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THE EFFECT OF GCMAF COMPLEXED WITH OLEIC ACID ON MULTIPLE MYELOMA CULTURES

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Abstract: Deglycosylated vitamin D-binding protein-derived macrophage-activating factor (GcMAF) is known to be a strong immune stimulatory natural molecule. Data in literature demonstrate that GcMAF has a direct role in decreasing cell proliferation of different cancer cell lines. In this study we evaluate the direct effect of GcMAF complexed with oleic acid (OA-GcMAF) on human multiple myeloma cells (KMS-12-BM), as well as the effect on the same cell line of human macrophages (CRL9853) previously activated by OA-GcMAF. Cell viability and living cell number were evaluated respectively by tetrazolium dye cell viability assay and by Trypan blue staining. Interactions between activated macrophages and myeloma cells were studied by time lapse photography. Our results show that OA-GcMAF decreases the cell viability of KMS-12-BM with a dose-dependent pathway. Furthermore OA-GcMAF activates human macrophages, which in turn phagocytise myeloma cancer cells. OA-GcMAF confirms its double effect on cancer cells: a direct inhibition of

their viability and, at the same time, an efficient macrophage activation leading to a significant depletion of cancer cell population. **Introduction:** In recent years the interest of vitamin D-binding protein-derived macrophage-activating factor (GcMAF) as a potent immunotherapeutic agent has increased. The GcMAF has been shown to be effective in stimulating murine macrophages *in vitro* to phagocytose human breast carcinoma cultures (1, 2), as well as inhibiting the growth of prostate cancer cells (3). It has also been the agent that has been referenced as reducing tumour burden in several clinical approaches (4, 5). In previous studies GcMAF has been used to stimulate Raw 264.7 cells (murine macrophage cell line) that were observed *in vitro* to phagocytose MCF-7 cells (human breast carcinoma). In this study we demonstrate the effect of GcMAF stabilized with oleic acid (OA-GcMAF) directly on KMS-12-BM multiple myeloma cells and on co-culture of stimulated human macrophages (CRL9853) and KMS-12-BM. **Materials and Methods:** Cell lines: KMS-12-BM: human multiple myeloma cell line was purchased from DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) and cultured in RPMI 1640 supplemented with 10% FBS and 2mM L-Glutamine (Life Technologies, Paisley, UK). Cultures were passaged every 3-4 days. CRL9853: human spleen macrophage was purchased from ATCC (American Type Culture Collection, Teddington, UK) and routinely cultured in IMDM supplemented with 10% FBS and 2mM L-Glutamine (Life Technologies). Cultures were passaged every 3-4 days. Prior to assay, CRL9853 cells were activated culturing them for 72h in the presence of OA-GcMAF at a concentration 100ng/ml in complete medium. Stimuli: OA-GcMAF, commercially available, was prepared at Immuno Biotech Ltd. (Guernsey, Channel Island) with a proprietary procedure previously described (6). Cell viability

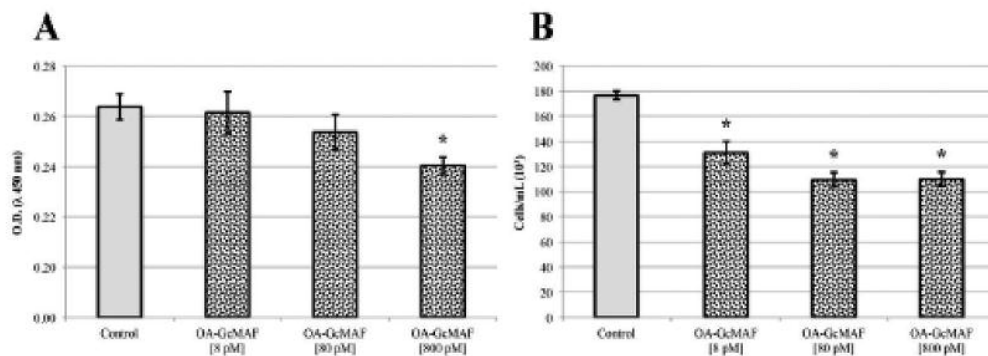


Figure 1. KMS-12-BM cell viability assay and Trypan Blue viable cell counts. (A) Results for (8 pM) demonstrate a slight decrease on cell proliferation which becomes significantly greater at (800 pM) indicating a direct effect on cell viability. (B) The decrease in viable cell number as the OA-GcMAF concentration increases corroborating the observed cell viability assay data. Results are expressed as mean value±S.E.M. for five replicates. (*p<0.01).

