Enhancement of cancer chemotherapy *in vitro* by intense ultrawideband electric field pulses

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Experiments have been performed to enhance the Jurkat cell-killing effects of the cancer chemotherapy agent bleomycin using electric field pulses of 50–200 kV/cm peak electric field strength, ~150 ns duration, and nanosecond rise time. Dramatic increases in cell killing (factors of ~1000) were observed with a low dose of bleomycin after treatment with trains of ten or more pulses at all electric field strengths tested, compared to pulse-only or drug-only treatments. Cell death occurred within 24 h for treated cells, with some evidence of membrane phosphatidylserine externalization at 6 h postexposure but no significant increase in caspase activity, indicating that the primary mode of cell death was not caspase-mediated apoptosis. © *2006 American Institute of Physics*. [DOI: 10.1063/1.2194115]

I. INTRODUCTION

An important goal of cancer chemotherapy is to increase the effectiveness of drugs with reduced doses to minimize adverse side effects to the patient. Recent experiments have demonstrated that a variety of membrane-impermeant antineoplastic drugs, chiefly the antitumor antibiotic bleomycin, can be loaded into cells using electroporation.[1] This type of treatment has utilized pulses with durations of order 1 ms and peak electric field strengths in the range of 1–2 kV/cm. Animal trials[2] and ongoing clinical trials have tested such methods for use with tumors of the skin, as well as tumors near the skin surface in the head and neck. The electric field is applied to the tumor using needle or paddle electrodes in such treatments.[3]

Bleomycin is a powerful antibiotic agent that reacts in the presence of metallic ions and oxygen to catalyze double-strand breaks (DSBs) in the DNA of living cells.[4] Some single-strand breaks (SSBs) are also created. This is the same function performed by ionizing photons in x-ray and gamma exposures. If the DNA that controls mitosis is damaged, the cell's next attempt to divide fails, the cell cycle arrests, and the cell dies. When sufficient DNA damage occurs to interrupt ordinary cellular functions, cell death rapidly ensues. Under ordinary conditions, the DNA of cells is protected from the effects of bleomycin as its motion is blocked by the intact cell membrane. At equilibrium, a cell's interior concentration of bleomycin is expected to be about 0.05%–0.1% of any introduced extracellular concentration. Even at these low rates of uptake, bleomycin has been shown to be reasonably effective in killing cancer cells due to their faster metabolism, compared to healthy tissue. Electroporation[5] has yielded dramatic improvement in this killing effectiveness.[6–9]

Agee proposed that cancer chemotherapy could be enhanced using ultrawideband (UWB) electric field pulses,[10,11] enabling other methods of electric field coupling to be used for emerging therapies. In the experiments presented here, the electroporation of mammalian cells by UWB pulses was tested to determine the effectiveness of such treatment. These pulses were of interest because, unlike conventional electroporation wave forms, they contained a large high-frequency energy content that could be coupled to tissue by methods not involving direct contact electrodes.

II. EXPERIMENTAL CONFIGURATION AND PROCEDURES

In the present experiments, ultrawideband electric field pulses were produced by a high-voltage peaking circuit generator (L-3-Titan Pulse Sciences, San Leandro, CA) coupled to a broadband tapered transmission line. The tapered line provided uniform 50 Ω impedance along its length, while the spacing of the electrodes increased from input to termination. The line was terminated by an array of four 200-Ω low-inductance Carborundum resistors to provide a 50-Ω resistive termination. The transmission line was enclosed in a copper-shielded box lined with microwave absorber material to trap and absorb radiated emissions. The final configuration was roughly equivalent to a gigahertz TEM (GTEM) cell.[12]

For biological cell exposures, a BTX electroporation cuvette (BTX-Genetronix, Holliston, MA) was connected across the broadband load and filled with cell suspension. This introduced an impedance mismatch between the genera-

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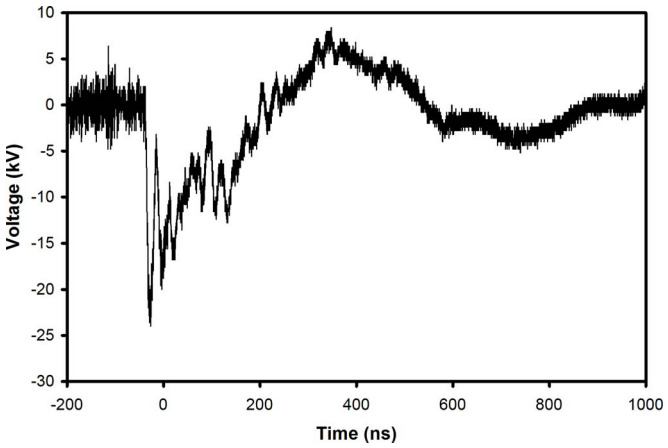

FIG. 1. UWB output voltage pulse measured across 4-mm electroporation cuvette and HBSS solution.

