Static Magnetic Field Interferes with the Physiological Removal of Circulating Apoptotic Lymphocytes

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Abstract— In various cellular systems, including cells of the immune system, a number of biological effects induced by static magnetic fields (SMFs) have been reported and different mechanisms have been proposed to explain these effects. Although a number of theoretical models have been proposed, the variety of experimental conditions (intensity, frequency and time windows of the fields, differing characteristics of the materials used- cell type, age, treatment) makes contradictory the data present in the literature and the possibility of the replication of the experiments. However, (S)MFs have been reported to perturb distribution of membrane proteins and sugars, cytoskeleton and trans-membrane fluxes of different ions, especially calcium \([\text{Ca}^{2+}]\). In turn, these alterations could interfere with specific physiological activities, like phagocytosis, which are based on receptors, cytoskeleton elements and motor proteins. In a previous work we found that, sinusoidal liver cells quickly recognized and engulfed human lymphocytes exposed for up to 72 h to 6 mT SMF, by using the same receptors that mediate the clearance of apoptotic cells. Thus, aim of the present work has been to decipher the modifications exerted by the SMF on lymphocytes and/or on the process of phagocytose. We analysed the cell surfaces of normal and apoptotic human lymphocytes in the presence or absence of 6 mT SMF by immunocytochemistry and biochemistry assays. SMF increases, in a time-dependent way, the expression of GD3 ganglyoside and cholesterol on the plasma membrane of normal lymphocytes and prevents GD3 removal on apoptotic-induced cells. Lipid peroxidation of plasma membrane was observed soon after lymphocytes induction of apoptosis and after 72 h of SMF exposure. The recognition and the engulfment of the control and apoptotic lymphocytes is modified by SMF exposure. In fact, normal exposed lymphocytes are recognized by the liver sinusoids at the same extent of the apoptotic non exposed cells. Conversely, the exposure to SMF promoted the binding but delayed the engulfment of apoptotic lymphocytes in \textit{in situ} as well as in \textit{in vitro} phagocytose assays. Further studies will clarify the eventual implication of SMF on human health.

1. INTRODUCTION
The development of modern society has been accompanied by a dramatic increase in the number of electronic devices, that, as consequence, have increased the exposure to static and/or electric fields of humans. An increasing bulk of evidence indicates that SMFs influence tissues and cells [1–3]. Indeed, in various cellular systems, including cells of the immune system, a number of biological effects have been reported and different mechanisms have been proposed to explain these effects. In our and other author’s studies, it was reported that the cellular and molecular modifications induced when magnetic fields interact with biological material depend on the duration of exposure, intensity, tissue penetration and the type of cells [1]. However, difficulties remain in resolving the contradictory results that arise from the multiplicity of experimental conditions [2, 3]. The plasma membrane is considered a primary site of SMF action. Thus, it is likely that, SMFs influence the diamagnetic properties of the plasma membrane that in turn distorts embedded ion channels (i.e., calcium ions channel) to the point of altering their function [3]. MFs affect the rotation of the membrane’s phospholipids and change protein distribution. Altogether, these alterations strongly interfere with physiological activities, linked to phagocytic mechanisms, like the recognition and clearance of circulating apoptotic cells. The ultimate and most favourable fate of almost all dying cells is engulfment by neighbouring or specialized cells. Efficient clearance of cells undergoing apoptotic death is crucial for normal tissue homeostasis and for the modulation of immune responses [4, 5]. Engulfment of apoptotic cells is finely regulated by a highly redundant system of receptors and bridging molecules on phagocytic cells that detect molecules specific for dying cells. The clearance of dying cells is an important fundamental process serving multiple functions in the regulation of normal tissue turnover and homeostasis. Aim of the present work has been to decipher the modifications exerted by 6 mT SMF on the process of clearance of apoptotic lymphocytes.
2. MATERIALS AND METHODS

Induction of lymphocytes apoptosis and exposure to SMF — Human lymphocytes were obtained after Ficoll gradient separation of buffy coats from blood donations of non-smoker healthy males, aged 25–50 as reported in [2]. Apoptosis was induced with $10^{-2}$ M cycloheximide (CHX) for 18 h.

SMF 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 with a range of operating temperature of 0°C to 50°C and an accuracy (at 20°C) of ±1% (Hall-effect gaussmeter, GM04 Hirst Magnetic Instruments Ltd, UK). The laboratory areas between incubators, worktops and tissue culture hood measured 0.08 µT to 0.14 µT (50 Hz) magnetic fields. In the room the background flux density was 10 µT (static) and the local geomagnetic field was approximately 43 µT (for exposure details see [12]).

