

Morphofunctional Study of 12-O-tetradecanoyl-13-phorbol Acetate (TPA)-Induced Differentiation of U937 Cells Under Exposure to a 6 mT Static Magnetic Field

Luciana Dini, * Majdi Dwikat, Elisa Panzarini, Cristian Vergallo, and Bernadetta Tenuzzo

*Department of Biological and Environmental Science and Technology (Disteba),
University of Salento, Lecce, Italy*

This study deals with the morphofunctional influence of 72 h exposure to a 6 mT static magnetic field (SMF) during differentiation induced by 50 ng/ml 12-O-tetradecanoyl-13-phorbol acetate (TPA) in human leukaemia U937 cells. The cell morphology of U937 cells was investigated by optic and electron microscopy. Specific antibodies and/or molecules were used to label CD11c, CD14, phosphatidylserine, F-actin and to investigate the distribution and activity of lysosomes, mitochondria and SER. $[Ca^{2+}]_i$ was evaluated with a spectrophotometer. The degree of differentiation in SMF-exposed cells was lower than that of non-exposed cells, the difference being exposure time-dependent. SMF-exposed cells showed cell shape and F-actin modification, inhibition of cell attachment, appearance of membrane roughness and large blebs and impaired expression of specific macrophagic markers on the cell surface. The intracellular localization of SER and lysosomes was only partially affected by exposure. A significant localization of mitochondria with an intact membrane potential at the cell periphery in non-exposed, TPA-stimulated cells was observed; conversely, in the presence of SMF, mitochondria were mainly localised near the nucleus. In no case did SMF exposure affect cell viability. The sharp intracellular increase of $[Ca^{2+}]_i$ could be one of the causes of the above-described changes. Bioelectromagnetics, 2009. © 2009 Wiley-Liss, Inc.

Key words: static magnetic field; monocyte; differentiation; plasma membrane; mitochondria

INTRODUCTION

The widespread use of medical diagnostic instrumentation such as NMR, and the generation of extremely low frequency electromagnetic fields by common electrical devices have introduced many sources of static magnetic fields (SMFs) and 'quasi'-SMFs into our living environment. The influence of SMFs on living organisms, tissues and cells is a topic of considerable interest, mainly due to their health implications [Rosen, 2003; Dini and Abbro, 2005; Feychting, 2005]. Indeed, SMFs are able to influence a number of biological systems, particularly those whose functions are linked to the properties of membrane channels [Rosen, 2003]. While exposure to SMFs alone has little or no effect on cell growth and genetic toxicity regardless of the magnetic density [Ikehata et al., 1999; Wiskirchen et al., 2000; Nakahara et al., 2002; Schiffer et al., 2003; Leszczynski, 2005], its bioeffects are stronger when applied in combination with other external factors such as ionising radiation and some chemicals [Zmyslony et al., 2000; Zhang et al., 2003]. Understanding the biophysical interaction of SMFs with living cells is a problem that is far from being

resolved. In addition, it is still difficult to resolve the problem of contradictory results due to the multiplicity of experimental conditions (magnetic flux density, exposure times, cell types, etc.) and to different response of the same cell line to different field intensity [Walleczek and Liburdy, 1990; Chionna et al., 2005; Ghibelli et al., 2006; Tenuzzo et al., 2006]. However, in vitro studies are yielding results that cannot be ignored, although they are often viewed with scepticism.

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*Correspondence to: Luciana Dini, Department of Biological and Environmental Science and Technology, University of Salento, Via per Monteroni, Lecce 73100, Italy.
E-mail: luciana.dini@unile.it

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Living cells respond to SMFs of moderate intensity (from 1 mT to 1 T) with intracellular and extracellular changes [Miyakoshi, 2005; Amara et al., 2007]. At the beginning of the cascade of the cellular changes, it is likely that SMFs influence the diamagnetic properties of the plasma membrane. The reorientation of diamagnetic anisotropic molecules in the cell membrane is capable in turn of distorting embedded ion channels, calcium ions in particular, sufficiently to alter their function [Rosen, 2003].

Calcium ion fluxes and modulation of intracellular concentration are of particular interest in many biological functions, due to the ion activation of signalling pathways. Changes in $[Ca^{2+}]_i$ have been reported during SMF exposure as well as after phorbol ester-induced macrophage differentiation of U937 cells [Flipo et al., 1998; Fanelli et al., 1999; Chionna et al., 2005; Tenuzzo et al., 2006]. The human leukaemia cell line, U937, has properties of monoblasts or immature monocytes [Sundström and Nilsson, 1976], and can be differentiated into more mature monocyte-macrophages by 12-*O*-tetradecanoyl-13-phorbol acetate (TPA) (the most effective one) [Hass et al., 1989, 1997], dimethyl sulfoxide (DMSO) [Nakamura et al., 1990], retinoic acid [Olsson and Breitman, 1982], vitamin D₃ [Olsson et al., 1983], cytokines [Harris et al., 1985] and low-concentration glutamine [Spittler et al., 1997; Pagliara et al., 2005]. Differentiation, which has been extensively studied, is a highly complex phenomenon, characterised by permanent alterations in a variety of cellular parameters/activities, like morphology, metabolic pathways, cell growth, adhesion to substrates, active phagocytosis, etc. On the contrary, few studies have looked at the effects of SMFs on cell differentiation, and the data they reported has tended to be contradictory, given the multiplicity of experimental conditions [Espinar et al., 1997; Hirai and Yoneda, 2004; Pagliara et al., 2005; Cornaglia et al., 2006; Huang et al., 2006; Tenuzzo et al., 2006; Amara et al., 2007]. In spite of the large number of studies that has been performed with the aim of deciphering alterations at molecular level (mostly ions, DNA integrity) exerted by *in vivo* exposure to EMFs, normally of high intensity and frequency [Somosy, 2000], few studies have been carried out on the possible modifications of the cellular compartments (organelles) and even fewer on the biological effects of moderate intensity SMFs. Therefore, to gain insight into the bioeffects of SMFs on the *in vitro* monocyte/macrophage differentiation of U937 cells, we investigated morphofunctional modifications of U937 cells during phorbol ester-induced differentiation under exposure to a 6 mT SMF for 3 days.

MATERIALS AND METHODS

Cell Culture and Differentiation

Human U937 myeloid leukaemia cells were cultured in RPMI-1640 medium (Cambrex, Verviers, Belgium), supplemented with 10% foetal bovine serum (FBS), 2 mM L-glutamine (Cambrex), 100 IU/ml penicillin and streptomycin solution (Sigma, St. Louis, MO) and 10³ U/ml amphotericin B (Cambrex), in a 5% CO₂ humidified atmosphere at 37 °C. 1 × 10⁶ cells/ml were maintained in 75 cm² flasks, changed every 3–4 days.

