Electromagnetic exposure: consequence on monocytes-macrophages differentiation and clearance of apoptotic lymphocytes

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The interest in the biological effects of non-ionizing electromagnetic fields (EMFs) on the whole organism, as well as on cellular systems, has considerably increased in recent years in consideration of their probable health risk for humans. EMFs are distributed everywhere in the environment since they are generated by natural and artificial sources (from the generation and transmission of electricity to domestic appliances and industrial equipment, to telecommunications and broadcasting). Findings on the effects of EMFs exposure on humans are often contradictory and, therefore, difficult to interpret. However, epidemiological studies have associated EMFs exposure with increased incidence of cancer, even if no direct evidence of tumorigenicity have ever been demonstrated. Contradictory are also the data in the literature concerning the biological effects of EMFs, mostly due to the multiplicity of experimental conditions (i.e., in vitro or in vivo models, intensity and type of field, oscillatory or static, time of exposure, metabolic state of the cells, etc).

However, converging data indicate that the primary site of action of EMFs is the plasma membrane [18]. The influence exherted by EMFs on plasma membrane has been described at different levels: on the electrochemical balance of the membrane [15], on its surface [16], on the distribution of membrane proteins [1] and membrane receptors, on the cell-cell and cell-matrix junctions [21], on the sugar residues on cell membrane [3, 4], and on transmembrane fluxes of different ions especially calcium [5, 24], in turn these perturbations influence the apoptotic rate, cellular shape and cytoskeleton [5, 6].

Taking into account a link between childhood leukemia and exposure to EMF and the fundamental role played by the physiological condition and/or the developmental story of the cells for the response to the (E)MFs exposure, our study was focused on the differentiation of the monocytic U937 cell line under continuous exposure to 6mT static MFs (SMFs). These cells preserve all characteristics of in vivo monocytes including the ability to differentiate in mature macrophages under the effects of different inducers. Differentiated U937 cells adhere to the culture plate and acquire phagocytic capability. Unluckily, data of literature regarding the effects of EMFs on cell adhesion and phagocytosis are scarce and very contradictory. Studies of Blumenthal et al. [2] showed an inhibition of cellular adhesion in the presence of EMFs and MFs for fibroblasts and human melanoma cells. Even more doubtful are the data on monocytes differentiation into macrophages by phorbol esters [20, 23] and those on phagocytosis [8, 20].

It is worth noting that many of the properties of the mature macrophages are plasma membrane mediated, and therefore influence of static MFs on the differentiation process of U937 (in particular for adhesion and phagocytosis) can be hypothesized. In this context aim of the present work has been the study of the macrophagic differentiation of human pro-monocytic U937 cells under static MFs exposure. In the present paper the effect of 6 mT SMFs on the macrophage differentiation of U937 cells, with respect to degree of differentiation, phagocytic activity, cell surface markers, cell shape and F-actin integrity, has been reported, giving evidence of biological effect of the SMFs exposure.

Design and Methods

Cells and cultures

The human monocytic U937 cells and peripheral blood lymphocytes (PBL) were cultured in RPMI 1640 medium at 37°C 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 of 5% CO_2; cells were used at the density of $1x10^6$ *cells/ml*. Peripheral mononuclear blood cells were obtained after gradient separation of buffy coats from nonsmoking healthy male blood donors. PBL were separated from monocytes by double adherence to plastic; they were over 95% pure as judged by morphological criteria. They were used on the first day of explant, at a cell density of $1x10^6$ *cells/ml*.

Magnetic field application

SMFs were produced by metal magnetic disks of known intensities. Unlike electric fielddriven, solenoid-generated MFs, SMFs do not induce any temperature in-

crease. Magnetic disks used had a magnetic induction of 6 mT (1T=10^4*G*). Magnets were placed under the culture Petri dish. Since MFs decreases according to the square of the distance, the actual field intensity on the cells was calculated considering the square of the thickness of the bottom of the Petri dishes (1.2 *mm*). MFs were applied concomitanly with differentiation inducer.

Macrophagic differentiation

U937 cells were differentiated on a substrate of fibronectin 10*μg/ml* left on the bottom of dishes for 18 *hrs* at 4°*C*, and then saturated with bovine serum albumin-phosphate buffer (BSA-PBS) 1% for 90 minutes at 12°C. Macrophage differentiation was induced with: 50 *ng/ml* 12-0-tetradecanoil-13-phorbol acetate (TPA), 10% dimethyl sulfoxide (DMSO), 100 *mM/L* Zn^{++}, and with low concentration of glutamine 0,05 *mM/L* (using a culture medium not supplemented with L-glutamine). Differentiation was monitored for 3 days from the addition of the inducers and its degree was evaluated by morphological criteria and by Nitro blue tretrazolyum test (NBT).

