Vaccine 33 (2015) 2289–2296

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Nanoemulsion-based mucosal adjuvant induces apoptosis in human epithelial cells☆

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ARTICLE INFO

Article history:
Received 26 November 2014
Received in revised form 13 February 2015
Accepted 2 March 2015
Available online 25 March 2015

Keywords:
Caspases
Necrosis
Necroptosis
Pyroptosis
Autophagy
RPMI 2650
FaDu

ABSTRACT

Nanoemulsions (NEs) are adjuvants that enhance antigen penetration of the nasal mucosa, increase cellular uptake of antigens by both epithelial and dendritic cells, and promote the migration of antigen-loaded dendritic cells to regional lymph nodes within 24-h of vaccine administration. The objective of this study was to elucidate cell death caused by W_{80}5EC NE and identify caspases and genes associated with death pathways. Consistent with this aim, we show that exposure of human epithelial cells (EC), both RPMI 2650 and FaDu, to NE results in the activation of caspases (1, 3/7, 6, 8, and 9) and the expression of genes involved in apoptotic as well as authophagy and necrosis pathways. Interestingly, the NE activates caspase 8 which promotes "immunogenic apoptosis". The rescue assay was employed to investigate the fate of RPMI 2650 cells treated with W_{80}5EC NE. After four-hour treatment with as little as 0.03% of NE no cells were rescued at 72 h. Remarkably, immediately after four-hour treatment, the cells morphologically resembled untreated cells and most of the cells were alive. Altogether, these results suggest that NE induces death of human ECs through multiple pathways. Epithelial cell death caused by W_{80}5EC may have further implications on antigen uptake, processing, and presentation by DC's.

1. Introduction

An adjuvant is defined as a substance that accelerates, prolongs, or enhance antigen-specific immune response [1]. It stimulates the immune system to augment the response to a vaccine, without having any specific antigenic effect *per se*. Adjuvants may act in several ways including: enhancing the uptake of the antigen by APCs, progressive release and delayed clearance and thereby longer exposure of antigen to the immune system, and finally inducing the production of various cytokines [2–4]. However, the safety of an adjuvant is paramount before assessing the advantages of the immune enhancement they provide. Shoenfeld and Agmon-Levin recently coined the term "ASIA – Autoimmune/inflammatory Syndrome Induced by Adjuvants" [5]. The most frequently reported symptoms include myalgias, myositis, arthralgias, neurological manifestations, fever, dry mouth and cognitive alterations [6]. These symptoms have been linked to side effects of some adjuvants. Therefore, the study of new adjuvants should be carefully designed to evaluate long-term safety issues to exclude the possibility that genetic compositions, external or endogenous environmental factors and interactions between adjuvant and antigen may predispose to the emergence of an autoimmune or an auto-inflammatory syndrome [7]. To this end, studies on the interaction between new adjuvants and host cells are urgently needed.

Abbreviations: NE, nanoemulsion; PI, propidium iodide; PS, phosphatidylserine; XTT, cell proliferation assay kit.

☆ This project has been funded in whole or in part with Federal funds from the National Institute for Allergy and Infectious Disease, National Institutes of Health, Department of Health and Human Services, under Contract No. HHSN272200900031C and by Wroclaw Research Center EIT+ under the project "Biotechnologies and advanced medical technologies – BioMed" (POIG 01.01.02-02-003/08-00) financed from the European Regional Development Fund (Operational Programme Innovative Economy, 1.1.2).

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http://dx.doi.org/10.1016/j.vaccine.2015.03.002
0264-410X/

Nanoemulsions (NEs) are oil-in-water emulsions (~400 nm droplet size), prepared using surfactants, solvent, soybean oil and water that are non-toxic to mucosa at biocidal concentrations [8]. Recent studies have documented that NE can also be used as a mucosal adjuvant when mixed with antigens [8–15]. When administered together with antigen, NE induces both humoral and cell-mediated immune (CMI) responses and protects against challenge with a pathogen [9,12,13,15,16]. Furthermore, in addition to Th1/Th2 responses, NE has also been shown to stimulate a Th17 response [17]. Finally, NE adjuvant was well tolerated and enhanced immune responses in humans [18].

Previously, we documented that a NE-based vaccine induced antigen uptake and provided evidence that the resultant antigen-primed ECs were engulfed by phagocytic DCs [19]. We also observed that epithelial cells (EC) treated with NE became increasingly apoptotic and necrotic over time.

The manner in which a cell dies is important for host immunological responses. Antigen Presenting Cells (APCs) are expected to phagocytose apoptotic cells and present apoptotic cell-derived antigens to T cells [20]. Uptake of the dying cells depends predominantly on phosphatidylserine (PS) recognition [21] which serves as an "eat me" signal for phagocytes. Necrotic cells, on the other hand, serve as natural adjuvants, activating DCs by endogenous signals [22].

Here we undertook an investigation to further elucidate cell death caused by W_{80}5EC NE and to identify caspases and genes associated with these death pathways.

