CD40- and HLA-DR-mediated cell death pathways share a lot of similarities but differ in their use of ADP-ribosyltransferase activities

Claire Leveille, Hakima Zekki, Reem Al-Daccak and Walid Mourad

Centre de Recherche en Rhumatologie et Immunologie, Le Centre Hospitalier de Quebec, Pavillon CHUL, 2705 Boulevard Laurier, T1-49, Sainte-Foy, Quebec G1V 4G2, Canada

Keywords: apoptosis, B lymphocytes, CD40, MHC class II

Abstract

CD40 and HLA-DR molecules are two major components of the immune system, and their engagement on several cell types leads to various cellular events that modulate cell function. In this study, we demonstrate that signaling via these molecules leads to a rapid B cell death. CD40-mediated cell death was mainly observed in Epstein–Barr virus (EBV)-transformed B cell lines, whereas, HLA-DR-induced response can be triggered in normal activated B cells as well as in EBV-transformed B cell lines. Cell death induced via both molecules does not require de novo protein synthesis, but involves the integrity of the cytoskeleton. The sensitivity of CD40- and HLA-DR-mediated cell death to various inhibitors is very similar to that previously reported for tumor necrosis factor receptor (TNFR)- and Fas-triggered apoptosis; however, caspases leading to poly(ADP-ribose) polymerase cleavage are not implicated in this response. Both B cell death forms do not involve Fas–Fas ligand and TNF–TNFR systems, but require LFA-1-independent cell–cell interactions mediated by still undefined molecules. Although CD40- and HLA-DR-mediated cell death appears to follow a common pathway, inhibitors of poly- and mono-ADP-ribosyltransferase activity differentially affect these responses. Defining the molecules involved in CD40- and HLA-DR-mediated death will provide a possible interrelation between the different B cell death programs that can lead to a better comprehension of regulation of B cell functions.

Introduction

The normal healthy immune system utilizes apoptosis to regulate the number of effector lymphocytes in the body, both prior and following exposure to foreign antigens. B cells in particular are subject to apoptotic cell death throughout their existence, although the receptors initiating apoptosis are somewhat characteristic of their stage of development (1,2). Classical triggers of B cell death include cross-linking of surface Ig, particularly in immature B cells (1–5), and ligation of CD95/Fas, particularly in activated B cells (6–10). Both forms follow well-established cell death programs sharing the specific morphological and biochemical traits including the decrease in cell volume, membrane blebbing, chromatin condensation and DNA cleavage into internucleosomal fragments (ladder).

Although the engagement of MHC class II molecules on human resting B cells and other cell types leads to their cellular activation (11), it has been recently reported that these molecules can also mediate cell death of human activated B cells (12,13). The signaling pathways involved in MHC class II-mediated cell death seem to involve some of the pathways implicated in other forms of B cell death such as protein kinase activation, Ca\(^{2+}\) mobilization and tyrosine phosphorylation of p72syk (14–16). In addition, this B cell death is also partially inhibited by the serine/threonine phosphatase inhibitor okadieic acid, indicating a signaling pathway that involves phosphorylation and dephosphorylation (13). CD40 belongs to the tumor necrosis factor receptor (TNFR) family and has an important role in B cell physiology. Similar to MHC class II molecules, positive (17) and negative signals have been reported upon CD40 ligation (18–21). Initially, signaling via CD40 has been reported as essential for B cell proliferation, differentiation and Ig isotype switching (22,23), and was latter demonstrated to have a role in the regulation of...
of B cell death (20, 21). Its engagement on immature B cells rescues from IgM-mediated death (24), whereas in mature B cells it increases cells’ susceptibility to Fas-mediated death by inducing Fas expression and/or sensitivity (7,9). It is worth noting that a similar inter-relations was reported between the MHC class II molecules and Fas, where ligation of the former increases B cell sensitivity to Fas-mediated apoptosis and/or induces Fas ligand (FasL) expression allowing the triggering of a Fas death program (14,25). Although, the cytoplasmic domain of the CD40 molecule does not contain a death domain like other members of the TNFR family, such as Fas and TNFR1, recent studies demonstrated that its engagement on transformed cells of mesenchymal and epithelial origin (26), on myelomas (21), and on B cell hybridomas (20) leads to cell death. The influence of transformation, maturation and activation status as well as the mechanisms underlying this CD40-mediated response are not yet established.

