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Programmed Necrotic Cell Death Induced by Complement Involves a Bid-Dependent Pathway

Lea Ziporen,* Natalie Donin,* Taisia Shmushkovich,* Atan Gross,† and Zvi Fishelson2*

The membrane attack complex (MAC) of the complement system induces a necrotic-type cell death. Earlier findings suggested that Bcl-2 protects cells from MAC-induced necrosis. Here we examined the involvement of Bid, a proapoptotic protein, in MAC-induced cytotoxicity. Bid knockout (Bid−/−) mouse embryonic fibroblasts (MEF) and primary fibroblasts were damaged by complement but to a significantly lower extent than wild-type (WT) fibroblasts. Bid silencing with small interfering RNA duplexes led to elevated resistance of mouse fibroblasts, human K562, and Jurkat cells to lysis by complement. Bid−/− MEF were also resistant to toxic doses of streptolysin O, melittin, and A23187. Analysis of complement protein deposition on fibroblasts demonstrated that less complement C3 and C9 bound to Bid−/− than to WT cells, even though expression of the membrane complement inhibitors Crry and CD59 was relatively reduced on Bid−/− cells. Bid was rapidly cleaved in WT MEF subjected to lytic doses of MAC. Pretreatment of the cells with the pan-caspase inhibitor z-Val-Ala-Asp(OMe)-fluoromethylketone reduced Bid cleavage and cell lysis. These results indicate that complement MAC activates two cell death pathways, one involving caspases and Bid and one that is Bid-independent. The Journal of Immunology, 2009, 182: 515–521.

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2 Address correspondence and reprint request to Dr. Zvi Fishelson, Department of Cell and Developmental Biology, Sackler School of Medicine, Tel Aviv University, Tel Aviv, Israel; and Department of Biological Regulation, The Weizmann Institute of Science, Rehovot, Israel

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Materials and Methods

Cell cultures

SV-40-transformed wild-type (WT) and Bid knockout (KO) mouse embryonic fibroblasts (MEF) were provided by Stanley J. Korsmeyer (Harvard Medical School, Boston, MA). MEFs were grown in high-glucose DMEM (Sigma-Aldrich), supplemented with 10% heat-inactivated FCS (Invitrogen) and 100 U/ml penicillin, 100 μg/ml streptomycin (Bio-Lab), 2 mM l-glutamine, and 1 mM sodium pyruvate.

Primary mouse fibroblasts were isolated from Bid KO and WT (designated WT2) C57BL/6 neonates. Briefly, after sterilization with polylyne and ethanol, skins were separated and incubated with 2.5% trypsin overnight. Dermis was separated from epidermis and incubated 1 h at 37°C with...
NORMAL HUMAN SERUM (NHS) WAS OBTAINED FROM HEALTHY DONORS. HEAT INACTIVATION (HIS) OF NHS WAS PERFORMED BY INCUBATION FOR 30 MIN AT 56°C. HUMAN SERA WERE KEPT FROZEN AT −70°C IN SMALL ALIQUOTS AND THAWED ONLY ONCE. C8-DEFICIENT HUMAN SERUM (C8D) WAS PREPARED FROM A C8D PATIENT. PURIFIED HUMAN C2, FACTOR B, C5b,6, C7, C8, AND C9 PROTEINS AND C2- AND FACTOR B-DEPLETED HUMAN SERA WERE PURCHASED FROM COMPLEMENT TECHNOLOGY.

RABBIT ANTI-HUMAN/MOUSE BID AB WAS PURCHASED FROM SANTA CRUZ BIO-TECNOLOGY. MONOCLONAL MOUSE ANTI-ACTIN WAS PURCHASED FROM CHEMICON INTERNATIONAL. POLyclONAL ANTI-AMARIN DIRECTED TO K562 OR JURKAT CELLS WERE PREPARED IN RABBITS. POLYCLONAL ABs DIRECTED TO HUMAN C3 AND C9 WERE PREPARED IN GOATS. ANTI-MOUSE CD59a WAS PROVIDED BY PAUL MORGAN (UNIVERSITY OF WALEs COLLEGE OF MEDICINE, CARDIFF, U.K.) AND MONOCLONAL RAT ANTI-MOUSE CRY WAS PURCHASED FROM BD BIOSCIENCES. FITC-CONJUGATED ANTI-Mouse directed to mouse, rabbit, and rat IgG AND Peroxidase-conjugated goat anti-mouse IgG WERE PURCHASED FROM JACkSON IMMUNORESEARCH LABORATORIES. Peroxidase-conjugated goat anti-rabbit IgG AND RABBIT ANTI-GOAT IgG ABs WERE PURCHASED FROM SIGMA-ALDRICH. Z-VAD-FMK (PAN-CASpASE INHIBITOR) WERE PURCHASED FROM R&D SYSTEMS.