U937 cells were differentiated with 50 ng/ml TPA, on a substrate of fibronectin (100 µg/10 ml phosphate buffered saline (PBS), 0.2 M, pH 7.4) left on the bottom of Petri dishes (diameter of 320 mm) for 18 h at 4 °C, and then saturated with 1% bovine serum albumin in PBS, pH 7.4 (BSA–PBS) for 90 min at 12 °C.

Differentiation, monitored for 3 days following the addition of TPA, was evaluated by Nitro blue tetrazolium (NBT) (Sigma) assay [Rook et al., 1985]. Briefly, cells incubated with NBT (0.1 mg/ml) culture medium (RPMI-1640) for 2 h at 37 °C were washed three times with absolute methanol. The amount of diformazan salts, determined by spectrophotometer (DU 640B Spectrophotometer, Beckman Coulter, Fullerton, CA) at 630 nm after dissolving crystals in 1 ml of KOH 2 M/DMSO solution (460 µl KOH and 540 µl DMSO), was directly related to the degree of differentiation.

Magnetic Field Application

The SMF was produced by Neodymium magnetic disks (10 mm in diameter and 5 mm in height) of known intensity supplied by Calamit (Milano, Italy), placed (South up) under the culture Petri dishes (diameter 320 mm). The intensity of the field generated by the magnet was checked by means of a gaussmeter with an operating temperature range of 0–50 °C and an accuracy (at 20 °C) of ±1% (Hall-effect gaussmeter, GM04 Hirst Magnetic Instruments, Tregonigge Falmouth, UK). The field intensity of 6 mT was recorded at 2.5 cm from the magnet. This distance was obtained by interposing between the magnetic disk and the Petri dish (the thickness of the bottom of Petri dish is 1.2 mm) two disks of the same diameter as the culture dish, that is, one metallic disk (iron, 5 mm thick, in order to minimise the differences in field intensity across the bottom of the dish) and one of inert material (polystyrene, the same material as the culture plates, 1.9 cm thick). The thickness of the inert layer was calculated considering that the medium containing the cells in suspension (volume 3 ml) filled the capsule to a depth of 6 mm. The field intensity was measured in

three different zones of the dish bottom: in the area from the centre of the dish to 5 mm, 6.00 ± 0.01 mT; in the area from 5 to 100 mm from the centre of the dish, 5.90 ± 0.06 mT; in the peripheral part (from 100 to 160 mm from the centre of dish), 5.90 ± 0.1 mT. The magnets did not produce temperature variation. Exposures were carried out in a blind manner. Culture dishes were always placed on the same two shelves in a tissue culture incubator where the ambient 50 Hz magnetic field inside the incubator was $0.95/0.62$ μ T (heater on/off) and the static magnetic flux densities were 5.5 μ T (vertical component). The magnetic field in the laboratory areas between incubators and worktops measured 0.08 – 0.14 μ T (50 Hz). In the room the background flux density was 10 μ T (static) and the local geomagnetic field was approximately 43 μ T.

All the experiments described below were carried out on cell samples after 24, 48 and 72 h of incubation with 50 ng/ml of TPA (Sigma) in the absence or presence of 6 mT static MF. Controls were undifferentiated U937 cells, in the presence (positive control) or absence (negative control) of SMF.

Morphology, Cytochemistry and Immunofluorescence

Cells were observed as fresh samples and after haematoxylin–eosin staining performed on cells adhering to glass coverslips and on non-attached cells. The cells were washed with bidistilled water and then fixed with Mayer's haematoxylin solution (Sigma) for 10 min. For adherent cells, after fixation, the glass coverslips were washed with water and then stained with an eosin acidic aqueous solution (0.1% eosin and 1.6 ml/L glacial acetic acid) for 10 min and washed again with bidistilled water. When dried, they were mounted on a slide for optic microscopy observation (Eclipse 80i microscope, Nikon, Tokyo, Japan) using a Nikon $100\times$ objective. Image acquisition was performed for each sample in order to analyse the cell shape in a blind fashion. Acquisition and visualization were completely computer-controlled with ACT software (Nikon/Coord Automatisering, The Netherlands). Image analysis software (Lucia 5.0, Nikon, Tokyo, Japan) was used. At least 300 cells for each experiment were scored.

For SEM, cells were quickly washed in cacodylate buffer (0.2 M pH 7.4), fixed with 2.5% glutaraldehyde in the same buffer, for 1 h at ice temperature and postfixed with 1% OsO_4 in the same buffer. After fixation and acetone dehydration, cells were further dehydrated, using the Critical Point Dryer 020 Balzer, and stub-mounted specimens were gold coated using

a Balzer 040 Sputter Coater. Cells were examined under a Philips XL50 scanning microscope operating at 20 kV.

To quantify the morphological modifications in SEM images, we considered roundness as a marker of cell shape. The roundness index was calculated as the ratio between the cell surface and square of the cell perimeter. The higher the index, the higher the roundness of the cell. In the case of perfect circularity the index is $1/4\pi$, deriving from the ratio $\pi r^2/(2\pi r)^2$.

The shape index of randomly selected cells obtained from samples exposed and non-exposed to 6 mT SMF during TPA-induced differentiation was compared (non-exposed + TPA 1 day, $n = 120$ cells; +TPA 2 days, $n = 131$; +TPA 3 days, $n = 109$ cells. SMF exposed + TPA 1 day, $n = 118$; +TPA 2 days, $n = 95$; +TPA 3 days, $n = 87$).

Cells, fixed with 4% formalin in PBS 0.2 M, pH 7.4 for 10 min, were analysed for surface localization of sugars using the FITC-conjugated lectins concanavalin A (conA, 40 μ g/ml, for α -mannose) and *Ricinus communis* (2 μ g/ml, for D-galactose) for 30 min in the dark at room temperature. F-actin (filamentous actin) was detected using 20 μ M FITC-conjugated phalloidin for 20 min in the dark at room temperature.

FITC-conjugated Annexin V (20 μ M for 20 min at room temperature in the dark) was used for observation of phosphatidylserine (PS) on the cell surface of fresh samples.

For surface antigen expression, cells were washed twice with ice-cold PBS before incubation at 4°C with a monoclonal mouse anti-human CD11c primary antibody ($1:50$ in PBS for 30 min; DAKO A/S, Glostrup, Denmark) and a FITC-conjugated anti-mouse-IgG secondary antibody ($1:50$ in PBS for 30 min; Sigma) and a FITC-conjugated monoclonal mouse anti-human CD14 primary antibody ($1:50$ in PBS for 30 min; DAKO A/S).