Lymphocyte characterization — Normal, apoptotic and SMF exposed lymphocytes (up to 72 h) were studied for their cell surface modifications by cytochemistry and immunocytochemistry of exposed saccharides (FITC conjugates lectins: 40 µg/ml Concanavalin-A, D-mannose/D-glucosamine and 2 µg/ml Ricinus Communis, D-galactose/D-galactosamine), cholesterol (0.05 mg/ml Filipin), GD3 (Ganglyoside-Preursor-Disialohematoside) ganglyoside (monoclonal Ab anti-GD3 1:100). Samples were observed under a fluorescent microscope, Nikon 80i. The thiobarbituric acid (TBA) test was used to evaluate the presence of malondialdehyde (MDA).

Phagocytosis test — The adhesion and internalization of normal, apoptotic and SMF exposed lymphocytes to liver sinusoidal cells was quantified after in situ injection of $1 \times 10^6$ Hoechst 33342 labeled normal and apoptotic lymphocytes, in the absence or in the presence of 6 mT SMF. The cells concentration represent a saturating concentration as evaluated by the presence of cells in the medium collected from the liver. Livers were perfused in a non-recirculating system at a flow rate of 1 ml/min at ice temperature for adhesion measurement and at 37°C for internalization evaluation. The adhesion specificity was tested in parallel inhibition experiments by adding appropriate inhibitors into the perfusion tube before adding apoptotic lymphocytes. Macrophages (3 days TPA-differentiated THP-1 cells) were co-incubated up to 6 h with normal and apoptotic exposed or not exposed lymphocytes, in a ratio of 10 apoptotic cells per phagocyte. Internalization of lymphocytes was allowed in the absence and in the presence of 6 mT SMF.

3. RESULTS

3.1. SMF Modifies the Exposure of Cell Surface Molecules on Control and Apoptotic Lymphocytes

Lymphocytes viability was more than 95% at the end of the isolation procedure and it was reduced to 30% when cells were induced to apoptosis by 18 h incubation with $10^{-2}$ M CHX. SMF exposure up to 24 h did not reduce cell viability or growth. SMF was able to reduce the yield of apoptotic cells (15–20% less) after CHX induction. Both the apoptotic treatment and the exposure to 6 mT SMF affect sugar expression as quantity and surface distribution; mannose/D-glucosamine and galactose/D-galactosamine residues were enhanced on the external surface of the plasma membrane (Tab. 1).

Table 1: Semiquantitative evaluation of FITC-conjugate lectins for the detection of carbohydrate residues on the cell surface of control (CTRL) and apoptotic (APO) lymphocytes in the absence and in the presence of 6 mT SMF.

<table>
<thead>
<tr>
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<th>APO+SMF</th>
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<td>Con-A</td>
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<td>Ricinus c.</td>
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CTRL = normal lymphocytes; APO = apoptotic lymphocytes, 18h

To verify if the SMF interferes with the plasma membrane glycoproteins distribution through the lipid alteration the measurements of thiobarbituric acid reactive substances (TBARS), the localization of GD3 ganglyoside and cholesterol have been performed. The data are reported in Figs. 1, 2. An extremely high increase of lipid peroxidation was measured (Fig. 1). GD3 ganglyoside, that is randomly distributed on the cell surface of control lymphocytes, was clustered at one pole...
Figure 1: (a) Level of thiobarbituric acid reactive substances (TBARS) in control and 6 mT SMF exposed human lymphocytes. O.D. optical density at 233 nm. (b) Fluorescence images of triple staining of nucleus (red, Hoechst 33342), cholesterol (blue, Filipin) and GD3 ganglyoside (green, primary Ab anti GD3, secondary Ab FITC conjugated) of 6 mT SMF exposed (a’) and control (b’) human lymphocytes. Bars = 5 µm. Arrowhead = GD3, thick arrow = cholesterol; thin arrow = nucleus.

Figure 2: Number of control or $10^{-2}$ M CHX treated lymphocytes in the absence or in the presence of 6 mT SMF for 24 h, adhering (white columns) or internalized (black columns) by liver sinusoidal cells. Statistical analyses were performed using Student’s $t$-test for unpaired data, and $P$ values < 0.05 versus CTRL were considered significant. Data are presented as mean ±S.D..

of the cells after induction of apoptosis or after 48 h of exposure to 6 mT SMF (Fig. 2(b)). A comparable behavior was observed for cholesterol distribution (Fig. 2(b)).

3.2. The Recognition and Engulfment of Apoptotic Lymphocytes is Modified under SMF

Phagocytosis of the apoptotic cells is the last, but very important, step of the apoptotic program. In vivo apoptotic cells are cleared by phagocytosis with a rapid and efficient process able to remove damaged cells and components by inhibiting inflammation and by modulating the immune system [5, 6].

During phagocytosis of apoptotic cells plasma membrane has a pivotal role for the recognition of dead cells and for their engulfment through connection with cytoskeleton [7]. The phagocytosis of apoptotic cells is a multi-steps process. Apoptotic cells are first recognized by phagocytes, to which they adhere; then apoptotic cells are internalized and finally degraded. These mechanisms assure an efficient clearance of dead cells in non-pathological conditions. The data obtained in in situ and in in vitro experiments indicate that SMF interfere with both the steps of recognition and of engulfment of the apoptotic lymphocytes (Figs. 2 and 3). Apoptotic lymphocytes were retained by sinusoidal liver cells at high degree, and when internalization was allowed, they were found inside the cells. Worth noting, control non exposed lymphocytes were never phagocytosed. However, when normal lymphocytes were exposed for at least 24 h to SMF, they were retained by sinusoidal walls (three times with respect to control) (Fig. 2) and internalized when allowed.