Based on various studies demonstrating a complementary and similarity between MHC class II- and CD40-mediated signaling (27,28), we designed these studies to investigate the interrelationship between CD40- and MHC class II-mediated cell death, and to determine their underlying mechanisms, in a panel of B cell lines presenting different maturation and/or activation stages. Our data indicate that CD40-mediated cell death occurs mainly in Epstein–Barr virus (EBV)-transformed B cell lines, while MHC class II-induced response can be triggered in activated B cells as well as transformed B cell lines. Although the cell death induced via both molecules shares a lot of similarities, they seem to follow distinct signaling pathways.

**Methods**

**Reagents**

Mouse anti-HLA-DR mAb L243, anti-CD18 mAb TS1/18, anti-CD40 mAb G28.5 and isotype-matched IgG1 mAb were purified from liquid ascites of hybridoma cells obtained from the ATCC (Rockville, MD). Anti-CD95 (Fas) mAb CH-11 was purchased from Upstate Biotechnology (Lake Placid, NY), neutralizing anti-Fas mAb ZB4 from Coulter/Immunotech (Coulter, FL) and neutralizing anti-TNF-α mAb from R & D Systems, (Minneapolis, MN). Mouse anti-poly(ADP-ribose) polymerase (PARP) mAb C2-10 was generously given by Dr G. Poirier (CHUL, Quebec, Canada). Purified goat anti-human IgM antibodies were from Sigma (St Louis, MO). Cycloheximide (CHX) (prepared in DMSO), cytochalasin B (20 mM stock in DMSO), EDTA, EGTA and DTT were purchased from Upstate Biotechnology (Lake Placid, NY), neutralizing anti-Fas mAb ZB4 from Coulter/Immunotech (Coulter, FL) and neutralizing anti-TNF-α mAb from R & D Systems, (Minneapolis, MN). Mouse anti-poly(ADP-ribose) polymerase (PARP) mAb C2-10 was generously given by Dr G. Poirier (CHUL, Quebec, Canada). Purified goat anti-human IgM antibodies were from Sigma (St Louis, MO). Cycloheximide (CHX) (prepared in DMSO), cytochalasin B (20 mM stock in DMSO), EDTA, EGTA and DTT were purchased from Sigma. Actinomycin D (10 mM stock in DMSO) was obtained from ICN (Montreal, PQ, Canada). The protease inhibitor DEX (100 mM stock in DMSO) was obtained from Calbiochem (San Diego, CA, USA). PMSF (250 mM stock in DMSO) from Sigma, TPCK (25 mM stock in ethanol) and TLCK (28 mM stock in methanol) from Boehringer Mannheim (Montreal, PQ, Canada), E-64 (10 mM stock in DMSO), calpain inhibitor II (50 mM stock in DMSO), pepstatin (50 mM stock in DMSO), N-ethylmaleimide (NEM) (400 mM stock in ethanol) and leupeptin (10 mg/ml in water) from ICN, and iodoacetic acid (prepared fresh in water) from Sigma. Caspase inhibitors YVAD-CMK, DEVD-CHO and Z-Asp-CH2-DCB were purchased from Bachem (Budendorf, Switzerland). 3-Aminobenzamide (3ABA), nicotinamide (NAM) and m-iodobenzylguanidine (MIBG) were from Sigma.

