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Nanoemulsion-based mucosal adjuvant induces apoptosis in human epithelial cells

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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-hours of vaccine administration. The objective of this study was to elucidate cell death caused by W₈₀5EC 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 autophagy 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 W₈₀5EC NE. After four hour treatment with as little as 0.03% of NE no cells were rescued at 72 hours. 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 W₈₀5EC may have further implications on antigen uptake, processing, and presentation by DC's.

Keywords

caspases; necrosis; necroptosis; pyroptosis; autophagy; RPMI 2650; FaDu

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.

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 W₈₀5EC NE and to identify caspases and genes associated with these death pathways.

Materials and Methods

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.

Reagents

Staurosporine was purchased from Sigma-Aldrich (Roche Diagnostics GmbH), and the Annexin V kit from BioVision (Milpitas, CA). W₈₀5EC 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.

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 W₈₀5EC for 2-24 hours. After each time point, treatment was removed and cells were washed with medium. Then cells were incubated for an additional 72 hours and were subjected to the XTT assay (Life Technologies).

Evaluation of apoptosis and/or necrosis

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

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% W₈₀5EC, 1 μ M staurosporine or 2.5 μ M doxorubicin for 6 hours using RNeasy[®] RT, as previously described [19]. RT-PCR analysis was performed using the RT² First Strand Kit, RT² 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_i Method [23].

Evaluation of caspases in RPMI 2650 and FaDu cells

RPMI 2650 and FaDu cells were cultured in 6-well plates, at a concentration of 2×10^5 cells/well in 2 ml medium. The next day, the cells were treated with W₈₀5EC, staurosporine or 1mM H₂O₂ for 6 hours. 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).

Results

Effect of W₈₀5EC NE treatment on early apoptosis of RPMI 2650 cells

The W₈₀5EC-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% W₈₀5EC 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 (Figure 1). These data are consistent with differing mechanisms of cytotoxicity to cells treated with W₈₀5EC and staurosporine.

W₈₀5EC 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 W₈₀5EC NE for six hours.

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 percentages of cells expressing caspase 6 which was paralleled by increasing percentages of caspase 6/PI and PI stained cells being observed.

Caspase 8—The highest percentage of cells expressing caspase 8 was reached at an NE concentration of 0.03% (Fig. 2A). The percentage of cells expressing caspase 8 was approximately two times higher (25%) than cells expressing caspases 3/7 or 6. Increasing percentages of caspase 8/PI and PI stained cells at higher concentrations of NE was observed, indicating that NE became cytotoxic.

Caspase 9—Activation of caspase 9 followed the same trend as observed for caspases 1 and 8. Fourteen percent of cells expressed caspase 9 was achieved at a concentration of 0.03% of NE (Fig. 2A). At concentrations of NE higher than 0.03% the percentage of the

cells expressing caspase 9 tended to decrease which was attended by an increased percentage of cells stained with caspase 9/PI and PI alone.

Figures 2B and C show cells treated with staurosporine and untreated cells, respectively.

Detection of caspases in FaDu cells treated with NE

To confirm that W₈₀5EC NE action is not exclusively specific to RPMI 2650 cells, FaDu cells were treated with increasing concentrations of W₈₀5EC and examined as to whether W₈₀5EC treatment induced activation of caspases 1 and 9. As shown in Figure 3, treatment with W₈₀5EC induced activation of caspase 1 and caspase 9 in 4.37% and 7.25% of cells, respectively. The percentage of caspase positive cells was NE concentration-dependent up to 0.03% of W₈₀5EC NE; at concentrations of NE higher than 0.03% caspase positive cells dramatically decreased due to the cytotoxic effects of NE inducing necrosis (data not shown).

RT-PCR analysis of genes involved in apoptosis induced by NE in RPMI 2650 cells

To distinguish between apoptotic, necrotic and autophagy events induced by NE in RPMI 2650 cells, we determined the expression profile of genes important for the mechanisms of cellular death. RPMI 2650 cells treated with either 0.05% W₈₀5EC, 2.5 μ M of doxorubicin (a death pathway inducer) or 1 μ M of staurosporine (apoptotic inducer) as well as untreated cells were evaluated using a panel of 84 human genes associated with the human death pathway finder. The data from Volcano plots for each treatment compared with untreated controls are shown in Table 2. W₈₀5EC NE activates pathways similar to both doxorubicin and staurosporine with the greatest involvement of apoptotic pathways and the presence of both autophagy and necrosis.

