Effects of ultra-wideband electromagnetic pulses on pre-neoplastic mammary epithelial cell proliferation

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Abstract. Electromagnetic ultra-wideband pulses (UWB) or nanopulses, are generated by a wide range of electronic devices used in communications and radar technology. However, the specific effects of nanopulse exposure on cell growth and function have not been extensively investigated. Here, studies have been conducted to determine the effects of prolonged exposure to non-ionizing, low to moderate intensity nanopulses on the growth of pre-neoplastic CL-S1 mammary epithelial cells *in vitro*. Cells were grown in culture and maintained in serum-free defined medium containing 10 ng/ml EGF and 10 µg/ml insulin as comitogens. Studies showed that 0.25–3.0 h exposure to nanopulses of 18 kV/m field intensity, 1 kHz repetition rate and 10 ns pulse width had no effect on CL-S1 cell growth or viability during the subsequent 72-h culture period. However, exposure to similar nanopulses for prolonged periods of time (4–6 h) resulted in a significant increase in cell proliferation, as compared to untreated controls. Additional studies showed that nanopulse exposure enhanced CL-S1 cell growth when cells were maintained in media containing only EGF, but had no effect on cells maintained in defined media that were mitogen-free or containing only insulin. Studies also showed that the growth-promoting effects of nanopulse exposure were associated with a relatively large increase in intracellular levels of phospho-MEK1 (active) and phospho-ERK1/2 (active) in these cells. These findings demonstrate that prolonged exposure to moderate levels of UWB enhanced EGF-dependent mitogenesis, and that this growth-promoting effect appears to be mediated by enhanced activation of the mitogen-activated protein kinase (MAPK) signalling pathway in pre-neoplastic CL-S1 mammary epithelial cells.

INTRODUCTION

Ultra-wide band pulses (UWB) have a wide-frequency bandwidth, a rapid pulse rise time, and short pulse duration. Nanopulses are UWB with duration of 1–10 ns, rise time between 0.1 and 1.0 ns, and approximate bandwidth of 1 GHz (Gos *et al.* 2000). More conventional electromagnetic pulses have a narrower bandwidth. Practical UWB application was first used by the military for

the development of new communication and radar technologies, and today UWB is used in high-powered microwave weapons, electronic warfare for jamming other radar and communication devices, ground and ocean penetrating radars, as well as intrusion detection and alert devices in security systems (Taylor 1991). There has been a substantial increase in the exposure of the general population to nanopulses as they are now extensively used in a wide range of communication (cell phones, pagers, radios), localization (precision geo-location, beacon and positioning devices) and detection radar (altimeter and obstacle avoidance radar, collision avoidance backup sensors, intrusion detection) devices (Taylor 1991; Gos *et al.* 2000). Nevertheless, the potential human health risk of chronic nanopulse exposure is unknown.

The effects of conventional forms of electromagnetic radiation on living organisms have been the subject of investigation for many years. Experimental evidence clearly demonstrates that electromagnetic fields can influence significantly biological tissue function and structure. In general, electromagnetic effects on living matter can be classified as either ionizing or non-ionizing. Ionizing effects result from ionization of atoms or molecules. Generally, ionization will occur only if the electric field intensity is very high. Once ionization has occurred, application of an electric field will result in forces experienced by the ions, causing them to move and generate an electric current. The resulting heat produced by this depends upon the inherent electrical resistance of the matter that the current is flowing in (Albanese *et al.* 1994). Electromagnetic-induced ionizing effects in living matter are associated with physical trauma, damage and/or destruction of biological tissues and/or cells.

In contrast, non-ionizing effects are caused by electromagnetic-induced changes in the structure and function of cellular and subcellular components, such as membranes, enzymes, transport systems and so forth (Albanese *et al.* 1994; Merritt *et al.* 1995; Sherry *et al.* 1995; Walters *et al.* 1995; Jauchem 1997; Pakhomov *et al.* 1998; Pakhomova *et al.* 1998). The magnitude and direction of non-ionizing effects are directly related to electromagnetic field variables, including waveform, frequency and pulse duration. Specific variations in the configuration and temporal exposure patterns of extremely weak electromagnetic nanopulses have been shown to produce highly specific biological responses, similar to those induced by pharmaceutical agents (Raslear *et al.* 1993; Seaman *et al.* 1998), and to stimulate cell proliferation (Rubik 1997; Scardino *et al.* 1998; Seaman *et al.* 1998; Satter Syed *et al.* 1999; Seaman *et al.* 1999; Binhi & Goldman 2000; Pletnev 2000; Nayci *et al.* 2001; Canedo-Dorantes *et al.* 2002; Nayci *et al.* 2003; Nelson *et al.* 2003; Stacey *et al.* 2003; Trostel *et al.* 2003). The following studies were conducted to determine the effects of low to moderate levels of non-ionizing UWB radiation on the growth of pre-neoplastic mouse CL-S1 mammary epithelial cells *in vitro*. Additional studies investigated the effects of nanopulse exposure on the activation of the mitogen-activated protein kinase (MAPK) mitogenic signalling pathway in these cells.

