SHORT PAPER

Poly I:C Delivery into J774.1 & RAW264.7 Macrophages Induces Rapid Cell Death

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ABSTRACT

Background: Cytosolic double-stranded RNA (dsRNA) is an important 'molecular signature' for the detection of intracellular viral infections. Although intracellular dsRNA is a known potent inducer of apoptosis, the optimal time and dose for the onset of dsRNA-mediated apoptosis have not been studied in detail. **Objective**: To perform an accurate temporal assessment of the cell death responses in dsRNA-dependent cytotoxicity. **Methods**: Poly I:C (PIC), a synthetic dsRNA molecule was delivered intracellularly into J774.1 and RAW264.7 murine macrophages via electroporation. Cell viability was measured using the MTT assay and apoptosis was determined by sub-G0/G1 DNA content using flow cytometry. **Results**: Loss of cell viability was seen as early as 3h post-electroporation of macrophages. A significant increase in the sub-G0/G1 DNA content consistent with apoptosis was observed in PIC-electroporated macrophages as early as 3h post electroporation. **Conclusion**: Intracellular PIC delivery induces rapid macrophage cell death.

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Keywords: Apoptosis, Double-Stranded RNA, Intracellular, Macrophages, Poly I:C

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INTRODUCTION

Upon sensing of pathogen-associated molecular patterns (PAMPs) through germline-encoded pattern recognition receptors (PRRs), the host immune system will mount an appropriate response against the invading pathogen such as viruses (1). Viral nucleic acids such as double-stranded (ds) RNA are one of the PAMPs recognized by PRRs. For RNA and some DNA viruses, the cytosolic compartment in particular represents an essential subcellular niche in its life cycle. dsRNA is a replication intermediate for RNA viruses and may also be formed in an infection by DNA viruses where the genome is used so economically that both strands are transcribed (2). The host immune system has evolved specialized receptors to detect the presence of microbial dsRNA in the cytosolic space. Retinoic-acid inducible gene-I (RIG-I)-like receptors RIG-I and melanoma differentiation-associated gene 5 (MDA5) recognise cytosolic dsRNA to induce a classical antiviral type I interferon (IFN) response (3,4) as well as apoptosis (5,6).
Cell death is one of the most fundamental immune defense mechanisms in response to a viral infection (7,8). As a consequence, viruses have evolved proteins to block responses. Recognition of viral RNA not only results in a type I IFN response, but also apoptosis, programmed cell death. It is known from early on that dsRNA transfection of HeLa cells induces apoptosis (9). Later work elucidated this mechanism showing dsRNA transfection in HeLa cells triggering caspase 8 activation via the formation of a death inducing signalling complex (DISC) leading to apoptotic death (10). Other work has also demonstrated the apoptotic capacity of cytosolic dsRNA in leukemic cells (11-14) including our recent work where we elucidated the exact time of onset of dsRNA-mediated apoptosis in U937 leukemia cells (15). However, an accurate temporal and dose assessment dsRMA-mediated cell death has not yet been investigated in detail in immune cells including macrophages. Macrophages play a critical role in mounting innate immune responses against intracellular viral infection. In this short study, we show that cytosolic dsRNA induces macrophage cell death in a time- and dose-dependent manner, where the onset of apoptosis occurs as early as 3 h.

MATERIALS AND METHODS

Cell culture. The murine macrophage cell lines, J774.1 and RAW264.7, were obtained from the American Tissue Culture Collection (Manassas, VA). Cells were cultured in RPMI 1640 medium (Invitrogen, Grand Island, NY) supplemented with 5% foetal calf serum (Invitrogen, Grand Island, NY), 2 mM L-glutamine (Invitrogen, Grand Island, NY), 20 U/ml penicillin (Invitrogen, Grand Island, NY), and 20 μg/ml streptomycin (Invitrogen, Grand Island, NY) at 37°C in a humidified atmosphere with 5% CO2.
Electroporation of nucleic acids into cells. Prior to electroporation, 4×10^6 cells/ml in medium were mixed with HEPES buffer (pH 7.0) to a final concentration of 10 mM. Cells (250 μl containing 1×10^6 cells) were transferred into 4-mm gap electroporation cuvettes (Bio-Rad, Hercules, CA) together with various amounts of PIC (Sigma, St. Louis, MO) or calf thymus DNA (CT DNA) (Sigma, St. Louis, MO) that were previously prepared in sterile nuclease-free water. Sterile nuclease-free water without poly I:C or CT DNA was used as a negative control for transfection. Electroporation was performed at 240 V with a capacitance of 975 μF at room temperature in a Gene Pulser XCell™ (Bio-Rad, Hercules, CA).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. After electroporation, the electroporated cells and control cells were seeded in a 96-well tissue culture plate (Corning, Tewksbury, MA) at 1×10^5 cells/well in quadruplicate for each type of treatment conditions and incubated at 37°C. The MTT assay was performed after appropriate incubation times by addition of 100 µl of 1 mg/ml MTT (Sigma, St. Louis, MO) to each well, followed by 1 h of incubation. The product was solubilised by addition of 200 µl of 10% SDS and 50% isopropanol overnight. The next day, the absorbance of the wells at 570 nm was analysed spectrophotometrically in a BioTek ELx808 ELISA plate reader (BioTek, Winooski, VT).