**Cell lines and cell culture**

Burkitt lymphoma Raji (EBV+), Ramos (EBV+) and pre-B cell line JM1 were obtained from the ATCC. The MHC class II- derivative of Raji, RM3, and LFA-1-deficient EBV-transformed B cell (LAD) (Dr R, Geha, Children Hospital, Boston, MA), EBV-transformed JY and LG2 cell lines (Dr J. Thibodeau, IRCM, Montreal, Canada) were also used in this study. All cells were cultured in RPMI 1640 (Celltech, Montreal, Canada) supplemented with 10% heat-inactivated FBS and penicillin/streptomycin (Life Technologies, Gibco/BRL, Burlington, Canada). Human tonsils were obtained from patients undergoing tonsillectomy and B cells were prepared as we previously described (29). CD40-activated normal B cells were generated by co-culturing peripheral blood B cells with L cells transfected with the CD40 ligand (CD40L) in the presence of IL-4 as previously described (30).

**Treatment procedures**

Cells were suspended at a concentration of 1×10⁶ cells/ml in RPMI supplemented with 10% FBS. The viability of the cells preceding treatment was confirmed to be >90% by Trypan blue exclusion. Aliquots (100 µl) of cellular suspensions were transferred to Titertubes and treated in triplicate with different antibodies or their isotype-matched controls for the indicated time periods. For Fas-induced cell death, CHX-treated (1 µg/ml) cells were incubated in the presence of anti-Fas (CD95) mAb CH-11 for 24 h in a 96-well plate. All treatments were carried out at 37°C in a humidified 5% CO₂ atmosphere. When the effects of different inhibitors were evaluated, drugs were added to the cells 1–3 h before the inducers of cell death. For the caspases inhibitors, cells were preincubated with inhibitors for 5 h before treatment with death inducers. Dissolve (DMSO, ethanol or methanol) had no measurable effect.

**Cell death assay**

Since cell death is accompanied by cell shrinkage and changes in membrane permeability, features of late stage apoptosis (31), cell death was evaluated by ethidium bromide uptake as previously described (12,32). Briefly, treated cells were resuspended and stained with 4 µg/ml ethidium bromide on ice for 5–30 min. The percentage of dead cells was then assessed by flow cytometry (FACSort; Becton Dickinson Canada, Mississauga, ON, Canada). Dead cells appeared as 90% by Trypan blue exclusion. Aliquots (100 µl) of cellular suspensions were transferred to Titertubes and treated in triplicate with different antibodies or their isotype-matched controls for the indicated time periods. For Fas-induced cell death, CHX-treated (1 µg/ml) cells were incubated in the presence of anti-Fas (CD95) mAb CH-11 for 24 h in a 96-well plate. All treatments were carried out at 37°C in a humidified 5% CO₂ atmosphere. When the effects of different inhibitors were evaluated, drugs were added to the cells 1–3 h before the inducers of cell death. For the caspases inhibitors, cells were preincubated with inhibitors for 5 h before treatment with death inducers. Dissolve (DMSO, ethanol or methanol) had no measurable effect.

**Western Blot analysis**

Western blot analysis was carried out to investigate PARP cleavage. Whole cell lysates were separated by 10% SDS-PAGE under reducing conditions, transferred to PVDF
CD40- and HLA-DR-mediated B cell death pathways share 721

Fig. 1. HLA-DR- and CD40-mediated cell death. (A) Representative experiment of HLA-DR-mediated cell death of the Raji cell line. Cells were stimulated with 5 μg/ml of anti-HLA-DR mAb or isotype control mAb for 1 h and cell death was evaluated by ethidium bromide uptake as described in Methods. (B) Raji cells triggered with variable concentrations of anti-HLA-DR mAb L243, 1 μg/ml of anti-CD40 mAb G28.5 or variable concentrations of anti-HLA-DR plus 1 μg/ml of anti-CD40 mAb G28.5 for 1 h. Cell death was then evaluated as above, and results are presented as percentage of specific cell death after subtracting the percentage of cell death detected with isotype control-treated cells that was always similar to the background level and represents <10%. Each point presents the mean of triplicates of a representative experiment where SD was always <5%.