MATERIALS AND METHODS

Cell culture and experimental treatments

All materials were purchased from Sigma Chemical Company (St. Louis, MO), unless otherwise stated. The CL-S1 pre-neoplastic mouse mammary epithelial cell line was derived from the hyperplastic D1 cell line that arose spontaneously in a BALB/c mouse (Danielson *et al.* 1980). CL-S1 cells are immortal in culture, but do not grow in soft agarose or form solid tumours upon transplantation back into the mammary gland (Anderson *et al.* 1979; Danielson *et al.* 1980). These pre-neoplastic CL-S1 mammary epithelial cells were serially passaged at subconfluent

cell density and maintained in serum-free defined control media consisting of DMEM/F12 containing 5 mg/ml bovine serum albumin (BSA), 10 µg/ml transferrin, 100 U/ml soybean trypsin inhibitor, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 10 ng/ml EGF, and 10 µg/ml insulin. For subculturing, cells were rinsed twice with sterile Ca²⁺- and Mg²⁺-free phosphate-buffered saline (PBS), and then incubated in 0.05% trypsin containing 0.025% EDTA in PBS for 5 min at 37 °C. The released cells were then diluted in DMEM/F12 media, pelleted by centrifugation, and the cell pellets were then resuspended in serum-free media, and counted by using a haemocytometer. The cells were plated at a density of 1×10^5 cells/well in 24-well culture plates for growth studies and at a density of 1×10^6 cells/100 mm culture plates for Western blot analysis.

Nanopulse generator and treatment chamber

Nanopulse exposure experiments were conducted at Louisiana Tech University. The exposure facility is equipped with a range of low- to high-intensity pulsers that can be used to test biological effects of different UWB variables and conditions, including various combinations of field intensity (kV/m), frequency (Hz) and exposure duration (h). In all experiments described here, the pulse width was approximately 10 ns and pulse rise time was approximately 0.1 ns. Biological samples were placed in the temperature-controlled (27 °C) gigahertz transverse electromagnetic mode (GTEM) cell (ETS-Lindgren, Glendale Heights, IL) and exposed to nanopulses of defined properties. These pulses are non-ionizing and do not cause sample heating (Naarala *et al.* 2004; Vernier *et al.* 2004; Simicevic & Haynie 2005). In all experiments, cells in control groups were treated in a similar manner as cells in treatment groups, except that the power to the pulser was not turned on. All experiments were repeated at least three times. The basic setup of the nanopulse exposure facility is shown in Fig. 1.

Figure 1. Generalized operational setup of the nanopulse exposure facility.

Measurement of viable cell number

Pre-neoplastic CL-S1 mammary epithelial cell number was determined in 24-well culture plates (six wells/group) by the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) colourimetric assay as described previously (Sylvester *et al.* 1994; McIntyre *et al.* 2000). On the day of assay, treatment media were replaced with fresh growth medium containing 0.83 mg/ml MTT, and the cells were returned to the incubator for 4 h. Afterwards, media were removed once again and MTT crystals were dissolved in 0.5 ml of isopropanol for appropriate use. The optical density of each sample was read at 570 nm on a microplate reader (Packard SpectraCount), against a blank prepared from cell-free cultures. The number of cells/well was calculated against a standard curve prepared by plating various concentrations of cells, as determined by haemocytometer, at the start of each experiment (Sylvester *et al.* 1994; McIntyre *et al.* 2000).