MEASUREMENTS OF NECROTIC CELL DEATH

CELL LYSIS WAS PERFORMED IN DULBECCO’S PBS CONTAINING 1 mM CaCl2 AND 1 mM MgCl2. FIBROBLASTS WERE HARVESTED BY TRYPsinIZATION AND LABELLED OR NOT WITH Na251CrO4 (DuPont Pharmaceuticals) IN CULTURE MEDIUM FOR 60 MIN AT 37°C. THEN THE CELLS WERE TREATED WITH NHS (AS A SOURCE OF COMPLEMENT) DILUTED IN PBS FOR 60 MIN AT 37°C. 51Cr-labeled CELLS WERE SEDIMENTED BY CENTRIFUGATION AND AN ALIQUOT OF THE SUPERNATANT WAS REMOVED FOR RADIOACTIVE COUNTING IN A γ-COUNTER. MAXIMAL 51Cr RELEASE (TOTAL CPM) WERE DETERMINED AFTER SOLUBILIZATION OF THE CELLS WITH 2% TRITON X-100. SPONTANEOUS 51Cr RELEASE WAS MEASURED WITH CELLS TREATED WITH HIS INSTEAD OF NHS. ALL EXPERIMENTS WERE CONDUCTED IN TRIPlicATES. RESULTS ARE EXPRESSED AS PERCENTAGE OF SPECIFIC LYSIS (51Cr RELEASE) CALCULATED AS FOLLOWS: [(EXPERIMENTAL CPM – SPONTANEOUS RELEASE CPM)/TOTAL CPM – SPONTANEOUS RELEASE CPM] x 100.

NECROTIC CELL LYSIS WAS ALSO MEASURED BY Trypan blue (0.1%) OR PROPIDiUM IODiDE (PI) INCLUSION AND YIELDED SIMILAR RESULTS TO THE 51Cr RELEASE ASSAY. FOLLOWING TREATMENT WITH COMPLEMENT, PI WAS ADDED TO A FINAL CONCENTRATION OF 0.5 μg/ml AND EDTA TO 1.5 mM. CELLS WERE KEPT ON ICE IMMEDIATELY EXAMINED IN A FACScan (BD BIOSciENCES). THE DATA WAS ANALYZED BY USING WINMDi 2.8 AND THE PERCENTAGE OF DEAD (PI POSITIVE) CELLS WAS CALCULATED. K562 AND JURKAT CELLS WERE INCUBATED WITH DILUTED RABBIT ANTI-K562 (1/20) OR RABBIT ANTI-JURKAT (1/100) ANTISERUM FOR 30 MIN AT 4°C AND THEN WITH COMPLEMENT (NHS OR HIS, 50%) FOR 60 MIN AT 37°C. CELL LYSIS WAS DETERMINED BY TRYPAN blue INCLUSION. THE MOUSE FIBROBLASTS WERE MORE SENSITIVE TO NHS THAN K562 AND JURKAT CELLS. THEREFORE, MEFS WERE TREATED WITH LOWER NHS CONCENTRATIONS (5–20%).

SIMILARLY, LYSIS OF CELLS BY SLO, MEltinIN, OR THE CALCIUM IONOPHORE A23187 IN PBS, AT VARIOUS CONCENTRATIONS, WAS MEASURED BY 51Cr RELEASE OR PI UPTAKE. SLO WAS PREACTIVATED BY TREATMENT WITH 10 mM DTt FOR 5 MIN AT ROOM TEMPERATURE. SPONTANEOUS RELEASE FOR THE SLO AND MEltININ ASSAYS WERE MEASURED IN CELLS TREATED WITH DTt OR PBS, RESPECTIVELY. CELLS WERE TREATED WITH THE CALCIUM IONOPHORE A23187 AT 25 μM IN DMSO FOR
60 min at 37°C. Spontaneous release for the A23187 assay was measured in cells treated with DMSO. Percentage of lysis was calculated as described above.