Results

Ligation of CD40 triggers cell death in human EBV⁺ B cell lines

As previously reported (13), ligation of HLA-DR molecules on Raji cells induced a rapid cell death response that resulted in cell shrinkage and ethidium bromide uptake within 60 min of stimulation (Fig. 1A). Similar results were obtained using the Annexin V/propidium iodide assay (data not shown). To determine the effect(s) of CD40 engagement on HLA-DR-mediated Raji cell death, cells were treated with anti-CD40 mAb G28.5 in the presence or absence of different concentrations of anti-HLA-DR mAb L243. Stimulation with anti-CD40 mAb G28.5 alone induced a remarkable cell death (25%) and increased the percentage of anti-HLA-DR mAb L243-induced cell death response when mAb L243 was used at suboptimal concentrations, demonstrating an additive effect between these two antibodies (Fig. 1B). To further assess the potential role of CD40 in the induction of cell death, dose–response and time course studies were conducted with Raji cells. A significant cell death response (20%) was detected after 30 min of anti-CD40 mAb treatment and reached a maximum by 1 h (Fig. 2A). When the kinetics of CD40-induced cell death was compared to that of HLA-DR-mediated death, it was clear that both programs are triggered in the same time frame (Fig. 2B), but with different magnitude. Both CD40- and HLA-DR-mediated cell death responses are dose-dependent and the maximal response induced via HLA-DR required a larger amount of the antibodies than the CD40-mediated cell death response (Fig. 2C and D).

DNA strand break and/or cleavage into low mol. wt nucleosomes (ladder) are characteristics of classical apoptosis (33). TUNEL assay was conducted to determine the effect of CD40 and HLA-DR engagement on DNA strand breaks, whereas agarose gel electrophoresis was performed to visualize DNA...
CD40- and HLA-DR-mediated B cell death

Fig. 3. CD40- and HLA-DR-mediated DNA strand breaks. Representative experiment of CD40- and HLA-DR-mediated DNA strand breaks in Raji cells stimulated with isotype control (1 µg/ml), anti-CD40 mAb (1 µg/ml) or anti-HLA-DR mAb (2 µg/ml) for 1 h. DNA strand breakage was then evaluated by flow cytometry using the TUNEL assay.

fragmentation into low mol. wt fragments. Similar to HLA-DR-mediated death, CD40-mediated death presented DNA strand breaks (Fig. 3). However, the typical DNA fragmentation into low mol. wt nucleosomes was not detected in CD40-treated cells (data not shown), as it has been previously reported for HLA-DR-mediated cell death (13).

Together these data indicate that CD40, similar to HLA-DR, can transduce death signal and the observed additive effect of these two cell death programs suggests common and/or complementary pathways.

To determine the possible targets of CD40-mediated death, we used a panel of B cell lines of different phenotypes and at different maturation/activation status, as well as purified tonsillar B cells that represent a pool of heterogeneous B cells at different stages of activation. CD40-mediated cell death occurred in EBV+ B cell lines, including the LFA-1− EBV-transformed LAD cell line and the EBV+ BL Raji cells along with its MHC class II− derivative RM3 cells, but not in EBV− pre-B, Ramos and tonsillar B cells (Fig. 4). The CD40-sensitive cell lines, except the MHC class II− RM3 cells, also underwent cell death following treatment with anti-HLA-DR mAb L243 (Fig. 4). Tonsillar B cells were also sensitive to HLA-DR-mediated cell death, as it has been previously reported for activated splenic B cells (13). The sensitivity of RM3 cells to CD40 ligation indicates that the CD40-induced cell death program is independent from the expression of MHC class II molecules. Furthermore, the susceptibility of LAD cells (LFA-1− cell line) indicates that the activation of the LFA-1−ICAM system is not an absolute requirement for CD40- and HLA-DR-mediated cell death.

