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Static magnetic field selects undifferentiated myelomonocytes from low-glutamine concentration stimulated U937 cells

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Abstract

Reduced glutamine (GLN) concentration in the culture medium of a U937 cell line caused them to be differentiated along the monocytic pathway; cells attached to the matrix and to each other by extending pseudopodia and acquired specific functional characteristics, such as the expression of α-naphthyl-acetate esterase and the capacity to reduce nitroblue tetrazolium, as well as becoming active phagocytes. When U937 cells were differentiated under continuous exposure to a 6 mT static magnetic field (MF) the overall differentiation process was perturbed. Surprisingly, after 5 days' exposure to the static MF, higher cell viability and differentiation were observed in cells cultured in a GLN-deprived medium than in cells grown in the same medium but in the absence of a static MF. The latter cells, particularly those that were still floating in the medium, were stimulated with TPA for a further 3 days. These cells differentiated and attached to the substrate. Conversely, the same treatment applied to cells cultured in GLN-deprived medium in the presence of the static MF resulted in resistance to TPA-induced differentiation. Indeed, these cells exhibited a round shape and in-suspension growth.

Keywords: Differentiation; Magnetic field exposure; Monocytes; Glutamine

1. Introduction

The established human cell line U937, having monoblastic and immature monocytic characteristics, can be induced to *in vitro* differentiation using various stimuli, including 12-O-tetradecanoyl-13-phorbol acetate, 9-*cis* retinoic acid, 1α, 25-dihydroxycholecalciferol, interferon γ, tumour necrosis factor α and low-concentration glutamine (GLN) (Nichols and Weinberg, 1989; Spittler et al., 1995, 1997).

GLN is the most abundant amino acid in the blood and in the free amino acid pool of the body, and is important in the growth of normal and neoplastic cells as well as in many types of cultured cells (Spittler et al., 1995, 1997). In the cells of the immune system, to which it is delivered from skeletal muscle after surgical procedures, trauma and starvation, GLN serves as an energy substrate, acts as a glucose precursor, counteracts acidosis and regulates intracellular water content (Oehler and Roth, 2003; Newsholme et al., 2003). GLN is essential for lymphocyte proliferation, influences the differentiation of B cells into plasma cells and induces phenotypical and functional differentiation of U937 myelo pro-monocytic cells (Spittler et al., 1995, 1997; Chwals, 2004). GLN deprivation induces irreversible differentiation of HL60 cells and when this stimulus is given in combination with the appropriate molecules which induce monocyte differentiation of HL60, it enhances their effects (Nichols and Weinberg, 1989). GLN deprivation is associated with apoptosis (Exner et al., 2002; Papaconstatntinou et al., 1998; Petronini et al., 1996) and this property has been used in GLN-related antineoplastic therapies (Medina, 2001a,b).

The influence of static MFs is becoming a topic of considerable interest, due to the widespread use of common electrical devices and medical diagnostic instrumentation. The last 20 years have seen increased interest in the possible effects of magnetic fields on health due to the substantial evidence of their influence on living organisms, tissues and cells (Tenforde, 1995; Zhadin, 2001; Rosen, 2003a; Dini and Abbro, 2005). Although a number of theoretical models have been proposed (Rosen, 2003b; Liburdy, 1992), the

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possible mechanisms of the induced effects are still not known, probably due to difficulties in the extrapolation of results and the replication of studies in different laboratories which result from various reported non-linearities in the intensity, frequency and time windows of the fields, differing characteristics of the materials used (cell type, age, treatment), and differences of experimental approach.

Reducing GLN concentration in the culture medium of U937 cells causes their differentiation along the monocytic pathway (Spittler et al., 1997). The maturation of monocytes along the monocytic pathway includes changes in the morphology of the cells and in the plasma membrane modulation of several antigens. Round U937 cells in suspension with smooth surfaces attach to the matrix and to each other by extending pseudopodia, subsequently developing three-dimensional aggregates upon exposure to the differentiating agents. Moreover, U937 cells acquire certain functional properties associated with maturating monocytes (Hass et al., 1997). These include the expression of *a*-naphthyl-acetate esterase and the capacity to reduce nitroblue tetrazolium. The cells also become active phagocytes. Furthermore, the expression of *c*-fms (M-CSF receptor), macrophage colony stimulating factor (m-csf), prostanoids, cytokines and cytokine receptors increases during TPA-induced differentiation (Hass et al., 1997). However, the intracellular signals of this differentiation program remain unclear, as does the influence of external factors such as exposure to static MFs.