Electrophoresis and Western blot analysis

Whole cell lysates and subcellular fractions obtained from the different treatment groups 24 h after treatment exposure were dissolved in Laemmli buffer (Laemmli 1970) and protein concentration in each sample was determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) according to the manufacturer's directions. Equal amounts of protein from each sample (50 µg/lane) in a given experiment were loaded on polyacrylamide minigels and were electrophoresed through a 7.5% resolving gel. Proteins were transblotted (25 V for 12–16 h) to PVDF membranes (Dupont, Boston, MA) according to the method of Towbin (Towbin *et al.* 1979). Membranes were blocked with 2% BSA in 10 mM Tris-HCl containing 50 mM NaCl and 0.1% Tween 20, pH 7.4 (TBST) and then were incubated with either antiphospho-MEK1 (active), anti-total ERK1/ERK2 (active and inactive), antiphospho-ERK1/2 (active) or anti-β-actin monoclonal antibodies (Santa Cruz Biotech, Santa Cruz, CA, USA). Membranes were then rinsed five times with TBST, and then incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies (Transduction Laboratories, Lexington, KY) in TBST with 2% BSA for 1 h. Afterwards, blots were rinsed five times with TBST and protein bands were visualized by chemiluminescence according to the manufacturer's instructions (Pierce, Rockford, IL). In each experiment, blots from each treatment group were exposed on the same piece of film (Kodak X-OMAT AR, Rochester, NY). Images were acquired with a Microtek 9600XL scanner (Microtek Laboratory Inc., Redondo Beach, CA) and were analysed with Scion software (Scion, Frederick, MD).

Statistical analysis

Differences among the various treatment groups were determined by analysis of variance followed by Duncan's multiple range test. A difference of $P < 0.05$ was considered to be significant, as compared to controls or as defined in the figure legends.

RESULTS

These pre-neoplastic CL-S1 mammary epithelial cells were exposed to a nanopulse electric field strength of 18 kV/m at a repetition rate in the range of 1–1000 kHz and an exposure time of 0–4 h. Afterwards, cells in all treatment groups were returned to the incubator and 72 h later viable cell number was determined using the MTT assay. The results of these studies are shown in Fig. 2. Exposure to nanopulses for 0.25–2.0 h over a 1000 kHz range of pulse repetition rates had no significant effect on cell growth during the subsequent 72-h culture period. However,

Figure 2. Effects of various nanopulse repetition rates and durations of exposure at a constant field intensity of 18 kV/m on growth of the pre-neoplastic CL-S1 mammary epithelial cells in culture. Cells were initially plated at a density of 1×10^5 cells/well in 24-well culture plates and then exposed to the respective nanopulse treatments. Vertical bars represent mean cell count/well $\pm$ SEM of 24 wells in each group 72 h following nanopulse treatment. * $P < 0.05$, as compared to untreated controls within each respective treatment group.

treatment for 4 h with nanopulses at 18 kV/m field intensity, and 1 or 10 kHz, but not 100 and 1000 kHz repetition rate resulted in a significant increase in CL-S1 cell growth during the subsequent 3-day culture period. Similar experiments conducted to determine the effects of a 4-h nanopulse treatment exposure at 0.18 kV/m and 1.8 kV/m intensities showed that these treatments had no effect CL-S1 cell growth (Fig. 3).

The effects of various durations of exposure to nanopulses of 18 kV/m field intensity and 1 kHz repetition rate on the subsequent growth of pre-neoplastic CL-S1 mammary epithelial cells are shown in Fig. 4. Nanopulse exposure for 0–3 h had no effect on cell growth (Fig. 4). In contrast, prolonged exposure for 4–6 h resulted in a significant increase in cell growth as compared to untreated control cells, yet growth-promoting effects of 4 h nanopulse treatment did not differ significantly from that observed after 5- or 6-h treatment exposure (Fig. 4).

The effects of 4-h nanopulse exposure on the mitogen-dependent pre-neoplastic CL-S1 mammary epithelial cell proliferation are shown in Fig. 5. The cells were divided into different treatment groups and fed serum-free defined media supplemented with or without 10 ng/ml EGF, 10 μ g/ml insulin or the combination of EGF and insulin. Cells in the different media treatment groups were then exposed to nanopulses for 0–4 h treatment at 18 kV/m field intensity and 1 kHz frequency, and were then returned to the incubator. Viable cell number was determined 72 h following nanopulse exposure. CL-S1 cells remained viable in mitogen-free media, but showed little or no growth after 3 days in culture, and nanopulse treatment did not alter this

Figure 3. Effects of a 4-h exposure to nanopulse treatment at various frequencies and intensities (0.18 kV/m, top and 1.8 kV/m, bottom) on the growth of pre-neoplastic CL-S1 mammary epithelial cells in culture. Cells were initially plated at a density of 1×10^5 cells/well in 24-well culture plates and were then exposed to nanopulses. Vertical bars represent mean cell count/well $\pm$ SEM of 24 wells in each group 72 h following nanopulse treatment. * $P < 0.05$, as compared to untreated controls within each respective treatment group.

response. Supplementation of culture media with insulin was found to stimulate CL-S1 proliferation over that of mitogen-free treated cells, but this stimulation was less than that observed in CL-S1 cells treated with insulin and EGF, and again nanopulse treatment had no effect on this response. In contrast, CL-S1 cells maintained in media containing only EGF remained viable, but did not display significant growth over that observed in untreated cells maintained in mitogen-free media. However, nanopulse treatment was found to significantly increase the number of cells treated with EGF alone or the combination of EGF and insulin, as compared to their respective untreated controls (Fig. 5).