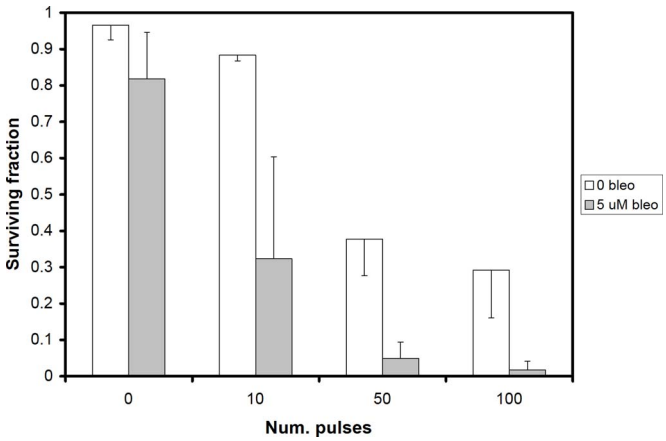

FIG. 2. Cytotoxicity of bleomycin and 95 kV/cm UWB pulses.

tor and load. Accordingly, peak voltages across the load were considerably lower than charging voltages. Cultured Jurkat cells (ATCC, Manassas, VA) were suspended in Hanks' balanced salt solution (HBSS) at a density of 10^6/ml and placed in cuvettes in the GTEM cell. Cell samples were subjected to trains of 10–100 UWB pulses at 1 Hz pulse repetition rate. The charging voltage was −40 kV and the output voltage was measured with a Northstar PVM-5 high-voltage probe connected to a Tektronix TDS7404 digital oscilloscope with data stored in a personal computer (PC) using LABVIEW. The peak electric field across each sample was selected by the use of a cuvette with a 1-, 2-, or 4-mm electrode gap.

The high-voltage output of the UWB generator, measured across a 4-mm electroporation cuvette loaded with HBSS, is depicted in Fig. 1. The generator charge voltage was −40 kV for this test, so some voltage reduction due to the impedance mismatch was evident. This pulse produced a 60 kV/cm peak electric field across the sample for this 4-mm electrode spacing.

Cells exposed to UWB pulses, both with and without extracellular bleomycin, were assayed for survival in 24 h postexposure by trypan blue staining and clonogenic assay. Clonogenic assay was prepared by serial dilution at 24 h and incubated for ten days to determine the population of cells capable of normal replication after treatment.

III. EXPERIMENTAL RESULTS

Data in Fig. 2 compare the survival of cells exposed to UWB pulse trains to those exposed to both UWB pulses and bleomycin at a 5 μM extracellular concentration, based on the trypan blue assay. Significant enhancement (>90% killed versus 20%) was observed by the application of pulses to cells together with a low dose of chemotherapy drug. This bleomycin concentration is 10% of the concentration typically used for significant cell killing in tissue culture (50 μM) and is roughly equivalent to the minimum doses typically used in human cancer chemotherapy. The 95 kV/cm pulsed fields were generated by placing cells in cuvettes with 2-mm electrode gap spacing.

Clonogenic assay results, showing the level of dilution required to exclude viable cells from a sample, and thus the number density of clonogenically viable cells are depicted in Fig. 3. These data show that for cells treated with 100 UWB pulses at 95 kV/cm peak electric field, in the presence of bleomycin, less than one cell in 10 000 was clonogenically viable. This represents a 1000-fold enhancement of electrochemotherapy over bleomycin alone.

The application of UWB electric field pulses dramatically increased the killing effect of a 5 μM bleomycin dose, resulting in a 1000-fold reduction in the number of viable cells capable of reproduction. Although much longer pulse trains were required to achieve this effect for UWB pulses compared to 100 μs pulses,[1] it was clear that for a pulse train resulting in slightly more than 50% killing, a very high degree of killing could be achieved with the addition of a very low concentration of bleomycin. This result suggested that the UWB pulses increased the permeability of the cell membranes to allow bleomycin to interact with cellular DNA.