Scanning electron microscopy

Cells, fixed with 2,5% glutaraldehide in cacodilate buffer 0.1 M pH 7.4 for 30 *min* on ice, were washed twice with the same buffer and post-fixed with 1% OsO_4 in cacodilate buffer for 1 *h* at 4°*C*. Dehydratation was done at 4°*C* by using increasing graded acetone up to 100%. Critical Point Drying was performed with a 020 Balzer CPD. Samples, mounted on conventional SEM stubs, were gold-coated by sputtering device (Sputter Coated 040 Balzer). Observations were carried out with a Philips XL 20 scanning electron microscope at 5-15 KV.

Lectins, phosphatydilserine and phalloidin cytochemistry

Cells, fixed with 4% formalin in phosphate buffer pH 7.4, 5 *min*, were labelled with lectins-FITC conjugates, i.e. Concanavalin-A (Con-A) (D-mannose) (40 *μg/ml*) and *Ricinus communis* (D-galactose) (2 *μg/ml*), 30 *min* in the dark. Phalloidin-FITC conjugate (10 *μM* for 20 *min*) was used to analyze F-actin. Annexin V–FITC (6 *μg/ml*), 30 *min* in the dark, was used to analyze phosphatydilserine (PS) exposed on the external layer of plasma membrane.

Phagocytosis of latex beads and apoptotic lympho cytes

Fluorescent latex beads, 2 *μm* in diameter, were added to the culture medium (in a rate of 10 beads for 1 cell) of U937 cells, differentiated in presence and absence of static MFs. After 15, 30 *min*., 1, 2, 3 *h* the cells were washed at least 10 times in PBS and then fixed with 4% formalin. U937 cells were marked with phalloidin-FITC conjugated. Samples were observed with conventional and confocal fluorescent microscopy (PCM 2000 Nikon microscope) and cells that had adhered or phagocyte latex beads were counted. Lymphocytes, induced to apoptosis with puromycin (PMC) 10 *μg/ml* for 4 *h* or with cycloexhimide (CHX) 10^{-2} M for 24 *h*, and labelled with Hoechst 33258 1 *μg/ml* for 5 minutes, were added to the culture medium (in a rate of 1:1) of U937cells, differentiated in presence or absence of SMFs for up to 3 *h* To inhibit phagocytocis, U937 cells were treated with a solution of 80 *mM* Mannose/D-galattose/N-acetilglucosammine for 20' before adding apoptotic lymphocytes. Samples were analyzed at 15, 30 min, 1, 2 and 3 *h* of phagocytosis with conventional and confocal fluorescent microscopy (PCM 2000 Nikon microscope). Apoptotic indices were calculated.

Results and their Discussion

U937 cells are round-shaped cells with short microvilli , scarce cytoplasm and a large beam-shaped nucleus. U937 cells can be induced to differentiation by a large number of substances (TPA, DMSO, Zn and low concentration of glutamine). In their differentiated stage these cells assume a typical macrophage aspect, they adhere to substrate, change morphology (more irregular shape with pseudopodia), acquire the phagocytic capability and a positive reaction to NBT (Fig.1).

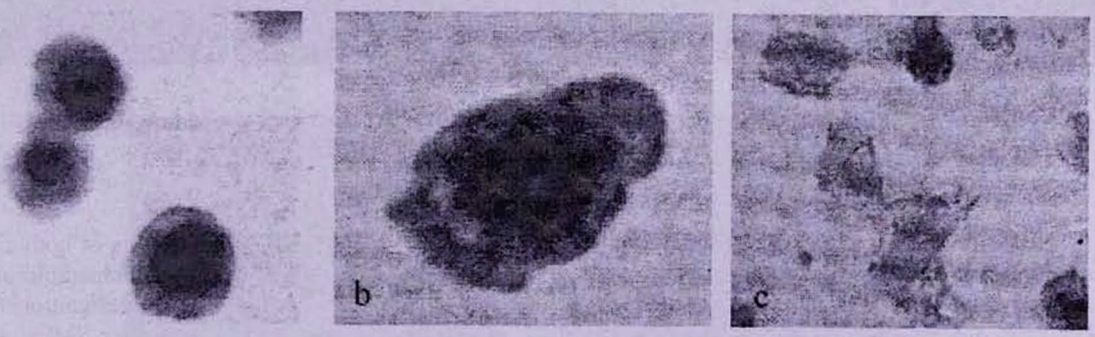

Fig.1. Light micrographs of hematoxylin/eosin stained undifferentiated (a) and TPA differentiated (b, 2 days; c, 3 days)U937 cells. Magnifications: a) 1000x; b) 1800x; c)800x