In a previous study we investigated the effect of moderate intensity static MF exposure on the differentiation process of U937 cells, as induced by various stimuli, obtaining data on the slowing of the process when simultaneously performed with phorbol ester and exposure to static MF (Pagliara et al., 2005).

The aim of the present study is to investigate the effect of a 6 mT static MF on monocyte differentiation as induced by low-concentration GLN considering its pro-apoptoptic activity and its role in the prevention of radiation-induced toxicity. Viability, death, adhesion to the substrate, phagocytosis index and re-differentiation of non-adherent cells were analysed.

2. Materials and methods

2.1. Cells and cultures

U937 monoblastic cells were cultured at 37 °C in RPMI 1640 medium supplemented with 10% inactivated fetal calf serum (FCS), 2 mM L-glutamine, 10.000 IU/mL nistatine, 100 IU/mL penicillin and streptomycin in a humidified atmosphere with 5% CO_2; cells were used at a density of 1×10^6 cells/mL.

2.2. Cell viability

Cell viability was assessed by membrane impermeability to trypan blue and by MTT assay.

Trypan blue dye exclusion test: Trypan blue (0.5% w/v) was added in equal volume to cell suspension and the cells were scored under phase contrast microscope. Dead cells take up the blue stain of trypan blue, whereas the live cells have yellow nuclei.

MTT assay: The culture medium was replaced with 1 mL/well of MTT (Sigma) solution (1 mg/mL in culture medium without phenol red). After 3 h of incubation at 37 °C the solution was removed, 1 mL/well of DMSO was added and the absorbance was read at 540 nm after 10 min of slow shaking.

2.3. Macrophagic differentiation

U937 cells were differentiated on a substrate of fibronectin 10 μg/mL previously left on the bottom of dishes for 18 h at 4 °C and then saturated with 1% bovine serum albumin–phosphate buffer pH 7.4 (BSA-PBS) for 90 min at 12 °C. Macrophage differentiation was induced with: 50 ng/mL 12-O-tetradecanoil-13-phorbol acetate (TPA), 10% dimethyl sulfoxide (DMSO), 100 mM Zn^{++} or low-concentration GLN 0.05 mM (using a culture medium not supplemented with Lglutamine). Differentiation was monitored for 3 days following the addition of the inducers and was evaluated by morphological criteria and the Nitro blue tretrazolyum test (NBT). NBT can be used to determine U937 differentiation by both light microscopy observations of diformazan crystals and by spectrophotometric analysis. At fixed times, 100 μL of 10 mg/mL NBT in PBS was added to 3 mL of cell medium; cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 for 30 min and for 2 h, before undergoing light microscopy and spectrophotometric analysis respectively. Cells were washed three times with PBS pH 7.4, fixed with 4% formalin in the same buffer and then stained with May-Grunwald dye for light microscopy observation. Cells with diformazan crystals (differentiated cells) were counted. Spectrophotometric analysis (absorbance $\lambda = 560$ nm) was performed with cells washed three times with PBS pH 7.4 in which diformazan crystals were dissolved in 2 mL of DMSO.

2.4. Static magnetic field application

The static MF was produced by Neodymium magnetic disks (10 mm in diameter and 5 mm in height) of known intensity supplied by Calamit Ltd. (Milano, Italy) placed under the culture Petri dishes. The intensity of the field generated by the magnet was checked by means of a gaussmeter (Hall-effect gauss meter, GM04 Hirst Magnetic Instruments Ltd., UK). A field intensity of 6 mT was obtained on the bottom of the culture dish at 2.5 cm from the magnet. This distance was obtained by interposing between the magnetic disk and the Petri dish two disks of the same diameter as the culture dish, one metallic disk (in order to minimize the differences in the field intensity across the whole bottom of the dish) and one of inert material. The field intensities measured in three different zones of the dish bottom are described in detail in Chionna et al., 2005. During the entire experiment no increase in temperature was observed. Dishes of cells were always placed on the same two shelves in a tissue culture incubator where the ambient 50 Hz magnetic field was 0.95/0.62 μT (heater on/off) and the static magnetic flux density was 5.5 μT. In the laboratory areas where the cells were processed (between incubators and worktop) the magnetic field ranged between 0.08 and 0.14 μT (50 Hz).

