EFFECTS OF VITAMIN D BINDING PROTEIN-DERIVED MACROPHAGE ACTIVATING FACTOR (GcMAF) ON HUMAN NEUROBLASTOMA CELLS AND PREDICTED MOLECULAR INTERACTION WITH THE VITAMIN D RECEPTOR

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Introduction 1

- From the historical perspective, the concept of immunotherapy of cancer is associated with the early work of Dr. William Coley.
- In modern times, it has been re-proposed since 1960.

Introduction 2

- As of today there are 57821 published papers on this topic, with an exponential growth in the number of publications.

- According to a recent study, "*… immunotherapies require* ***activation of macrophages*** *to be effective*" (Int J Cancer. 2008 Jan 15;122(2):461-7).

Introduction 3

- The central role of macrophages in the immunotherapy of cancer has been further highlighted in the article pasted below.

Introduction 4

- Since 1994, it has been demonstrated that macrophage activation requires Gc protein-derived Macrophage Activating Factor (GcMAF) (J Immunol. 1994 May 15;152(10):5100-7).
- Therefore, GcMAF has become a stronghold in the immunotherapy of cancer and, as today, there are scores of studies on this subject.

Introduction 5

- We and others demonstrated that GcMAF, in addition to stimulating tumoricidal macrophages, acts directly on cancer cells and inhibits tumor-induced angiogenesis (1).
- In particular, GcMAF modifies the malignant phenotype and reduces the metastatic potential of human prostate (2) and breast (3) cancer cells in culture.
- In this study we present data concerning the effect of GcMAF on human neuroblastoma cells.

- References.
- 1. Inhibition of angiogenesis by vitamin D-binding protein: characterization of anti-endothelial activity of DBP-maf. Kalkunte S, Brard L, Granai CO, Swamy N. Angiogenesis. 2005;8(4): 349-60. Epub 2006 Jan 7.
- 2. Vitamin D binding protein-macrophage activating factor directly inhibits proliferation, migration, and uPAR expression of prostate cancer cells. Gregory KJ, Zhao B, Bielenberg DR, Dridi S, Wu J, Jiang W, Huang B, Pirie-Shepherd S, Fannon M. PLoS One. 2010 Oct 18;5 (10):e13428.
- 3. Effects of vitamin D-binding protein-derived macrophage-activating factor on human breast cancer cells. Pacini S, Punzi T, Morucci G, Gulisano M, Ruggiero M. Anticancer Res. 2012 Jan;32(1):45-52.

Materials and Methods 1

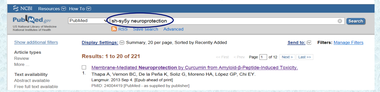
- **GcMAF**.
- Commercially available, highly active purified GcMAF was obtained from Immuno Biotech Ltd, Guernsey, Channel Isles.
- GcMAF was purified according to the procedure recently described in Autism Insights (2012; 4:31-8).
- At the end of the production process, GcMAF was checked for sterility in-house and externally by independent laboratories. Its safety and biological activity were tested in human and mouse monocytes, human breast cancer cells, human neuronal cells, and chick embryos.
- Comparative analysis demonstrated that this GcMAF had the highest biological activity in comparison with other preparations obtained from major researchers (Cancer Immunol Immunother. 2011 Apr;60(4):479-85).

Materials and Methods 2

- **Cell cultures**
- Human neuroblastoma cells SH-SY5Y, were obtained from the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna, Brescia, Italy. Cells were routinely maintained at 37°C in a humidified atmosphere of 5% CO_2 in Eagle's minimum essential medium in Earle's Balanced salt solution (45%), Ham's F12 medium (45%), foetal bovine serum (FBS) 10%.
- In some experiments, as described below, the cells were starved for 24h prior to the experiment that was then conducted in serum-free medium. Other experiments, designed to study inhibition of cell proliferation, were conducted in the presence of 1% serum in order to study actively proliferating cells.

Materials and Methods 3

- SH-SY5Y human neuroblastoma cells originally derived from a metastatic bone tumor biopsy (Methods Mol Biol. 2013;1078:9-21).
- They represent a model system to study the effects of anti-cancer therapies aimed at neuroblastoma (PLoS One. 2013 Jun 18;8 (6):e66430. Chemotherapy. 2012;58(1):44-51).
- However, since they are able to differentiate, they also represent a model system to study the neurobiology of neurodegenerative diseases (Chin Med J (Engl). 2010 Apr 20;123(8):1086-92. Review).
- Because of this, SH-SY5Y cells have also been used to study the neuroprotective effects of a variety of treatments.