Sub-G0/G1 DNA content analysis. At each time point post-electroporation, cells were pelleted at 700 g for 5 min, PBS-washed and resuspended in 70% ethanol for overnight fixation. Fixed cells were pelleted at 700 g for 5 min and resuspended in a DNA staining solution (38 mM Sodium citrate (Sigma, St. Louis, MO), 5 µg/ml RNase (Promega, Madison, WI) and 69 µM propidium iodide (Invitrogen, Grand Island, NY)). Cells were stained overnight at 4°C in the dark before analysing in a BD Accuri® C6 Flow Cytometer (BD Bioscience, San Jose, CA).

Results And Discussion

Cytosolic dsRNA is both a very potent cellular activator (i.e. IFN and cytokines) and cell death stimulant. Although cytosolic dsRNA is a known apoptotic stimulant, we wanted to further investigate the onset of cell death. The synthetic analogue, PIC, is used to mimic the effects of dsRNA. We chose electroporation as the main transfection method as this method would ensure the direct introduction of molecules, such as nucleic acids into the cytosolic space (16). To assess the effects of various doses of PIC on macrophage cell viability over time, the cells were electroporated with or without various doses of PIC and then analysed by MTT assay, a colorimetric assay that measures the metabolic activity of cells, at various time points. PIC-mediated cell death as seen as early as 3 h post-electroporation and maximal at 6 hr post-electroporation in both J774.1 and RAW264.7 cells (Figure 1). In addition, the relative cell viability was higher using 10 µg PIC compared with 1 µg PIC at 6h post-electroporation (Figure 1). Electroporation of calf thymus DNA (CT DNA), to mimic the effects of foreign dsDNA, resulted in the cell death of J774.1, but not RAW264.7 cells (Figure 1). This was not surprising as cytosolic dsDNA is known to induce rapid pyroptotic cell death via activation of the AIM2 inflammasome complex (17,18). Given that RAW264.7 cells lack the adaptor protein essential for inflammasome responses, apoptosis associated speck-like protein containing a carboxyl terminal CARD (ASC) (19), these cells are resistant to dsDNA-mediated toxicity (20). We show PIC-mediated toxicity to be comparable between J774.1 and RAW264.7 cells (Figure 1). Although this could suggest that ASC is not required for dsRNA-mediated cytotoxicity, we have yet to definitively demonstrate this. There is growing evidence to suggest that cytosolic dsRNA can trigger inflammasome responses (see review by Idris *et al.* (21)), potentially resulting in pyroptotic cell death. However, the current data is conflicting and inconsistent. Future work investigating dsRNA-mediated cell death responses in inflammasome component-deficient macrophages is critical to elucidate the role of inflammasomes in eliciting these responses.

The apoptotic effects of cytosolic dsRNA delivery is well known in a range of cell types (5,6,9-15).

