Requirement of Vav for anti-IgM-induced apoptosis and the decline in mitochondrial membrane potential in CH31 B lymphoma cells

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ABSTRACT

Engagement of membrane immunoglobulin (mlg) in B cells results in induction of self-tolerance probably through apoptosis or anergy unless provided with appropriate second signal(s) from T cells. Since anti-IgM induces apoptosis in CH31 cells, these cells would be a useful model to analyze B cell tolerance. Anti-IgM-induced apoptosis, accompanied by a reduction in mitochondrial membrane potential ($\Delta\Psi_{m}$), was preceded by tyrosine phosphorylation of several proteins including Vav. To address whether Vav is involved in anti-IgM-induced apoptosis, CH31 cells were transfected with expression vector pMKIT-Neo containing anti-sense vav, followed by selection in media containing G418. Three representative CH31 lines (CH31-13, 22, 24) deficient in Vav expression were assessed by apoptosis and $\Delta\Psi_{m}$ in response to anti-IgM. All the Vav-deficient lines failed to demonstrate apoptosis or a reduction in $\Delta\Psi_{m}$ after anti-IgM stimulation. Since the membrane IgM density was unaltered in the Vav-deficient lines compared with controls, the decreased induction of apoptosis was not attributed to a change in mlg density. All these findings suggest that Vav is involved in mlg-induced apoptosis in B cells.

Introduction

The interaction of self-antigen with corresponding B cells results in self-tolerance, which is mediated through induction of apoptosis or anergy in the context of development, activation stage of B cells, and antigen strength1-3. Because CH31 B lymphoma cells, representing immature B cells, demonstrated a transient growth arrest in the G1 phase accompanied by apoptosis upon engagement of membrane immunoglobulin (mlg), these cells would be suitable for analysis of B cell tolerance. Considerable evidence implicates mitochondria in apoptotic cell death in response to a variety of stimuli4. Cells have been demonstrated to undergo a reduction in mitochondrial membrane potential ($\Delta\Psi_{m}$), prior to onset of characteristic features of nuclear apoptosis (chromatin condensation and endonuclease-mediated DNA fragmentation) in a variety of cells including B cells5-8.

Engagement of mlg on B cells induces a tyrosine phosphorylation of a variety of proteins including Vav9. Vav family proteins consist of three members Vav, Vav 2, and Vav 3. Vav protein, expressed exclusively in hematopoietic cell lineages, functions as a GDP/GTP exchange factor involved in cytokine production, cytoskeletal reorganization, and proliferation of cells9. Vav becomes associated with Syk, She-Grb2, and Crk upon mlg ligation10,11. The tyrosine-phosphorylated Vav induced by lck or ZAP-70 had the capacity to provoke Rac-1 activation leading to the activation of c-Jun N-terminal kinase (JNK) signaling pathway in COS-7 and T cells12,13. Spleen cells from vav-/- mice exhibit-
ed an impaired proliferative response, accompanied by a lack of Ca\textsuperscript{2+} signal, following the stimulation of T and B cells by antigen\textsuperscript{1,2,20}. Moreover, a substantial defect in positive and negative selection of thymocytes has been found in vav- deficient mice\textsuperscript{1,2,20}.

To address whether Vav is implicated in antigen-mediated clonal selection in B cells, we established Vav-deficient cell lines from CH31 cells utilising a vector containing anti-sense vav. The Vav-deficient cells displayed an impaired response compared with wild-type (wt) cells, as assayed by apoptosis and \( \Delta \Psi \text{m} \). These observations would provide some understanding of mIg-induced apoptosis in B cells. Possible mechanism(s) for the antigen-induced apoptosis are discussed.

Materials and Methods

Cell line

B lymphoma cells CH31 (from Dr. Geoffrey Haughton, University of North Carolina, Chapel Hill, NC) were maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS), 50 \( \mu \)M 2-mercaptoethanol, 2 mM glutamine, and 100 \( \mu \)g/ml kanamycin at 37°C in humidified air with 5% CO\textsubscript{2}. Cells were stimulated with 1 \( \mu \)g/ml of anti-IgM (Bet-2)\textsuperscript{21}, a gift from Dr. William E. Paul (NIH, Rockville, MA), which was purified from ascites of scid mice by ammonium sulfate precipitation. The other reagents were of standard grade unless otherwise stated.