Results

- GcMAF treatment of SH-SY5Y cells resulted in different effects depending on the proliferative activity of the cells.
- In actively proliferating cells (in 1% serum) GcMAF inhibited cell proliferation in a dose-dependent manner and induced their apoptosis (Fig. 1).
- In serum-starved, quiescent cells, GcMAF induced morphological changes indicating differentiation (Fig. 2 A, B).
- The effects of GcMAF were mediated by cAMP production (Fig. 3), possibly through cross-talk with the vitamin D receptor (VDR).

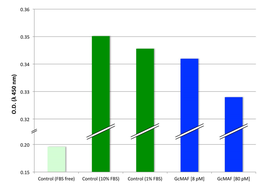


Fig. 1. SH-SY5Y cells proliferation was stimulated by 10% and 1% FBS to a similar extent. GcMAF, in the presence of 1% FBS, inhibited cell proliferation in a dose-dependent manner.

Assessment of cell proliferation was determined by Cell Counting Kit - 8 (Sigma Aldrich). Results are expressed as optical density (absorbance) at 450 nm.

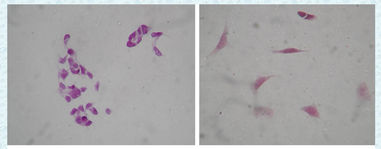


Fig. 2 A
Un-stimulated SH-SY5Y cells, quiescent after serum-starvation, at different magnification.
Cells, stained with haematoxylin-eosin, appeared as round, small, undifferentiated cells with large nuclei and no cytoplasmic elongations.

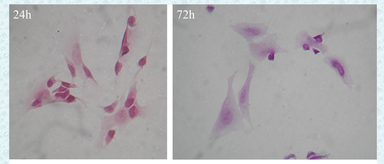


Fig. 2 B
After 24-72 h stimulation with 8 pM GcMAF, serum-starved, quiescent cells showed a significant change in morphology that was consistent with the induction of differentiation.
The cytoplasm was enlarged and several cytoplasmic elongations could be observed. The effect was time-dependent.

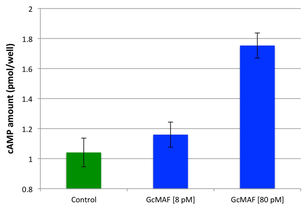


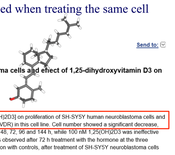
Fig. 3. GcMAF-induced cAMP formation in SH-SY5Y cells.

Discussion 1

- The results presented here demonstrate that GcMAF inhibits actively proliferating human neuroblastoma cells, whereas it induces the differentiation of serum-starved (quiescent) human neuroblastoma cells.
- The concentration of GcMAF necessary to inhibit proliferation of actively proliferating cells was 10 fold higher than that required to induce differentiation of quiescent cells.

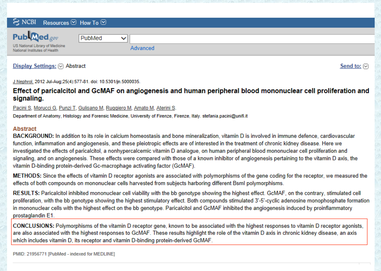
Discussion 2
GcMAF and the vitamin D receptor (VDR)

- *It has not escaped our notice that …* the effect of GcMAF on actively proliferating human neuroblastoma cells was superimposable to that observed when treating the same cell type with vitamin D3.

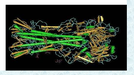


- This is not surprising since GcMAF shows a strict interconnection with vitamin D signaling.
- In fact, GcMAF is a member of the so-called vitamin D axis since it derives from de-glycosylation of Vitamin D-Binding Protein or Gc protein.
- Consistent with this notion, we had previously demonstrated that polymorphisms of the VDR gene, known to be associated with the highest responses to VDR agonists, were associated also with the highest responses to GcMAF.

- For review on the vitamin D axis and GcMAF, please see: European Nephrology, 2011;5(1):15-19



- These observations raise the question of whether there could be a direct molecular interaction between GcMAF and the VDR.
- This question might appear odd at first, since it had been postulated that VDR was localized in the cytoplasm and in the nucleus, whereas GcMAF could not cross the plasma membrane and therefore had to be recognized by a surface receptor, possibly a lectin-type receptor.
- (J. Biol. Chem, 1999, 274(16), 10697-10705)

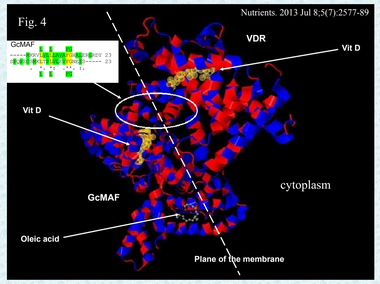


- However, in support for the hypothesis of a direct GcMAF/VDR interaction, there is the observation that VDR translocates to the plasma membrane, and plasma-membrane associated VDR is responsible for the rapid, non-genomic, effects of vitamin D.
- Thus, in order to verify the possibility of a molecular interaction between GcMAF and VDR, we compared the amino acid sequences corresponding to their respective vitamin D binding sites.