Figure 4 directly compares the killing effects of trains of ten UWB pulses at varied electric field strengths on bleomycin-treated Jurkat cells. Viability was assessed by trypan blue staining at 24 h. These data indicated that a train of ten UWB pulses at 50 kV/cm caused sufficient electroporation to deliver a maximum lethal dose of bleomycin to cells from a 5 μM extracellular solution, and higher electric field strengths and longer pulse trains did not further enhance kill-

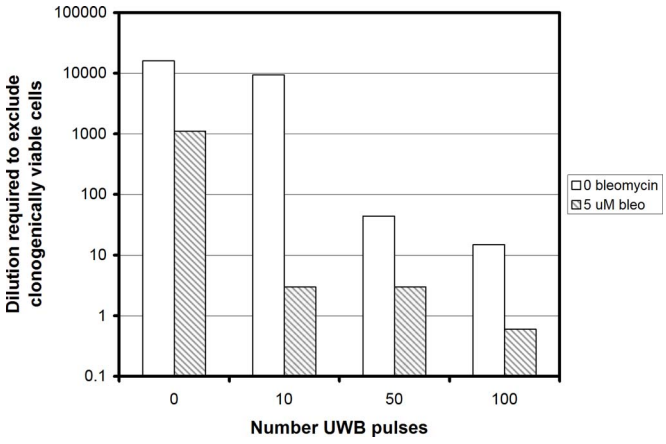

FIG. 3. Clonogenic viability (at 24 h postexposure) of Jurkat cells exposed to 95 kV/cm UWB pulse trains and/or 5 μM bleomycin.

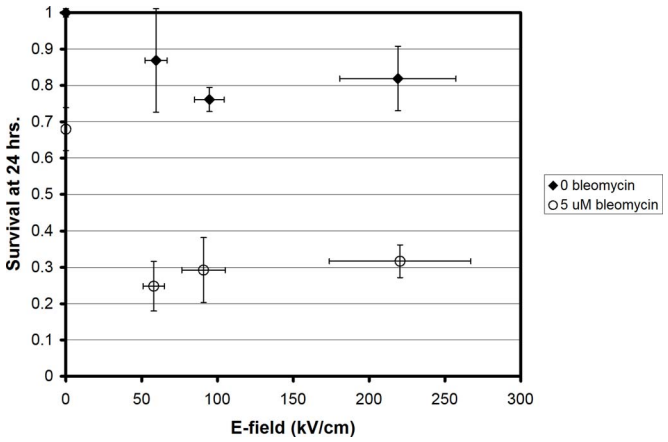

FIG. 4. Viability (at 24 h postexposure) of Jurkat cells exposed to single trains of ten UWB pulses with and without bleomycin for a range of peak electric fields (50–195 kV/cm).

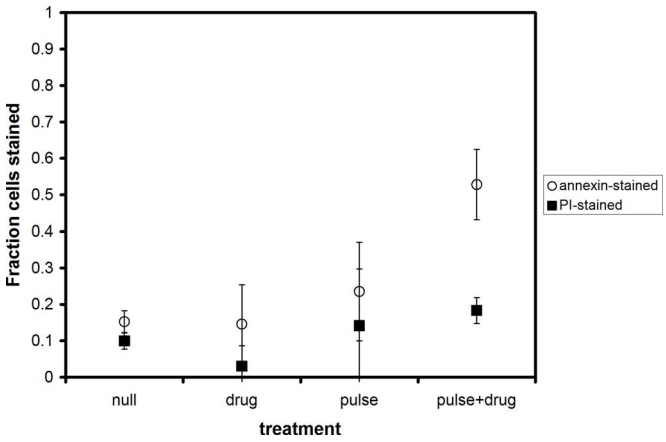

FIG. 5. Phosphatidylserine inversion on Jurkat cells 6 h after exposure to 5 μM bleomycin and/or a train of ten UWB pulses (50 kV/cm).

ing. Interestingly, these data also indicated that direct killing of Jurkat cells by trains of ten UWB pulses (without bleomycin) was not significantly improved by increasing the average peak electric field strength from 50 to 195 kV/cm, even though increasing the number of pulses (pulse train length) did markedly increase the cell killing (Figs. 2 and 3).

To better understand the biological mechanism by which cell death occurred in the experiments, exposed cells were screened for several features of apoptosis. Phosphatidylserine inversion on the plasma membrane and activation of caspases have been observed in mammalian cells exposed to nanosecond-duration pulses at fields of 50–200 kV/cm.[13,14] For the combined UWB/bleomycin treatment experiments, caspase activity was measured using the Promega (Madison, WI) Caspase-Glo 3/7 homogeneous luminescent assay for caspase-3 and caspase-7 activation. Phosphatidylserine (PS) inversion to the exterior of the plasma membrane is a standard cellular assay for apoptosis and was detected with the PS-binding protein Annexin as the fluorescent Annexin V-FITC (fluoroscein isothiocyanate) conjugate stain from Sigma (St. Louis, MO) in conjunction with propidium iodide (PI) to demonstrate cell viability via plasma membrane integrity.

The staining of treated Jurkat cells by Annexin V-FITC and PI is plotted in Fig. 5. At 6 h, none of the treatments caused a significant number of cells to lose membrane integrity, and the level of PI staining was not significantly different for control and treatment samples. However, the combined UWB pulse and bleomycin treatment resulted in a significant elevation of PS inversion and, consequently, Annexin V-FITC staining. As observed by fluorescence microscopy, approximately 50% of the cells in the assay sample expressed inverted PS at 6 h. It was noted that when separate aliquots from this treatment group were stained and observed at 24 h, approximately 50% of the cells stained positively for both PS inversion and loss of membrane integrity, suggesting that the cells that were killed by the combined treatment within 24 h were expressing the PS signal at 6 h.