Samples were mounted on a slide using a glycerol–PBS solution ($2:1$) and dried at room temperature. The cells were observed by fluorescent microscopy using a Nikon microscope with Plan Fluor objectives (Nikon). Quantitative fluorescence cell image analyses were acquired using an Eclipse 80i microscope (Nikon) coupled to a digital camera (DXM 1200 F, Nikon), with the corresponding filters for capturing images. The data were analysed using the public domain Scion Image software program (<http://www.scioncorp.com/index.htm>). In one experiment (triplicated), between 100 and 300 measurements were made for each treatment condition and time. For each cell, the image processing software provided a density profile of the fluorescence colours represented in the graphs as arbitrary absorbance units. The differences

in the values obtained were analysed using Student's *t*-test. The significance level was set at $P < 0.05$.

Mitochondrial $\Delta\psi_m$, Lysosomal Activity and Smooth Endoplasmic Reticulum Localization

Changes in the inner mitochondrial transmembrane potential ($\Delta\psi_m$) were determined by incubation of cells with 10 $\mu\text{g/ml}$ of Rhodamine 123 (Calbiochem, Darmstadt, Germany) for 30 min and JC-1 (Cell Technology, JC-1 Kit, Mountain View, CA) for 15 min, both at 37 °C in the dark. JC-1 was dissolved in DMSO (Sigma), stored and used according to the manufacturer's instruction. The monomeric form of the lipophilic cation JC-1 stains the cytoplasm green, while the dimeric form enters those mitochondria with intact membrane potential and stains them red. The percentage of the organic solvent in the samples never exceeded 1% (v/v). At the end of each incubation time, the slides were rapidly washed with PBS, drained to prevent formation of buffer salt crystals, and air-dried. Once completely dried, the slides were mounted with Histovitrex mounting medium (Carlo Erba, Milan, Italy) with no additional treatment and subjected to fluorescence analysis.

Lysosomal activity was performed by neutral red assay. Cells were incubated for 30 min at 37 °C in a 5% CO₂ moist atmosphere, with 0.01% Neutral red solution in culture medium.

The fluorescent probe, 3,3'-dihexyloxycarbocyanine iodide (DiOC₆) was used for the localization of the smooth endoplasmic reticulum. Cells were incubated for 10 min at 37 °C in a 5% CO₂ humidified atmosphere in the dark with 0.5 $\mu\text{g/ml}$ DiOC₆ in culture medium.

Measurements of Ca²⁺ Levels

Cells were washed twice with loading buffer (107 mM NaCl, 5 mM KCl, 7 mM NaHCO₃, 3 mM CaCl₂, 1 mM MgSO₄·6H₂O, 20 mM Hepes, 10 mM glucose, 0.1% BSA), resuspended at a concentration of

$1 \times 10^6/\text{ml}$ and then incubated with 4 μM Fura-2 acetoxymethyl ester (Fura2-AM; Sigma) for 45 min at 37 °C in a 5% CO₂ humidified atmosphere. After two washes with the loading buffer, cells were resuspended in the same freshly made buffer at a final concentration of 7×10^6 cells/ml, and stored at room temperature until use. Cells were pre-warmed at 37 °C for 20 min before measuring Fura-2 fluorescence with a Jasco FP-750 spectrofluorometer equipped with an electronic stirring system and a thermostabilised (37 °C) cuvette holder and monitored by a personal computer running the Jasco Spectra Manager software for Windows 95 (Jasco Europe s.r.l., Lecco, Italy). The excitation wavelengths were 340 and 380 nm and the emission wavelength was 510 nm; the slit widths were set to 10 nm. Two millilitres of cell suspension, at a final concentration of 1.4×10^5 cells/ml, were placed in a glass cuvette. Fluorescence values were converted to [Ca²⁺]_i values according to Grynkiewicz et al. [1985].

RESULTS

Degree of Differentiation

The degree of TPA-induced monocyte/macrophage differentiation increased with the time of phorbol ester incubation, with a sharp increase during the second day of treatment. Differentiation coincided with an increasing number of substrate-attached cells. After 72 h of incubation, few undifferentiated cells remained in suspension. The simultaneous administration of TPA and SMF decreased the degree of differentiation and the number of substrate-attached cells (Table I). Conversely, the differentiation degree of cells that were exposed to SMF alone for 72 h was unexpectedly high (Table I).

Cell Shape

U937 cells have a round shape, many short microvilli, scarce cytoplasm, large bean-shaped

TABLE I. Percentage of Differentiated TPA-Unstimulated and -Stimulated U937 Cells and Number of Substrate-Adhering U937 Cells per Light Microscope Field (40×)

Time (h)	Percentage of differentiation of U937 cells		Percentage of differentiation of TPA-stimulated U937 cells		Number of substrate-adhering U937 cells/light microscope field (40×) ^a	
	-SMF	+SMF	-SMF	+SMF	-SMF	+SMF
24	8 ± 1.2	18 ± 1.2	50 ± 1.2	38 ± 0.8	72 ± 1.3	79 ± 1.5
48	10 ± 1.2	27 ± 1.2	78 ± 2.1 ^b	56 ± 0.9 ^b	48 ± 0.5 ^b	51 ± 0.9 ^b
72	9 ± 1.2	55 ± 1.2	96 ± 2.3 ^b	58 ± 0.8 ^b	46 ± 0.3 ^b	43 ± 0.3 ^b

Each experiment was done in triplicate. Data are average ± SD of 100 fields examined for each experiment.

^aTPA-stimulated cells.

^b $P < 0.05$ compared to corresponding experiment at 24 h.

nucleus and grow in suspension. Differentiated U937 cells show the typical macrophagic phenotype, with many cytoplasmic protrusions and long pseudopodia and grow in adhesion. The simultaneous exposure to SMF and incubation with TPA prevented cell attachment to the substrate and induced time-dependent cell shape modification (i.e., maintenance of round shape and appearance of large blebs) (Fig. 1).

Cell Surface

Differentiation was accompanied by expression of a specific set of molecules on the cell surface (including integrins, adhesion molecules, PS and sugar residues,

etc.). Representative images of the results are shown in Figures 2 and 3.

TPA induced time-dependent expression of CD11c (marker of macrophage differentiation) and the loss of CD14. Conversely, SMF exposure decreased the expression of CD11c, but not of CD14, which was unaffected. Indeed, TPA-treated cells were negative for CD14 staining [Cutolo et al., 2001], since CD14 is used as substrate adhesion molecule during differentiation. Randomly distributed CD14 binding sites were observed only on cells not attached to the substrate; SMF caused the polarization of CD14 to one pole of the cells.