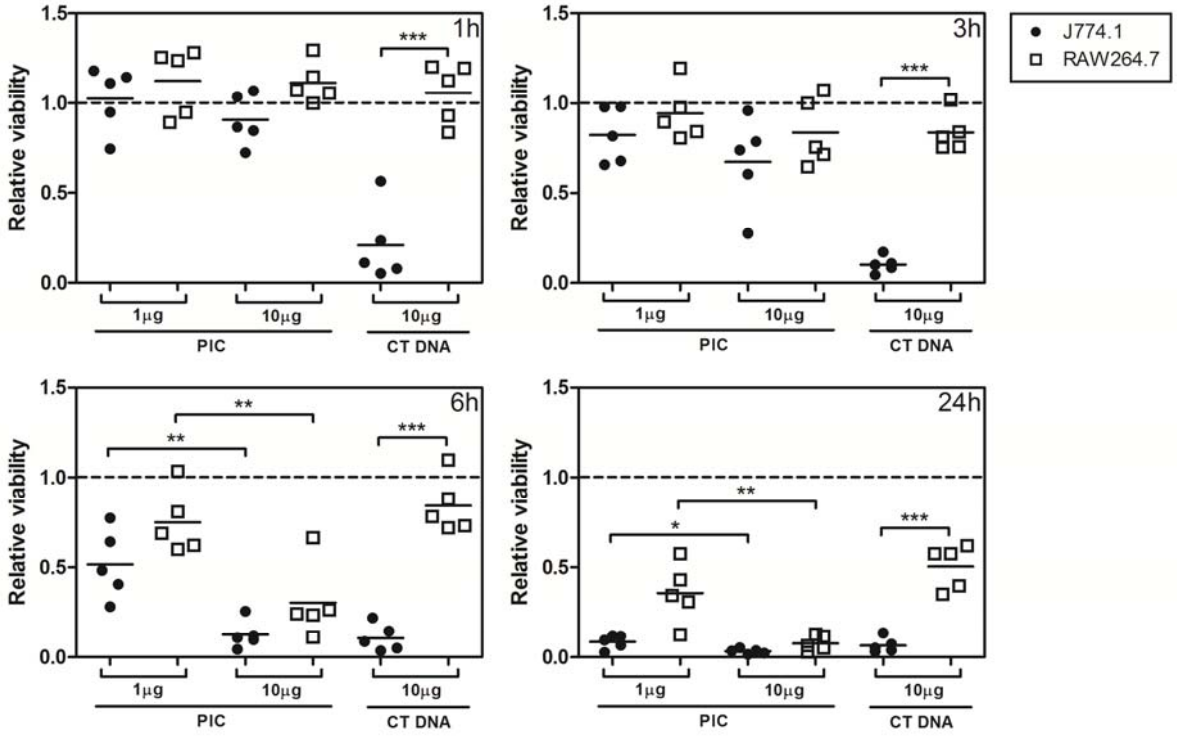

Figure 1. J774.1 and RAW264.7 murine macrophages die in response to PIC transfection in a time dependent manner. J774.1 and RAW264.7 cells were electroporated with indicated amounts of PIC or CT DNA. Cell viability was determined by MTT reduction, a measure of mitochondrial activity, at the indicated times post electroporation (1,3,6 and 24h). Results from each time point were normalised to samples electroporated alone (Control). The dotted line indicates no loss of viability relative to this control. Each data point shown is the result of an independent experiment and is the average of two separate electroporations within that experiment. *P<0.01, **P<0.001 and ***P<0.0001, Student's t-test.

However, the time onset of apoptosis in response to PIC is not well studied, let alone in macrophages. One major hallmark of apoptosis is cleavage of DNA. This can be measured by the appearance of cells with an amount of DNA less than that found in G0/G1 cells. Experimental conditions in dsDNA-electroporated primary murine macrophages to study apoptotic events via sub-G0/G1 DNA content analysis was previously optimised and found that the presence of sub-G0/G1 DNA corresponded with Annexin-V positive staining indicating apoptotic events (22). Compared to cells electroporated alone, analysis of DNA content showed sub-G0/G1 DNA, consistent with apoptosis appearing in PIC-electroporated J774.1 and RAW264.7 cells as early as 3h and reaching maximal levels at 6h (Figure 2).

