Gc-protein-derived macrophage activating factor counteracts the neuronal damage induced by oxaliplatin

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Oxaliplatin-based regimens are effective in metastasized advanced cancers. However, a major limitation to their widespread use is represented by neurotoxicity that leads to peripheral neuropathy. In this study we evaluated the roles of a proven immunotherapeutic agent [Gc-protein-derived macrophage activating factor (GcMAF)] in preventing or decreasing oxaliplatin-induced neuronal damage and in modulating microglia activation following oxaliplatin-induced damage. The effects of oxaliplatin and of a commercially available formula of GcMAF [oleic acid-GcMAF (OA-GcMAF)] were studied in human neurons (SH-SY5Y cells) and in human microglial cells (C13NJ). Cell density, morphology and viability, as well as production of cAMP and expression of vascular endothelial growth factor (VEGF), markers of neuron regeneration [neuromodulin or growth associated protein-43 (Gap-43)] and markers of microglia activation [ionized calcium binding adaptor molecule 1 (Iba1) and B7-2], were determined. OA-GcMAF reverted the damage inflicted by oxaliplatin on human neurons and preserved their viability. The neuroprotective effect was accompanied by increased intracellular cAMP production, as well as by increased expression of VEGF and neuromodulin. OA-GcMAF did not revert the effects of oxaliplatin on microglial cell viability. However, it increased microglial activation following oxaliplatin-induced damage, resulting in an increased expression of the markers Iba1 and B7-2 without any concomitant increase in cell number. When neurons and microglial cells were co-cultured, the presence of OA-GcMAF significantly counteracted the toxic effects of oxaliplatin. Our results demonstrate that OA-GcMAF, already used in the immunotherapy of advanced cancers, may significantly contribute to neutralizing the neurotoxicity induced by oxaliplatin, at the same time possibly concurring to an integrated anticancer effect. The association between these two powerful anticancer molecules would probably produce the dual effect of reduction of oxaliplatin-induced neurotoxicity, together with possible synergism in the overall anticancer effect. *Anti-Cancer Drugs* **00:000–000 © 2014 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

Anti-Cancer Drugs 2014, **00**:000–000

Keywords: cancer, Gc-protein-derived macrophage activating factor, immunotherapy, microglia, neurons, oxaliplatin, vitamin D

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Received 11 April 2014 **Revised form accepted** 4 September 2014

Introduction

Oxaliplatin is a platin-based anticancer agent that is used in the chemotherapy of a variety of cancers [1]. Although oxaliplatin-based regimens appear to be effective in metastasized advanced cancers [2], a major limitation to the widespread use of this chemotherapeutic agent is its neurotoxicity, which leads to peripheral neuropathy. This adverse effect manifests itself as a progressive, enduring and often irreversible tingling numbness, intense pain and hypersensitivity to cold, beginning in the hands and feet and sometimes involving the arms and legs, often with deficits in proprioception [3].

These initial symptoms evolve into chronic pain, a condition that significantly reduces patient compliance with oxaliplatin-based regimens and represents one of the factors for discontinuing treatment [4,5].

Knowledge on oxaliplatin neurotoxicity is mainly focused on neuronal involvement, whereas knowledge on modulation of central nervous system glial cells during chemotherapy-induced neuropathy is still quite limited. Growing evidence ascribes pathologic effects such as neuronal hyperexcitability and chronic inflammation to glial cells; morphologic features and pathogenic glial activation in rat oxaliplatin-dependent neuropathic pain [6] support the case for cerebral glial involvement in oxaliplatin-dependent neuropathy.

As a matter of fact, a growing number of studies provide evidence that the glia have a number of housekeeping functions necessary for healthy neuronal communication as well as for neuroprotection [7], and the notion that activation of glia in the central nervous system, and in particular of microglia, also leads to beneficial outcomes has recently gained consensus. Thus, microglia respond and contribute to a local immune environment that is beneficial for nearby healthy glia and neurons. These beneficial roles of microglia include the release and maintenance of anti-inflammatory factors, and processes that serve to protect against neurotoxicity [8]; thus, it has

0959-4973 © 2014 Wolters Kluwer Health | Lippincott Williams & Wilkins DOI: 10.1097/CAD.0000000000000177

been postulated that the fine balance between proinflammatory and anti-inflammatory events after damage could be controlled by varying the expression of the 'self-associated molecular pattern' and 'altered self' proteins in microglia [9]. It appears that, without appropriate clearance of dying cells and of cellular debris by activated phagocytic microglia, toxicity and cellular damage readily spread in the central nervous system [10]. Generally, microglia, congruent with typical macrophage and dendritic cell activity, act as a first line of defence against pathogen invasion by recognizing, sequestering and processing antigens. Once microglia are activated, they produce and release several signalling molecules that activate nearby astrocytes and neurons.

It has been recognized for many years that one of the most prominent endogenous regulators of macrophage function is the vitamin D-binding protein-derived macrophage activating factor, also designated as Gc-globulin-derived macrophage activating factor (GcMAF) [11].

In addition to stimulating macrophages, GcMAF has a number of other biological properties that contribute to its well-assessed therapeutic effects. These range from inhibition of angiogenesis [12] to direct inhibition of human cancer cell proliferation and metastatic potential [13,14]. We have recently demonstrated that these multifaceted effects of GcMAF can be interpreted considering the interconnection between the GcMAF and the vitamin D receptor (VDR) signalling pathways [15]. The in-vitro and in-vivo results recently published [16] are consistent with a direct effect of GcMAF on human neuronal viability and metabolic activity, as well as on inflammation-associated pain.

As GcMAF-based integrative anticancer regimens appear to be very effective in controlling metastasized advanced cancers [17], we postulated that GcMAF could be used in combination with oxaliplatin-based therapeutic regimens to counteract the neurotoxicity of these treatments with possible synergism in the anticancer effects.

In fact, the neuroprotective compounds used to prevent chemotherapy-evoked neuropathy are poorly effective [5] and, importantly, they do not contribute to anticancer effects. Therefore, molecules that are able to counteract the cell modifications associated with neuropathic pain at the same time contributing to anticancer effects would represent a significant improvement over existing regimens. Consistent with these premises, in this study we evaluated the roles of a commercially available preparation of GcMAF [oleic acid-GcMAF (OA-GcMAF)] commonly used in anticancer treatment in preventing neuron modifications and inducing microglia activation following oxaliplatin-induced damage.

Methods

Cell lines

The SH-SY5Y cell line was purchased from Istituto Zooprofilattico dell'Emilia e della Romagna, Brescia, Italy. The human microglial C13NJ cell line was kindly donated by Dr Jean Mazella (Institut de Pharmacologie Moléculaire et Cellulaire, Centre National de la Recherche Scientifique, Université de Nice-Sophia Antipolis, Valbonne, France).

We chose the SH-SY5Y cell line because of its dual role in oncology and neuropharmacology. This line was originally derived from a metastatic bone tumour biopsy [18] and represents a model to study the effects of anticancer therapies aimed at neuroblastoma [19,20]. However, this cell line also represents a model to study the neurobiology of neurodegenerative diseases and neuropathies [21]. Because of this, SH-SY5Y cells have been extensively used to study the neuroprotective effects of a variety of treatments [22]. In our experiments, we exploited these latter features of SH-SY5Y cells and appropriately serum-starved the cultures before the experiments, as described [23].

The cell lines were maintained, respectively, in DMEM/Ham's F12 and in DMEM supplemented with 10% foetal bovine serum (FBS) and 2 mmol/l L-glutamine at 37°C in a humidified atmosphere with 95% air/5% CO_2. The growth medium was changed every 2 or 3 days. The co-culture (C13NJ/SH-SY5Y) was maintained in DMEM supplemented with 10% FBS and 2 mmol/l L-glutamine. Cell media, FBS and L-glutamine were purchased from EuroClone S.p.a. (Milan, Italy).

Before each treatment, the cell lines and the co-culture were starved for 24 h in appropriate medium without FBS (starvation medium); thereafter, fresh starvation medium containing stimuli was added for 24 or 48 h. For the experiments on co-culture, C13NJ and SH-SY5Y cells were mixed at densities of 50 000 and 500 000 cells/ml, respectively.

Stimuli

Oxaliplatin (Sigma Aldrich, Milan, Italy) was dissolved in PBS and concentrations of 1, 10, 100, 500 μmol/l and 1 mmol/l were used.