The percentage of lymphocytes induced to apoptosis under exposure to SMF bound to sinusoidal liver cells was higher than the apoptotic ones. Correspondingly the number of internalized apoptotic lymphocytes was increased. The induction of apoptosis and SMF exposure had a synergic effect.

Time course (1, 2, 3, 4 h) of in vitro phagocytosis of apoptotic cells is reported in Fig. 3. Phagocytosis is progressively achieved with time of TPA-induction (i.e., from 1 to 3 days): 2% of THP-1 cells at 1 day, 25% at 2 days and more than 50% at 3 days. Only differentiated THP-1 cells could bind and internalize apoptotic cells. Non-differentiated THP-1 cells could only bind apoptotic cells at very low rate (5–7%) but not engulf them. Surprisingly, at the end of 4 h of phagocytosis, in the presence of SMF a very small percentage of non-differentiated THP-1 cells bore phagosomes containing apoptotic cells. In presence of SMF the percentage (about 25%) of non-differentiated THP-1 cells able to bind apoptotic cells increased. Exposure of 3 day-differentiated THP-1 cells to SMF during phagocytosis promoted engulfment of apoptotic cells preventing their binding (Fig. 3).
Figure 3: Percentage of the phagocytosis index (black columns) and of binding (white columns) of THP-1 cells, differentiated with TPA 50 ng/mL for 3 days in the presence (+SMF) and absence (−SMF) of 6 mT SMF. The values are the mean ± SD of three independent experiments. The values −SMF are significantly different (p < 0.05) with respect to +SMF. The phagocytosis index and percentage of binding was measured scoring at least 500 cells for each experimental time. Images show contrast phase (left) and fluorescent (right) micrographs showing THP-1 cells at the third day of differentiation with TPA, internalizing (arrows) Hoechst 33342 stained apoptotic U937 cells after 4 h of phagocytosis assay in the absence (a), (a′) and in the presence (b), (b′) of 6 mT SMF. Bars = 10 µm.

4. CONCLUSIONS

Our data support the bioeffects of the exposure to 6 mT SMF on the phagocytosis of human normal and/or apoptotic lymphocytes. SMF induced modifications of the plasma membrane of normal exposed lymphocytes, making these cells much more similar to the apoptotic ones. As a consequence, they are fast and silently removed by phagocytes. We showed the strong and reproducible effects that the exposure to SMF exerts on the process of liver phagocytosis of apoptotic lymphocytes. Liver clearance of apoptotic lymphocytes is a complex mechanism that can be influenced at least at three different levels: phagocyte receptors, apoptotic cell quality and SMF exposure. On the side of the phagocyte receptors, in general, the efficiency of phagocytosis is proportional to the number of expressed receptors. In the liver this aspect is particular evident, since the receptors involved in the recognition of apoptotic cells are modulated in relation to the type of the cells, to the physiological or pathological status of the organ and to the cell localization inside the lobule [7].

The extent of the SMF influence is also related to the degree of macrophage maturation. Since 6 mT SMF exposure interferes with monocyte/macrophage TPA-induced differentiation of U937 promonocytes and THP-1 monocytes (respectively: 20% increment and 15% decrement) [8], the delayed macrophage maturation could explain the defects in phagocytosis. Impairment of phagocytosis could also be due to the alteration of cytoskeleton following SMF exposure. Ruffling of plasma membrane, that subsequently close to form macropinosomes, requires reorganization of the actin filament network to the cell periphery, that has been reported to be altered by SMF exposure [9]. Microtubules polymerization is under the control of concentration of Ca$^{2+}$, that are modulated under SMF exposure. Ca$^{2+}$ are also very important for signals transduction during TPA differentiation and cytoskeleton modifications during phagocytosis. Ca$^{2+}$ intracellular concentration in U937 cells increases under exposure to SMF as well as during TPA treatment also in the presence of SMF [10, 11]. The modulation of the index and rate of phagocytosis of apoptotic cells could further be influenced by modifications of cell surface molecules exerted by SMF exposure. These modifications, involving a large set of molecules, can, in turn, affect related functions like cell-cell recognition, interaction and attachment; all activities that are very important during phagocytosis of apoptotic cells.

Altogether, our data gave evidence of a strong influence of SMF on phagocytosis. The implications for human health needs to be deeply investigated, because the pathological and/or physiological implications are not yet understood and need to be further investigated. In particular, further studies will clarify if SMF exposure could be used as a novel therapeutic tool to produce “apoptotic dressed cancer cells” and thus promote their elimination.
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REFERENCES