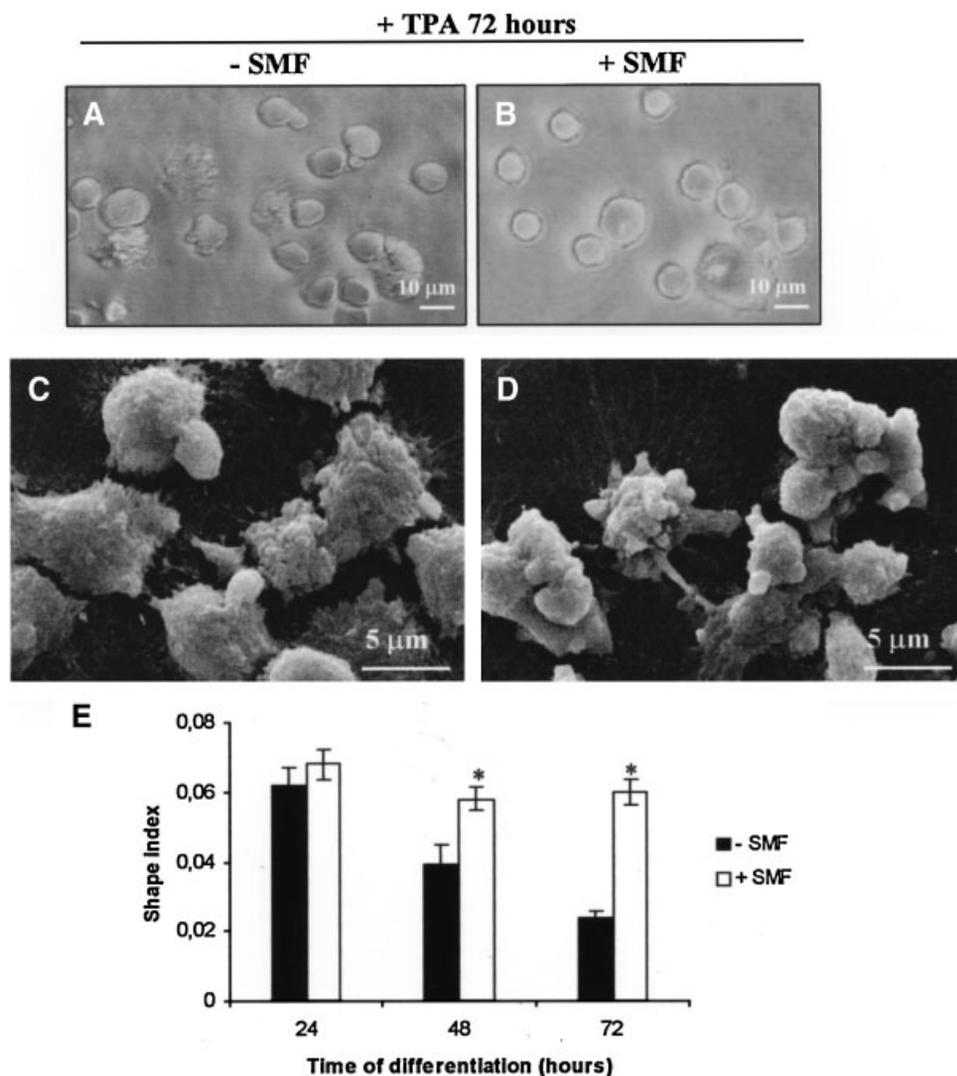


Fig. 1. LM (A,B) and SEM (C,D) micrographs showing U937 cells differentiated for 3 days with TPA in presence and absence of 6 mT SMF. Formation of large blebs and scarce substrate adhesion are clearly visible; TPA = 12-O-tetradecanoylphorbol-13-acetate; SMF = static magnetic field. E: Quantitative data for SEM observation of U937 cells during 72 h of TPA-induced differentiation are shown. Statistically significant modification to cell shape is produced by exposure to SMF. *P < 0.05 compared to non-exposed cells.

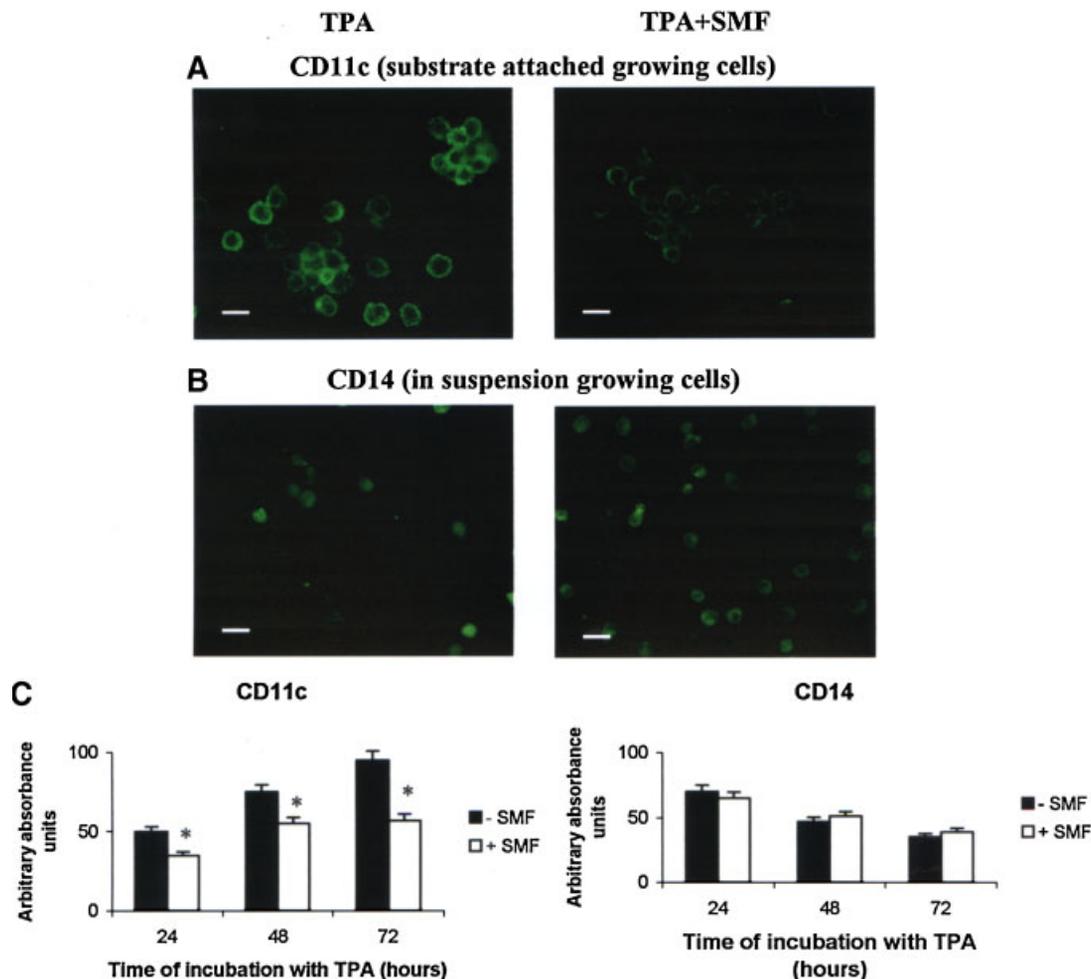


Fig. 2. Fluorescence light microscopy micrographs of FITC-labelled anti CD11c (A), anti CD14 (B) antibodies to U937 cells differentiated with 50 ng/ml TPA in presence and absence of 6 mT SMF after 72 h. Bars = 5 μ m; TPA = 12-*O*-tetradecanoylphorbol-13-acetate; SMF = static magnetic field. C: Quantitative fluorescence cell image analysis (fluorescence expressed as arbitrary absorbance units). Statistical analysis of different values obtained from