OA-GcMAF is commercially available and was prepared in-house at Immuno Biotech Ltd following a proprietary procedure. Briefly, GcMAF was purified according to the procedure described previously [24]. Vitamin D-binding protein was isolated from purified human serum, obtained from the American Red Cross, by either 25-hydroxyvitamin D3–sepharose high-affinity chromatography or actin–agarose affinity chromatography. The bound material was eluted and then further processed by incubation with three immobilized enzymes as described [25]. The resulting GcMAF was filter-sterilized. The protein content and concentration were assayed using the standard Bradford protein assay method. Purity was assessed by SDS-PAGE and western blotting analysis performed after each step of the preparation procedure [16]. 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 monocytes, human breast cancer cells and chick embryo chorionallantoic membranes [12, 14,15]. Highly purified OA (molecular weight, 282.46, molecular formula $C_{18}H_{34}O_2$; Acros Organics, Geel, Belgium) was complexed with GcMAF in accordance with the molecular structures and modelling described [15]. The optimal conditions for the preparation of the complexes were established according to the principles described [26]. OA-GcMAF had a classic dose-dependent effect on all the different cell lines used to test its efficacy; the 800 pmol/l concentration was chosen as it was the highest concentration that induced a positive cell response just before the plateau doses [16].

Cell viability assay

Cell viability was evaluated using the reduction of a tetrazolium salt, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium monosodium salt (WST-8), as an index of cell dehydrogenase activity. SH-SY5Y and C13NJ cells were plated into 96-well plates at densities of 20 000 and 10 000 cells/well, respectively, in their appropriate complete growth medium. Cells were starved for 24 h and then treated with different concentrations of oxaliplatin and/or OA-GcMAF. Because of the stronger sensitivity of C13NJ, oxaliplatin concentrations used for this cell line were higher than those used for SH-SY5Y. Cells were treated with oxaliplatin alone for 24 h and with OA-GcMAF alone for 48 h. When investigating the neuroprotective effect of OA-GcMAF, OA-GcMAF was added to the starvation cell medium 24 h before oxaliplatin was added for 24 h; thus, OA-GcMAF remained in the culture medium for a total of 48 h. At the end of each treatment, the medium was replaced with 100 µl of fresh starvation medium, along with 10 µl of the chromogenic solution (WST-8). After incubation of the wells for 3 h at 37°C, the absorbance was directly measured at 450 nm (optical density) using a Multiscan FC photometer (Thermo Scientific, Milan, Italy). Each experimental point was performed in quintuplicate, and each experiment was performed three times.

Cell density and morphology evaluation

SH-SY5Y and C13NJ cells were seeded on coverslip glasses in their appropriate complete growth medium. Cells were starved for 24 h and then treated with different concentrations of oxaliplatin and/or OA-GcMAF for 24 or 48 h.

At the end of the treatments, the medium was removed and two washes with cold PBS were performed. One ml of 0.5% paraformaldehyde was added to each coverslip glass for 10 min. After two washes with cold PBS, the coverslip glasses were dried for 1 h (at room temperature) and then stained with haematoxylin–eosin dye following a standard procedure.

Coverslip glasses were observed under a light microscope and pictures were taken at different magnifications using a digital camera (Nikon Coolpix 990; Nikon Instrument S.p.A., Milan, Italy).

The cell density was quantified by measuring the cell surface/mm^2 with ImageJ software (National Institutes of Health, Bethesda, Maryland, USA; *http://imagej.nih.gov/ij*, 1.47t).

cAMP assay

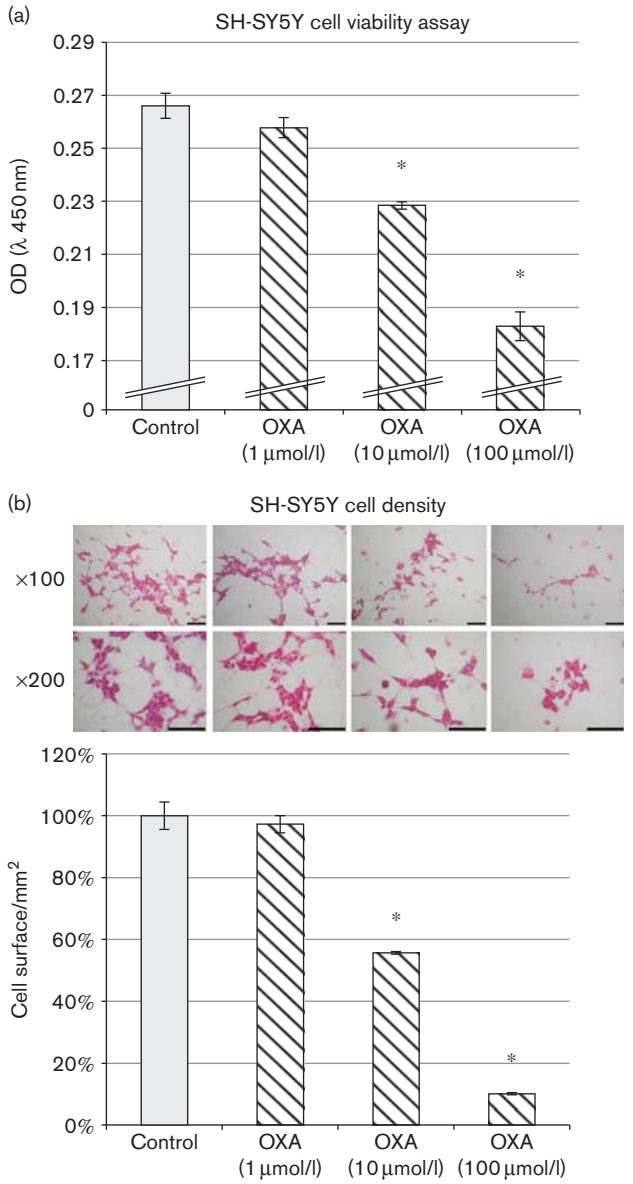
SH-SY5Y (10×10^6 cells/well) and C13NJ (4×10^6 cells/well) cells were plated in Petri dishes in their appropriate complete growth medium; after 24 h of starvation, the cells were treated for 25 min with oxaliplatin and/or OA-GcMAF. Thereafter, the medium was removed and 1.5 ml of HCl (0.1 mol/l) was added for 20 min to allow cell lysis and degradation of phosphodiesterases. Cells were scraped and centrifuged at room temperature for 10 min at 1800 rpm. The supernatants were directly measured in duplicate by performing a cAMP competitive enzyme-linked immunosorbent assay (ELISA) (Thermo Scientific).

Western blotting

SH-SY5Y (10×10^6 cells/well) and C13NJ (4×10^6 cells/well) cells were plated in Petri dishes in their appropriate complete growth medium; after 24 h starvation, cells were treated for 24 or 48 h with oxaliplatin and/or OA-GcMAF. Thereafter, the medium was removed, two washes with phosphate-buffered saline (PBS) were performed and the cells were scraped from the surface of the dishes. The cell suspension was centrifuged at 1000 rpm for 10 min at room temperature. The pellets were treated with lysis buffer containing protease inhibitors (Sigma Aldrich) for 30 min at 4°C, vortexing every 5 min. The homogenates were centrifuged at 4°C for 10 min at 12 000 rpm and the supernatants were used to evaluate the protein concentration using the Bradford method. Equal amounts of protein (30 µg) were subjected to 12% polyacrylamide gel electrophoresis and then transferred onto a nitrocellulose membrane (Porablot NPC, MACHEREY-NAGEL; Carlo Erba Reagents, Milan, Italy). After blocking for 1 h with 5% bovine serum albumin (BSA) in Tris-buffered saline (TBS) containing 0.1% Tween 20 at room temperature, the blot was incubated overnight at 4°C with the primary antibody for vascular endothelial growth factor (VEGF), B7-2 and neuromodulin [growth associated protein-43 (GAP-43); Santa Cruz Biotechnology, Santa Cruz, California, USA] at a 1 : 500 dilution and for ionized calcium binding adaptor molecule 1 (Iba1) (Wako Chemicals GmbH, Neuss, Germany) at a 0.5 µg/ml concentration. The goat anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology) was added at a 1 : 5000 dilution for 1 h at room temperature and VEGF, B7-2 and GAP-43 were detected with the

Amersham ECL Plus Western Blotting Detection Reagent (EuroClone, Milan, Italy). In contrast, the goat anti-rabbit HRP-conjugated secondary antibody (Santa Cruz Biotechnology) was added at a 1 : 5000 dilution for 1 h at room temperature and Iba1 was detected with the Amersham ECL Plus Western Blotting Detection Reagent (EuroClone). Protein expression levels were then quantified using image analysis software (ImageJ; National Institutes of Health; *http://imagej.nih.gov/ij*, 1.47t).