Construction of anti-sense vav plasmid

The mouse vav gene was amplified by reverse-transcription-polymerase chain reaction (RT-PCR) from Balb/C spleen total RNA with KOD polymerase (Toyobo, Tokyo, Japan) following the primers: forward, 5'-ATTCTCGAGAGAGGCCGGCAGCCA-CCATG-3'; reverse, 5'-GTCTTACAGGGCACGAGGCTCACGCAATATTCCGGA-3', generating fragments of 2575 bp. The sequence of the PCR products was confirmed to be homologous to the sequence reported previously\textsuperscript{22}. The wt vav cDNA were subcloned at XhoI in reverse orientation into the expression vector pMKIT-Neo, which was generously provided by Dr. K. Maruyama (Tokyo Medical and Dental University, Tokyo). Recombinant DNA was sequenced to confirm the fidelity of each construct.

Transfections and generations of stable cell lines expressing antisense vav

Cells were transfected by electroporation using a gene pulser (300 V, 960 \( \mu \)F) from Bio-Rad (Hercules, CA, USA) with 50 \( \mu \)g of either pMKIT-Neo-anti-sense vav, or vector-alone control (Neo) plasmids. Transfectants (5.0×10\textsuperscript{4} cells/well) were cultured in 96-well plates for 48 h and then selected in the RPMI-1640 medium with Geneticin (G418, 1 mg/ml) (Life Technologies, Inc., Grand Island, NY, USA). Several individual clones were obtained by limiting dilution. The level of Vav protein was determined by Western blotting, as described below.

Western blotting

Western blotting was done as described by Yanase et al\textsuperscript{23}. Briefly, samples were separated by 7.5% SDS/PAGE. Proteins were transferred to PVDF membranes (Millipore, Bedford, MA, USA), blocked with 5% non-fat dry milk, and then washed with PBS/0.5% nonfat dry milk/0.05% Tween 20. The membranes were blotted with mouse anti-Vav monoclonal antibody (mAb) (Upstate Biotechnology Associates, Lake Placid, NY, USA), followed by incubation with secondary horse-radish-peroxidase (HRP)-labeled goat anti-mouse IgG (Cappel Research Products, Durham, NC). After washing, membrane-bound HRP-conjugated Ab was visualized with enhanced chemiluminescence (Amersham Life Science, Buckinghamshire, UK).

Flow cytometric analysis of apoptosis, mitochondrial membrane potential, and membrane density of IgM and class II molecule

To evaluate apoptosis, hypodiploid DNA was analyzed using a flow cytometer (FACSCalibur, Nippon Becton Dickinson Company Ltd., Tokyo, Japan) using CELL Quest software (Becton Dickinson Immunocytometry System, San Jose, CA, USA), as previously described\textsuperscript{24}. To determine \( \Delta \Psi \text{m} \), fluorochrome 3,3'dihexyloxacarbocyanine iodide (DiOC \textsubscript{6}) (Molecular Probes, Eugene, OR) was used as previously described\textsuperscript{25}. Briefly, the cells stimulated with or without anti-IgM for various times were loaded with DiOC \textsubscript{6}, followed by analysis on a flow cytometer. As a positive control for dissipation of \( \Delta \Psi \text{m} \), cells in RPMI-1640 medium were incubated with an uncoupling reagent carbonyl cyanide m-chlorophenylhydrazone (mCICCP, 50 \( \mu \)M) (Sigma, St. Louis, MO, USA), a protonophore, which disrupts \( \Delta \Psi \text{m} \). To evaluate membrane density of IgM or MHC class II molecule, cells were stained with anti-IgM (Bet-2) or M5/114 (from American Type Culture Collection, VA, USA), followed by incubation with fluorescein-labeled mouse anti-rat k mAb (from Dr. William E. Paul; NIH, Rockville, MA, USA), and analyzed using the flow cytometer.
Results

Prevention of endogenous Vav expression through stable expression of vav anti-sense RNA in CH31 cells

Vav has been demonstrated to undergo tyrosine phosphorylation after anti-IgM stimulation in B cells. To assess whether anti-IgM-induced apoptosis involves Vav activation, a genetic approach was employed. CH31 cells were transfected with pMKIT-Neo-anti-sense-vav, a vector expressing anti-sense vav transcript using an electroporation method, followed by selection in G418 for 14 days. As a control for non-specific effect of the anti-sense plasmid backbone, the cells were transfected with pMKIT-Neo vector alone (Neo). To verify the capacity of the anti-sense vav to prevent Vav expression, the levels of endogenous Vav protein were determined by Western blotting. Three cell lines (CH31-#13, #22, #24) out of nine G418-resistant cell lines demonstrated a decreased level in Vav expression by more than 50% compared with controls (wt and Neo) (Fig. 1), and they were selected for further analysis.

