Abstract: Vitamin E (VitE) is a potent antioxidant and contributes as an apoptosis inhibitor by preventing apoptotic death by suppressing cell membrane scrambling with phosphatidylserine translocation and caspase activities. Fas ligand (FasL) is well known to induce cell apoptosis. Activation of phosphoinositide 3 kinase (PI3K) signalling is stimulated by VitE. The present study addressed the effects of VitE on survival of mouse dendritic cells (DCs) and signalling molecules underlying. To this end, mouse bone marrow cells were isolated and cultured to attain bone marrow-derived DCs (BMDCs). The cells were treated with FasL in the presence or absence of VitE. Western blotting and FACS analysis were performed to determine expression of signalling molecules and their involvement in DC apoptosis. As a result, FasL treatment resulted in activation of caspase 8 and an increased number of Annexin V+ cells, the effects were significantly suppressed when VitE was present in the cell culture. Importantly, the anti-apoptotic effects of VitE were abolished by using pharmacological inhibition of PI3K signalling with LY294002. Our results showed that VitE inhibited FasL-mediated DC apoptosis through PI3K signalling, the effect is expected to facilitate the survival of DCs and promote the immune response against pathogens.

Keywords: Caspase, Dendritic cell; Fas ligand; PI3K and vitamin E.
1. Introduction

Dendritic cells (DCs) are the most potent antigen-presenting cells in the immune system by presenting pathogen-derived antigens to T cells to elicit the effective adaptive immune response [1]. The interaction of DCs with antigens induces activation and maturation of DCs, which are characterized by upregulations of costimulatory and antigen-presenting molecules as well as release of inflammatory cytokines. These mature DCs are then induced to undergo apoptosis and disappear from the lymph nodes (LNs) [2]. The apoptosis plays a major role in regulating and maintaining a balance between cell proliferation and cell death, therefore abnormal expression of apoptotic markers such as phosphatidylserine exposure and caspase activities, may lead to a variety of human pathologies. Therefore, the inhibition of DC apoptosis is thought to induce immune response against pathogens [1].

Fas ligand (FasL) is one of the members of the tumor necrosis factor (TNF) superfamily and induces apoptosis by cross-linking of the death-inducing receptor Fas [3]. FasL also triggers mitochondrion-dependent intrinsic apoptosis [4] that is regulated by Bcl-2 family proteins [5,6]. Increased expression of anti-apoptotic molecules and decreased expression of pro-apoptotic molecules block the mitochondrial depolarization [6] to promote cell survival. FasL involves not only in the regulation of cell survival but also in inflammatory response as it is a stimulator of functional and phenotypic maturation of immature DCs [7], whereas enhances the activation of regulatory DCs to induce immunosuppressive response [8].

Vitamin E (VitE) is the most important lipid-soluble antioxidant to protect cells from damage [9], whereas displays anticancer effects by inducing apoptosis and suppressing the growth of cancer cells [10]. VitE inhibits inflammation and exerts anticancer effects by promoting cell cycle arrest, differentiation, and apoptosis through activations of phosphoinositide 3 kinase (PI3K) and nuclear factor-κB (NF-κB) signalings [11,12]. Thus, VitE deficiency is associated with increased risk of cancer and dysfunction of the immune system [12]. The effect of VitE on apoptosis of DCs has not been reported, although pro-apoptotic effects of VitE on cancer cells are well documented. Thus, the present study explored whether VitE influences survival of DCs. To this end, bone marrow derived mouse DCs (BMDCs) were treated with FasL in the presence or absence of VitE and different hallmarks of apoptosis were determined.

2. Materials and Methods

2.1. Mice

BALB/c mice were purchased from Taconic Farms (Hudson, NY, USA) and housed in a specific pathogen-free facility at Institute of Genome Research. The animals had free access to food and drinking water. Animal care and experimental procedures were performed according to the Vietnamese law for the welfare of animals and were approved by the institutional review board of Institute of Genome Research.