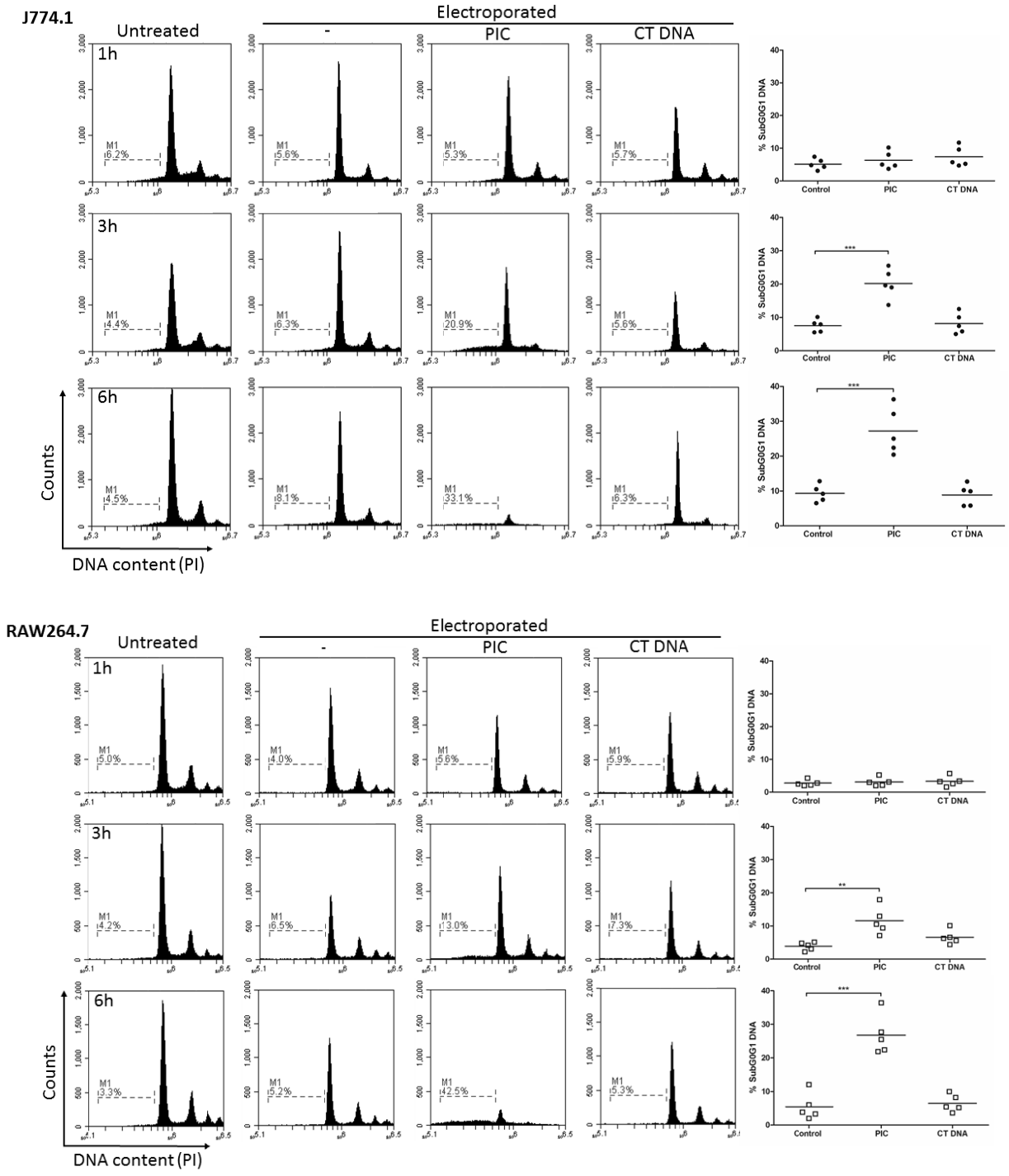

Figure 2. Cell death seen in PIC-transfected murine macrophages is consistent with apoptosis. J774.1 and RAW264.7 cells were electroporated with or without (- or control) 10 µg of PIC or CT DNA. Electroporated cells were assessed for sub-G0/G1 DNA content by flow cytometry at indicated times post electroporation (1,3 and 6h). Examples of primary flow cytometric data are shown, with a graph showing data from five independent experiments. **P<0.001 and ***P<0.0001, Student's t-test.

Sub-G0/G1 DNA content in CT DNA-electroporated RAW264.7 and J774.1 cells were comparable to that of cells electroporated alone suggesting that apoptosis was not induced in these cells (Figure 2). Electroporation of DNA with a similar dose of DNA (10 μg) in primary macrophages is known to induce pyroptotic cell death, rather than apoptosis, at early time points (18). However, from our experiments we cannot ascertain whether the cells are undergoing pyroptosis. More detailed assays (e.g. Annexin V/Propidium iodide staining and lactate dehydrogenase assay) have to be done in future studies to definitively show whether cells are undergoing apoptosis, as sub-G0/G1 DNA content analysis alone is not sufficient for this.

In summary, we show that PIC can induce macrophage cell death in a time- and dose-dependent manner and that PIC-mediated apoptosis was seen as early as 3 h post-electroporation. Future research in this field can contribute to the better understanding of how cells respond to an RNA virus infection. By understanding the onset of macrophage apoptosis in response to cytosolic dsRNA, we can devise early therapeutic interventions during the beginning stages of an RNA virus infection.

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