A qualitative observation of the morphology of the cells was made by light microscopy at 6 h, and the proportion of cells exhibiting unusual morphology (swelling, micronuclei formation, shrinkage, and wrinkled appearance) agreed very closely with the Annexin V-FITC staining trend. Apparently, at 6 h, affected cells expressed both PS inversion on the membrane and other morphological changes in anticipation of cell death.

Figure 6 displays the results of the caspase activity assay. The assay produced light from a luciferase in the presence of activated caspase from cell lysate. Caspase activity was measured at 6 h after exposure of cells to bleomycin and UWB pulses. While a slight elevation of caspase was evident for the samples treated with ten UWB pulses, the difference was less than a two-fold improvement. For an enzyme activity assay, typically a 10–30-fold increase would be expected from a significant event or treatment. For the samples treated with 50 or 100 pulses, there was no significant change in caspase activity compared to the control samples.

IV. DISCUSSION

It appears that the cells that were killed by the combined treatment with bleomycin and UWB pulses progressed through a physiological cell death, rather than undergoing immediate death due to a direct effect of the UWB pulse treatment. The cell death featured the physiological PS inversion response and in most cases was likely complete within

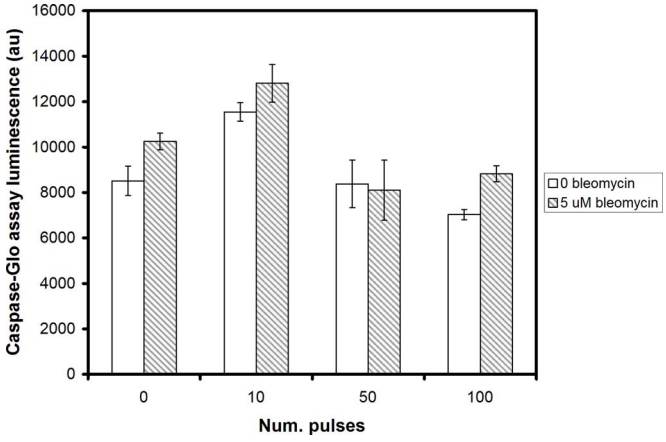

FIG. 6. Activation of caspase-3 and caspase-7 in Jurkat cells 6 h after exposure to 5 μM bleomycin and/or a train of ten UWB pulses (50 kV/cm).

about 24 h after the exposure, although the data suggest that some cells retained membrane integrity at 24 h but were not, in fact, clonogenically viable. This cell death did not exhibit activation of caspases, which is a prominent feature of apoptotic cell death. However, cells did exhibit morphological changes which were consistent with apoptosis.

It has been proposed[15] that although the duration of an UWB pulse is several orders of magnitude shorter than the charging time of a cell membrane, if a pulse of sufficiently high voltage is applied, a transmembrane voltage of 0.2–1 V may still be induced, giving rise to pores of ~1 nm in diameter. It is estimated that pores of roughly 1.5 nm in diameter would be required to admit bleomycin molecules, and it is likely that such a "supraelectroporation" mechanism was responsible for the increased action of bleomycin in cells subjected to UWB pulses. Furthermore, recent work with bleomycin electrochemotherapy[16] has revealed that there appear to be multiple regimes for bleomycin effects in cells, depending on intracellular concentration. In particular, the results reported by Tounekti *et al.* suggested that at low rates of bleomycin uptake, cells were able to repair the DNA damage caused by the drug, but above a threshold number of DNA double-strand breaks (DSB) or in the presence of a significant number of single-strand breaks (SSB), apoptosis occurred. It was also reported that above a second threshold, for very high numbers of DSB, cell death (termed "pseudoapoptosis") occurred featuring some, but not all, of the features of apoptosis. Similar results were observed[17] in experimental tumor model systems. This mechanism is supported by the data in the experiments reported here.

The majority of work reported to date on UWB and similar ultrashort electrical pulses has focused on the effects of coupling the electric field pulses to intracellular membranes[14] and modulating physiological functions in cells.[18] However, the effect of such UWB pulses on the plasma membrane has been noted but not previously investigated thoroughly. The present experiments have demonstrated that ultrashort pulses of 60 ns duration or greater are useful for modulating cellular uptake of very small molecular species in addition to the intracellular effects reported in previous studies. It is expected that such transmembrane uptake would be possible for even shorter pulses (approximately 10 ns in duration), although the electric field strengths required to achieve membrane permeabilization might be considerably higher than in the present study.

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