2.2. Bone marrow-derived DCs

BALB/c mice were anesthetized with isoflurane gas and bone marrow cells were flushed out of the cavities from the femur and tibia with PBS. Cells were washed twice with RPMI-1640 and seeded out at a density of 4 x 10⁶ cells per 60-mm dish. Cells were cultured for 8 days in RPMI-1640 (GIBCO) containing: 10% FCS, 1% penicillin/streptomycin, 1% glutamine, 1% non-essential amino acids (NEAA) and 50µm β-mercaptoethanol. Cultures were supplemented with GM-CSF (35 ng/mL, Sigma Aldrich) and fed with fresh medium containing GM-CSF on days 3 and 6. Nonadherent and loosely adherent cells were
harvested after 8 days of culture. BMDCs were treated with FasL (500ng/ml, Sigma Aldrich) or LPS (100ng/ml, Sigma-Aldrich) in the presence or absence of VitE (α-tocopherol, 100µM, Sigma Aldrich). A PI3K pharmaceutical inhibitor LY294002 (100 nM, Sigma-Aldrich) was used to determine signaling molecules underlying FasL-induced DC apoptosis.

2.3. Immunoblotting

DCs (2 x 10⁶ cells) were washed twice in PBS, then solubilized in lysis buffer (Pierce) containing protease inhibitor cocktail (Sigma-Aldrich). Samples were stored at -80°C until use for western blotting. Cell lysates were separated by 10% SDS-PAGE and blotted on nitrocellulose membranes. The blots were blocked with 5% nonfat-milk in triethanolamine-buffered saline (TBS) and 0.1% Tween-20. Then the blots were probed overnight with monoclonal antibodies directed against either p-IκB-α or p-MAPKp38 or p-ERK1/2 or FasL or GAPDH (Cell signaling) diluted 1:1000 in blocking buffer, washed 5 times, probed with secondary antibodies (anti-mouse or anti-rabbit, GE healthcare) diluted 1:5000 for 1 h at room temperature and washed final 5 times. Antibody binding was detected with the enhanced chemiluminescence (ECL) kit (Amersham). Densitometer scans of the blots were performed using Quantity One (BioRad).

2.4. Caspase 8 activity assay

Caspase 8 activity was determined using a kit from Biovision according to the manufacturer’s instructions. Briefly 1x10⁶ cells were washed twice with cold PBS, fixed and permeabilized with ‘Cytotox/Cytoperm’ solution and then washed twice with ‘Perm/Wash’ buffer. Then cells were stained with FITC conjugated anti-active Caspase 8 antibody in ‘Perm/Wash’ buffer for 60 mins. After 2 washing steps, the cells were analyzed by flow cytometry (FACSARia Fusion, BD Biosciences).

2.5. Phosphatidylserine translocation

Apoptotic cell membrane scrambling was evidenced from annexin V binding to phosphatidylserine (PS) at the cell surface. The percentage of PS-translocating cells was evaluated by staining with fluorescein isothiocyanate (FITC)-conjugated Annexin V. In brief 5 x10⁵ cells were harvested and washed twice with Annexin washing buffer (AWB). The cell pellet was resuspended in 100 µl of Annexin-V-Fluos labelling solution (Roche) (20µl Annexin-V-Fluos labelling reagent in 1 ml AWB) and incubated for 15 min at room temperature. After washing with AWB, the cells were analyzed by flow cytometry.

2.6. Statistics

Data are provided as means ± standard error of the mean (SEM). All experiments were performed at least three times. Statistical significance was determined using Student’s two-tailed unpaired t-test or ANOVA. For all statistical analysis, *P < 0.05, **P < 0.01, and ***P < 0.001 were considered statistically significant.

3. Results

3.1. Effect of VitE on the expression of FasL and PI3K signalling in DCs

To explore the modulation effect of VitE on FasL expression, BMDCs were cultured with GM-CSF for 8 days and subsequently treated with VitE in the presence or absence of TLR4 ligand (LPS), which induces inflammation-mediated DC maturation and differentiation [13]. The results obtained in accordance with data of Jin et al. [14] that treatment of mouse DCs with VitE or LPS downregulated by 1.73- or 2.25-fold in the expression level of FasL protein, respectively. Importantly, there was further a 2.76-fold decrease of the FasL
expression in LPS-stimulated DCs when treated with VitE (Figure 1A-B).

Moreover, to examine expression of several signaling molecules in VitE-treated DCs, we observed that VitE enhanced the phosphorylation level of PI3K 1.8-fold (Figure 1A and C), but not p38MAPK and ERK1/2 in LPS-stimulated DCs (Figure 1A). The evidence suggested that PI3K activity might partially modulate DC survival upon VitE treatment.