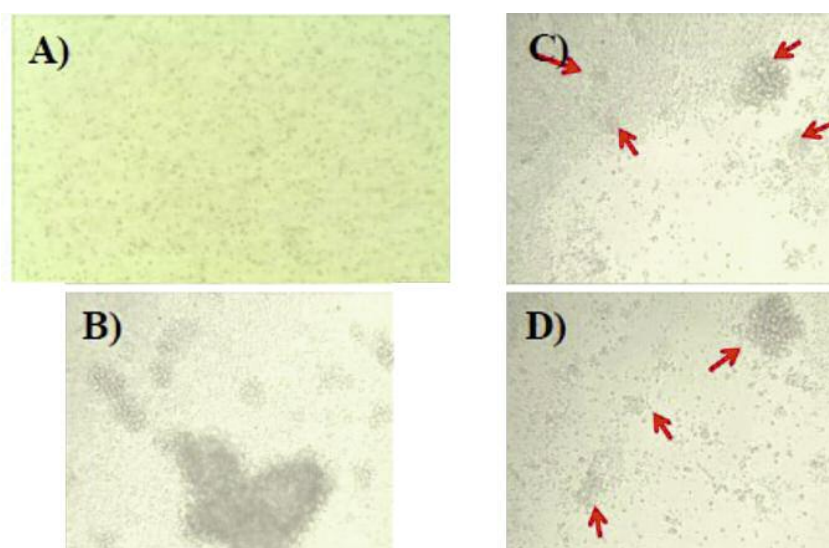


Figure 2. Selected frame shots over time of KMS-12-BM cells in the presence of OA-GcMAF-stimulated CRL9853 human macrophages. (A) KMS-12-BM suspension culture typical growth pattern. (B) KMS-12-BM cells 24h post addition of OA-GcMAF activated human macrophages. CRL9853 cultures as large masses of suspension cells and the dense collection of cells can be observed sitting on the KMS-12-BM suspension cells. The OA-GcMAF-activated macrophages are extremely motile under observation and move around clearing the KMS-12-BM cells. During the phagocytosis smaller dense “clusters” of cells break off and scavenge the KMS-12-BM cells quite rapidly. (C) KMS-12-BM cells 48h post addition of OA-GcMAF-activated human macrophages. CRL9853 cells now have a few large cell mass “clusters” and many smaller “clusters” as can be observed (red arrows). The number of KMS-12-BM cells has been decreased and the clearance of cells can be easily observed. (D) KMS-12-BM cells 72h post addition of OA-GcMAF-activated human macrophages CRL9853. OA-GcMAF-activated CRL9853 cells are still extremely active with the phagocytotic activity and are clearing the KMS-12-BM cells from the culture. This image shows the smaller clusters of cells as indicated by the red arrows. The clusters have become smaller and rapid moving seeking out and destroying the remaining KMS-12-BM cells.

assay: Cell viability was evaluated by the reduction of a tetrazolium salt (WST-8) as an index of cell dehydrogenases' activity. KMS-12-BM cells were seeded into a 96-well plate at a density of 3×10^4 cells/well in their appropriate starvation medium (without FBS). After incubation for 24h the cell line was treated for 24h with the following different concentrations of OA-GcMAF (8-80-800 pM). At the end of the treatment, the medium was replaced with 100 μ l of fresh starvation medium plus 10 μ l of WST-8. The 96-well plate was incubated for 3h at 37°C and the optical density (O.D.) was directly measured at A450nm by Multiscan FC photometer (ThermoScientific, Milano, Italy). Cell counting – Trypan blue assay: To corroborate the results obtained by cell viability assay, a viable cell count was performed. Briefly, KMS-12-BM cells were plated into a 6-well plate at a density of 2×10^5 cells/well in starvation medium. After 24h incubation, human multiple myeloma cells were treated with OA-GcMAF at the increasing concentrations (8-80-800 pM) for 24h. At the end of the treatment, a volume of cell suspension was collected and the viable cell number was counted by Trypan Blue staining.