Fig. 1



Cell viability and cell density of SH-SY5Y cells after treatment with oxaliplatin. (a) Cell viability, evaluated using the WST-8 assay. Histograms show a decrease in the metabolic activity of SH-SY5Y cells after 24 h of treatment with oxaliplatin at different concentrations. (b) Cell density, evaluated by light microscopy and quantification, after haematoxylin–eosin staining. Pictures show a decrease in the cell density of SH-SY5Y cells after 24 h of treatment with oxaliplatin at different concentrations. Upper panels: total magnification × 100; lower panels: total magnification × 200. Bar = 100 μm. The histograms represent quantification of the cell density using ImageJ. They show the mean area occupied by the SH-SY5Y surface in five different fields (1 mm^2) on each coverslip. Two coverslips were counted in a blinded manner for each experimental point. Results are expressed as mean value ± SEM for two replicates (*$P < 0.05$ vs. control); each experiment was performed three times. OD, optical density; OXA, oxaliplatin.

Detection of DNA fragmentation

DNA integrity of the SH-SY5Y/C13NJ co-culture was evaluated by using the DNA fragmentation detection kit (FragEL Fragmentation Detection Kit; Fluorescent-TdT Enzyme; Calbiochem, Life Technology, Milan, Italy). The principle of fluorescein-FragEL is that terminal deoxynucleotidyl transferase (TdT) catalyses the addition of fluorescein-labelled and unlabelled deoxynucleotides to the 3′-OH ends generated by endonucleases during apoptotic degeneration. SH-SY5Y and C13NJ cells were seeded at densities of 100 000 and 20 000 cells, respectively, on coverslip glasses in their appropriate complete growth medium. After 24 h of starvation cells were treated with different concentrations of oxaliplatin and/or OA-GcMAF for 24 or 48 h. At the end of the treatments the medium was removed and two washes with cold PBS were performed. The cell lines were first fixed in 0.5% paraformaldehyde in PBS for 10 min and then permeabilized for 2 min in cold acetone at room temperature. After three washes in TBS, the slides were incubated in 1× TdT equilibration buffer for 30 min at room temperature. Thereafter, the slides were incubated in staining solution consisting of 57 μl fluorescein-FragEL TdT labelling reaction mix + 3 μl TdT enzyme solution for 1.5 h at 37°C. Three more washes in TBS for 1 min at room temperature were performed and the slides were eventually mounted onto a microscope slide with a drop of mounting medium. Images were taken under a motorized Leica DM6000 B microscope equipped with a DFC350FX camera (Leica, Manneheim, Germany) at × 200 magnification, and the signal intensity was analysed using ImageJ software.

Results

Treatment of SH-SY5Y cells with oxaliplatin and OA-GcMAF: cell viability, morphology, cAMP, VEGF and neuromodulin expression

It is well known that oxaliplatin induces cell damage of SH-SY5Y cells, deeply affecting their viability [21]. To assess the most appropriate oxaliplatin concentration inducing reversible damage we evaluated the cell viability of SH-SY5Y cells after 24 h of treatment with oxaliplatin at different concentrations. Treatment for 24 h was chosen as the most appropriate time period for treatment, sufficient to induce significant and, at the same time, reversible cell damage. As shown in Fig. 1a, cell viability, evaluated using the WST-8 assay, decreased, as expected, with an increase in oxaliplatin concentration. Significant differences ($P < 0.05$) in comparison with control, untreated cells were observed when oxaliplatin concentrations of 10 and 100 μmol/l were used.

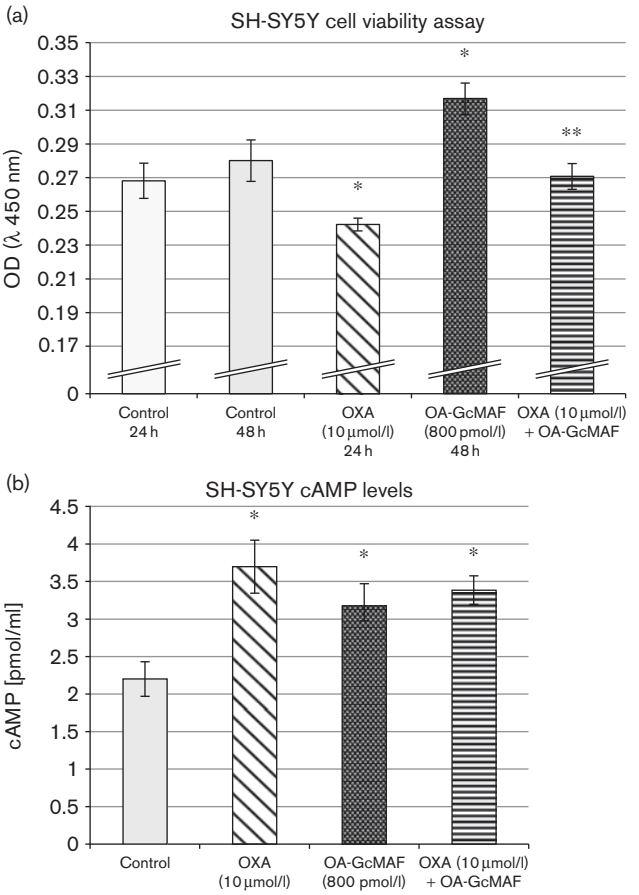
A lower concentration of oxaliplatin such as 1 μmol/l did not significantly affect SH-SY5Y cell viability.

The decrease in oxaliplatin-induced cell viability was associated with a significant decrease in cell density, evaluated by morphological analysis (Fig. 1b). Cell density variations, observed by light microscopy, were then quantified using appropriate software and expressed as cell surface/mm^2 (Fig. 1b). Cell density was significantly decreased ($P < 0.05$), consistent with cell viability assay results, when SH-SY5Y cells were treated with 10 and 100 μmol/l oxaliplatin. Data obtained from both the cell viability assay and the morphological analysis indicated that the most appropriate concentration of oxaliplatin that induced reversible cell damage was 10 μmol/l. Therefore, this concentration was the one we focused on in the present study.

When SH-SY5Y cells were treated for 48 h with OA-GcMAF (800 pmol/l), a significant increase ($P < 0.05$) in cell viability was observed (Fig. 2a). This concentration and time period of treatment (48 h) were chosen as the most appropriate after preliminary experiments in which several doses and different time periods of treatment were tested (data not shown). Cell viability after the treatment with OA-GcMAF was higher in comparison with cell viability of the cells cultured for 48 h in the same medium but without OA-GcMAF. The cell viability induced by treatment for 24 h with 10 μmol/l oxaliplatin was, as expected, significantly decreased compared with that of control cells cultured in the same medium for 24 h but without oxaliplatin. The decrease in cell viability induced by 10 μmol/l oxaliplatin was completely reversed when OA-GcMAF was added 24 h before the treatment, as shown in Fig. 2a ($P < 0.05$). At this experimental point, OA-GcMAF was still present when cells were treated with oxaliplatin; thus, OA-GcMAF remained in the cell medium for a total of 48 h.

It is well assessed that GcMAF increases the intracellular concentration of cAMP in human mononuclear cells, and this signal transduction pathway has been implicated in the immunostimulating effects of GcMAF [12]. As cAMP mediates the neuroprotective effects of a variety of substances [27], we decided to study whether cAMP was also involved in the neuroprotective effect exerted by OA-GcMAF in SH-SY5Y cells. Our results demonstrate that cAMP levels significantly increased ($P < 0.05$) in comparison with control untreated cells when SH-SY5Y cells were treated with 800 pmol/l OA-GcMAF (Fig. 2b). However, oxaliplatin alone, as well as the combination of oxaliplatin and OA-GcMAF, induced, to the same extent, the activation of the cAMP signal transduction pathway (Fig. 2b). These results confirm that in SH-SY5Y cells, as well as in other cell lines, OA-GcMAF activates a cAMP-dependent signal transduction pathway. However, as the treatment with oxaliplatin alone activates, as expected [28], the same pathway, no differences were observed when SH-SY5Y cells were treated with OA-GcMAF and oxaliplatin simultaneously.