![Figure 1](image)

**Figure 1.** Effect of VitE on the expression of FasL and PI3K signalling in DCs. (A) Original Western blot of DCs, which were either treated with LPS in the absence or presence of VitE or left untreated (control). Protein extracts were analyzed by Western blotting using antibodies directed to FasL, p-PI3K, p-p38 and p-ERK1/2. Protein loading was controlled by GAPDH antibody. (B-C) Arithmetic mean ±SEM (n = 4) of the abundances of FasL (B) and p-PI3K (C) proteins as the ratios of target proteins/GAPDH. *(p<0.05) represent significant difference from VitE-untreated DCs, ANOVA.

### 3.2. Effect of VitE on caspase 8 activity in DCs

The next experiments was performed to determine the effect of VitE on caspase 8 activation. Treatment of cells with FasL for 24h was followed by activation of caspase 8, the effect was significantly attenuated by 1.44-fold when the cells were exposed to VitE (Figure 2A-B). In addition, to ask whether the regulation of the caspase 8 activation is mediated via PI3K signaling, FasL-induced BMDCs were treated with LY294002 and followed by exposure to VitE for 24h. As noted in Figure 2A-B, the inhibitory effect of caspase 8 activity by VitE was abolished in the presence of LY294002, suggesting that activation of PI3K signaling contributed to the suppressing effect of FasL-induced caspase 8 activity by VitE in BMDCs.

### 3.3. Effect of VitE on cell membrane scrambling in DCs

The activation of caspases is expected to trigger DC apoptosis, additional experiments were performed to examine the effect of VitE on cell membrane scrambling reflecting PS exposure at the cell surface. The PS exposure was determined by annexin V binding. As illustrated in Figure 3A-B, exposure of DCs to FasL was followed by stimulation of annexin V binding, the effect was reversed by 1.72-fold when VitE was present in the cell culture. Similar to caspase 8 activity, the increased annexin V binding in FasL-treated DCs was also blocked by addition of LY294002 (Figure 3A-B). Therefore, the contribution of VitE on suppressing effect of increased number of apoptotic DCs triggered by FasL was sensitive to the activation of PI3K signaling.
Figure 2. Effect of VitE on caspase 8 activity in DCs. (A) Histograms of caspase 8 activity is obtained by FACS analysis in a representative experiment. (B) Arithmetic means ± SEM (n = 4) of the percentages of control-(white bar) and FasL-treated (black bars) DCs with activated caspase 8 are untreated (control) or treated with VitE in the absence or presence of LY294002. * (p<0.05) represents significant difference from FasL-treated DCs and # (p<0.05) represents significant difference from FasL and VitE-treated DCs, ANOVA.

Figure 3. Effect of VitE on cell membrane scrambling in DCs. (A) Histograms of annexin V binding as obtained by FACS analysis in a representative experiment. (B) Arithmetic means ± SEM (n = 4) of the percentage of control- (white bars) and FasL-treated (black bars) DCs with annexin V binding are untreated or treated with VitE in the absence or presence of LY294002. ** (p<0.01) represents significant difference from FasL-treated DCs and # (p<0.05) represents significant difference from FasL and VitE-treated DCs, ANOVA.

4. Discussion

The present study reveals that treatment of mouse DCs with FasL caused the apoptotic cell death as was evidenced by activation of caspase 8 and the augmented percentage of annexin V+ cells, these effects were reversed when VitE was present in the cell culture. FasL has previously been shown to trigger apoptosis in a wide variety of cells including cancer cells, macrophages, B and T lymphocytes [15-19]. The role of Fas signaling in the modulation of DC apoptosis remains to be elucidated. Several studies have indicated that the interaction
between Fas and FasL, results in activation of caspase cascade and suicidal cell death [17, 19], whereas the interaction with agonistic anti-Fas antibody leads to the apoptosis resistance in DCs [20]. Previous investigations on B cell and macrophages showed that apoptosis in these cells is triggered by Fas signalling [15,21]. Importantly, FasL was observed in this study to stimulate caspase 8 activity and PS exposure in mouse DCs (Figure 2-3). Differently, FasL induces cell apoptosis through other pathways such as JNKs/p38 MAPK [22] or protein kinase C [23].