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<th>Cell Lines</th>
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Fig. 1. Inhibition of endogenous Vav expression levels by plasmid encoding anti-sense message for vav. CH31 cells were transfected with pMKIT-Neo-anti-sense-vav or control Neo and selected with RPMI-1640 medium containing G418 (1 mg/ml), followed by limiting dilution to obtain an individual clone. Levels of endogenous Vav proteins in three clones and control Neo were determined by Western blotting. The Vav levels in the clones were expressed as multiples of the level from wt CH31 cells.

Failure to induce apoptosis in the CH31 cell lines deficient in endogenous Vav protein following anti-IgM stimulation

Anti-IgM causes CH31 cells to induce a transient growth arrest at G1, followed by apoptosis. Since cells transfected with vector alone displayed essentially identical results to wt cells as assayed by apoptosis, mitochondrial membrane potential, and mlg density (data not shown), the wt cells were used as controls for those with anti-sense vav. Apoptosis was assessed by the PI staining method. Approximately 38% of CH31 cells underwent apoptosis at 24 h after stimulation with 1 μg/ml anti-IgM, whereas a considerable degree of prevention of anti-IgM-induced apoptosis was found in all the Vav-deficient cell lines during 24 to 48 h (Fig. 2). These observations suggest that Vav appeared to be involved in anti-IgM-induced apoptosis in B cells.

Fig. 2. Anti-IgM fails to induce apoptosis in the Vav-deficient cells. The Vav-deficient (CH31-#13, #22, #24) or wt cells were assayed for apoptosis by PI staining method at 24 h or 48 h after stimulation with 1 μg/ml anti-IgM or medium alone. The percentage of apoptosis in cells cultured with medium alone was less than 5%.

Failure to induce a decline in mitochondrial membrane depolarisation in the Vav-deficient cells following anti-IgM stimulation

Anti-IgM-induced apoptosis has been shown to accompany a reduction in ΔΨm in B cells. To check ΔΨm, cells were loaded with amphiphilic cationic fluorochrome DiOC6, and retention of fluorescence was monitored in a flow cytometer. As expected, cells treated with the mitochondrial uncoupling agent mCICCP for 30 min displayed a decreased fluorescence compared with untreated control (Fig. 3A). Anti-IgM induced a reduction in ΔΨm (ΔΨm10%) in approximately 35% of cells at 24 h after stimulation, whereas the anti-IgM-induced reduction in ΔΨm was substantially prevented in the Vav-deficient cell lines (CH31-#13, CH31-#24) (Fig. 3B), suggesting the involvement of Vav protein in the mlg-induced mitochondrial change in B cells.
Requirement of Vav for anti-IgM-induced apoptosis

Unaltered expression of IgM and MHC class II molecules in the Vav-deficient Cells

To determine whether the failed anti-IgM-induced apoptosis and reduction in $\Delta\Psi$m resulted from an altered expression of membrane IgM in the Vav-deficient cells, the IgM densities in the cell lines were assessed using a flow cytometer. Comparable expression of IgM and MHC class II molecules was found between wt CH31 and the Vav-deficient line CH31-#24 (Fig. 4). Essentially similar profiles were obtained from two other cell lines, CH31-#13 and CH31-#22, except for somewhat enhanced expression of class II molecules in CH31-#13 cells (data not shown). These observations suggest that a decreased anti-IgM-induced apoptosis is not due to an altered membrane IgM density.

Discussion

The interaction of an antigen with mIg plays a crucial role in differentiation, proliferation, and apoptosis during B cell development, which processes are preceded...
ed by a complex biochemical signaling pathway through B cell receptor complexes. The engagement of mlg results in rapid tyrosine phosphorylation of several proteins including Vav protein. To address whether Vav is implicated in anti-IgM-induced apoptosis, a genetic approach using anti-sense vav transcript was used. The Vav-deficient cell lines were established from CH31 cells, which have been used as a model for analysis of antigen-induced apoptosis in B cells, through transfection with anti-sense vav plasmid, and were found to be defective in apoptosis induction following anti-IgM stimulation.

Vav, exclusively expressed in hematopoietic cells, is implicated in lymphocyte activation and cytoskeletal change. The spleen B cells from vav-/- mouse revealed a reduced proliferation and intracellular Ca²⁺ signal in response to an antigen, whereas mitogen or CD40-L-induced proliferation was intact, suggesting that Vav protein plays a crucial role exclusively on antigen receptor-induced signaling in B cells. Our observations suggest that Vav may be required for mlg-induced apoptosis as well as for proliferation. Indeed, the requirement of Vav for thymocyte selection has recently demonstrated.