The static MF was applied concomitantly with differentiation inducers. Exposures were carried out in a blind manner. Experiments without magnets were performed simultaneously as controls.

2.5. Light microscopy

Cell morphology was observed by light microscopy and scanning electron microscopy.

For light microscopy observation, cell samples were fixed for 10 min with 4% formaldehyde in PBS 0.2 M, pH 7.4 at 4 °C and stained with hematoxylin eosin. Samples were observed by light microscopy, using an Eclipse 80i microscope (Nikon, Japan).

2.6. Phagocytic rate

The phagocytic capacity of U937 cells, differentiated in the presence and absence of the static MF, was measured by incubating cells (2×10^5) for 1 and 3 h at 37 °C (time established in preliminary experiments) with fluorescent latex beads (2 μm in diameter), added to the culture medium in a ratio of 10 beads to 1 cell.

After 1 and 3 h, cells were extensively washed in PBS and then fixed with 4% formalin. U937 cells were further stained with FITC-conjugated phalloidin. Samples were observed with a NIKON PCM 200 fluorescent microscope with Plan Fluor objectives (Nikon, Japan).

The phagocytic rate was measured as the number of cells containing particles out of 500 cells scored.

3. Results

3.1. Monocyte differentiation

The degree of differentiation, evaluated by NBT test and by counting the cells attached to the culture plate, correlated with the differentiation agent and with the period of stimulation (Tables 1 and 2). Indeed, TPA was the most effective, while the least was DMSO. A high degree of differentiation was already reached after 2 days of incubation with TPA. By analysing the degree of differentiation in the fractions of culture plate-attached and in-suspension cells, TPA was found to induce both differentiation and adhesion, whereas low-concentration GLN and Zn^{++} (in that order), induced differentiation but little adhesion. Indeed, after 3 days of stimulation with low-concentration GLN, the number of differentiated in-suspension cells was greater (according to the

Table 1
Number of substrate plate attached versus in suspension U937 cells upon differentiating stimuli

Days	TPA				Low-GLN conc.				DMSO				Zn^{++}			
	a	*a* + MF	*s*	*s* + MF	*a*	*a* + MF	*s*	*s* + MF	*a*	*a* + MF	*s*	*s* + MF	*a*	*a* + MF	*s*	*s* + MF
1	12	150	20	20	8	3	25	30	Nd	Nd	23	40	Nd	Nd	22	25
2	30	160	19	18	48	52	24	40	10	Nd	30	39	16	Nd	21	15
3	230	160	6	22	106	28	20	36	0	0	0	0	5	0	0	0

The data are the mean of three independent experiments. S.D. does not exceed 5%.
a: cells attached to the substrate; *s*: cells floating in the medium; TPA: 50 ng/mL 12-O-tetradecanoil-13-phorbol acetate; low-GLN conc: low-glutamine concentration, 0.05 mM/L; DMSO: 10% dimethyl sulfoxide; Zn^{++}: 100 mM/L.

Table 2
Semiquantitative evaluation of differentiation by NBT test observed at light microscope

Cell fractions		1 day		2 days		3 days	
		− sMF	+ sMF	− sMF	+ sMF	− sMF	+ sMF.
TPA	Suspension	+	++	+++	+	++++	+
	Adhesion	+++++	+++++	+++++	++++	+++++	+++
Zn^{++}	Suspension	+	++	+	++	+	++
	Adhesion	+	+	++	+	++	+
DMSO	Suspension	−	−	−	+−	−	−
	Adhesion	−	+−	+−	+	+	+
Low-GLN conc.	Suspension	+	+	+++	+++	++++	++++
	Adhesion	+	+	+++	++	+++	+++

The data represent one out of three independent experiments.
Adhesion: cells attached to the substrate; suspension: cells floating in the medium; TPA: 50 ng/mL 12-O-tetradecanoil-13-phorbol acetate; low-GLN conc: low-glutamine concentration, 0.05 mM/L; DMSO: 10% dimethyl sulfoxide; Zn^{++}: 100 mM/L.