Analysis of cell lysates by Western blotting

Cell pellet (0.5 × 10⁶) was mixed with 20 μl of lysis buffer composed of 100 mM Tris (pH 7.5), 10 mM EDTA, protease inhibitor mixture (Sigma-Aldrich) and 0.7% Triton X-100. After three cycles of freezing and thawing, the cell lysate was subjected to centrifugation for 15 min at 14,000 g and the supernatant was collected for further analysis. Protein concentration was analyzed with the BCA protein assay kit (Pierce). Cell lysates (0.5 × 10⁶ cells/lane) were subjected to SDS-PAGE under reducing conditions (50 mM DTT), in a 12% acrylamide gel. The proteins were transferred onto a nitrocellulose membrane (Schleicher & Schuell Microscience). The membrane was blocked with 5% skim milk (Tnuva) in TBST (Sigma-Aldrich) for 1 h at room temperature. The membrane was treated with a specific first Ab and then with a peroxidase-conjugated secondary Ab. Bands were developed with an ECL reagent (Pierce) and exposed to a SuperRX film (Fuji).

Analysis of C3 and MAC deposition and complement regulators by flow cytometry

Fibroblasts were harvested by trypsinization, washed, and plated at 8000 cells/well in a 96-well plate. The cells were first treated with purified C5b,6 (1.5 μg) for 5 min at 37°C. C7 (1.5 μg) was then added for 15 min at 37°C. After a brief washing with PBS, the cells were treated with C8 (0.5 μg) and C9 (5 μg) for 15 min at 37°C. Cell lysis was measured by glucose 6-phosphate dehydrogenase release by using a Vibrant cytotoxicity assay kit (Invitrogen/Molecular Probes).

Silencing of Bid by RNA interference

A 25 nucleotides duplex oligonucleotide (Stealth siRNA, Invitrogen) specific to mouse Bid mRNA sequence 426 – 450 (5’-3’ sense: GGA GAA CGA CAA GGC CAU GCU GAU A (designated siRNA-426) and a similar small interfering RNA (siRNA) specific to human Bid mRNA (siRNA1: GGG AAG AAU AGA GGC AGA UUC UGA A) were used. A scramble control duplex oligonucleotide was used as negative control (5’-3’ sense: GCC UCC UAA CUA ACC AAG GGC GAA U). Before transfection, cells were grown for 24 h in DMEM without antibiotics. A day before the transfection, WT MEF were seeded in a 12-well plate.
Statistical analysis

Student’s paired t tests were used to determine the statistical significance of differences between various data sets. Results are expressed as arithmetic means and statistical significance was assumed when \( p < 0.05 \).

Results

Bid deficiency is associated with resistance to complement-mediated lysis

The involvement of Bid in complement-mediated lysis was investigated. MEF derived from WT or Bid KO mice were tested with complement and cell death was analyzed by measuring uptake of trypan blue (data not shown) and PI or by a \( ^{51} \)Cr release assay. As shown in Fig. 1, lysis of Bid \(-/-\) fibroblasts, both SV-40 immortalized (Fig. 1, A and C) and primary cells (Fig. 1B), was significantly lower than that of WT cells. Western blot analyses confirmed the absence of Bid in the corresponding cells (Fig. 1D). Our data indicated that lysis of the MEF by human complement was initiated via the classical complement pathway. This claim is supported by experiments showing that cell lysis was inhibited by Mg-EGTA (2.5 mM Mg\(^{2+}\), 10 mM EGTA) (percentage of lysis was reduced from 83.3 \( \pm \) 6.1 to 17.0 \( \pm \) 1.5, \( p < 0.05 \)) and in the absence of C2 (100% lysis by NHS vs 39.0% \( \pm \) 3.0 lysis by C2-depleted human serum, \( p < 0.05 \)). Restoration of C2-depleted serum with human C2 to its physiological concentration markedly increased MEF lysis. In contrast, factor B-depleted human serum induced the same level of MEF lysis as NHS (98.6% \( \pm \) 4.4 vs 99.3% \( \pm \) 3.5, respectively), and restoration with factor B had no effect on cell lysis. Blocking Abs anti-human MBL had no significant effect on MEF lysis (anti-MBL Abs: 93.7% \( \pm \) 4.6 vs control: 98.0% \( \pm \) 2.2).