three different experiments was performed using Student's *t*-test. Data are represented as mean values (with corresponding standard deviations) of about 100 measurements for each experimental group. Significance level was set at $P < 0.05$ (* significantly different compared to corresponding time for non-exposed cells). [The color figure for this article is available online at www.interscience.wiley.com.]

PS is another macrophage marker, since it is not expressed on cell surface of undifferentiated monocytes [Callahan et al., 2000]. Indeed, U937 cells were labelled with AnnexinV-FITC only after Triton permeabilization, while the differentiated cells showed fluorescent decorated surfaces, whose intensity increased with time of TPA treatment, preferentially localised in the cytoplasmic protrusions. PS surface expression was largely induced by SMF exposure (Fig. 3).

During the monocyte/macrophage differentiation, the sugar residues exposed on the cell surface progressively changed as detected by lectin cytochemistry (ConA-FITC conjugates for α -mannose residues and *R. communis*-FITC conjugates for D-galactose residues). ConA-FITC conjugates were randomly distributed on non-differentiated U937 cell surface.

Conversely, the intensity of fluorescence of differentiated cells increased, however with a lower increment in the presence of SMF. Differentiated cells were Triton-treated to localise cytoplasmatic mannose residues that were found in the vicinity of the plasma membrane; a remarkable decrement of fluorescence intensity was found under SMF exposure (Fig. 3).

On the cell surface of differentiated cells, a clear fluorescent ring of *R. communis*-FITC conjugates was observed, whose fluorescence intensity sharply decreased under SMF exposure.

Actin Filaments

De-arranged F-actin microfilaments and F-actin concentrated in the numerous protrusions and pseudo-podia localised at one pole of the cells were seen in 24 h

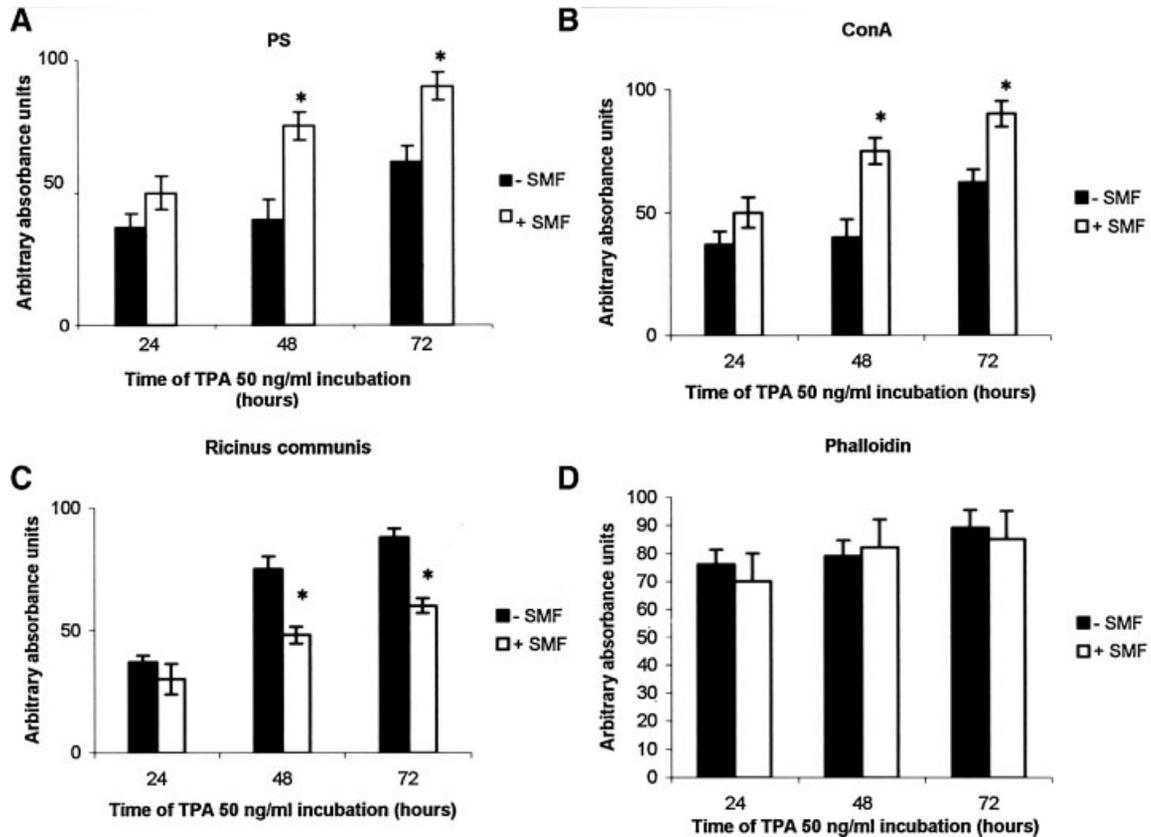


Fig. 3. Time course (from 24 to 72 h) of quantitative fluorescence cell image analysis (fluorescence expressed as arbitrary absorbance units) of PS (A), ConA-FITC (B), *Ricinus communis*-FITC (C) and phalloidin-FITC conjugates (D) to U937 cells differentiated with 50 ng/ml TPA in presence and absence of 6 mT SMF. Statistical analysis of different values obtained from three different experiments was performed using Student's *t*-test. Data are

represented as mean value (with corresponding standard deviations) of about 100 measurements for each experimental group. Significance level was set at $P < 0.05$ (* significantly different compared to corresponding time for non-exposed cells). TPA = 12-*O*-tetradecanoylphorbol-13-acetate; SMF = static magnetic field; PS = phosphatidylserine; Con-A = concanavalin A.

SMF-exposed U937 cells and in SMF-exposed differentiated cells, respectively. An SMF exposure induced modifications of actin filaments and their localisation was not statistically significant (Fig. 3).