2. Materials and methods

2.1. Cells

RPMI 2650 (CCL-30), a human nasal septum squamous cell carcinoma cell line, and FaDu (HTB-43), a human pharyngeal squamous cell carcinoma cell line, were obtained from ATCC. Cells were maintained as recommended by ATCC. Cells were passaged two to three times per week to keep them in log phase growth.

2.2. Reagents

Staurosporine was purchased from Sigma–Aldrich (Roche Diagnostics GmbH), and the Annexin V kit from BioVision (Milpitas, CA). W_{80}5EC NE, an oil-in-water emulsion made from ingredients that are Generally Recognized As Safe (GRAS) was provided by the NanoBio Corporation (Ann Arbor, MI). Caspases 1, 6, 8, 9 and 3/7 were assessed using caspase kits (ImmunoChemistry Technologies, Bloomington, MN) and the CaspaTag Caspase 3/7 kit (EMD Millipore, MA), respectively.

2.3. Rescue assay

RPMI 2650 cells were cultured in 96-well flat bottom plates at a concentration of 3×10^3 cells/well. Eighteen hours later cells were treated with increasing concentrations of W_{80}5EC for 2–24 h. After each time point, treatment was removed and cells were washed with medium. Then cells were incubated for an additional 72 h and were subjected to the XTT assay (Life Technologies).

2.4. Evaluation of apoptosis and/or necrosis

For evaluation of early apoptosis, the annexin V (BioVision, Milpitas, CA) assay was employed as described previously [19].

2.5. RT-PCR analysis

Gene expression analysis was performed with real-time RT-PCR. Total RNA was isolated from RPMI 2650 cells untreated and treated with 0.05% W_{80}5EC, 1 μM staurosporine or 2.5 μM doxorubicin for 6 h using RNAzol®RT, as previously described [19]. RT-PCR analysis was performed using the RT^2 First Strand Kit, RT^2 Profiler ™PCR Array, a panel of 84 human genes associated with the human death pathway finder (Qiagen, PAHS-212A). Data analysis was performed using the DDC_t method [23].

2.6. Evaluation of caspases in RPMI 2650 and FaDu cells

RPMI 2650 and FaDu cells were cultured in 6-well plates, at a concentration of 2×10 cells/well in 2 ml medium. The next day, the cells were treated with W_{80}5EC, staurosporine or 1 mM H_2O_2 for 6 h. After the treatment all cells were harvested, washed, and stained using the caspase kits. To determine dead cells, the cells were stained with PI and analyzed using an EPICS XL MCL flow cytometer (Beckmann-Coulter) and Expo32 software or a BD LSRFortessa Cell Analyzer (BD Biosciences, Mississauga, ON).

3. Results

3.1. Effect of W_{80}5EC NE treatment on early apoptosis of RPMI 2650 cells

The W_{80}5EC-treated RPMI 2650 cells were stained with annexin V and PI and analyzed by flow cytometry. Six-hour treatment of RPMI 2650 cells with 0.04% W_{80}5EC resulted in approximately 3.3% early apoptotic cells (no change as compared to untreated control) and 10.9% late apoptotic cells (annexin V and PI fluorescence). In contrast, staurosporine-treated cells showed 16.1% early apoptotic cells and 7.4% late apoptotic cells (Fig. 1). These data are consistent with differing mechanisms of cytotoxicity to cells treated with W_{80}5EC and staurosporine.

3.2. W_{80}5EC NE treatment of RPMI 2650 cells induces activation of caspases

We tested the activation of initiator caspases: 1, 8, 9 and effector caspases 3/7, 6 (Table 1) in RPMI 2650 cells after treatment with increasing concentrations of W_{80}5EC NE for 6 h.

Caspase 1. In NE treated cells the activation of caspase 1 increased with increasing concentrations of NE reaching plateau at a concentration of 0.03% (Fig. 2A). At the highest concentration tested (0.07%) the percentage of cells expressing active caspase 1 tended to decline. Interestingly, the percentage of caspase 1/PI stained cells increased over the range of NE concentration (0.03–0.06%). The percentage of dead cells (exclusively stained with PI) increased at a concentration of NE higher than 0.06% (data not shown).

Caspase 3/7. Activation of caspases 3/7 showed a similar pattern as caspase 1 albeit to a lesser degree. Activation of caspases 3/7 reached plateau at a concentration of 0.03% after which the percentage of active caspase 3/7 positive cells gradually decreased. The percentage of caspase 3/7 and PI stained cells increased over the range of NE concentrations tested. The percentage of dead cells increased with increasing concentration of NE but never exceeded 10% of the treated cells.

Caspase 6. Almost the same pattern of caspase 6 activation was seen as for the activation of caspases 3/7 (Fig. 2A). At a concentration of 0.03% of NE, approximately 10% of cells expressed caspase 6. Higher concentrations of NE resulted in decreasing