**EBV− B cells are resistant to CD40-mediated cell death**

The failure of certain B cell lines to undergo apoptosis upon ligation of their CD40 or HLA-DR molecules and the differential sensitivity of tonsillar B cells to both cell death triggers raised the possibility whether the cells’ activation status is important for the induction of these cell death pathways. To address this issue, Ramos cells were stimulated for 48 h with known triggers of B cell activation, then cell death was induced by anti-CD40 mAb G28.5, anti-HLA-DR mAb L243 or both of them. Activation of Ramos cells with anti-CD40 mAb alone or with phorbol myristate acetate overcame their unresponsiveness status to HLA-DR-mediated death but did not alter their resistance to CD40-induced cell death (Fig. 5A). Simultaneous treatment of these cells with both anti-HLA-DR and anti-CD40 mAb did not induce any significant increase in the percentage of cell death as compared to stimulation with
Fig. 5. Activation of Ramos cells renders them sensitive to HLA-DR- but not to CD40-mediated cell death. (A) Ramos cells were stimulated for 48 h with anti-IgM antibodies (10 µg/ml), anti-CD40 mAb G28.5 (1 µg/ml), anti-IgM + anti-CD40 antibodies (10 and 1 µg/ml respectively), anti-HLA-DR mAb L243 (1 µg/ml) or phorbol myristate acetate (10 ng/ml). Cells were then treated for 1 h with isotype control (10 µg/ml), anti-HLA-DR mAb (10 µg/ml), anti-CD40 mAb (1 µg/ml) or with anti-HLA-DR + anti-CD40 mAb. Cell death was then evaluated and results expressed as mean of the percentage of specific cell death of triplicate cultures. (B) Peripheral blood B cells from three different normal donors were activated in the presence of CD40L-transfected L cells and IL-4 as described (30). Activated B cells were collected and treated immediately or after overnight incubation in culture medium (rest), with anti-CD40 mAb G28.5 (1 µg/ml), anti-HLA-DR mAb L243 (10 µg/ml) or mouse IgG (10 µg/ml) for 1 h. Cell death was evaluated and results are presented as described above.

mAb L243 alone. It is worth noting that a large number of CD40 molecules on anti-CD40-pretreated Ramos cells were still free, because staining of these cells with FITC-labeled anti-CD40 mAb was only slightly decreased when compared to untreated cells. However, to confirm the absence of CD40-mediated death following pre-activation of Ramos via CD40, cells were co-cultured with CD40L-transfected fibroblasts for 32 h, and then were collected and triggered with anti-CD40 mAb G28.5 for 1 h. Although Ramos cells became sensitive for HLA-DR-mediated death, they remained unresponsive to CD40-mediated death (data not shown). As previously reported (24,34), Ramos B cells activated with anti-IgM antibodies undergo apoptosis and co-stimulation through CD40 rescues these cells from anti-IgM-induced cell death (Fig. 5A). Stimulation of anti-IgM-pretreated Ramos cells with anti-HLA-DR or anti-CD40 mAb did not increase the cell death response. However, anti-IgM-pretreated Ramos cells that were rescued by CD40 signal were very sensitive to HLA-DR-mediated cell death but remained resistant to subsequent CD40 stimulation. Together, these data indicate that, although activated Ramos cells are sensitive to HLA-DR-mediated cell death, they do not trigger cell death signal in response to CD40 ligation.

Following these observations, we wondered whether the activation of normal B cells can render them sensitive to CD40-mediated cell death. Purified peripheral blood B cells

Downloaded from https://academic.oup.com/intimm/article-abstract/11/5/719/710429/CD40- and-HLA-DR-mediated-cell-death-pathways-share by guest on 08 October 2017
(that are resistant to CD40- and HLA-DR-mediated cell death; data not shown) were activated in a co-culture system with CD40L-transfected fibroblasts in the presence of IL-4, as previously described (30). Cells were then collected, and either triggered immediately with anti-CD40 or anti-HLA-DR, or re-cultured, alone, in fresh medium (rest), overnight, and then triggered with anti-CD40 or anti-HLA-DR mAb L243 immediately after their activation in the CD40 system induced a significant cell death response which, in one case, was remarkably increased when CD40-activated cells were re-cultured overnight in fresh medium. This increase in the sensitivity following re-culture in fresh medium without IL-4 may result from incomplete depletion of IL-4 during the activation step. In contrast, anti-CD40 mAb G28.5 induced only a modest cell death response (7–12%) under both conditions. The level of CD40 expression is not a limiting factor, because activated B cells express a high level of CD40 that is similar to that detected in the investigated B cell lines (data not shown). These results confirm that HLA-DR-mediated B cell death is highly influenced by the activation status of B cells, while CD40-mediated death does not seem to be under the same influence.