[Ca²⁺]_i

[Ca²⁺]_i increased during the entire TPA-induced differentiation of U937 cells, doubling the values of undifferentiated cells between 25 and 48 h of induction. The simultaneous administration of SMF and TPA gave rise to a threefold increase in cytosolic free [Ca²⁺]_i with respect to control cells. [Ca²⁺]_i decreased during the 49–72 h of treatment (Fig. 4). A parallel trend was seen for the endoplasmic reticulum [Ca²⁺]_i (Fig. 4).

Lysosomes, RES, Mitochondria

Lysosomes were preferentially localised at one pole of the undifferentiated cells, showing a brilliant red colour produced by the enzyme content. During differentiation, lysosomes were progressively characterised

by a reduced intensity and brilliance of the dye. Exposure to SMF during differentiation dramatically increased the intensity of staining, indicating a high enzyme content (Fig. 5).

The fluorescent probe DiOC₆ (3,3'-dihexyloxacarbocyanine iodide) that stains SER, localised differently in differentiated as well as undifferentiated cells. SER was found at the cell periphery during differentiation only in the absence of SMF (Fig. 5).

Mitochondria distribution and membrane potential changed, as detected by Rhodamine 123 and JC-1 (Fig. 6). In the U937 cells, all the mitochondria had an intact membrane potential and localised randomly in the cytoplasm. At the beginning of differentiation, mitochondria moved from the vicinity of the nucleus towards the cell periphery, with a preferential localization inside cell protrusions (Fig. 6). Differentiation produced a progressive reduction of the number of mitochondria with intact membrane potential. When differentiation was induced in the presence of SMF,

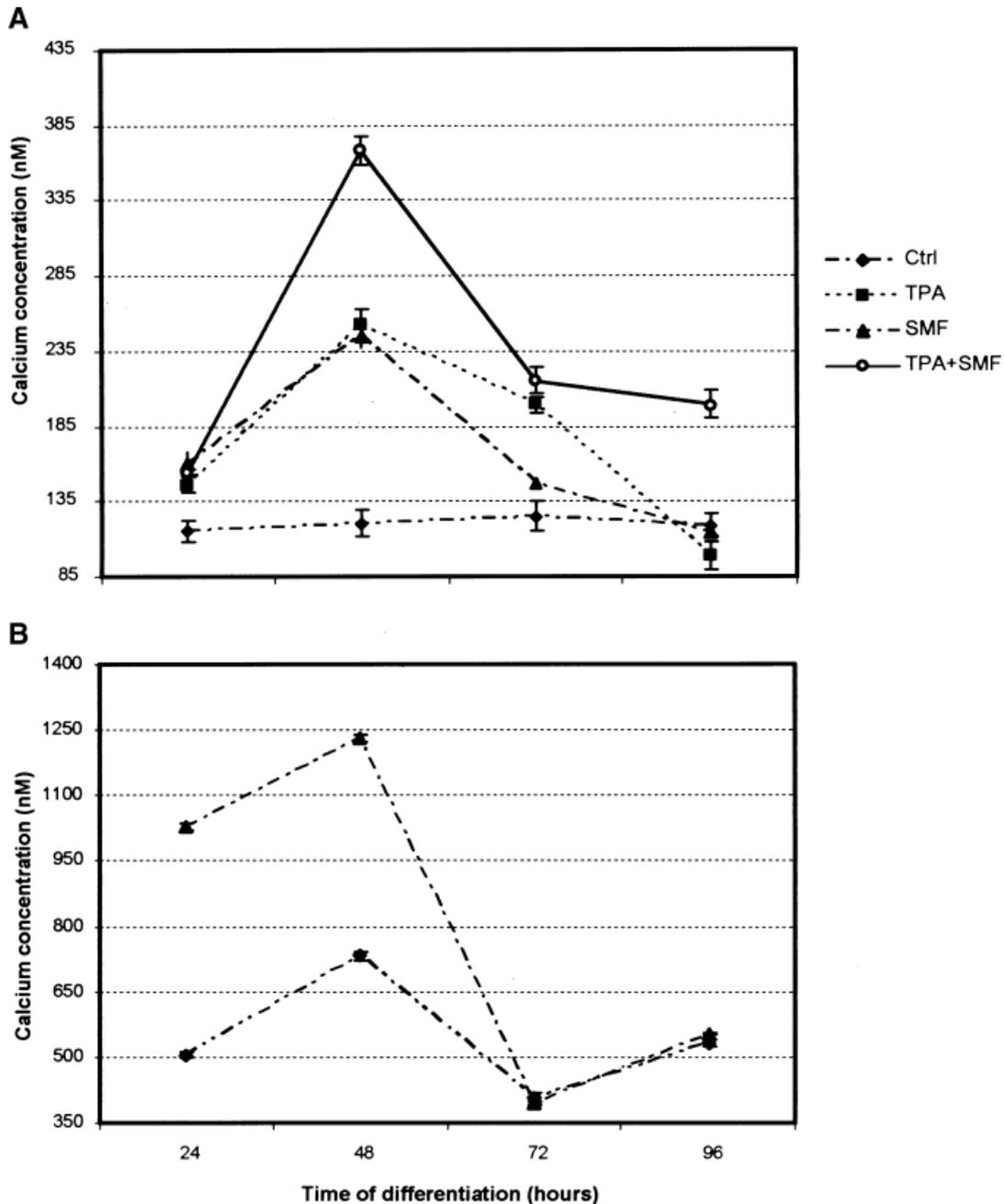


Fig. 4. Time course (24–96 h) of $[Ca^{2+}]$, (**A**=total cytosolic free $[Ca^{2+}]$; **B**=endoplasmic reticulum $[Ca^{2+}]$) measured with Fura-2 in U937 cells differentiated with TPA 50 ng/ml in presence or absence of 6 mT SMF. Data are average \pm SD of three independent experiments. TPA = 12-O-tetradecanoylphorbol-13-acetate; SMF = static magnetic field.

mitochondria localised near the nucleus and the number of those with a normal membrane potential increased.