Cell salvage after NE treatment

To examine the fate of RPMI 2650 cells after NE treatment, the cell rescue assay was employed. Cells were treated with increasing concentrations of W₈₀5EC for 2, 4, 6, 8, and 24 hours, then cells were washed and incubated in fresh medium for an additional 72 hours. Cell viability was measured using the XTT assay. As shown in Table 3 cell survival was dependent on both the concentration of NE and treatment time. No cells were rescued after six-hour treatment with 0.03% of W₈₀5EC.

Discussion

Apoptosis is important in several immunological responses. In particular, APCs are thought to engulf apoptotic cells and present cell-derived antigens to T cells [20]. PS translocation in apoptotic cells is an important phagocytic signal for APCs [21]. Necrotic cells, on the other hand, serve as natural adjuvants to activate DCs by endogenous signals [22]. However, many recent studies have demonstrated that the mode of cell death does not impact uptake and presentation of cell-associated antigen by DCs or their maturation [24].

In our previous studies we observed that NE treatment of cells for time periods in the range of 24-72 hours resulted in high percentages of both late apoptotic and necrotic cells and only

8% of early apoptotic cells after 48 hour treatment [19]. In this study we used annexin V and PI to distinguish early and late apoptotic cells treated with NE for only 6 hours (Fig. 1). We observed that cells treated with increasing concentrations of NE became progressively double-stained (annexin V/PI). This observation indicates that most of the cells either succumb to apoptotic necrosis, oncotic necrosis, or both. This observation is consistent with data obtained by Lecoeur, et al [25], who documented that external PS exposure is not exclusive to apoptotic cells, but also occurs in oncotic cells. All combined data suggest that NE treatment may induce transient early apoptosis in NE treated cells which subsequently become late apoptotic and die.

Our earlier reports indicate activation of pro-apoptotic pathways in ECs by W₈₀5EC when evaluated both *in vitro* in cell based [19] and *in vivo* in mouse models [26]. In the present studies, the pathways for NE-mediated cell death were evaluated with respect to caspases 1, 3/7, 6, 8 and 9 using RPMI 2650 cells after treatment with increasing concentrations of W₈₀5EC. Cells were treated for 6 hours (longer exposure resulted in a significantly higher percentage of dead cells). Caspases classified as initiators and effectors of cell death (Tab. 1) were evaluated.

The treatment of RPMI 2650 cells with 0.03% of W₈₀5EC for six hours induced the highest activation of measured caspases in NE-treated cells with cell death never exceeding 6% (Fig. 2A). Interestingly, initiator caspases (1, 8 and 9) were activated in a higher percentage of RPMI 2650 cells than effector caspases (3/7 and 6), 14.2-25.4% versus 10.6-11.2%. Since activation of initiator caspases temporally precedes the activation of effector caspases during the course of apoptosis, it is possible that after activation of initiator caspases non-specific damage of NE to cells decreases the optimal activation of effector caspases. The relatively high percentage of cells expressing caspase 1 (>19%) supports this idea since caspase 1 is involved in pyroptotic [27] and necrotic cell death [28, 29]

Caspase 8 promotes “immunogenic apoptosis” and is one of the first enzymes to be activated in cells treated with FasL or TNF- α and regulates either cell death (apoptosis, necroptosis) or cell survival [30]. In the immunogenic apoptotic pathway, early activation of the endoplasmic reticulum (ER)-stress kinase PERK leads to phosphorylation of the translation initiation factor eIF2 α , followed by partial activation of caspase-8. Caspase-8-mediated cleavage of the ER protein BAP31 and conformational activation of Bax and Bak stimulates calreticulin that transmits the Golgi apparatus to be secreted by SNARE-dependent exocytosis [31]. Recently, it has been demonstrated that NEs elicit immunogenic apoptosis of nasal mucosal epithelial cells *in vivo* following nasal treatment in mice as determined *in situ* by probing for calreticulin [26].

Here, we probed for caspase 8 in RPMI 2650 cells treated with W₈₀5EC NE. After 6-hour treatment with increasing concentrations of NE we observed an NE-dependent increase in the number of cells expressing caspase 8. The higher concentrations of NE (>0.045%) inhibited expression of caspase 8, suggesting that these cells may die due to necrosis rather than apoptosis. The increasing number of PI positive cells supports this notion. Our observation is consistent with data obtained by others who documented that apoptotic and necrotic effects of emulsion-based adjuvants play a pivotal role in antigen delivery and

presentation [32-34]. The presence of dead or dying cells presumably generates danger signals to stimulate migration of APCs, facilitates antigen uptake, and induces the maturation of dendritic cells [20, 35].