Figure 6 shows Western blot analysis of various intracellular proteins associated with the MAPK mitogenic-signalling cascade 24 h after nanopulse treatment exposure. Nanopulse exposure was for 0 or 4 h at 18 kV/m field intensity and 1 kHz repetition rate. The cells were maintained in culture media containing either 10 ng/ml EGF or the combination of 10 ng/ml EGF and 10 μ g/ml insulin. Untreated CL-S1 control cells maintained in defined serum-free

Figure 4. Effects of different durations of exposure to nanopulse treatment at 18 kV/m field intensity and 1 kHz repetition rate on the growth of pre-neoplastic CL-S1 mammary epithelial cells in culture. Cells were initially plated at a density of 1×10^5 cells/well in 24-well culture plates and were then exposed to their respective nanopulse treatments. Vertical bars represent mean cell count/well $\pm$ SEM of 24 wells in each group 72 h following nanopulse treatment. * $P < 0.05$, as compared to untreated controls within each respective treatment group.

Figure 5. Effects of nanopulse exposure for 4 h at 18 kV/m field intensity and 1 kHz repetition rate on the growth of the pre-neoplastic CL-S1 mammary epithelial cells in culture grown in serum-free defined media supplemented with or without 10 ng/ml EGF, 10 µg/ml insulin, or the combination of EGF and insulin. Cells were initially plated at a density of 1×10^5 cells/well in 24-well culture plates and exposed to their respective nanopulse treatments. Vertical bars represent mean cell count/well $\pm$ SEM of 24 wells in each group 72 h following nanopulse treatment. * $P < 0.05$, as compared to untreated controls within each respective treatment group.

media containing EGF alone or the combination of EGF and insulin showed a low-intensity Western blot band for phospho-MEK1 (active) and phospho-ERK1/2 (active), and nanopulse treatment induced a large increase in the relative band intensities of these kinases. The relative levels of total (active and inactive) ERK1/2 showed little or no difference among the different treatment groups (Fig. 6).

Figure 6. Western blot analysis of intracellular protein levels associated with MAPK mitogenic signalling in pre-neoplastic CL-S1 mammary epithelial cells grown in serum-free defined media supplemented with either 10 ng/ml EGF or the combination of 10 ng/ml EGF and 10 µg/ml insulin, 24 h following 0 or 4 h nanopulse treatment at 18 kV/m field intensity and 1 kHz repetition rate. Cells were initially plated at a density of 1×10^6 cells/well in 100-mm culture plates and were then subjected to respective culture medium and nanopulse treatments. The next day whole cell lysates (50 µg/lane) from the different treatment groups were collected and then subjected to fractionated by SDS-PAGE and Western blot analysis for phospho-MEK1 (active), total (active and inactive) and phospho-ERK1/2 (active) levels.

DISCUSSION

Results in this study demonstrate that prolonged exposure to moderate intensity UWB can significantly stimulate mitogen-dependent pre-neoplastic CL-S1 mouse mammary epithelial cell proliferation *in vitro*. Cells maintained in serum-free defined media containing either 10 ng/ml EGF as a mitogen or 10 ng/ml EGF plus 10 µg/ml insulin as comitogens, displayed significantly greater cell growth than untreated control cells during the subsequent 72 h incubation period following a 4-h nanopulse treatment of 18 kV/m field intensity and 1 kHz repetition rate. However, these growth-promoting effects were not observed when cells were maintained in serum-free defined media that was mitogen-free or contained only 10 µg/ml insulin as a mitogen. These results indicate that activation of an EGF-receptor mitogenic signal is required for expression of nanopulse-induced enhancement of CL-S1 cell proliferation. The MAPK or Ras/Raf/MEK/ERK pathway is a major signalling conduit associated with EGF-induced mitogenesis in normal, pre-neoplastic, and neoplastic mammary epithelial cells (Sylvester *et al.* 2002). Cells maintained in serum-free defined media containing EGF alone or the combination of EGF plus insulin, showed a relatively large increase in intracellular levels of phospho-MEK1 (active) and phospho-ERK1/2, 24 h following nanopulse exposure. However, this effect was not observed in cells maintained in media that were mitogen-free or contained only insulin as a mitogen. Therefore,

these findings strongly suggest that nanopulse-induced stimulation of pre-neoplastic CL-S1 mammary epithelial cell growth is mediated through enhanced EGF-dependent activation of the MAPK mitogenic signalling pathway.