Silencing of Bid expression in mouse fibroblasts and in K562 and Jurkat cells

The effect of Bid silencing in WT cells with a Bid specific siRNA on resistance to lysis by complement was tested. To this end, WT MEFs were transiently transfected with Bid siRNA-426 or a control scrambled siRNA. After 48 h, cell extracts were analyzed by Western blotting with anti-Bid Abs and Bid level was quantified after determination of band OD. The level of Bid protein in siRNA-426 transfected cells was found to be \( \sim \)35% of that in cells transfected with scrambled control siRNA (Fig. 1E). Bid and control siRNA-transfected cells were then tested for their sensitivity to lysis by complement (NHS) in a \(^{51} \)Cr release assay. As shown in Fig. 1F, reduction in the level of Bid was accompanied by a significant decrease in cell sensitivity to complement-mediated lysis. Similarly, K562 and Jurkat human tumor cells were treated for 48 h with siRNA directed to human Bid. Reduction in level of expression of Bid in these cells was accompanied by a decrease in the lysis of these cells by Ab and NHS (50%). For example, in a representative experiment lysis of K562 cells was reduced from 41.0 to 17.5% (\( n = 4, p < 0.05 \)) and lysis of Jurkat cells was reduced from 28.6 to 10.3% (\( n = 5, p < 0.01 \)).

Complement is activated more efficiently by WT than by Bid KO MEF

To compare the relative capacity of WT and Bid KO cells to activate complement, we studied deposition of C3 and C9 on cells treated with complement. To avoid excess cell death, the C3 deposition assay was performed with C8D. K562 cells (transfected with Bid or control siRNA) were treated with Ab and C8D and washed. Mouse fibroblasts (Bid \(-/-\) and WT) were similarly treated with human serum, as a source for complement, and washed. The cells were then treated with anti-C3 Abs and FITC-labeled second Abs and analyzed by Flow Cytometry. As shown in Fig. 2A, less C3 was deposited on K562 cells that were subjected to Bid silencing relative to cells transfected with control siRNA. Similarly, Bid \(-/-\) MEF bound less C3 than WT MEF (Fig. 2B). In addition, lysates of MEFs subjected to complement treatment were analyzed by Western blotting with anti-C3 or anti-C9 Abs. Complement activation is generating a C3 convertase that cleaves C3, thus leading to its activation and deposition of C3b onto the cell surface (1). Fig. 2C shows the cell bound C3b forms. As can be seen, on both WT and Bid \(-/-\) cells most of the bound C3b was immediately cleaved, probably by serum factor I, into iC3b, and the prevalent bands seen are the iC3b \( \alpha \)-, \( \alpha \)-2, and \( \beta \)-chains. Higher levels of C3b/iC3b were deposited on WT than on Bid \(-/-\) cells. C9 deposition was similarly analyzed in cells treated with normal human serum. As shown in Fig. 3A, larger quantities of C9 were seen on WT than on Bid \(-/-\) MEF. C9 deposition on WT cells was maximal after 10 min and then decayed, whereas on Bid \(-/-\) cells small quantities of cell bound C9 were observed only after 15 and 30 min. Formation of oligomerized, SDS-resistant poly C9 was not detected under the conditions of this experiment. Cell bound C5b-9 complexes were quantified by FACS with monoclonal anti-neo
conditions known to cause cleavage of Bid, fibroblasts were treated with NHS under
To determine whether or not complement-mediated cell death trig-
Complement induces a caspase-dependent cleavage of Bid
WT cells.

C5b-9 Abs (clone aE11). Larger amounts of C5b-9 complexes
Bid is required for cell death induced by pore formers
To rule out possible effects of serum proteins other than the
enlarged expression of the complement regulatory proteins on the

Cell resistance to complement-mediated lysis may result from
elevated expression of the complement regulatory proteins on the

To test whether the absence of Bid renders cells more resistant
to cell death induced by other pore formers and by a calcium iono-

Complement induces a caspase-dependent cleavage of Bid
To determine whether or not complement-mediated cell death trig-
gers cleavage of Bid, fibroblasts were treated with NHS under
conditions known to cause ~50% necrotic cell death and the level
of Bid within the cells was determined by Western blotting. The

full-length 22-kDa Bid was detected with the anti-Bid Abs (Fig.
As early as 5 min after mixture of the cells with complement,
Bid level dropped to ~10–20% of control (HIS treated cells). It
then remained at the same low level. Only at the 60 min time point
when the cells became necrotic and the actin level in them dropped
too, Bid level was reduced even further (Fig. 7A). Under sublytic
conditions (12% cell death), Bid level started dropping only after
15 min and after 30 min reached about half of the control level (not
shown).