Additionally, we have observed an NE dose-dependent increase in the percentage of caspase/PI stained cells. These observations suggest that the cells do not necessarily undergo a single death pathway during the course of NE treatment but may die by pathways associated with apoptosis (activation of caspases 3/7 and 8), pyroptosis and/or necrosis (activation of caspase 1) or necroptosis (activation of caspase 8). Our observations are consistent with the interpretation that emulsion-based adjuvants lead to apoptosis and necrosis [32-34]. These pathways likely contribute to the generation of danger signals, antigen uptake, processing, presentation and immunogenicity [20, 35, 36].

The human epithelial FaDu cells were used to confirm that NE-mediated cell death is not cell line specific. We examined whether NE induces activation of caspase 1 and 9 in these cells. As shown in Figure 3 W₈₀5EC NE induced activation of caspase 1 and 9 in 4.4% and 7.3% of cells, respectively. The percentage of cells expressing these caspases was much lower when compared to RPMI 2650 cells (19.5 and 14.4%, respectively). The variance in the percentage of caspase expressing cells is likely due to the different origin of these two cell lines but more studies are warranted to explain this phenomenon.

Our earlier reports indicate activation of pro-apoptotic pathways by W₈₀5EC when evaluated both *in vitro* [19] and *in vivo* [26]. In the present studies we aimed to distinguish between induction of apoptotic, necrotic or autophagy events induced by NE in ECs, using RT-PCR analysis. We observed that transcripts involved in apoptosis specifically include genes such as BIRC2 (c-IAP2), GADD45A, BIRC3 (c-IAP1); in apoptosis and autophagy such as TP53; in autophagy such as SQSTM1; and necrosis such as DENND4A. This observation indicates that W₈₀5EC activates pathways similar to both doxorubicin and staurosporine with the greatest involvement of apoptotic pathways together with the presence of both autophagy and necrosis. The data are consistent with caspase flow cytometry studies and further support the notion that NE treated cells may die by pathways associated with apoptosis and/or necrosis.

Finally, we examined the fate of RPMI 2650 cells after NE treatment using a rescue assay. The data suggest that cells treated with as little as 0.03% of W₈₀5EC for 4 hours were preset to die. Interestingly, they bore morphological resemblance to untreated cells and most of the cells were alive (cell death did not exceed 6% at the time of measurement) as analyzed using flow cytometry (Fig. 2A).

Our present data elaborating different types of cell death caused by W₈₀5EC NE is relevant especially in light of the observation that a failure of the immune system to remove apoptotic cells can lead to systemic lupus erythematosus (SLE) and other inflammatory diseases [37]. Moreover, research on Th17 cells has suggested a crucial role in autoimmunity [38]. In a previous study we documented that W₈₀5EC NE is a potent adjuvant, inducing strong Th1 and Th17 immune responses [17]. The Th17 response, while constituting a protective arm defending the body against various infections, also functions as

a double-edged sword constituting a risk factor that mediates the induction of autoimmune diseases [39]. Therefore, it is important to exclude the possibility that cell death caused by W₈₀5EC NE may be involved in development of Th17 response associated with autoimmune or chronic inflammatory diseases. The study using animal models that address the potential of W₈₀5EC NE in causing autoimmune and/or chronic inflammatory disease are warranted [40].

Overall, the observation that NE induces cell death after 4 hour treatment and cells dying through multiple pathways suggests that W₈₀5EC NE may have important consequences for augmentation of immune response *via* uptake and presentation of antigen by APC's [20, 35, 36].

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Abbreviations used in this article

NE	nanoemulsion
PI	propidium iodide
PS	phosphatidylserine
XTT	cell proliferation assay kit

The objective of this study was to elucidate cell death caused by W₈₀5EC nanoemulsion (NE) and identify caspases and genes associated with death pathways. Consistent with this aim, we show that exposure of human epithelial cells 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 autophagy and necrosis. Altogether, these results suggest that NE induces death of human ECs through multiple pathways. Based on our data, we consider that cell death caused by the NE adjuvant is critically associated with uptake, processing and presentation of antigen by DC's.