Fig. 2



Cell viability and cAMP level of SH-SY5Y cells after treatment with oxaliplatin and OA-GcMAF. (a) Treatment of SH-SY5Y cells with oxaliplatin for 24 h negatively affects cell viability in comparison with control untreated cells (control 24 h). To allow OA-GcMAF to act as a protective factor, cells were cultured with OA-GcMAF for 24 h before adding oxaliplatin. When oxaliplatin was added to the culture (for 24 h), OA-GcMAF was still present in the medium, such that, all in all, it remained in the cell medium for 48 h. Histograms show that, in this case, cell viability is restored to significantly higher levels compared with those seen in the oxaliplatin (alone)-treated sample. (b) cAMP levels, evaluated using the ELISA assay, measured after 25 min of OA-GcMAF, oxaliplatin and OA-GcMAF + oxaliplatin treatment, suggest that both OA-GcMAF and oxaliplatin activate the same signal transduction pathway. Results are expressed as mean value ± SEM for five replicates (*$P < 0.05$ vs. control; **$P < 0.05$ vs. OXA alone). Each experiment was performed three times. OA-GcMAF, oleic acid-Gc-protein-derived macrophage activating factor; OD, optical density; OXA, oxaliplatin.

Furthermore, we investigated whether the observed effect of counteraction of the toxic effects of oxaliplatin by OA-GcMAF was due to neurotrophic/neurogenic factors released by neurons. Data in the literature interestingly show that, in addition to the traditional neurogenic and neurotrophic factors, VEGF also plays the role of a neurogenic factor involved in cell homeostasis [29]. Therefore, VEGF expression, after the treatment of

SH-SY5Y cells with OA-GcMAF and with oxaliplatin, was studied. Treatment of SH-SY5Y cells with OA-GcMAF induced an increase in the expression of VEGF in comparison with control, untreated cells ($P < 0.05$), as demonstrated by western blotting and densitometric analysis (Fig. 3a). In contrast, treatment of cells with oxaliplatin induced a significant decrease in VEGF expression in comparison with control, untreated cells. When cells were cultured for 24 h in the presence of OA-GcMAF and then triggered with oxaliplatin for 24 h, VEGF expression was superimposable to the level of VEGF expression in control, untreated cells and significantly higher than VEGF expression induced by the treatment with oxaliplatin alone. These results confirm the fact that OA-GcMAF by itself induces the expression of neurotrophic/neurogenic factors in SH-SY5Y cells and, at the same time, it counteracts the negative effects induced by oxaliplatin. These data are consistent with the described observation of a molecular interaction between cAMP signalling and VEGF expression [30] and suggest that both the cAMP signal transduction pathway and the expression of VEGF are implicated in the neuroprotective effects of OA-GcMAF.

To evaluate whether the neuroprotective effects observed after the treatment of neurons with OA-GcMAF were associated with the expression of markers suggesting neuron regenerative responses, we investigated the role of neuromodulin or GAP-43, a crucial proteic component present at high levels in neuronal growth cones during development and axonal regeneration [31,32]. Figure 3b shows the expression of neuromodulin after the treatment of SH-SY5Y cells with OA-GcMAF and with oxaliplatin. Treatment of SH-SY5Y cells with OA-GcMAF induced an increase in the expression of neuromodulin, as demonstrated by western blotting analysis. The densitometric analysis revealed that neuromodulin expression was significantly higher in cells treated for 48 h with OA-GcMAF in comparison with control, untreated cells ($P < 0.05$). This result underlines the neurotrophic effect of OA-GcMAF. When cells were treated with oxaliplatin, a significant decrease in neuromodulin expression was observed; this result was consistent with the morphological observations as well as with the previously shown data on cell viability describing cell damage induced by oxaliplatin. When cells were pretreated for 24 h with OA-GcMAF and then stimulated with oxaliplatin, no significant recovery was observed in the expression of neuromodulin.

These results suggest that OA-GcMAF is directly involved in neuronal pathfinding and branching during regeneration; however, in this case, it does not seem to revert the negative toxic effect induced by the treatment with oxaliplatin.

Treatment of C13NJ cells with oxaliplatin and OA-GcMAF: cell viability, morphology, cAMP, VEGF, Iba1 and B7-2 expression

C13NJ microglial cell viability after 24 h of treatment with oxaliplatin at different concentrations was evaluated using the WST-8 assay. As shown in Fig. 4a, cell viability decreased, as expected, with an increase in oxaliplatin concentration. Significant differences ($P < 0.05$) in comparison with control, untreated cells were observed for all the tested oxaliplatin concentrations (100, 500 μmol/l and 1 mmol/l).

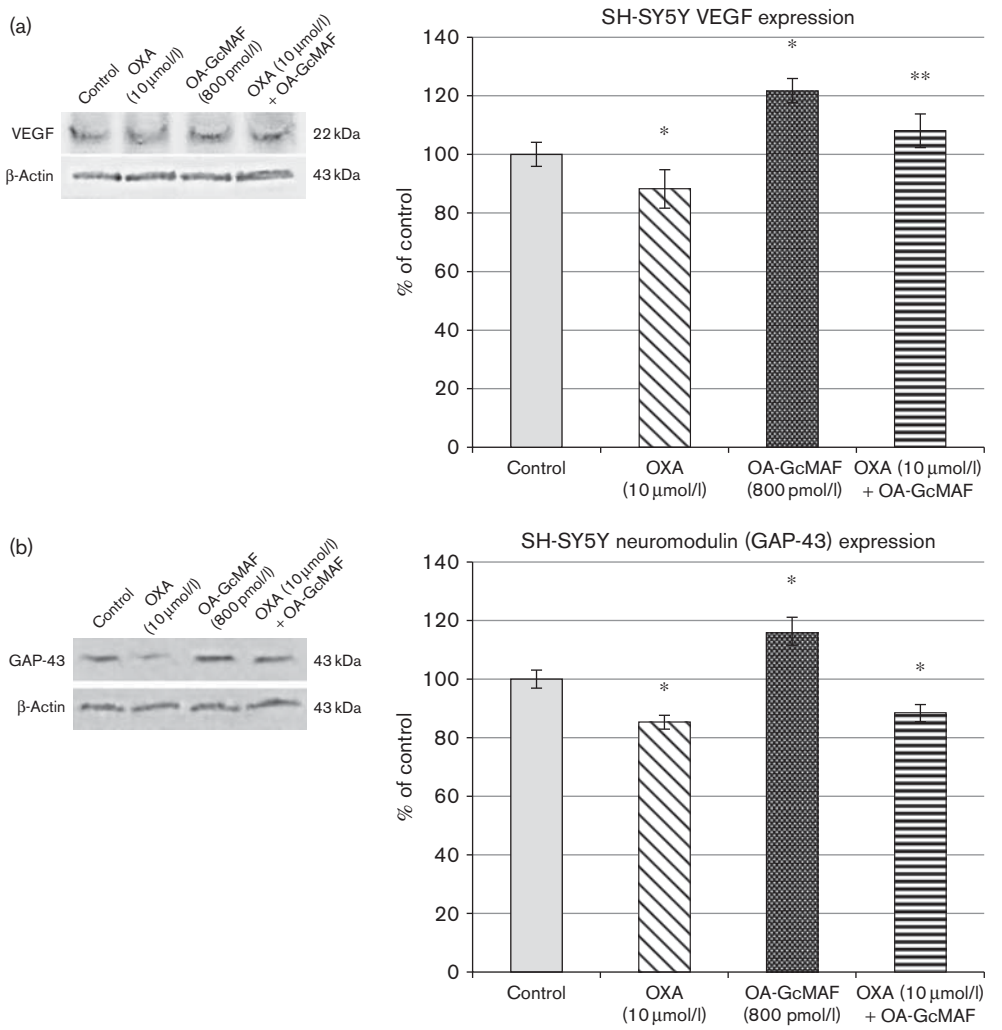
The decrease in oxaliplatin-induced cell viability was associated with a significant decrease in cell density, evaluated by morphological study (Fig. 4b). Cell density variations observed on light microscopy were then quantified using appropriate software and expressed as cell surface/mm^2 (Fig. 4b). Cell density was significantly decreased ($P < 0.05$), consistent with the results obtained by the cell viability assay, when the cells were treated with all the tested oxaliplatin concentrations. In contrast to the results observed in SH-SY5Y cells, the data obtained from both the cell viability assay and the morphological analysis indicated that the most appropriate concentration of oxaliplatin that induced reversible cell damage of C13NJ cells was 100 μmol/l. This concentration was the one we focused on in the present study.