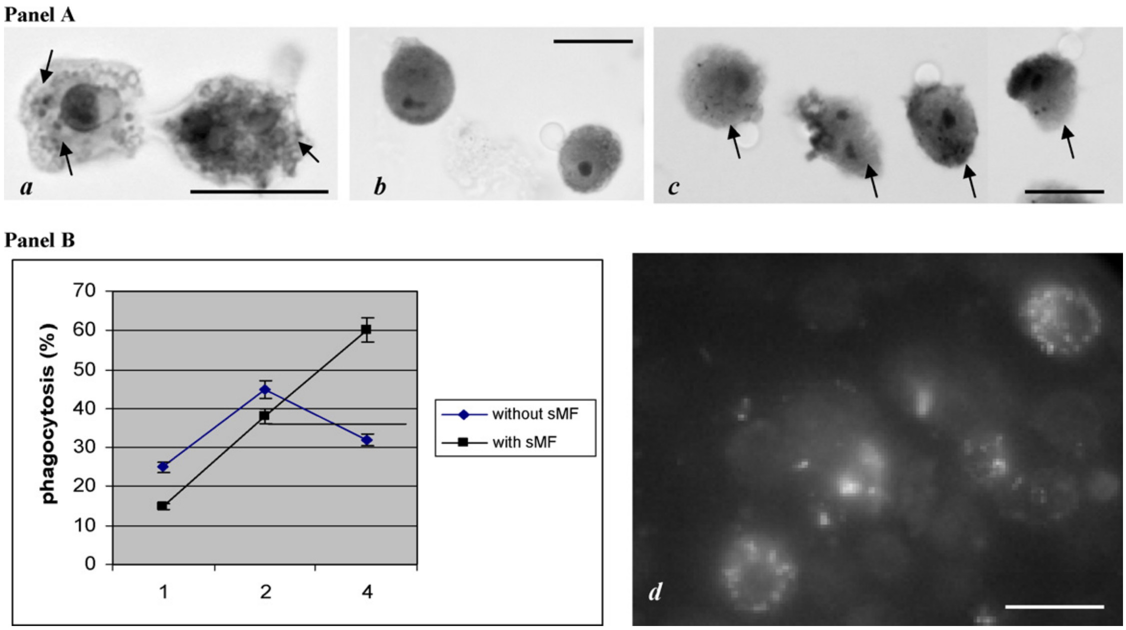

Fig. 1. Panel A: light micrographs showing diformazan salts (arrows) inside the cytoplasm representative of fully differentiated, substrate-attached U937 cells (a); in suspension undifferentiated U937 cells (b); in suspension differentiated U937 cells (c). NBT staining. Bars = 2 μm.
Panel B: percentage of phagocytosis of 3 days low-GLN concentration stimulated monocytes in the presence and in the absence of static MF (d). Fluorescence microscope image showing the active phagocytosis of fluorescent latex beads of differentiated U937 cells. Bar = 2 μm.

NBT test, Table 2, Fig. 1 panel A) than the number of differentiated adherent cells (Table 2).

The viability of the cell fraction floating in the culture medium was high up to the second day of stimulation and dropped off at the third day, with the exception of TPA and low-concentration GLN, which saw a drop of only 15% (Table 3). Apoptosis did not increase above basal value (about 5–8%).

When the U937 cells were differentiated with TPA under continuous exposure to a 6 mT static MF an overall slowdown of the differentiation process was measured (Table 2). Non-differentiated characteristics were also observed under light microscope: cells remained round and the percentage of substrate-attached cells decreased. Conversely, GLN-deprived cells differentiated under static MF showed a high degree of viability and differentiation but low adhesion with respect to those cultured in the absence of the static MF (Compare Tables 1, 2 and 3, Fig. 1). The phagocytosis rate correlated with the degree of differentiation (Fig. 1, panel B). However, after 4 h of incubation of cells with fluorescent latex beads under exposure to the static MF, the phagocytosis rate was higher than in the absence of the static MF.