Fig. 1. Orzechowska et al. Nanoemulsion-based mucosal adjuvant induces apoptosis in human epithelial cells

Fig. 2A. Orzechowska et al. Nanoemulsion-based mucosal adjuvant induces apoptosis in human epithelial cells

Fig. 2B. Orzechowska et al. Nanoemulsion-based mucosal adjuvant induces apoptosis in human epithelial cells

Fig. 2C. Orzechowska et al. Nanoemulsion-based mucosal adjuvant induces apoptosis in human epithelial cells

Fig. 3.
Orzechowska et al. Nanoemulsion-based mucosal adjuvant induces apoptosis in human epithelial cells

Table 1
Brief characterization of caspases tested in current study

Caspase	Role	Function	References
1	Initiator	Induces necrosis or pyroptosis; activates IL-1 β	[1-3]
3	Effector	Activated by death ligand; interacts with caspases 8 and 9, similar to caspase 7	[4, 5]
6	Effector	Executes apoptosis; interacts with caspase 8	[6, 7]
7	Effector	Executes apoptosis; interacts with caspase 8, survivin, XIAP	[8-11]
8	Initiator	Initiates apoptosis induced by Fas and other apoptotic stimuli; interacts with many apoptosis-related proteins; is involved in alternative “immunogenic apoptotic” pathway	[12, 13]
9	Initiator	Activated by cytochrome C and apaf-1; interacts with caspase 8	[9, 11]

Table 2

Transcript activation for apoptosis, autophagy, and necrosis pathways in RPMI 2650 cells after exposure to W₈₀5EC, doxorubicin or staurosporine for 26 hours.

Treatment	Gene regulated	Apoptosis		Apoptosis and autophagy	Apoptosis and necrosis	Autophagy	Necrosis
		Pro-apoptotic	Anti-apoptotic				
0.05% W ₈₀ 5EC	Up	BIRC2 (c-IAP2), GADD45A,	BIRC3 (c-IAP1)	TP53	none	SQSTM1	DEND4A
	Down	BCL2L11, CASP1 (ICE)		CASP3	none	none	none
2.5 μ M Doxorubicin	Up	APAF1, CASP1 (ICE), GADD45A, NOL3, TNFRSF10A (TRAIL-R)	BCL2A1, (Bfl-1/A1), BIRC3 (c-IAP1)	CASP3, FAS (TNFRSF6, TNF	SYCP2	CTSB, IRGM, MAP1LC3, SNCA, SQSTM1	DEFB1, DPYSL4, FOXI1, JPH3, KCNIP1
	Down	none	none	none	none	none	COMMD4, PARP2
1 μ M Staurosporine	Up	BCL2L11, CD40LG (TNFSF5), TNFRSF10A (TRAIL-R)	BCL2A1, (Bfl-1/A1), MCL1	AKT1	SYCP2	CTSS, ESR1 (ER α), GAA, IFNG, INS	DEFB1, KCNIP1, MAG
	Down	none	XIAP	none	CYLD	none	DEND4, EIF5B

Table 3

Cell rescue assay. RPMI 2650 cells were treated with different concentrations of NE for 2, 4, 6, 8, and 24 hours. After the treatment cells were washed and incubated in NE-free medium for additional 72 hours then cultures were subjected to XTT assay. Background optical density (medium alone) at the wavelength of 492 nm was subtracted from optical density of the wells containing control and treated cells.

Concentration of NE (%) N=4	Time treatment (h)				
	2	4	6	8	24
	O.D. SE	O.D. SE	O.D. SE	O.D. SE	O.D. SE
0	N.D.	N.D.	N.D.	N.D.	1.07 +/- 0.03
0.005	0.74 +/- 0.01	0.76 +/- 0.02	0.68 +/- 0.02	0.56 +/- 0.01	0.33 +/- 0.01
0.01	0.64 +/- 0.01	0.57 +/- 0.01	0.41 +/- 0.03	0.21 +/- 0.02	0.03 +/- 0.01
0.02	0.47 +/- 0.07	0.14 +/- 0.01	0.02 +/- 0.00	0.01 +/- 0.00	0.00 +/- 0.00
0.03	0.13 +/- 0.01	0.01 +/- 0.00	0.00 +/- 0.00	0.00 +/- 0.00	0.00 +/- 0.01
0.04	0.05 +/- 0.00	0.00 +/- 0.00	0.03 +/- 0.01	0.01 +/- 0.00	0.01 +/- 0.00