When C13NJ cells were treated for 48 h with OA-GcMAF (800 pmol/l), a significant increase in cell viability was observed in comparison with control, untreated cells (Fig. 5a) cultured for the same time period in the same medium but without OA-GcMAF. However, at significant variance with that observed in neurons, the decrease in cell viability induced by 100 μmol/l oxaliplatin was not reversed by pretreatment with OA-GcMAF. In this case, the presence of OA-GcMAF, added 24 h before the treatment with oxaliplatin and still present during the treatment with oxaliplatin, did not restore C13NJ cell viability (Fig. 5a).

Consistent with this lack of effect in restoring microglial cell viability, no significant differences in comparison with control untreated cells were observed in cAMP production when C13NJ cells were treated with OA-GcMAF (800 pmol/l), as shown in Fig. 5b. Oxaliplatin alone as well as in combination with OA-GcMAF induced a significant decrease in the level of cAMP, suggesting that the treatment with oxaliplatin with or without OA-GcMAF is associated with the activation of the signal transduction pathway, leading to cell suffering. The absence of stimulation of the cAMP pathway by OA-GcMAF suggests the possibility that the role of OA-GcMAF in activating the cAMP pathway could be cell-line dependent.

Thus, as microglia produce VEGF [29,33], we investigated the role of VEGF in the C13NJ cell line after treatment with oxaliplatin and/or with OA-GcMAF (800 pmol/l) for 48 h. Results showed that the treatment of C13NJ cells with OA-GcMAF induced a significant decrease in the expression of VEGF, as demonstrated by western blotting and densitometric analysis. As shown in Fig. 6a, VEGF expression is not significantly affected when C13NJ cells are treated with oxaliplatin alone or

Fig. 3



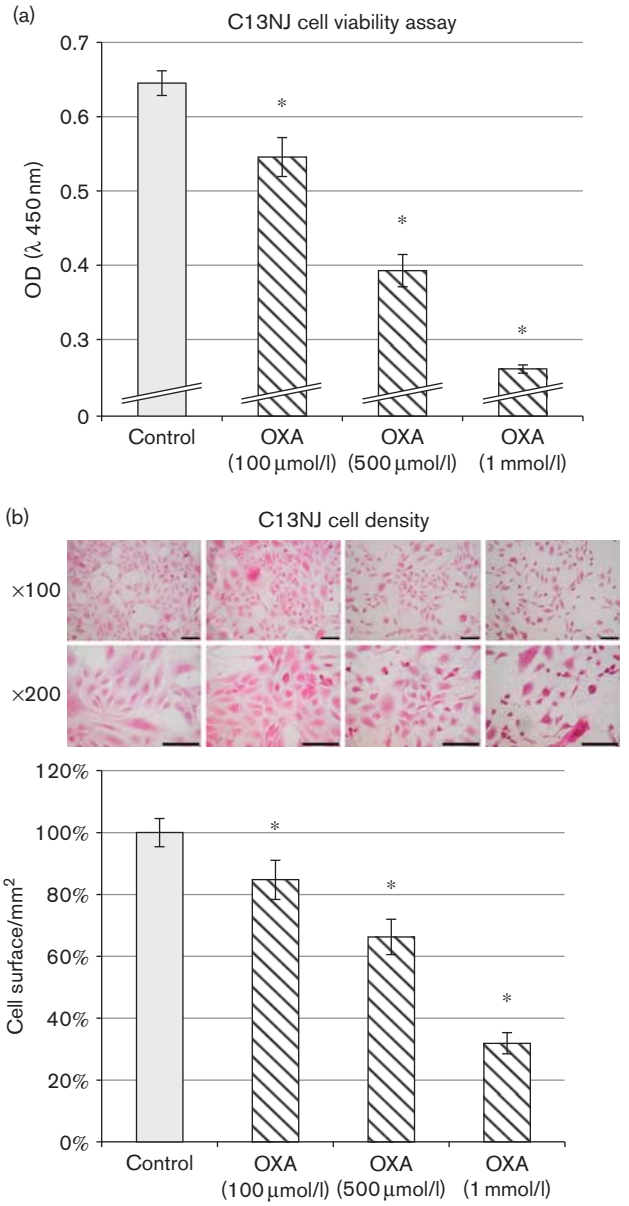
VEGF and neuromodulin (GAP-43) expression in SH-SY5Y cells after treatment with oxaliplatin and OA-GcMAF. (a) Expression of VEGF, evaluated by western blotting analysis and quantified using ImageJ software. Treatment of SH-SY5Y cells with OA-GcMAF induces an increased expression of VEGF in comparison with control, untreated cells (*$P < 0.05$). In contrast, treatment of cells with oxaliplatin induces a significant decrease in VEGF expression in comparison with control, untreated cells. When cells are cultured for 24 h in the presence of OA-GcMAF and then triggered with oxaliplatin for 24 h, VEGF expression is superimposable to the level of VEGF expression in control, untreated cells and significantly higher than VEGF expression induced by treatment with oxaliplatin alone (**$P < 0.05$ vs. OXA). (b) Expression of neuromodulin (GAP-43), evaluated by western blotting analysis and quantified using ImageJ software, shows a significant increase after the treatment of SH-SY5Y cells with OA-GcMAF (800 pmol/l). When cells are treated with oxaliplatin, a significant decrease in neuromodulin expression is observed in comparison with controls. When cells are pretreated for 24 h with OA-GcMAF and then stimulated with oxaliplatin, no significant recovery is observed in the expression of neuromodulin in comparison with treatment with oxaliplatin alone. Results are expressed as mean value ± SEM for two replicates (*$P < 0.05$ vs. control). Each experiment was performed three times. GAP-43, growth associated protein-43; OA-GcMAF, oleic acid-Gc-protein-derived macrophage activating factor; OXA, oxaliplatin; VEGF, vascular endothelial growth factor.

with the combination of oxaliplatin and OA-GcMAF in comparison with control cells.

Taken together, these results indicate that OA-GcMAF, at variance with that observed in neurons, did not positively stimulate the cAMP/VEGF signalling pathway in microglial cells, nor did it protect these cells from oxaliplatin-induced damage.

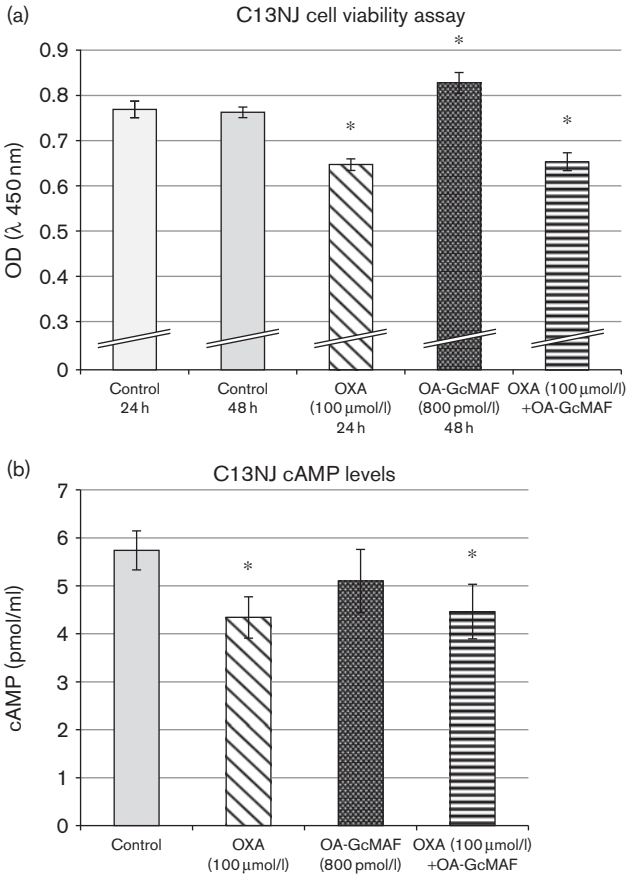
To evaluate the possible induction of microglial cells by OA-GcMAF, we investigated the expression of two markers of microglial activation, Iba1 and B7-2, by western blotting analysis and image quantification by densitometric analysis. Iba1 is a protein specifically expressed in macrophages/microglia and is upregulated during the activation of these cells [34]. B7-2 is a protein that is upregulated in activated microglia and is involved in breaking synaptic contacts of impaired neurons to allow nerve regeneration and neuritogenesis [35]. After 48 h of treatment with OA-GcMAF, the expression of Iba1 and B7-2 was not significantly different in

Fig. 4



Cell viability and cell density of C13NJ cells after treatment with oxaliplatin. (a) Cell viability, evaluated using the WST-8 assay. Histograms show a decrease in the metabolic activity of C13NJ cells after 24 h of treatment with oxaliplatin at different concentrations. (b) Cell density, evaluated by light microscopy, after haematoxylin–eosin staining. Pictures show a decrease in the cell density of C13NJ cells after 24 h of treatment with oxaliplatin at different concentrations. Upper panels: total magnification ×100; lower panels: total magnification ×200. Bar = 100 µm. Cell density quantification was performed using ImageJ software. The histograms show the mean area occupied by the C13NJ surface in five different fields (1 mm^2) on each coverslip. Two coverslips were counted in a blinded manner for each experimental point. Results are expressed as mean value ± SEM for two replicates (*$P < 0.05$ vs. control); each experiment was performed three times. OD, optical density; OXA, oxaliplatin.