- J. Cell. Biochem. 2002, 86(1), 128-135.
- Calcif. Tissue Int. 2013, 92, 151-162.

- There are 23 hydrophobic amino acids near the amino terminus of GcMAF
- (-----MKRVLVLLLAVAFGHALERGRDY)
- and 23 amino acids near the carboxyl terminus of the VDR
- (SFQPECSMKLTPLVLEVFGNEIS-----).
- These are the sequences that bind vitamin D.

- If these two sequence are aligned (Fig. 4), it is possible to observe not only that in both proteins there is a long stretch (13-14) of hydrophobic amino acids (highlighted in green in Fig. 4, insert), but that 4 hydrophobic amino acids are identical (L L FG; indicated in yellow and in green above and under the alignment).
- 11 amino acids have similar functional valence (as indicated by the conventional symbols [*], [.] and [:].

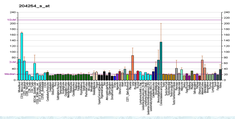


- A molecular interaction between the two proteins can therefore be proposed (Fig. 4).
- According to this model, the last 23 hydrophobic amino acids of VDR (VDR is on the right of Fig. 4), located at the inner part of the plasma membrane (represented as a dotted line), could interact with the first 23 hydrophobic amino acids of the GcMAF (GcMAF is on the left of Fig. 4) located at the external part of the plasma membrane, with vitamin D (represented in yellow) sandwiched in between the two vitamin D-binding proteins.

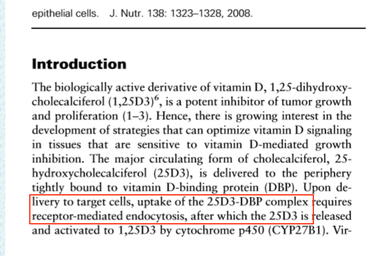
- Oleic acid, taken as an example of an unsaturated fatty acid bound to GcMAF, could stabilize the complex at the level of the plasma membrane.
- In fact, both vitamin D and oleic acid are located in a shallow cleft of the GcMAF protein that makes them accessible to the plasma membrane.
- Biochem. Biophys. Res. Commun. 1988, 153 (3), 1019-1024.

- According to this model, the presence of vitamin D and oleic acid should facilitate the interaction between GcMAF and VDR.
- This hypothesis is further strengthened by the observation that activated macrophages generate enough biologically active vitamin D so as to be detectable in the general circulation

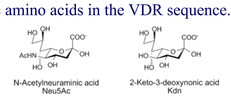
Arch Biochem Biophys. 2012; 523: 95-102.

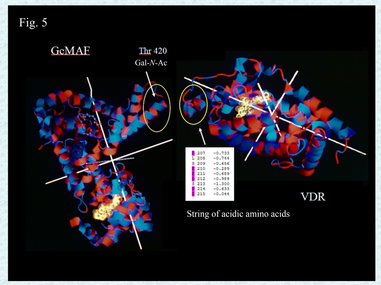


- In addition to this proposed mode of interaction at the level of the plasma membrane (Fig. 4), the interaction between GcMAF and VDR could also occur **inside the cell**.
- At variance with what previously postulated, vitamin D does not cross freely the membrane, and the Gc protein/vitamin D complex is endocytised through a receptor-mediated mechanism.
- Therefore, Gc protein (and hence GcMAF) can be found inside the cell.



- Once inside the cell, GcMAF could interact with VDR because of the presence of a string of acidic amino acids in the VDR sequence that bind the Gal-*N*-Ac moiety of GcMAF, but not to the non-deglycosylated (inactive) Gc protein (Fig. 5).
- In fact, inactive Gc protein, with sialic acid bound to Thr 420, cannot bind the string of acidic amino acids in the VDR sequence.





- The interaction between GcMAF and VDR helps explaining the multiplicity of effects attributed to GcMAF and the variety of clinical applications ranging from cancer to autism.
- In fact, VDR is expressed in a great number of cell types (including SH-SY5Ycells) and regulates a wide array of genes involved in the control of the major cell functions.