3.2. Glutamine deprivation-induced differentiation

The high degree of differentiation and viability observed in non-attached cells after low-concentration GLN induction was further analysed. We performed a time course experiment (from 1 to 6 days of treatment with low-concentration GLN) in the presence and absence of a 6 mT static MF, by analysing the morphology, viability and degree of differentiation of the non-attached cell fraction. Cell viability decreased progressively, falling more rapidly in the last two days. By the 6th day of culture, 90% of the cells had died. Surprisingly, in the presence of the static MF, 80% of the cells were still viable on the 6th day (Fig. 2, panel c).

After 5 days of static MF exposure, the degree of differentiation was significant (Fig. 2, panel B). Diformazan salts

Table 3
Percentage of cell viability (MTT assay)

Treatments	1 day		2 days		3 days	
	− sMF	+ sMF	− sMF	+ sMF	− sMF	+ sMF
TPA	96	97	88	84	78	69
Zn^{++}	88	89	83	85	20	15
DMSO	24	5	10	2	5	2
Low-GLN conc.	83	87	80	80	69	74

The data are the mean of three independent experiments. S.D. does not exceed 5%. The data refer to the entire (in suspension and attached) cell population. TPA: 50 ng/mL 12-O-tetradecanoil-13-phorbol acetate; Low-GLN conc: low-glutamine concentration, 0.05 mM/L; DMSO: 10% dimethyl sulfoxide; Zn^{++}: 100 mM/L.