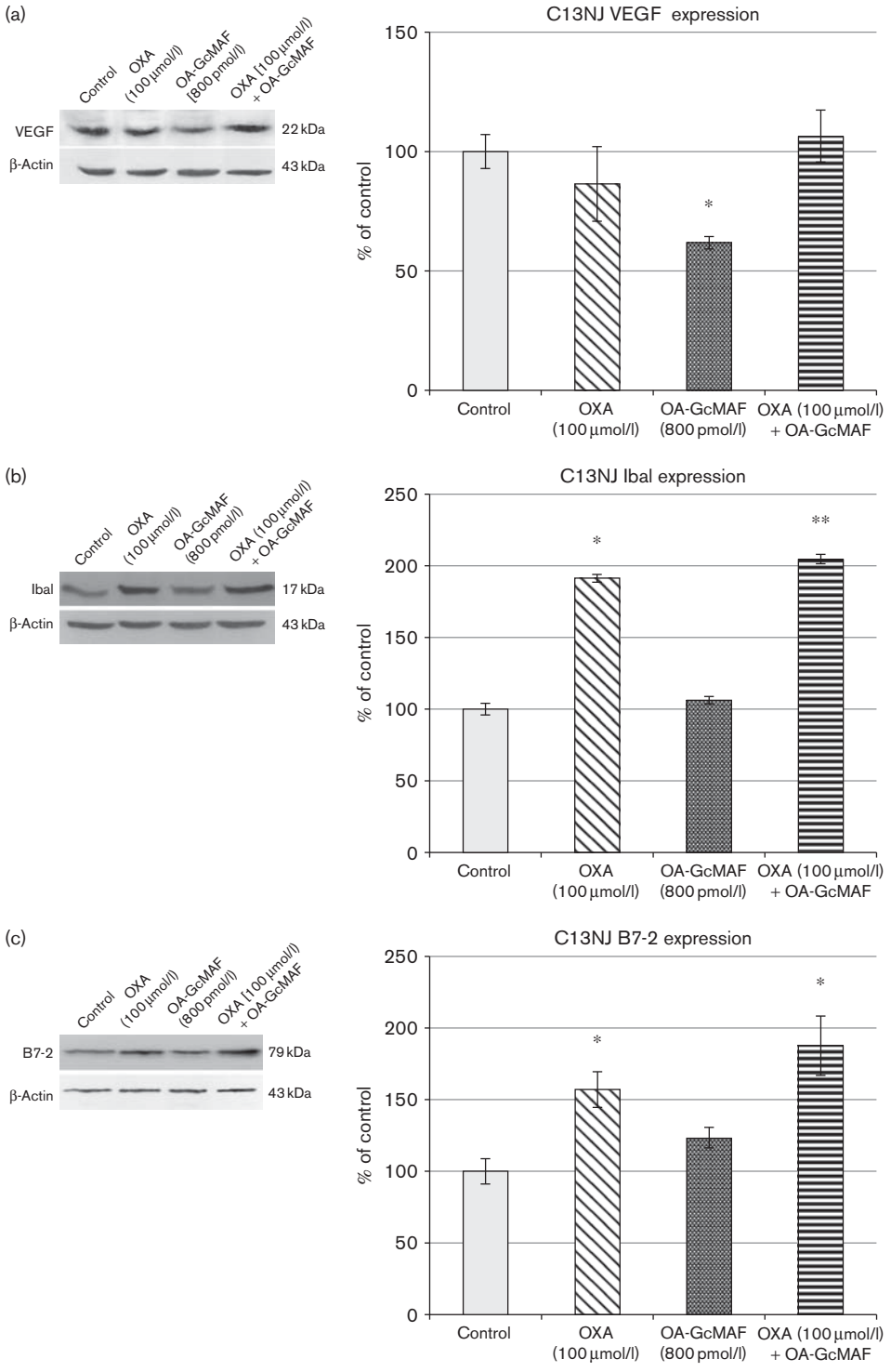
Fig. 5



Cell viability and cAMP level of C13NJ cells after treatment with oxaliplatin and OA-GcMAF. (a) Treatment of C13NJ cells with OA-GcMAF for 48 h induces a significant change in cell viability. Treatment with oxaliplatin, as expected, significantly decreases the cell viability in comparison with that of controls. Pretreatment for 24 h with OA-GcMAF before adding oxaliplatin does not significantly affect cell viability in comparison with oxaliplatin treatment alone. (b) cAMP levels, evaluated using the ELISA assay after 25 min of OA-GcMAF (800 pmol/l) treatment, suggest that OA-GcMAF does not activate the cAMP signal transduction pathway. In contrast, oxaliplatin induces a significant decrease in the cAMP level, which is not modified by the presence of OA-GcMAF. Results are expressed as mean value ± SEM for three replicates (*$P < 0.05$ vs. control). Each experiment was performed three times. ELISA, enzyme-linked immunosorbent assay; OA-GcMAF, oleic acid-Gc-protein-derived macrophage activating factor; OD, optical density; OXA, oxaliplatin.

comparison with control, untreated cells (Fig. 6b and c), suggesting that OA-GcMAF in the presence of a steady-state microenvironment does not affect basal activation of microglial cells. In contrast, treatment with oxaliplatin, as expected in the case of toxicants [6], induced a significant increase in both Iba1 and B7-2 expression. The expression of both markers was further increased when OA-GcMAF and oxaliplatin acted at the same time on C13NJ cells. In particular, as shown in Fig. 6b, the expression of Iba1, as well as of B7-2, was significantly ($P < 0.05$) higher in comparison with that observed after the treatment with oxaliplatin alone. These data suggest that OA-GcMAF exerts its strongest efficacy in activating microglial cells only when they are triggered by a toxicant. The damage induced by oxaliplatin, in the presence of OA-GcMAF, could lead to strong microglial activation, in turn leading to faster clearance of dying cells and cellular debris.