DISCUSSION

Monocyte/macrophage differentiation leads to the permanent alteration of many cellular parameters: morphology, metabolic pathways, cell growth and

function. All these changes are necessary for the best performance of macrophage activities. Modifications of the cell surface promote cell adhesion to the substrate, and integrity of cytoskeleton allows active phagocytosis. Well-developed lysosomes and energy producing organelles favour the digestion of ingested materials (debris as well as pathogens). Any factor perturbing these properties, as exposure to SMF, either delays or

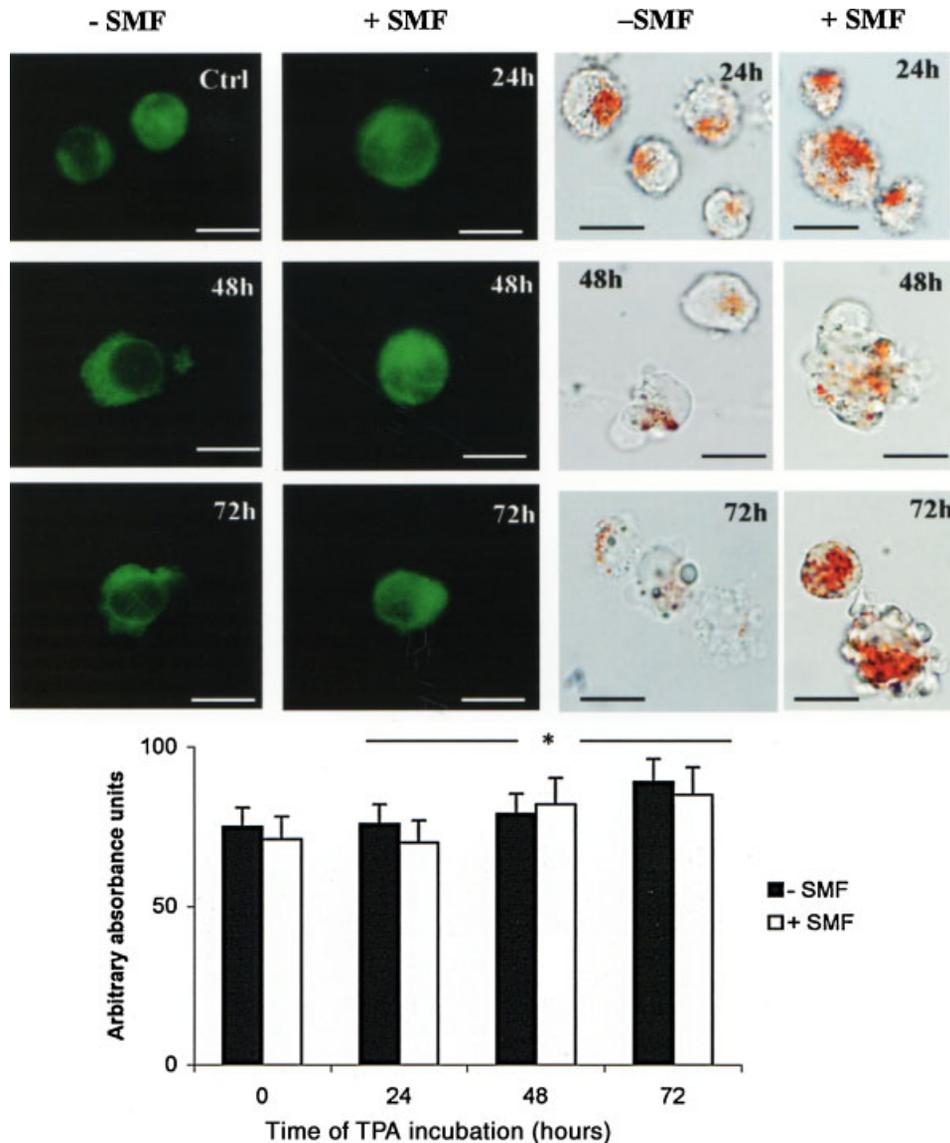


Fig. 5. Time course (24–72 h) of stained U937 cells differentiated with TPA 50 ng/ml in presence and absence of 6 mT SMF for SER (micrographs left side; fluorescent dye DiOC₆) and lysosomes (micrographs right side, neutral red dye). Quantitative fluorescence cell image analysis (fluorescence expressed as arbitrary absorbance units). Statistical analysis of different values obtained from three different experiments was performed using Student's

t-test. Data are represented as mean value (with corresponding standard deviations) of about 100 measurements for each experimental group. Significance level was set at $P < 0.05$ (* significantly different compared to corresponding time in non-exposed cells). Bars = 5 μ m; TPA = 12-*O*-tetradecanoylphorbol-13-acetate; SMF = static magnetic field. [The color figure for this article is available online at www.interscience.wiley.com.]

suppresses the macrophage function. The increasing studies aimed at defining the effects of exposure to SMFs have highlighted various bioeffects but only few of them were focused on differentiation, including monocyte/macrophage differentiation. Tao and Henderson [1999] described an increase in differentiation of HL-60 cells with low doses of TPA under exposure to 60 Hz EMF 100 μ T, and Huang et al. [2006] reported that osteoblast maturity was upregulated by 72 h exposure to a 0.4 T SMF. Indeed, exposure for up to 72 h to 6 mT SMF only promotes differentiation of U937 cells, while

simultaneous exposure to SMF and TPA resulted in a negative effect on differentiation. It may be hypothesised that the biochemical pathways involved during SMF exposure alone are different if co-administered with chemicals. Several experiments have been carried out to explore the interaction between MFs and biological systems [Gartze and Lange, 2002; Rosen, 2003], proposing various models. However, the studies of effects of MFs at a cellular level are still few and they are not able to univocally clarify the pathway [Dini and Abbro, 2005]. A number of nonlinearities (window

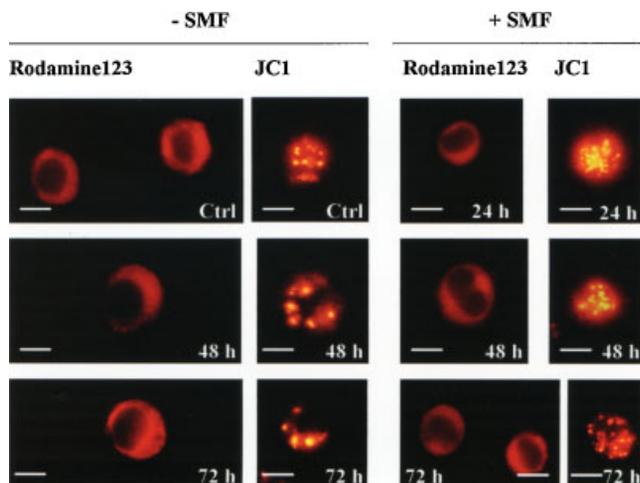


Fig. 6. Time course (24–72 h) of U937 cells differentiated with TPA 50 ng/ml in presence and absence of 6 mT SMF stained with 10 $\mu\text{g/ml}$ Rodamine 123 for 30 min and with JC1 1% (v/v). Bars = 5 μm ; TPA = 12-*O*-tetradecanoylphorbol-13-acetate; SMF = static magnetic field. [The color figure for this article is available online at www.interscience.wiley.com]

effects with respect to frequency, amplitude and duration) and peculiarities (cell type, age and treatment) in the experimental approach [Berg, 1999; Tenuzzo et al., 2006] may explain the differences in the results of these studies.