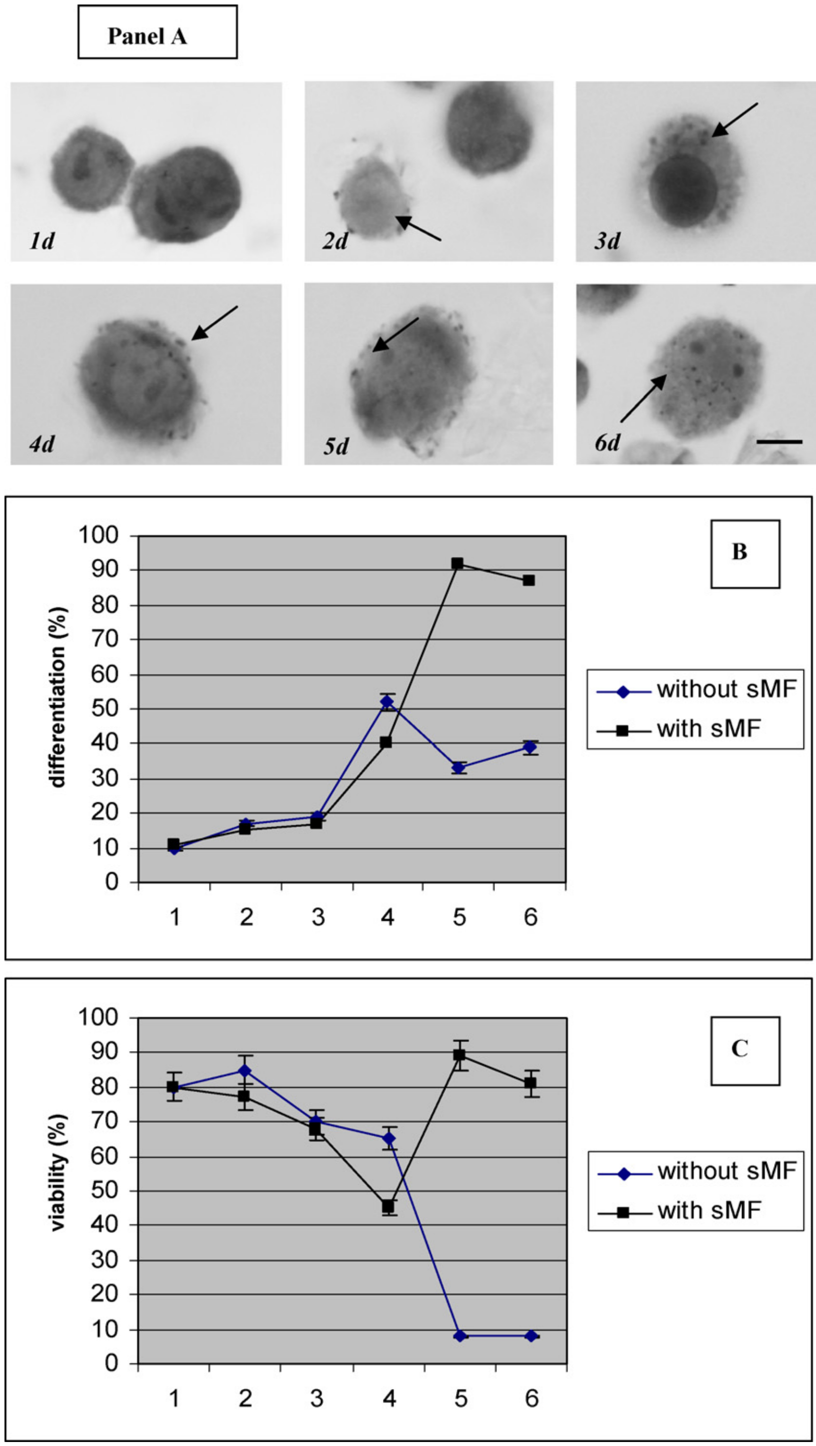

Fig. 2. Panel A: time course light micrographs showing the presence (arrows) of diformazan salts in U937 cells from 2 to 6 days (d) of culture in a medium containing low-glutamine concentration. Bar = 1 μm.
Panel B: time course of the differentiation degree of U937 cells, expressed as percentage of the undifferentiated cells, cultured for 6 days in a medium containing low-glutamine concentration.
Panel C: time course of the viability of U937 cells, expressed as percentage of the undifferentiated cells, cultured for 6 days in a medium containing low-glutamine concentration.

were observed under light microscope in low-GLN-treated cells at all experimental times (Fig. 2, panel A).

The fraction of in-suspension cells, after having been cultured with low-concentration GLN in the presence or absence of the static MF for 5 days, was stimulated with TPA for a further 3 days. As a control, U937 cells left in culture with complete medium without any changes or addition of fresh medium for 5 days, were stimulated with TPA for 3

Table 4
Percentage of viability, adhesion and differentiation of U937 cells after the re-differentiation with TPA for 3 days experiments

Treatments	Viability (%)	Adhesion (%)	Differentiation (%)
Low-GLN conc. −SMF	73	40	45
Low-GLN conc.+SMF	88	10	13
Control (deprived medium)	69	60	67

The data are the mean of three independent experiments. S.D. does not exceed 5%. The data refer to the entire (in suspension and attached) cell population.

days in the absence of the static MF. At the time of TPA addition, the viability of the control was about 65% and the degree of differentiation was 10–15%, due to the deposition of cells onto the plastic plate. These were the cells that showed the highest degree of differentiation after TPA-induction (Table 4). A good degree of differentiation and adhesion was also observed after TPA-induction in cells cultured for 5 days in low-concentration GLN medium in the absence of a static MF. Conversely, the GLN-deprived cells under exposure to the MF were the least responsive to further differentiation. These cells were highly viable and highly proliferating as indicated by MTT. The majority of the cells were floating in the medium, maintaining a round shape (Table 4). A low degree of differentiation and substrate adhesion (only 10%) was observed.