Studies have shown that non-ionizing electromagnetic fields can directly influence living tissues. (Bassett 1989, 1993; Raslear *et al.* 1993; Rubik 1997; Scardino *et al.* 1998; Seaman *et al.* 1998; Satter Syed *et al.* 1999; Seaman *et al.* 1999; Binhi & Goldman 2000; Pletnev 2000; Nayci *et al.* 2001; Canedo-Dorantes *et al.* 2002; Nayci *et al.* 2003; Nelson *et al.* 2003; Stacey *et al.* 2003; Trostel *et al.* 2003). In whole animal investigations, exposure to high-intensity UWB has been found to cause no genotoxic effects on either circulating peripheral blood or bone marrow cells (Vijayalaxmi *et al.* 1999). In addition, high-intensity nanopulse exposure had no adverse effects on animal physical activity, performance, blood chemistry or decision-making and cognitive function (Raslear *et al.* 1993; Walters *et al.* 1995). In cell culture studies, high-intensity nanopulse exposure has been found to not affect cellular survival, nor influence mitotic activity of mammalian cells (Stacey *et al.* 2003), and also was not mutagenic to yeast (Pakhomova *et al.* 1998).

In contrast, exposure to nanopulses has been found to induce significant biological effects, for example stimulation of cell proliferation in wound healing. During the past two decades, use of pulsed electromagnetic fields in stimulating bone repair has become widely accepted. Studies have shown that pulsed electromagnetic fields and capacitive coupling induce fields through soft tissue, resulting in low-magnitude voltage and currents at the fracture site, and greatly enhance fracture healing and patient recovery (Bassett 1989, 1993; Rubik 1997; Satter Syed *et al.* 1999; Nelson *et al.* 2003). Exposure to nanopulses has also been shown to significantly increase the healing of sutured and open skin wounds in rats, yet cause no adverse pathological, histological or bacteriological effects (Rubik 1997; Scardino *et al.* 1998; Trostel *et al.* 2003). Some studies have also shown that low frequency electromagnetic fields stimulate Ca^{++} mobilization, activate signal transduction cascades, promote cytokine synthesis and stimulate proliferation and differentiation of peripheral blood mononuclear cells (Canedo-Dorantes *et al.* 2002). Furthermore, when peripheral blood mononuclear cells were exposed to UWB and then applied locally on an ulcer surface, there was a significant promotion in the healing of chronic arterial and venous leg ulcers (Canedo-Dorantes *et al.* 2002).

The exact intracellular mechanisms involved in mediating growth-promoting effects of exposure to non-ionizing UWB are not presently understood. However, various forms of radiation have been shown to stimulate different classes of protein kinases, such as the Ras/Raf/MEK/ERK pathway (Denhardt 1996; Kolch 2000; Sylvester *et al.* 2002). EGF activates specific membrane-bound tyrosine kinase receptors that undergo dimerization and autophosphorylation at multiple tyrosine residues that are required for direct interaction between the receptor and effector molecules involved with intracellular signal transduction. One of the initial events in receptor tyrosine kinase mitogenic signalling is Ras activation (Denhardt 1996; Kolch 2000). A downstream effector of Ras is c-Raf-1, a serine/threonine kinase, which subsequently phosphorylates and activates the MAPK pathway (Denhardt 1996; Kolch 2000). Downstream effectors of ERKs are nuclear transcription factors such as Myc and Elk that ultimately induce various biological responses, including mitogenesis and anti-apoptotic responses associated with cell survival, by directly influencing gene expression (Denhardt 1996; Kolch 2000). Some forms of intense radiation have been shown to activate membrane-bound receptor-tyrosine kinases, ultimately leading to ERK1/2 activation (Dent *et al.* 2003). Results in the present study show that moderate nanopulse exposure does not induce a mitogenic response in the absence of EGF, but does potentiate EGF-induced mitogenic-responsiveness. This effect was associated with a corresponding increase in MEK1 and ERK1/2 activation.

In summary, prolonged exposure to moderate levels of non-ionizing UWB electromagnetic pulses promotes CL-S1 pre-neoplastic mammary epithelial cell growth. Although these findings do not directly associate UWB pulse exposure with an increased cancer risk, nanopulse-induced accelerated pre-neoplastic cell growth may ultimately promote neoplastic progression (Russo *et al.* 1989). Because of growth in the application and use of nanopulse-generating devices, the general population is being exposed to an ever-increasing amount of environmental UWB. The present findings indicate that additional studies are required to fully characterize the biological effects and intracellular mechanisms of action caused by prolonged nanopulse exposure, in order to clearly understand the possible impact of UWB exposure on human health.

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