$[\text{Ca}^{2+}]_i$ is increased during differentiation [De Coursey et al., 1996] and SMF exposure [Alvarez et al., 1989; Fanelli et al., 1999], but to different extents. Indeed, $[\text{Ca}^{2+}]_i$ increased in U937 cells after TPA administration, exposure to SMF and during the simultaneous co-administration of both, when the $[\text{Ca}^{2+}]_i$ was three times the control value, in line with literature data. Ca^{2+} has an important intracellular signalling function, regulating almost all cellular activities [Thul et al., 2008]. Specifically, maxi-K channels (IBK) are Ca^{2+} -activated soon after phorbol ester-induced differentiation of THP-1 human monocytic leukaemia cells [De Coursey et al., 1996]. This finding is consistent with the hypothesis that the reorientation of diamagnetic anisotropic molecules in the cell membrane is capable of distorting embedded ion channels to the point where it alters their function [St Perre and Dobson, 2000; Rosen, 2003]. A 150 s exposure to a 125 mT SMF affects voltage-activated Na^+ channels in GH3 cells, using the whole cell patch clamp method [Rosen, 2003]. The plasma membrane is considered a primary site of SMF action. Many efforts have been made to understand the cellular events that mediate the Ca^{2+} increase at plasma membrane level resulting from SMF exposure, but none of them has provided a definitive explanation. The recently presented theory of microvillar Ca^{2+} signalling and the

discovery of cable-like ion conductance in actin filaments [Lange, 1999, 2000; Gartzke and Lange, 2002] have prompted us to focus our attention on the morphofunctional modifications of plasma membrane, cell shape and cell organelles during TPA-induced macrophage differentiation under SMF. F-actin is one of the Ca^{2+} storage compartments in the cells; removal of Ca^{2+} causes depolymerisation of F-actin and the de-arrangement of the actin network, leading to modification of both cell shape and surface molecule distribution. Lange [2000] suggests that actin filaments create a diffusion barrier between membrane microvilli and the interior of the cell. Gelsolin, which depolymerises the microvilli filaments, is activated by the influx of Ca^{2+} into the cell, which in turn allows additional influx of Ca^{2+} from the microvilli. Indeed, SMF-exposed U937 cells bear numerous lamellar microvilli [Pagliara et al., 2005]. Our data suggest that SMF exposure induces the de-arrangement of the actin network. Disruption of F-actin and intermediate filaments has already been observed in different cell types exposed to fields of varying types and intensity (from EMFs, to ELF-MFs, to SMFs) [Popov et al., 1991; Santoro et al., 1997; Chionna et al., 2003, 2005].

SMFs may induce polarisation of many cellular components and favour their rearrangement [Somosy, 2000; Testorf et al., 2002]. A flat irregular cell shape with many cytoplasmic protrusions and long pseudopodia was observed in TPA-differentiated monocytes while an irregular shape, in which protrusions and pseudopodia were present only at one pole of the cells, was seen in TPA-differentiated monocytes under SMF exposure. The question of whether and how these morphological changes induce more or less significant perturbations of some cellular activities remains to be resolved.

SMF exposure induced a general redistribution and polarization at the cell periphery of the SER, mitochondria and lysosomes and in some cases changes of function, in agreement with previous reports [Somosy, 2000]. Considering that the SER is the most important cellular store compartment for Ca^{2+} , the general redistribution of the SER may be due to increased cytoplasmic $[\text{Ca}^{2+}]_i$.

The movement of mitochondria from the vicinity of the nucleus towards the cell periphery during differentiation has been linked to Ca^{2+} channels and Ca^{2+} influxes [Quintana et al., 2006]. In addition to their well-known functions in cellular energy transduction, mitochondria play an important role in modulating the amplitude and time course of intracellular Ca^{2+} signals. The work of Hoth et al. [2000] shows that mitochondria regulate ion channels by preventing the inactivation of the calcium release-activated

calcium (CRAC) channel that results from prolonged Ca^{2+} influx. It is known that sustained Ca^{2+} influx across the plasma membrane through the CRAC channel is required for T-cell development [Quintana et al., 2006]. For this purpose, mitochondria are translocated to the plasma membrane as a consequence of Ca^{2+} influx, and this directed movement is essential for sustaining Ca^{2+} influx through CRAC channels. Thus, movement of mitochondria during U937 cell differentiation may also serve to control Ca^{2+} -dependent functions. It is worth noting that differentiation of monocytes into macrophages rather than dendritic cells involves the activation of several caspases; however, TPA-induced caspase activation is not associated with apoptosis and involves mitochondria [Sordet et al., 2002]. Indeed, in this study exposure of U937 cells to TPA in the absence of SMF involved the mitochondria to a large extent. A decrease in $\Delta\psi_m$ without induction of apoptosis was also observed, which requires a better knowledge of the underlined biochemical pathways.

Membrane changes (i.e., expression of differentiation markers) accompany both monocyte differentiation and exposure to MFs. Modifications of cell surface morphology [Paradisi et al., 1993; Chionna et al., 2003; Tenuzzo et al., 2006] and the redistribution of plasma membrane proteins, receptors [Bersani et al., 1997] and sugar residues [Bordiushkov et al., 2000; Chionna et al., 2003; Tenuzzo et al., 2006, 2008] have been reported as a consequence of exposure to MFs of varying intensities and types. The quality and quantity of alterations is strictly related to the type of molecules and cells investigated. Thus, SMF-induced alterations in surface expression (as indicated by changes in glucidic residues, such as D-galactose) may not be limited to the differentiation markers, but may also include adhesion molecules, antigens and recognition-specific receptors. Alterations to these molecules may in turn lead to impairment of macrophage-specific activities regulated by the cell membrane. The cell surface changes observed in SMF-exposed cells are unlikely to result from reorganization of F-actin filaments alone. Lipid peroxidation could be a further cause of plasma membrane modification, since it has been observed as a consequence of exposure in THP-1 leukaemia cells [Cutolo et al., 2001; Amara et al., 2007]. Preliminary data of a study currently underway in our laboratory show a high level of lipid peroxidation in U937 cells exposed to SMF.

At the current state of knowledge, it is difficult to assess whether it is the reduced degree of differentiation in the presence of SMF that allows morphological modifications or vice versa. However, morphological modifications similar to those described here, have already been reported in various cell lines as a

consequence of field exposure [Santoro et al., 1997; Chionna et al., 2003, 2005; Teodori et al., 2006; Tenuzzo et al., 2008]. The effect of exposure on gene expression cannot be excluded either.

In summary, the simultaneous exposure of U937 cells to TPA and SMF does not have a synergic effect on the process of macrophage differentiation but causes numerous cellular and subcellular modifications. Whether and to what extent SMF-induced morphofunctional modifications to cellular and subcellular compartments have harmful consequences for human health, for example, by interfering with gene expression, needs to be clarified.

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