4. Discussion

The amino acid GLN has received considerable attention as a potential therapeutic adjuvant in critical illness and in improving postoperative clinical outcomes. Most studies of the role of GLN in cellular physiology have historically focused on its anabolic roles in specific cell types and its contribution to growth in cancer cells. However, an emerging body of work which examines the consequences of GLN deprivation on cellular survival and gene expression has constructed a new paradigm for this amino acid, in which limited extracellular GLN modulates stress and apoptotic responses (Mates et al., 2006; Fuchs and Bode, 2006). Indeed, GLN deprivation is associated with apoptosis in rat intestinal epithelial RIE-1 and lymphoma–leukaemia cells (Papaconstatntinou et al., 1998; Petronini et al., 1996). Furthermore, starvation-induced apoptosis of mouse B-lymphocyte hybridoma cells can be suppressed by the addition of GLN (Franek and Sramkova, 1996). States of physiological stress, including those resulting from the treatment of malignant diseases, are characterized by a relative deficiency of GLN. Supplementation with this inexpensive dietary supplement may be beneficial. Indeed, oral GLN supplementation may enhance the benefits of therapy by protecting normal tissues from (and sensitising tumour cells to) chemotherapy and radiation-related injury (Savarese et al., 2003).

The data reported in this study indicate that GLN deprivation induces differentiation of U937 cells but prevents their attachment to the substrate, which is a natural feature of macrophage differentiation when exposed to a static MF. Low-concentration GLN has already been reported to induce differentiation of U937 cells as well as HL60 cells (Spittler et al., 1997; Nichols and Weinberg, 1989). U937 cells are widely used as an *in vitro* experimental model in many different types of study, including differentiation. The differentiated cells attached to the substrate contained more vacuoles. They were able to reduce diformazan salts, and mitotic figures were significantly reduced, indicating fewer proliferating monocytes. Exner et al. (2002) also reported that U937 cells cultured in a GLN-deprived medium are more susceptible to specific apoptosis triggers. Thus, from the point of view of its anti-tumour effects, the fact that GLN is an effective regulator of cell behaviour, influencing cell growth and differentiation, supports the clinical usefulness of glutaminase, the major enzyme of GLN hydrolysis (Medina, 2001a, 2001b; Griffiths et al., 1996). Tumours elicit a specific response in the host's nitrogen metabolism, i.e., they mobilize and augment its circulating glutamine (Medina, 2001a, 2001b). There is a net flux of glutamine from host to tumour, which is possibly due to a higher ratio of glutamine synthetase (GS) to glutaminase (GA). Over the last few decades, two glutamine-related antineoplastic therapies have raised great expectations: glutamine clearance and the use of glutamine analogues to kill tumour cells by exhausting their provision of glutamine. However, clinical trials indicate multiple toxic effects, lack of specificity and/or ineffectiveness (Medina, 2001a, 2001b). Our data, showing that culture in a low-concentration GLN medium under exposure to a static MF selected a U937 cell subpopulation, was unexpected. Thus, from the point of view of human health, the selection of a population of undifferentiated cells when GLN deprivation is combined with exposure to static MF indicates that the clinical use of glutaminase could lead to unwanted outcomes. It is therefore necessary to conduct careful monitoring of the environmental conditions, uncontrolled elements of which could behave as pro-tumour stimuli. MFs are one of the environmental factors that should be kept under control. The widespread use of diagnostic medical instrumentation, such as NMR, and the generation of extremely low frequency electromagnetic fields by common electrical devices have introduced into our living environment many sources of SMFs and "quasi SMFs".