The most efficient molecule in our experiments, inducing macrophage differentiation of U937 cells, has been TPA, followed in decrescent order by low glutamine concentration, Zn^{++} and DMSO, as evaluated at light and electron microscopy and with the NBT. The degree of U937 differentiation is time-dependent; it increased during the second and the third day of treatment. When U937 cell differentiation was allowed under SMFs exposure, a decreased degree of differentiation was found at all times measured and with all inducers (Fig.2a,b).

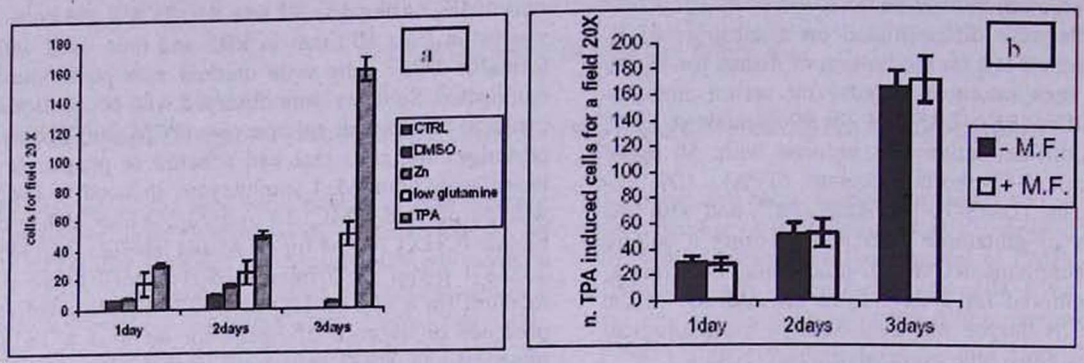

Fig.2. (a) Percentage of differentiated U937 cells after treatment with 50 *ng/ml* 12-0-tetradecanoil-13-phorbol acetate (TPA), 10% dimethyl sulfoxide (DMSO), 100 *mM/L* Zn^{++}, and with low concentration of glutamine 0,05 *mM/L* (using a culture medium not supplemented with L-glutamine). (b) Percentage of differentiated U937 cells after treatment with 50 *ng/ml* TPA in presence or absence of 6mT static MFs

Differences regarding cellular shape but not adhesion rate were also measured in macrophage differentiated cells in presence of SMFs. Exposure to SMFs altered both cell (U937 cells and lymphocytes) surface expression (i.e. ConA and *Ricinus communis* binding sites, PS) as well as F-actin distribution (Fig.3). Interestingly, when lymphocytes were simultaneously induced to apoptosis and exposed to SMFs, the modifications exerted by the apoptotic program were preventing by SMFs exposure: the labeling was similar to those observed for untreated lymphocytes in absence of SMFs (Fig.4).

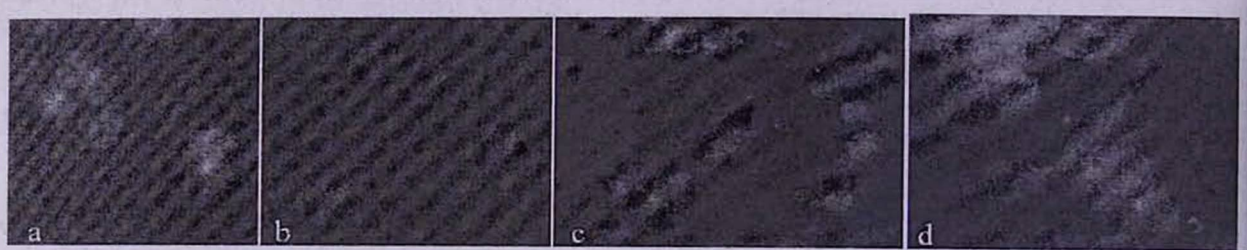

Fig. 3. ConA-FITC binding to undifferentiated (a,b) and differentiated (c,d) U937 cells in presence (b,d) or absence (a,c) of 6mT static MFs. Magnification: 800x

We tested the capability of TPA differentiated U937, in presence or absence of SMFs for 3 days, to phagocytose apoptotic lymphocytes and latex particles. Differentiated U937 cells are very active in the phagocytosis of apoptotic lymphocytes, while the exposure to SMFs reduced, up to 2 *h*, the number of cells internalizing apoptotic lymphocytes.