Three lines with diminished levels of Vav expression, ranging from <10% to 50% compared to wt cells, showed a comparable degree of inhibition of apoptosis upon mlg ligation, suggesting that a moderate level of inhibition of Vav expression is sufficient to achieve a phenotypic change. Since the anti-IgM Bet-2 we used in this experiment showed a relatively low avidity to IgM, it is possible that a degree of mlg engagement is critical for the requirement of Vav. Indeed, proliferative defect in vav-/- B cells in response to non-repetitive antigen was partially restored by an increase in the degree of mlg cross-linking using repetitive antigen. These findings might reflect that Vav is associated with other molecules such as Lyn, Blink, and Crk upon mlg engagement, and delicate balance among these molecules may be important for adjusting the threshold for mlg-mediated signaling depending on an antigenic feature.

Mitochondria are thought to play a crucial role in executing apoptotic cell death in various cell types including B cells. We have recently shown that anti-IgM-induced reduction in Ψm occurred just before the onset of apoptosis, which latter was independent of the Fas-Fas-L signaling pathway. Both anti-IgM-induced apoptosis and mitochondrial membrane dissipation was substantially inhibited in Vav-deficient CH31 cells, further suggesting that Vav functions upstream of mitochondria responsible for mlg-induced apoptosis. Interestingly, Vav-deficient thymocytes are also resistant to peptide-induced apoptosis, acting up-stream of mitochondrial changes.

Vav has been demonstrated to lead JNK activation through Rac. The mlg-mediated sustained increase in JNK activity has been demonstrated to correlate with induction of apoptosis in mouse as well as human B lymphoma cells. Moreover, CH31 cells expressing dominant-negative (dn) JNK1 displayed a substantial degree of resistance to anti-IgM-induced apoptosis. The coincidence of the resistance to anti-IgM-induced apoptosis between the cells expressing dnJNK1 and Vav-deficient cells may imply that Vav-mediated activation of JNK is required for mlg-induced apoptosis in B cells. It is of course necessary to prove that the Vav activation results in activation of JNK in B cells. Interestingly, such an activation of Vav protein led to JNK activation in T and COS-7 cells.

The present study demonstrated that Vav appeared to be implicated in anti-IgM-induced apoptosis, probably upstream of mitochondrial change and has some implications for understanding of mlg-induced apoptosis in B cells.

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References

7) Takada E, Toyoda H, Suzuki J, Mizuguchi J: Prevention of anti-IgM-induced apoptosis accompanying Gl arrest in B lymphoma cells overex-
CH31 リンフォーマ細胞で観察される抗原受容体を介するアポトーシス・ミトコンドリア膜電位低下における Vav の役割

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【要旨】B 細胞は、T 細胞からのシグナルが存在しない状況下で、抗原受容体を介して指原シグナルを受け取るとアポトーシスを誘導する。"CH31 リンフォーマ細胞株は抗-IgM 抗体で刺激されると G1 期に停止し、その後ミトコンドリア膜電位の低下やアポトーシスに陥るので、B 細胞受容体を分子レベルで解析するモデルとして有用である。これらの生物学的に重要な面を考慮して、Lyn, Syk, Btk, Vav などの酪酸リン酸化が誘導される。我々は、アポトーシス誘導における Vav の役割を解析するため、CH31 細胞を MALDI-TOF 質量分析で同定した、pMKIT-Neo ベクターに vav 遺伝子をアンチセンス方向に導入したベクターを CH31 細胞に導入し、G418 含有培地で培養し、トランスフォーマントを得た。これらの薬剤耐性を示す細胞株の中からウェスタンプロット法を用いて、Vav タンパク質の発現レベルが低下している細胞株を 3 種類 (CH3113, CH3122, CH3124) 検出した。親株を抗 IgM で刺激するとミトコンドリア膜電位の低下およびアポトーシスが誘導されるが、アンチセンス Vav 遺伝子を導入した細胞株では、これらの変化は認められなかった。また、Vav 発現低下細胞株と親株との間には抗原受容体の発現レベルの差は認められなかったことより、このアポトーシス抵抗性は抗原受容体の膜電位の低下によるものではないと考えられ、以上より、抗原受容体を介するアポトーシスおよびミトコンドリアの膜電位の低下に Vav が関与しているということが示唆された。

<Key words> B 細胞、B 細胞抗原受容体、Vav、ミトコンドリア膜電位、アポトーシス