. Our second-generation GcMAF-based immunotherapy (Fig. 4B) is characterized as; 1) the artificial exogeneous preparation of GcMAF using a sample of the patient's serum or serum from his or her close relatives, 2) examination of patient's GcMAF level and immunity status before GcMAF treatment, 3) the optimized GcMAF preparation is administered to the cancer patient.

We hypothesized that the GcMAF precursor (preGcMAF) — the full Gc protein, only lacking the galactosyl moiety — could be converted to GcMAF *in vivo* by processing with constitutive sialidase. We recently found that 1f1f-subtype of preGcMAF, as well as GcMAF itself, could be used as an effective precursor-type macrophage activator *in vivo*[13]. These results make it clear that β-galactosidase-treatment is a common modification for all subtypes of Gc protein to prepare GcMAF *in vivo*. Morita

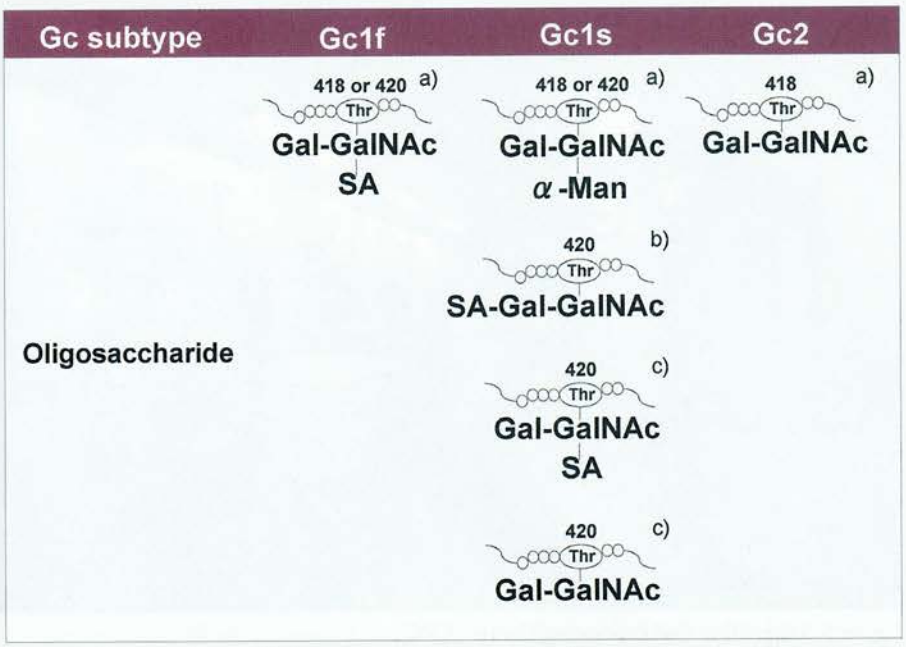

Figure 3: Proposed sugar chain structure of Gc protein subtype. a) Sugar structure proposed by Dr Nobuto Yamamoto. b) Sugar structure proposed by Dr C.R. Borges with LC-MS/MS analysis. c) Sugar structure proposed by Dr. Yoshihiro Uto and Dr. Hitoshi Hori with lectin analysis. GalNAc: N-acetylgalactosamine; Gal: galactose; SA: sialic acid; α-Man: α-mannose.

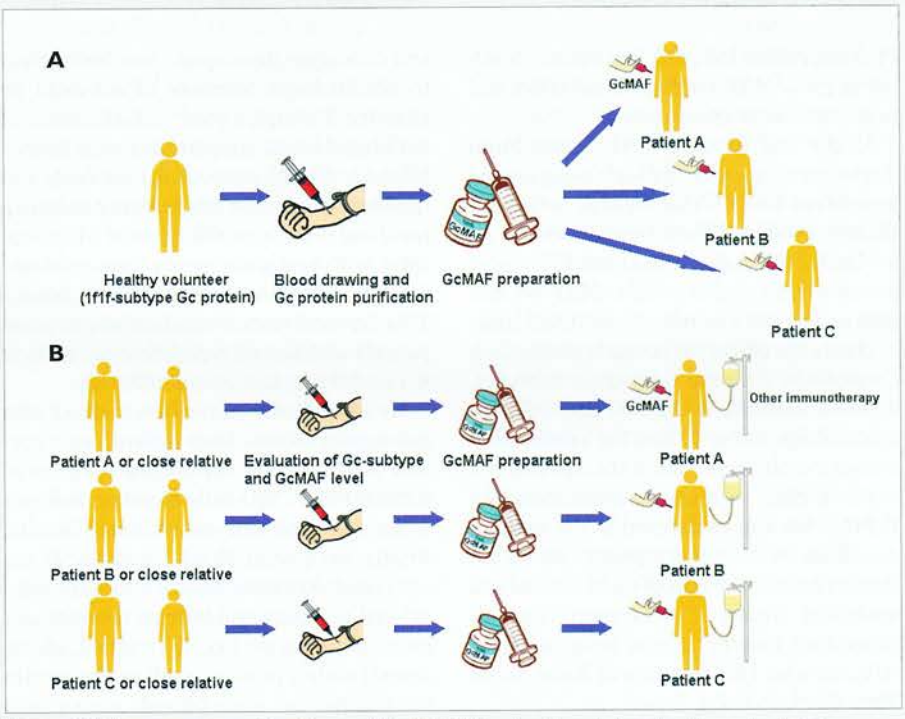

Figure 4: The treatment of patients with GcMAF using blood donated by volunteers.
(A) First-generation GcMAF is prepared from the serum of a healthy volunteer having 1f1f-subtype Gc protein. This GcMAF is then used to treat unspecified patients.
(B) Second-generation GcMAF is prepared from the serum of the cancer patient or his or her close relatives and used to treat that particular patient specifically.