However, the phagocytic indices of both exposed and non exposed cells were almost comparable after 3 *h* of phagocytosis. The reduced rate of recognition, observed at the short time of phagocytosis under SMFs, was not dependent on the inducer used to trigger apoptosis of lymphocytes (Fig.5, 6).

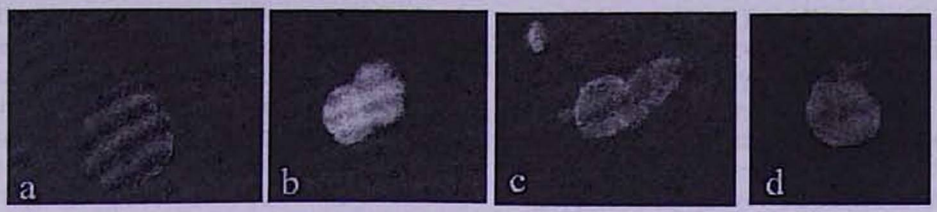

Fig. 4. ConA-FITC binding to normal (a,b) and apoptotic (c,d) lymphocytes in presence (b,d) or absence (a,c) of 6mT static MFs. Magnification: 1400x

In fact, the same results were obtained by using lymphocytes triggered to apoptosis by CHX or puromycin. To verify if this delay was specific for the phagocytosis of apoptotic lymphocytes or was a common alteration of the phagocytic process under SMFs, internalization of latex particles was tested. Indeed, similar data were observed for phagocytosis of latex particles. Therefore, our data suggest that exposure to SMFs delay the process of phagocytosis. Conversely, if we compare the percentage of cells that are phagocyting latex particles and the percentage of cells that have latex particles adhering to the cell surfaces, we see that adhesion is favoured under SMFs, in particular at longer time examined.

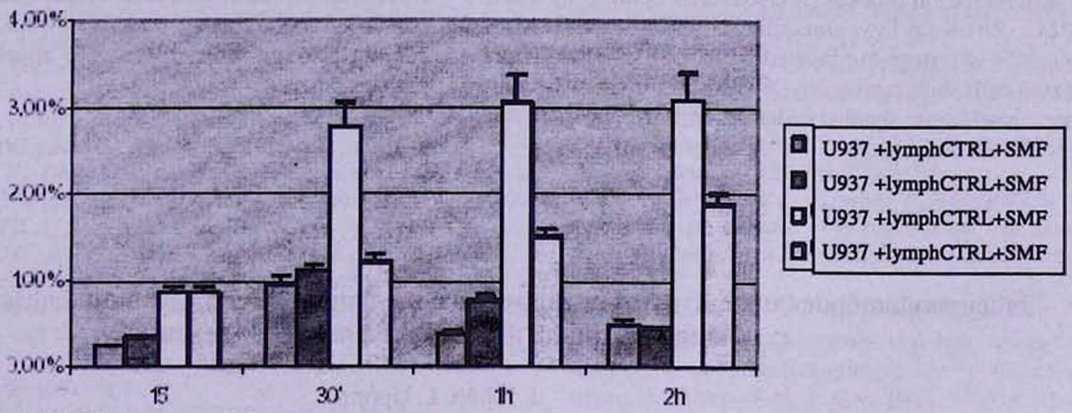

Fig.5. Time course of the percentage of phagocytosis of apoptotic lymphocytes by differentiated U937 cells in presence or absence of 6mT static MFs

Therefore, there is an increase in the percentage of cells that bind but not ingest apoptotic lymphocytes. It appears that SMF exposure favour the adhesion but slow down the efficiency of ingested particles. By using a galactose-mannose solution as inhibitor of phagocytosis, we surprisingly found a diminishing percentage of phagocytosis in the presence of this solution, thus indicating a recognition and a removal partially mediated by lectin like interactions. Moreover, the reduction of the phagocytic activity exerted by the SMFs is not cell type-dependent since we obtained the same results also for HepG2 cells.