Our recent study indicated that in DCs, VitE negatively regulates ROS formation through the presence of Klotho [24]. However, in that investigation we used LPS as an inhibitor of DC apoptosis and the signalling molecule linked to this physiological activity is NF-kB. In other study, VitE is indicated to induce activation of PI3K signalling pathway [10], which negatively regulates activation of DCs as well as pro-inflammatory response in these cells in the exposure to LPS [14]. Therefore we conducted experiments to determine the involvement between PI3K signalling and FasL-induced DC apoptosis. Consistently, we also showed that VitE enhanced activation of LPS-stimulated PI3K pathway (Figure 1). Importantly, we showed for the first time that the inhibitory effects of VitE on FasL-induced DC apoptosis were mediated through PI3K signalling (Figure 2 and 3). Differently, activation of STAT signaling facilitates survival as well as growth of cancer cells [25]. Clearly, the signaling of suicidal death is different between DCs and cancer cells, as cancer cells grow and divide in an uncontrolled manner [23] whereas mature DCs are induced to undergo apoptosis [2].

In conclusion, VitE inhibited FasL-mediated DC apoptosis through PI3K signalling, the effect is expected to facilitate the survival of DCs and promote the immune response against pathogens.

Acknowledgements

This research is funded by Vietnam Academy of Science and Technology for a project TDTBG0.00/21-23 under Part V grant number TDTBG0.05/21-23.

References

[1] J. Banchereau, R.M. Steinman, Dendritic cells and the control of immunity, Nature 392 (1998) 245-52.
[2] E. Ingulli, A. Mondino, A. Khoruts, M.K. Jenkins, In vivo detection of dendritic cell antigen presentation to CD4(+) T cells, J Exp Med 185 (1997) 2133-41.
[3] C. Yang, H.Z. Liu, Z.X. Fu, PEG-liposomal oxaliplatin induces apoptosis in human colorectal cancer cells via Fas/FasL and caspase-8, Cell Biol Int 36 (2012) 289-96.
[4] Q.G. Yan, J.G. Shi, F. Zhang, Q.T. Zhao, X.W. Pang, R. Chen, P.Z. Hu, Q.L. Li, Z. Wang, G.S. Huang, Overexpression of CYP2E1 enhances sensitivity of hepG2 cells to fas-mediated cytotoxicity, Cancer Biol Ther 7 (2008) 1280-7.
[5] A. Hamai, C. Richon, F. Meslin, F. Faure, A. Kauffmann, Y. Lecluse, A. Julli, L. Larue, M.F. Avril, S. Chouaib, M. Mehrpour, Imatinib enhances human melanoma cell susceptibility to TRAIL-induced cell death: Relationship to Bcl-2 family and caspase activation, Oncogene 25 (2006) 7618-34.
[6] S. Lucken-Ardjomand, J.C. Martinou, Regulation of Bcl-2 proteins and of the permeability of the outer mitochondrial membrane, C R Biol 328 (2005) 616-31.
[7] M. Rescigno, V. Pignut, B. Valzasina, S. Lens, R. Zubler, L. French, V. Kindler, J. Tschopp, P. Ricciardi-Castagnoli, Fas engagement induces the maturation of dendritic cells (DCs), the release of interleukin (IL)-1beta, and the production of interferon gamma in the absence of IL-12 during DC-T cell cognate interaction: a new role for Fas ligand in inflammatory responses, J Exp Med 192 (2000) 1661-8.
[8] C. Qian, L. Qian, Y. Yu, H. An, Z. Guo, Y. Han, Y. Chen, Y. Bai, Q. Wang, X. Cao, Fas signal promotes the immunosuppressive function of regulatory dendritic cells via the ERK/beta-catenin pathway, J Biol Chem 288 (2013) 27825-35.
[9] L. Bo, S. Jiang, Y. Xie, H. Kan, W. Song, J. Zhao, Effect of Vitamin E and Omega-3 Fatty Acids on Protecting Ambient PM2.5-Induced Inflammatory Response and Oxidative Stress in Vascular Endothelial Cells, PLoS One 11 (2016) e0152216.

[10] K.S. Ahn, G. Sethi, K. Krishnan, B.B. Aggarwal, Gamma-tocotrienol inhibits nuclear factor-kappaB signaling pathway through inhibition of receptor-interacting protein and TAK1 leading to suppression of antiapoptotic gene products and potentiation of apoptosis, J Biol Chem 282 (2007) 809-20.