**CD40- and HLA-DR-mediated cell death require LFA-1-independent cell–cell adhesion but do not involve Fas–FasL or TNF–TNFR systems**

Engagement of HLA-DR or CD40 molecules on human B cell lines leads to LFA-1-dependent and -independent homotypic cell–cell adhesion that can modulate cell function (35). Molecules involved in LFA-1-dependent adhesion are well known, whereas those involved in LFA-1-independent adhesion are not yet defined. The sensitivity of LAD cells to HLA-DR- and CD40-mediated cell death indicates that the observed cell death is not critically dependent on the activation of the LFA-1–ICAM system. To determine the role of the LFA-1-independent cell–cell adhesion in this response, cells were resuspended by pipetting for 10–15 s at 5 min intervals during stimulation to prevent their homotypic aggregation. Our results demonstrate that preventing prolonged cell–cell interactions completely abolishes HLA-DR- and CD40-mediated cell death in Raji cells (Fig. 6A) and in LAD cells (data not shown), indicating the necessity of cell–cell interactions for both forms of B cell death. As expected from these results, the cell death response of Raji and LAD cells to HLA-DR and CD40 mAb occurs more rapidly in cells stimulated in round-bottom tubes or wells than in cells incubated in flat-bottom wells (data not shown).

Controversial data have been reported concerning the role of Fas–FasL in HLA-DR-mediated cell death (16,25). Based on the requirement of cell–cell adhesion for CD40- and HLA-DR-mediated response, and the kinetics of the this response, one can favor the Fas–FasL involvement. To verify this issue, and to determine its implication in CD40-mediated cell death, Raji cells were stimulated with anti-HLA-DR and anti-CD40 mAb in the presence of the blocking anti-Fas mAb ZB4. The percentage of cell death following triggering of both molecules was not affected by the presence of the blocking anti-Fas mAb (Fig. 6B), indicating that Fas–FasL interaction is not involved in this CD40- and MHC class II-mediated cell death.

Similar results were obtained with LAD cells (data not shown). As expected from the sensitivity of LAD cells to both forms of cell death, anti-CD18 antibodies did not block HLA-DR- or CD40-mediated cell death. Stimulation of Raji (Fig. 6B) or LAD cells (data not shown) with HLA-DR or CD40 mAb in the presence of anti-CD40 or anti-HLA-DR mAb or re-cultured, alone, in fresh medium overnight, and then triggered with anti-CD40 or anti-HLA-DR mAb L243 immediately after their activation in the CD40 system induced a significant cell death response which, in one case, was remarkably increased when CD40-activated cells were re-cultured overnight in fresh medium. This increase in the sensitivity following re-culture in fresh medium without IL-4 may result from incomplete depletion of IL-4 during the activation step. In contrast, anti-CD40 mAb G28.5 induced only a modest cell death response (7–12%) under both conditions. The level of CD40 expression is not a limiting factor, because activated B cells express a high level of CD40 that is similar to that detected in the investigated B cell lines (data not shown). These results confirm that HLA-DR-mediated B cell death is highly influenced by the activation status of B cells, while CD40-mediated death does not seem to be under the same influence.

**CD40- and HLA-DR-induced cell death requires homotypic cell–cell interactions.** (A) Raji cells were treated with anti-HLA-DR, anti-CD40 or isotype control mAb, and were resuspended (R) by pipetting at 5 min intervals. Cell death was assessed by ethidium bromide uptake after 30 and 60 min of stimulation. Results are expressed as percentage of specific cell death as described in Fig. 1(B).