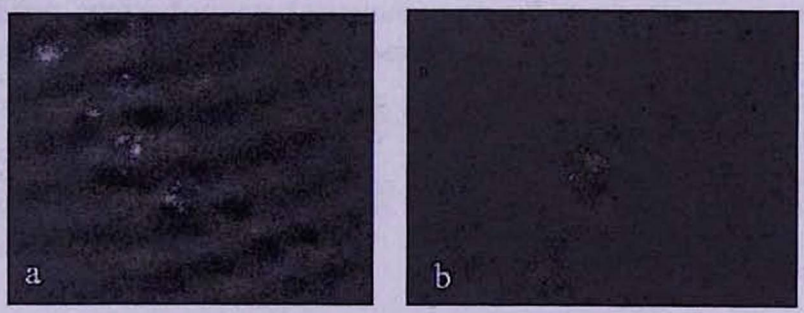

Fig.6. Fluorescent apoptotic lymphocytes phagocytosed after 30 *min* of coincubation by differentiated U937 cells in absence (a) or presence (b) of 6mT static MFs

Conclusions

In the present paper the effect of 6 mT static MFs on the macrophage differentiation of U937 cells, with respect to degree of differentiation, phagocytic activity, cell surface markers, cell shape and F-actin integrity, has been reported, giving evidence of biological effect of the static MFs exposure.

The most efficient molecule to induce macrophage differentiation of U937 cells, among those tested, has been TPA. Phorbol ester is indeed well known to induce with a high efficacy differentiation of monocytic cells, most likely through interaction with protein kinase C (PKC) [7,9–11, 14, 17, 22].

Our data, conversely, are not in agreement with those of other authors showing that a substrate of fibronectin saturated with BSA induced U937 cells differentiation. Much more controversial are the data regarding the effects of SMFs on the U937 cells differentiation, cell adhesion and phagocytosis. Our data indicate that SMFs influenced the differentiation process of U937 cells induced by TPA, DMSO, Zn++ or low glutamine concentration. In fact, static MFs exposure diminished the degree of TPA differentiated cells, but, conversely, not the adhesion to the substrate. In addition, from our data it derives that TPA and SMFs have not an additive effect on U937 differentiation. SMFs exposure gave rise to other modifications on differentiated U937 cells that were related to: cell surface and shape, F-actin distribution and expression of sugar residues and PS. However, these modifications exherted by static MFs exposure are not directly linked to the differentiative process, since modifications of cell shape, F-actin and plasma membrane have been reported also for cells not under the differentiation process, further supporting the fact that plasma membrane is the primary site for SMFs action. Phagocytosis under static MFs or EMFs has been reported to increase as well as to decrease [20].

We showed a reduction of the phagocytic rate at short time (up to 2 *h*), that reached control value at the third hour. Phagocytosis is a very complex phenomenon and phagocytosis of apoptotic cells is even more complex, due to the ability to prevent inflammation or immunoresponses [19]. Many consecutive causes can converge and lead to a reduced phagocytic index, like variation of Ca^{++} concentration that modulates cytoskeleton that can affect engulfing capability on one side, while modifications of cell surface can affect binding to apoptotic lymphocytes or latex particles.

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Էլեկտրամագնիսական դաշտի ազդեցությունը մոնոցիտների մակրոֆագային դիֆերենցման և ապոպտոզային լիմֆոցիտների կլիրենսի վրա

Լ. Դինի, Լ. Աբբրո

In vivo պայմաններում ուսումնասիրվել է մարդկային պրոմոնոցիտային U937 բջիջների մակրոֆագերի դիֆերենցման ընթացքը հաստատուն մագնիսական դաշտի ներգործության պայմաններում: Բացահայտվել է դրա բացասական ազդեցությունը բջիջների դիֆերենցման աստիճանի, նրանց ֆագոցիտար ակտիվության, բջջային մակերեսի ցուցիչների և F-ակտինի ամբողջականության վրա:

Влияние электромагнитного излучения на моноцит-макрофагную дифференциацию и клиренс апоптозных лимфоцитов

Л. Дини, Л. Аббро

В экспериментах in vivo проведено исследование процесса дифференциации макрофагов человеческих промоноцитных U937 клеток под воздействием постоянного магнитного поля. Выявлено его отрицательное влияние на степень дифференциации клеток, их фагоцитарную активность, маркеры клеточной поверхности и целостность F-актина.

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