Figure 5: Saisei Mirai Cell Processing Center (CPC).
(A) Cell culture room in CPC. Each room has an air lock. To prevent contamination of outside air, dust and microbes, each room is maintained at a set room pressure. The conditions in each room and important equipment is monitored 24 hour a day. (B) Treatment of cell culture in the safety cabinet. The worker wears special clothes and shoes. We treat cells under aseptic conditions and observe the condition of the culture every day under a microscope. (C) The cell-banking system introduced with liquid nitrogen tank. Cells such as T lymphocytes from the patient are kept for long periods for use in the future.

Pharmaceutical Ind., Ltd. is currently developing preGcMAF for preclinical trials and towards clinical application.

At the end of April 2011, Saisei Mirai started treating cancer patients using second generation GcMAF macrophage activation therapy produced from human serum. As at March 2012, Saisei Mirai have 137 cancer patients registered as using GcMAF. We also plan to conduct a comparative clinical study to clarify the efficacy of several combination therapies of different Gc protein subtypes, different content of GcMAF, and different macrophage status to find the relationship between each combination therapy and the curative effect of human serum including GcMAF. We aim to determine the optimal condition of the macrophage activation therapy from the results of these clinical and analytical studies. Furthermore, second-generation therapy is now being used for HIV and also HCV patients at Kobe Saisei Mirai Clinic in Kobe, Japan.

Hyper T/NK cell Therapy

In a previous study, tumor-infiltrating lymphocytes (TIL) have been reported to be effective in experimental and clinical research of advanced cancer[14,15]. TIL recognize specific antigens expressed by autologous tumor cells. However, this specific antigenicity is too low to achieve a high degree of antigenicity in therapeutic use. In order to solve this problem, Sekine and colleagues developed a feasible method to obtain large numbers of activated or effective T lymphocytes[16,17]. Cultivation of peripheral blood lymphocytes with immobilized anti-CD3 monoclonal antibody and human recombinant interleukin-2 induces a rapid and massive proliferation of T lymphocytes and greatly augments their cytotoxic activity. Administration of these expanded TILs demonstrates clinical activity in some patients with several types of cancer, making it a useful adoptive immunotherapy.

We developed a cultivation method with autologous plasma from patients with specific antibodies for membrane antigens of natural killer (NK) cells based on Sekine's techniques and our own clinical results. Briefly, peripheral blood lymphocytes are cultivated with immobilized anti-CD3 monoclonal antibody and human recombinant interleukin-2 as per Sekine's method, adding several matrix proteins such as fibronectin in plasma or immobilized monoclonal antibody such as CD161 for NK cells[18]. This cultivation method is used not only to obtain activated T lymphocytes, but also 'Hyper T cells' and NK cells. Hyper T cells, a name we coined, are unique immature multipotent T cells with various capabilities. Populations of these cells deplete as we age. In particular, a significant decline in number is evidnent in people over 50 years of age. Hyper T cells have a broader specificity for antigens expressed by autologous tumor cells, are able to proliferate and maintain their activity for long periods *in vivo*[19]. Therefore, expansion of Hyper T cells has the potential to be a suitable and important factor of adoptive immunotherapy against cancer. NK cells are a unique subset of lymphocytes, distinct from T lymphocytes. They contribute to essential immune systems such as host antimicrobial and antitumor immunity without requirement for prior immune sensitization of the host[20]. NK cells are promising effector cells for immunotherapy against cancer. Three cell types T lymphocytes, Hyper T cells, and NK cells, are cultured simultaneously. Methods to expand one cell type have been developed by several researchers. However, established methods of cultivation are problematic with respect to cost, complexity and safety, such as the risks of using biological tissue of animal or human origin including human serum albumin. Therefore, in spite of its possible effectiveness, it has not been entirely accepted in general medical practice. We developed a simple method of simultaneously culturing — T lymphocytes, Hyper T cells, and NK cells — which together seem to be important for an effective immune therapy. Another advantage of our method is free of animal and human material. Each cell is shown to be effective in cancer, but many problems are also overlooked. For example, it is said that NK cells have difficulty permeating deep into the tumor tissue. Accordingly, it is necessary

for NK cells to attack a tumor tissue after making a tumor is reduced in size by T lymphocytes and Hyper T lymphocytes. Such a sensible strategy makes it an effective immune therapy. Using these cells in combination allows the advantage of one cell type compensate for the disadvantage of using the other alone — their synergistic actions contribute to the eradication of tumor cells.

Actually, we tested this combination therapy with systemically administered T lymphocytes, Hyper T cells and NK cells in more than 100 cancer patients. This method can be of benefit to patients and is a promising immunotherapy. We expect that the described immunotherapy will play a central role in future treatments against certain human cancers, both alone and in combination with other therapies such as GcMAF and/or hyperthermia treatment.

Cell Processing Center

Saisei Mirai Cell Processing Center (CPC) is a special clean facility for cell culture and preparation of medicine for the patient (Fig 5). To maintain safety and high quality, only medical specialists with knowledge and skills for cell-culture work are employed at the CPC. In our tissue-engineered products, such as GcMAF, Hyper T NK cells, and cancer vaccines are manufactured.

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