Fig. 6

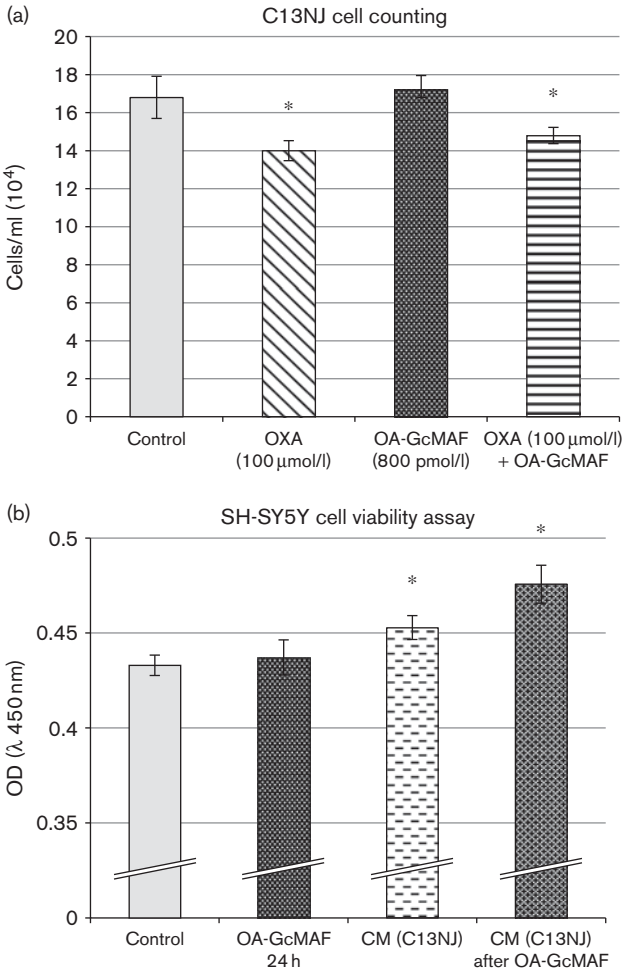


VEGF, Iba1 and B7-2 expression in C13NJ cells after oxaliplatin and OA-GcMAF treatment. (a) Expression of VEGF, evaluated by western blotting analysis and quantified using ImageJ, significantly decreases after C13NJ cell treatment with OA-GcMAF (800 pmol/l), whereas treatment with oxaliplatin or oxaliplatin + OA-GcMAF does not significantly affect VEGF expression in comparison with controls. (b) After treatment with OA-GcMAF, the expression of Iba1 is not significantly increased in comparison with control, untreated cells. However, treatment with oxaliplatin induces a significant increase in Iba1 expression. The expression of this marker is further increased (*$P < 0.05$) when OA-GcMAF and oxaliplatin act at the same time on C13NJ cells. (c) B7-2 expression shows a similar trend as that shown by Iba1. In fact, treatment with OA-GcMAF alone does not significantly affect B7-2 expression in comparison with control, untreated cells. In contrast, treatment with oxaliplatin induces a significant increase in B7-2 expression, which is further increased when OA-GcMF is present. Results are expressed as mean value ± SEM for two replicates (*$P < 0.05$ vs. control; **$P < 0.05$ vs. OXA alone). Each experiment was performed three times. Iba1, ionized calcium binding adaptor molecule 1; OA-GcMAF, oleic acid-Gc-protein-derived macrophage activating factor; OXA, oxaliplatin; VEGF, vascular endothelial growth factor.

However, to rule out the possibility that microglia induction could result in an increase in microglial cell number, cell counting was performed after oxaliplatin and OA-GcMAF treatment. As shown in Fig. 7a, no increase in cell number was observed when C13NJ cells were treated with OA-GcMAF. As expected, the cell number was significantly decreased in the presence of oxaliplatin with or without OA-GcMAF. These results are concordant with the decrease in C13NJ cell viability observed after the previously described treatment with oxaliplatin, and they indicate that OA-GcMAF increases microglial activation without leading to a concomitant, and potentially harmful, increase in cell number.

To assess whether microglial cell activation occurring after OA-GcMAF treatment led to the synthesis and release of neuroprotective factors, we tested the effects of the conditioned medium (CM) of C13NJ cells before and after their treatment for 48 h with OA-GcMAF (800 pmol/l) on SH-SY5Y cell viability. A cell viability assay was performed on SH-SY5Y cells after their treatment for 24 h with the CM of C13NJ cells. Results showed that C13NJ microglial cells, by themselves, released some neuroprotective factors in the culture medium, inducing a significant increase in neuronal cell viability (Fig. 7b). However, SH-SY5Y cell viability was not increased further on treatment with CM harvested from C13NJ cells treated for 48 h with OA-GcMAF at 800 pmol/l (Fig. 7b). Treatment with OA-GcMAF alone for 24 h does not affect, as expected, SH-SY5Y cell viability. This result suggests that the positive effect, in terms of cell viability, observed when SH-SY5Y cells are treated with OA-GcMAF is mainly due to a direct effect of this molecule on neurons rather than to an indirect effect mediated by microglia neurotrophic factors.

Fig. 7

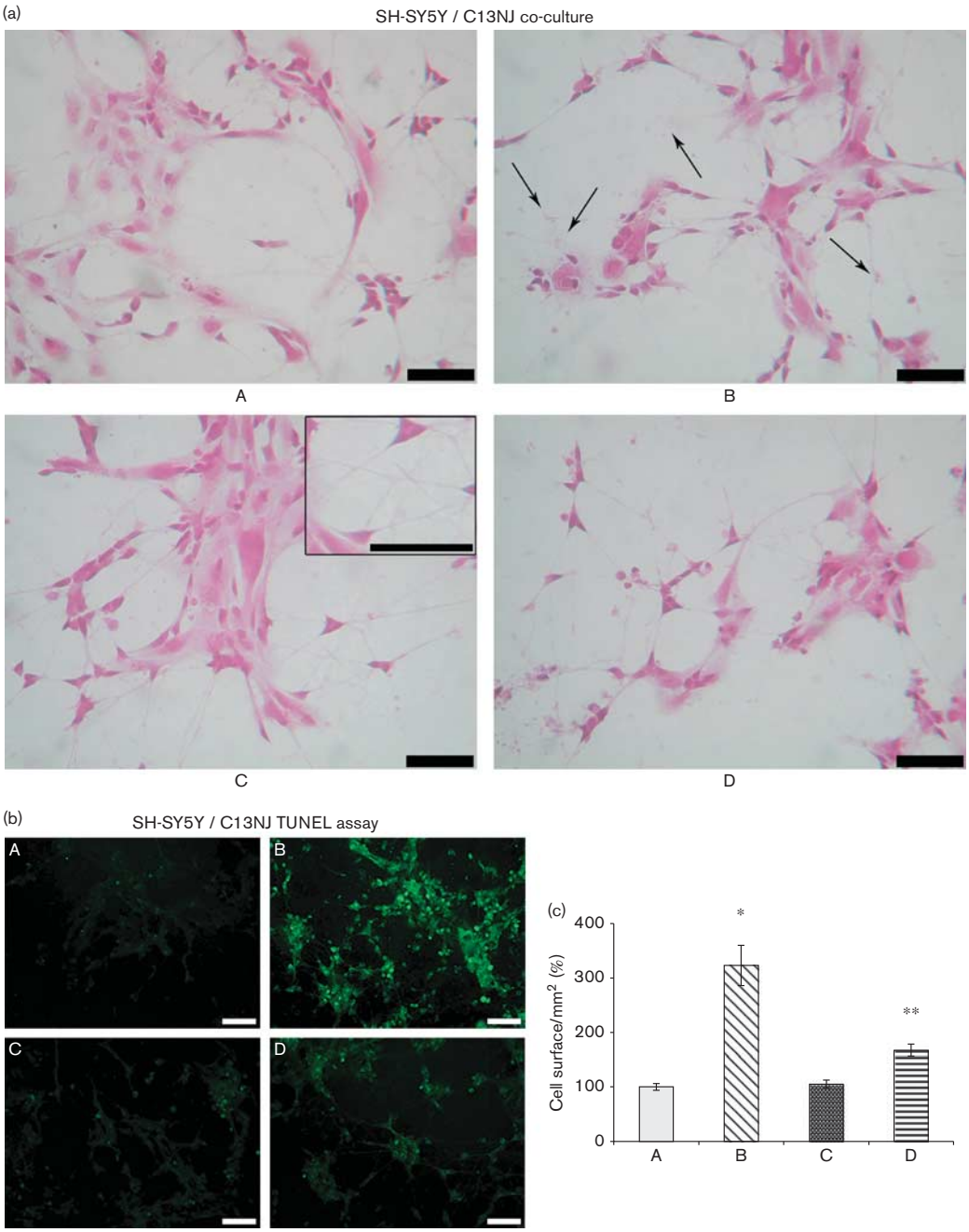


C13NJ cell counting and SH-SY5Y cell viability after treatment with conditioned medium of C13NJ cells. (a) C13NJ cell counting. Treatment with oxaliplatin with or without OA-GcMAF induces a significant decrease in cell number, whereas treatment with OA-GcMAF alone does not affect the cell number in comparison with control cells. (b) SH-SY5Y cell viability, evaluated using the WST-8 assay, significantly increases at the same extent as when SH-SY5Y cells are treated for 24 h with conditioned medium (CM) of C13NJ cells obtained after 48 h of OA-GcMAF treatment or directly harvested from untreated C13NJ cells. Results are expressed as mean value ± SEM for five replicates (*$P < 0.05$ vs. control). Each experiment was performed three times. OA-GcMAF, oleic acid-Gc-protein-derived macrophage activating factor; OD, optical density; OXA, oxaliplatin.