Video-time lapse photography: KMS-12-BM cells were seeded into a 24-well plate at a density of 1×10^6 cells/well with a 1ml volume. The cells were allowed to settle for a minimum of 2h prior to the addition of the OA-GcMAF-activated CRL9853 macrophages. The staging mat was set at a temperature of 37°C and allowed to equilibrate prior to placement of the 24-well plate. The OA-GcMAF-activated CRL9853 macrophages were added to a final concentration of 5×10^5 cells per well in 1ml. HEPES (Fisher Scientific, Loughborough, UK) was added to each well to provide a final concentration of 25mM to stabilize the culture pH. Once activated macrophages and the HEPES were added, the 24-well plate was observed microscopically and an image selected. An initial frame was taken and a time-lapse film initiated. A frame was taken every 3 minutes until filming was stopped. *Results:* Cell viability assay: Cell viability (Figure 1), evaluated both by tetrazolium dye cell assay (A) and by Trypan blue staining (B), decreased when KMS-12-BM cells were treated with increasing concentrations of OA-GcMAF. In particular, when cells were treated with OA-GcMAF (800 pM) a significant reduction ($p < 0.01$) in cell

viability was observed in comparison to the untreated control cells. *Discussion:* It has been shown that Gc-MAF activates Raw 264.7 murine macrophages to phagocytose and destroy MCF-7 human breast carcinoma cells (1). An identical effect has been recorded for the first time with the stimulation of human macrophages CRL9853 on KMS-12-BM cell line. The OA-GcMAF-stimulated human macrophages seek out surround and phagocytose the myeloma cell lines destroying them. This demonstrates that the CRL9853 behave as postulated against KMS-12- BM cell line. In addition the effect of increased cell death in the presence of OA-GcMAF alone as indicated by direct evaluation of viable cell counts and viability assay also provides supports to the previous data generated (1). This study provides some inferred evidence to support the *in vivo* clinical data that has recently been published (6) providing some insight into the method of potential tumour removal by stimulated macrophages. In conclusion OA-GcMAF has demonstrated two major effects: a direct decrease of KMS-12-BM cell viability and an efficient activation of human macrophages, which become able to phagocytose and destroy the myeloma cells. The studies will be expanded further to encompass additional cancer cell lines.

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**SENESCENCE LINKED INFLAMMATION,
 REPLICATION STRESS, FRAGILE SITES AND DNA
 METHYLATION CHANGES IN SPORADIC
 BREAST AND PROSTATE CANCER.**

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Hypermethylation tends to occur near fragile sites in cancer (1). Persistent daughter strand gaps near fragile sites appear to play a key role in this process in human breast and prostate cancer. Like reactive oxygen species, 5-Azacytidine and its primary breakdown product deoxyriboguanylurea produce replication stress that induces fragile sites (2). The recently elucidated repair processes invoked by replication stress at fragile sites involving Mus81-EME and ERRC1 (3) coupled with the enzymology of DNA methyltransferases provide a clear model for methylation change at these sites during carcinogenesis. Evidence obtained with nanotechnology strongly suggests that an M1 inflammation is present at the invasive edges of human prostate tumors and that it produces persistent replication stress at this interface through the production of reactive oxygen species. Persistent daughter strand gaps at a hot spot for DNA methylation near Fragile site 11J in human breast and prostate tumor specimens provide additional evidence for inflammation's role in epigenetic changes seen in human tumors. The data will be discussed in terms the possible link between the Senescence Associated Secretory Phenotype (SASP) (4), the Senescence Messaging Secretome (SMS) (5) and Proliferative Inflammatory Atrophy (6) seen in prostate cancer.

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**VACCINATION WITH WT1 MRNA-
 ELECTROPORATED DENDRITIC CELLS
 TO PREVENT RELAPSE IN 30 ACUTE
 MYELOID LEUKEMIA PATIENTS**

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