The TPA-induced differentiation of U937 cells in the presence of a static MF was lower than the TPA-induced differentiation in the absence of the field; however, the rate of differentiation with low-concentration GLN was not significantly altered by the presence of the magnetic field. These data are in contrast with those of studies (Tao and Henderson, 1999) reporting an enhancement of the differentiation effect of low doses of TPA on HL60 cells when administered under a 60-Hz EMF at 1 G. On the other hand, it is worth noting that in our experimental model of U937 cells, the depressive response to exposure is only observed when the static MF and TPA are co-administered to the cells. Indeed, exposure of U937 cells to a static MF is by itself sufficient to promote differentiation (submitted manuscript). This ability of static MFs was also reported by Huang et al. (2006), who showed that osteoblast maturity is upregulated by exposure for up to 72 h to a static MF of 0.4 T. The discrepancies in these data are not surprising, given the multiplicity of experimental conditions, e.g., field intensity and type, time of exposure, *in vitro* and *in vivo* models, etc., and the types of cell used (Tenuzzo et al., 2006). The difficulty of obtaining non-contradictory data has generated a certain scepticism. On the other hand, the results of these studies cannot simply be ignored. Living cells are able to respond to SMFs of moderate intensity with intracellular and extracellular changes (Miyakoshi, 2005; Amara et al., 2007), whose underlying mechanisms remain to be fully elucidated. The prominent role of Ca^{++} could explain why exposure to SMFs interferes with drug-induced differentiation of monocytes into macrophages. Indeed, it is known that MF exposure promotes Ca^{++} fluxes and affects ion channels, which in turn regulate many cellular functions and differentiation steps. Maxi-K (IBK) channels are Ca^{++} activated soon after phorbol ester induction in THP-1 human monocytic leukaemia cells (De Coursey et al., 1996). On the other hand, a 150 s exposure to a 125 mT static magnetic field affects the voltage-activated Na(+) channels in GH3 cells, using the whole cell patch clamp method (Rosen, 2003b). This finding is consistent with the hypothesis that reorientation of diamagnetic anisotropic molecules in the cell membrane is capable of distorting imbedded ion channels to the point of altering their function. The administration of TPA to U937 cells results in a modulation of the concentration of $[Ca^{++}]i$, which is further modulated when phorbol ester is administered in the presence of a static MF for up to 24 h (submitted manuscript). Ca^{++} could be also responsible for two other alterations, i.e., morphology and phagocytosis rate, by interfering with the polymerisation of microtubules and the assembly of actin filaments (Tenuzzo et al., 2006; Dini and Abbro, 2005; Simko et al., 2001; Flipo et al., 1998). Many inorganic and virtually all organic molecules have diamagnetic properties. Phospholipids have a greater diamagnetic anisotropy than polypeptides or proteins, most of which is generated by their acyl chains and biological membranes, with their highly ordered phospholipid bi-layer structure. Distortion of the intra-membranous portion of imbedded proteins is thought to derive from the reorientation of membrane phospholipids induced by exposure to moderate intensity static MFs. Many cell surface modifications have been described, including expression of surface antigens (e.g., CD14 and CD11c), of sugar moieties and lipid peroxidation (Hass et al., 1994; Cutolo et al., 2001; Amara et al., 2007). Specifically, lipid peroxidation could also play an important role in plasma membrane modifications and consequently in substrate adhesion, cell-cell adhesion and phagocytosis. The lack of correlation between differentiation and adhesion rates measured in the low-GLN concentration treated U937 cells in the presence of the static MF cannot be explained solely with reference to the alterations caused by the MF on the cell surface. Some as yet undeciphered molecules could be involved. Indeed, the simultaneous action of low-concentration GLN and static MF exposure, leading to an increase in the fraction of viable cells, could be due to as yet unrecognized pathways, activated most likely by Ca^{++} mobilisation. Perturbation in gene expression cannot be excluded either, since this amino acid has been shown to regulate the expression of several genes (such as p47phox, p22phox, gp91phox, alpha-actin and fibronectin) and to activate several proteins (such as ASK1, *c*-myc, *c*-jun and p70s6k) (Curi et al., 2005; Curi et al., 2007). In addition, static MFs have been reported to influence the expression of some genes (Goodman and Blank, 1998; Pipkin et al., 1999; Potenza et al., 2004; Hirose et al., 2003).

The most intriguing data is, however, the limited response to a subsequent differentiating stimulus of the in-suspension U937 cells after 5 days of culture in a GLN-deprived medium under static MF exposure. Indeed, these cells were highly viable and showed the shape of undifferentiated and proliferating monocytic cells. Conversely, in the absence of the MF, the corresponding cell fraction after 5 days of culture in GLN-deprived medium, or in a medium that was naturally deprived of nutrients (5 days of culture without changes with fresh medium), was highly responsive to the TPA stimulus. The limited differentiation after 5 days culture is a specific outcome of the combined action of low-concentration GLN and static MF exposure; GLN deprivation alone or the progressive loss of nutrients during 5 days of culture did not have the same effect. Taken together, these factors could negatively affect therapy, and potentially alter its efficacy.

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