Treatment of C13NJ and SH-SY5Y cell co-cultures with oxaliplatin and OA-GcMAF: morphology and apoptosis

Data obtained on co-cultures of C13NJ and SH-SY5Y cells showed that the morphology of and the interactions between neurons and microglial cells were deeply affected by the treatment with oxaliplatin (Fig. 8a). An oxaliplatin concentration of 10 µmol/l was used for co-culture experiments, as this was the concentration used to treat neurons that are more sensitive to oxaliplatin than are C13NJ cells. As shown in Fig. 8a, after the treatment with oxaliplatin, a decrease in microglia–neuron connections and a significant decrease in neuron density in comparison with controls and OA-GcMAF-treated cells were observed. Microglia–neuron connections were restored when the co-cultures were treated with OA-GcMAF for 24 h before adding oxaliplatin for 24 h (Fig. 8a); the neuron density was further reduced because of the toxic effect of oxaliplatin, and a lot of debris was still detected around the cells. To better investigate and quantify cell damage, a Tunel assay was performed. As shown in Fig. 8b, in control cells, as well as in cells treated with OA-GcMAF, a low number of apoptic cells were detected, as expected for cell cultures. However, when the cells were treated with 10 µmol/l oxaliplatin, a significant increase in the number of apoptotic cells was observed; apoptosis was significantly reduced when the co-cultures were pretreated for 24 h with OA-GcMAF before adding oxaliplatin. Variations in apoptosis, observed by light microscopy, were then quantified using appropriate software and expressed as cell surface/mm^2 (Fig. 8c). As shown using histograms, considering the level of apoptosis in control cells to be

Fig. 8



Co-culture morphology after treatment with oxaliplatin (10 μmol/l) and OA-GcMAF. (a, A) Control, untreated cells. Neurons show a typical triangular body, an evident nucleus, a wide cytoplasm and several long cytoplasmic elongations. Microglia are mostly represented by round cells, sometimes forming clusters. Elongations arising from the glial cells and neurons form a sort of intricate net that links microglia and neurons to each other. Bar = 100 μm. (B) Oxaliplatin (10 μmol/l)-treated cells. Most of the cell connections are lost or deeply damaged and only a few neurons are present. A lot of debris (arrows), a clear sign of cell degeneration, is present. Bar = 100 μm. (C) OA-GcMAF-treated cells. No significant differences are observed, either in microglia or in neurons, when the co-culture is treated with OA-GcMAF, in comparison with control, untreated cells. Numerous cell connections are present (details at a higher magnification in the square). Bars = 100 μm. (D) OA-GcMAF and oxaliplatin-treated cells. Cell connections are increased in comparison with oxaliplatin treatment alone. Neuron density is still decreased because of the toxic effect of oxaliplatin, and some debris is still present. Bar = 100 μm. (b) Tunel assay. In (A) control cells, as well as in (C) cells treated with OA-GcMAF, only a basal low level of apoptosis is detectable. When (B) cells are treated with oxaliplatin (10 μmol/l), a significant increase in the number of apoptotic cells is observed; (D) apoptosis is significantly reduced when the co-cultures are pretreated for 24 h with OA-GcMAF before adding oxaliplatin. (c) Variations in apoptosis are quantified using appropriate software and expressed as cell surface/mm^2. Results are expressed as mean value ± SEM for five replicates (*$P < 0.05$ vs. control). Each experiment was performed three times. OA-GcMAF, oleic acid-Gc-protein-derived macrophage activating factor.

100%, oxaliplatin increased the level of apoptosis by more than three times; when the co-culture was pretreated with OA-GcMAF and then with oxaliplatin the level of apoptosis significantly decreased in comparison with that observed after treatment with oxaliplatin alone.

Discussion

Oxaliplatin is a platin organic drug with good therapeutic effects in several solid tumour types, but it has a characteristic pattern of neurotoxicity. Such a toxicity, characterized by cold-exacerbated paresthesias, muscle spasms and fasciculations, sometimes leads to functional impairment and even ataxia, which frequently compromise the efficacy of chemotherapy. Therefore, there is a need for the investigation of agents that may ameliorate or modulate the neurotoxic symptoms induced by such oxaliplatin-based chemotherapy regimens. It is of fundamental importance that, to be clinically useful, the antineuropathic agents must reduce the neurotoxic effect of the chemotherapeutic agent without hampering its full antitumour efficacy. In this study, we evaluated the role of a molecule that has immunotherapeutic properties by itself, OA-GcMAF, in directly counteracting the toxic effects of oxaliplatin on human neurons and in inducing microglia activation.

Our results demonstrate that a commercially available formula of GcMAF, OA-GcMAF, which has been shown to be successful in the integrated immunotherapy of advanced cancers [17], may significantly contribute to neutralizing the neurotoxicity induced by oxaliplatin, at the same time possibly concurring to an overall anticancer effect.

Thus, we demonstrated that OA-GcMAF reverses the neuronal damage inflicted by oxaliplatin and preserves neuronal viability, possibly through the cAMP/VEGF neuroprotective signalling pathway.

It is also interesting to note that the observed effects of OA-GcMAF on the cAMP/VEGF cascade are consistent with the well-established association between GcMAF and VDR. Thus, activation of VDR in neurons is known to involve cAMP signalling [36], and cAMP signalling mediates the protection of neurons and cerebral vascular endothelial cells by VEGF [37]. The following signalling cascade can therefore be proposed: GcMAF activates VDR, which in turn stimulates cAMP signalling, resulting in the induction of VEGF expression, which is responsible for neuronal protection against oxaliplatin-induced damage. Besides its role in reversing oxaliplatin-induced damage and in the activation of the cAMP/VEGF cascade, OA-GcMAF directly induces an increase in the expression of neuromodulin, a well-known protein associated with filopodial extension and neurite branching, clearly allowing growth of a developing or regenerating neuron.

This proposed mechanism of action underlying the neuroprotective effect of OA-GcMAF is paralleled by the effects on microglial cells. At variance with its effects on neurons, OA-GcMAF does not appear to protect microglial cells against the damage induced by oxaliplatin and, consistently, it does not increase cAMP levels, nor does it induce the expression of VEGF. However, as microglial cells express the receptor associated with OA-GcMAF signalling, that is VDR [38], other biological effects of OA-GcMAF can be expected in these cells. Consistent with this hypothesis, we demonstrated that OA-GcMAF, in the presence of important cell damage induced by oxaliplatin, increased the expression of specific markers of microglial cell activation (Iba1 and B7-2), not associated with an increase in the cell number, thus lending credit to the hypothesis that OA-GcMAF-stimulated microglial cells contribute to the overall neuroprotective effect by scavenging oxaliplatin-damaged cells. This hypothesis is consistent with the notion that microglial cell activation is directly engaged in processes involved in restoring normal pain signalling and protecting against neurotoxicity [8].

In addition to these considerations, and consistent with an active role of microglial cells in preserving neuronal function and restoring neuronal viability, we observed that the culture medium of C13NJ cells contained neurotrophic and neuroprotective factors that may further contribute to the preservation of neuronal viability. Oddly enough, the release of neuroprotective factors in the CM of C13NJ cells was not affected by OA-GcMAF. Although puzzling, these observations further demonstrate that the effects of OA-GcMAF are not only cell specific but are also very selective as far as the intracellular signalling pathways are concerned. Further studies will be aimed at elucidating the precise role of OA-GcMAF in inducing microglial cell activation and in the overall context of neuroprotection. In fact, the therapeutic efficacy of OA-GcMAF in treating neurological conditions such as autism spectrum disorders and mylagic encephalomyelitis [16,25] provides indirect evidence for a significant neuroprotective effect *in vivo*. Consistent with this indirect clinical evidence, preliminary results appear to indicate that intraperitoneal administration of OA-GcMAF significantly reduces inflammatory pain in rats [16].

If these observations obtained *in vitro* are replicated *in vivo*, it can be hypothesized that GcMAF could be included in oxaliplatin-based regimens. The association between these two powerful anticancer molecules would probably produce the dual effect of reduction of oxaliplatin-induced neurotoxicity and possible synergism in the overall anticancer effect.

Acknowledgements

Stefania Pacini and Marco Ruggiero received grants from the University of Firenze and the Project PRIN 2009.

The authors thank Dr Jean Mazella (Institut de Pharmacologie Moléculaire et Cellulaire, UMR 7275, Centre National de la Recherche Scientifique, Université de Nice-Sophia Antipolis, Valbonne, France) for kindly donating the cell line C13NJ to us.

Conflicts of interest

M.R. is the consulting scientific director of Immuno Biotech Ltd (the company that isolates and purifyes the GcMAF protein and produces oleic acid-GcMAF). However, M.R. had no prior knowledge of the experimental protocols and did not participate in the actual planning of the individual experiments. He participated in the analysis of the results only after they had been verified and validated by the other authors. Funding supporting this study is derived from the University of Firenze, Italy.

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