The possibility that complement-induced Bid cleavage is
caused by caspases was examined next. As shown in Fig. 7B,
pretreatment of the cells with zVAD a pan-caspase inhibitor,
largely prevented Bid cleavage in cells treated with NHS. The
inhibitors acted more effectively on cells subjected to low (5%)
than to high (10%) NHS concentration. Interestingly, zVAD also reduced necrotic death of WT MEF by complement, but not death of Bid−/− MEF (Fig. 7C).

Discussion
The cause of necrotic cell death activated upon membrane insertion of the complement membrane attack complex is still not well characterized. Involvement of elevated intracellular calcium ion concentrations (19), mitochondrialdamage, and ATP depletion (20) has been implicated in the cell death. Results presented here demonstrate that Bid is involved in necrotic cell death induced by complement. Bid−/− fibroblasts are partially protected from complement-mediated cell death relative to WT cells. Furthermore, silencing of Bid in fibroblasts and in K562 and Jurkat cells with siRNA protects them from complement-mediated cell death. Cells lacking Bid are still lysed by complement, albeit to a lower degree, suggesting that complement MAC activates in these cells two distinct cell death pathways, a Bid-dependent and a Bid-independent pathway. It still remains to be determined whether the Bid-pathway is an independent pathway or an amplification pathway to the Bid-independent necrotic pathway. Interestingly, Bid-sufficient cells were better complement activators than Bid-deficient cells. Thus, cells lacking Bid deposited lower amounts of C3b and C5b-9 (MAC) on their surface. The level of the membrane regulators that inhibit complement deposition was apparently not elevated, and even reduced, on Bid−/− cells as compared with WT cells. This finding fits well with our earlier description of reduced deposition of C3b on Jurkat cells overexpressing Bcl-2 (13) and requires further investigation.

Involvement of Bid in complement-mediated lysis implies that at least partly complement MAC activates a programmed necrotic cell death. Programmed necrosis is a term introduced in recent years to indicate a type of cell death that depends on activation of an intracellular signaling cascade, much like apoptosis, but culminates in cell swelling, massive organelles damage and plasma membrane rupture (21–23). Thus, caspase-independent necrosis initiated via activation of Fas, TNF, or Fas-associated death domain protein was described and was shown to be dependent on the receptor-interacting protein kinase (24–27). Involvement of Bid in induction of necrosis has not been indicated before. Our results suggest that Bid may play a role in necrotic cell death induced not only by complement but also by other pore formers such as SLO and melittin and by toxic doses of calcium ionophores. Similarities between the lytic pathways induced by complement MAC, SLO, melittin, and calcium ionophore have been suggested (28, 29).

Upstream and downstream components of the necrotic Bid pathway are being looked for. Because Bcl-2 can confer on cells protection from the lytic action of complement (13), it is a candidate target for the action of Bid. Bcl-2 is known to regulate the permeabilization of the mitochondrial outer membrane in response to intrinsic apoptotic death signals (30). Bid-Bcl-2 binding has been shown to induce Bak- or Bax-mediated induction of apoptosis but so far not necrosis (31). Alternatively, Bid activated by complement or by other pore formers may acquire a necrotic form and act directly on intracellular organelles. In this case, Bcl-2 binding to the active Bid moiety will be protective and will inactivate Bid as it inactivates Bax and Bak (reviewed in Refs. 32 and 33). We propose that in cells attacked by complement, Bid, and/or Bid fragments inflict damage to certain intracellular organelle(s), leading to production of some toxic substances that trigger from within a necrotic-type cell death.

Activation of apoptotic Bid depends on its cleavage by caspase 8 or granzyme B, resulting in formation of an active truncated 15-kDa fragment (tBid) (34–36). tBid targets the mitochondria causing leakage of apoptogenic proteins. Caspase 2 (37) and calcium/calcipain (38) can also activate Bid’s proapoptotic activity by limited cleavage. In addition, full-length Bid can directly associate with the mitochondrial membrane and induce apoptosis (39). As shown here, complement-induced Bid cleavage and necrosis was attenuated by pretreatment with the pan-caspase inhibitor zVAD. This suggests that complement MAC-mediated activation of caspases is essential for the Bid-dependent necrotic pathway. The mode of caspase activation by complement in the studied fibroblasts is still not known. Caspase activation by complement MAC was shown to induce in rat mesangial cells apoptosis (12). In contrast, treatment of oligodendrocytes cultured in serum-free medium with sublytic doses of MAC led to inhibition of caspase 8 activation, reduced Bid cleavage, and protection from apoptosis (40). It emerges that different cell types respond differently to MAC inserted into their plasma membrane.
Disclosures

The authors have no financial conflict of interest.

References