[11] E. Pierpaoli, V. Viola, F. Pilolli, M. Piroddi, F. Galli, M. Provincialia, Gamma- and delta-tocotrienols exert a more potent anticancer effect than alpha-tocopheryl succinate on breast cancer cell lines irrespective of HER-2/neu expression, Life Sci 86 (2010) 668-75.

[12] A.A. Albaharni, R.F. Greaves, Fat-Soluble Vitamins: Clinical Indications and Current Challenges for Chromatographic Measurement, Clin Biochem Rev 37 (2016) 27-47.

[13] E. Shumilina, N. Zahir, N.T. Xuan, F. Lang, Phosphoinositide 3-kinase dependent regulation of Kv channels in dendritic cells, Cell Physiol Biochem 20 (2007) 801-8.

[14] X. Jin, L. Song, X. Liu, M. Chen, Z. Li, L. Cheng, H. Ren, Protective efficacy of vitamins C and E on p,p'-DDT-induced cytotoxicity via the ROS-mediated mitochondrial pathway and NF-kappaB/FasL pathway, PLoS One 9 (2014) e113257.

[15] B.C. Richardson, N.D. Lalwani, K.J. Johnson, R.M. Marks, Fas ligation triggers apoptosis in macrophages but not endothelial cells, Eur J Immunol 24 (1994) 2640-5.

[16] J. Tschopp, M. Irmler, M. Thome, Inhibition of fas death signals by FLIPs, Curr Opin Immunol 10 (1998) 552-8.

[17] J. Chung, Y.O. Yoon, J.S. Lee, T.K. Ha, S.M. Ryu, K.H. Kim, M.H. Jeong, T.R. Yoon, H.K. Kim, Inulin induces dendritic cells apoptosis through the caspase-dependent pathway and mitochondrial dysfunction, Biol Pharm Bull 34 (2011) 495-500.

[18] S. Kreuz, D. Siegmund, J.J. Rumpf, D. Samel, M. Leverkus, O. Janssen, G. Hacker, O. Dittrich-Breiholz, M. Kracht, P. Scheurich, H. Wajant, NFkappaB activation by Fas is mediated through FADD, caspase-8, and RIP and is inhibited by FLIP, J Cell Biol 166 (2004) 369-80.

[19] S. Buonocore, S. Van Meirvenne, F.X. Demoor, F. Paulart, K. Thielemans, M. Goldman, V. Flamand, Dendritic cells transduced with viral interleukin 10 or Fas ligand: no evidence for induction of allotolerance in vivo, Transplantation 73 (2002) S27-30.

[20] D. Ashany, A. Savir, N. Bhardwaj, K.B. Elkon, Dendritic cells are resistant to apoptosis through the Fas (CD95/APO-1) pathway, J Immunol 163 (1999) 5303-11.

[21] D. Ashany, X. Song, E. Lacy, J. Nikolic-Zugic, S.M. Friedman, K.B. Elkon, Th1 CD4+ lymphocytes delete activated macrophages through the Fas/APO-1 antigen pathway, Proc Natl Acad Sci U S A 92 (1995) 11225-9.

[22] S. Qi, W. Fu, C. Wang, C. Liu, C. Quan, A. Kourouma, M. Yan, T. Yu, P. Duan, K. Yang, BPA-induced apoptosis of rat Sertoli cells through Fas/FasL and JNKs/p38 MAPK pathways, Reprod Toxicol 50 (2014) 108-16.

[23] L.P. Eberl, G. Egidy, F. Pinet, L. Juillerat-Jeanneret, Endothelin receptor blockade potentiates FasL-induced apoptosis in colon carcinoma cells via the protein kinase C-pathway, J Cardiovasc Pharmacol 36 (2000) S354-6.

[24] N.T. Xuan, P.T. Trang, N. Van Phong, N.L. Toan, D.M. Trung, N.D. Bac, V.L. Nguyen, N.H. Hoang, N. Van Hai, Klotho sensitive regulation of dendritic cell functions by vitamin E, Biol Res 49 (2016) 45-54.

[25] M. Baskiewicz-Masiuk, B. Machalinski, The role of the STAT5 proteins in the proliferation and apoptosis of the CML and AML cells, Eur J Haematol 72 (2004) 420-9.