**Fig. 6.** CD40- and HLA-DR-induced cell death requires homotypic cell–cell interactions. (A) Raji cells were treated with anti-HLA-DR, anti-CD40 or isotype control mAb, and were resuspended (R) by pipetting at 5 min intervals. Cell death was assessed by ethidium bromide uptake after 30 and 60 min of stimulation. Results are expressed as percentage of specific cell death as described in Fig. 1(B).

Since TNF-α was recently shown to induce cell death in an EBV+ B cell line (36) and is known to be produced upon MHC class II ligation (37), we investigated its role in HLA-DR- and CD40-mediated cell death. Stimulation of Raji (Fig. 6B) or LAD cells (data not shown) with HLA-DR or CD40 mAb in the presence of neutralizing anti-TNF-α mAb did not affect either cell death responses. It is worth noting that blocking antibodies were used at concentrations capable of inhibiting Fas- or TNF-induced cell death (data not shown).

Together these data indicate that LFA-1-independent cell–cell interactions are required for CD40- and HLA-DR-mediated cell death; however, the Fas–FasL and TNF–TNFR systems are not involved in this response.
CD40-induced B cell death does not involve de novo protein synthesis but requires the integrity of the cytoskeleton and the presence of divalent cations

It was previously demonstrated that HLA-DR-mediated B cell death is independent of de novo protein synthesis, but requires the integrity of the cytoskeleton and the presence of divalent cations (13). Hence, to investigate the role of these factors in CD40-mediated cell death, Raji and LAD cells were first pretreated with various inhibitors, and then stimulated with anti-CD40 mAb. Treatment of LAD cells with actinomycin D or CHX did not have any effect on CD40-mediated apoptosis (Table 1), indicating that the CD40-mediated cell death program, similar to that triggered through HLA-DR, does not require de novo protein synthesis (38) and suggesting that both cell death programs utilize pre-existing apoptotic effector molecules. In contrast, the integrity of the cytoskeleton is required for CD40- and HLA-DR-mediated cell death, since both responses were completely abolished in cells pretreated with cytochalasine B (Table 1). Similar to HLA-DR-, CD40-mediated cell death was inhibited by Ca2+, Mg2+ chelators, with remarkable inhibition observed with 1 mM of the reducing agent and Zn2+ chelator DTT (Table 1), indicating that the divalent cations play a critical role in HLA-DR- and CD40-mediated cell death. Similar results were also obtained with Raji cells (data not shown).

Serine and cysteine proteases but not caspases are involved in HLA-DR- and CD40-induced cell death

Recent studies have enlightened the important role of proteases in the execution phase of apoptosis (39,40). The data presented in Table 1 demonstrate that the serine protease inhibitor DCI, and the serine/cysteine protease inhibitors TLCK and TPCK inhibited both cell death responses; however, DCI was less effective in abolishing the HLA-DR-mediated apoptosis. In contrast, the serine/cysteine protease inhibitors PMSF and leupeptine, and the aspartic protease inhibitor pepstatin did not have any effect on both CD40- and HLA-DR-mediated apoptosis. Only the cysteine protease inhibitor iodoacetic acid was able to abolish these cell death responses, while other known cysteine protease inhibitors, E-64 and NEM, and the calpain inhibitor, CI-II, did not have any effect. Although the use of protease inhibitors does not allow the elucidation of the type of proteases involved in the cell death process, the observed inhibitors’ effects suggest the involvement of serine and cysteine proteases in CD40- and HLA-DR-mediated cell death.

The involvement of the cysteine protease caspases in various models of apoptosis is well documented (41). Accordingly, the role of caspases in HLA-DR- and CD40-mediated death was evaluated using three well-characterized caspase inhibitors, the caspase-1 protease inhibitor Z-Asp-DCB, the caspase-3 protease inhibitor DEVD-CHO and the caspase inhibitor caspase-1 protease inhibitor Z-Asp-DCB (42). Cells were pretreated with caspases’ inhibitors and cell death was induced by incubating the cells with mAb L243 or G28.5 for 1 h, or with anti-Fas mAb CH-11 for 24 h, as a control. All three inhibitors did not affect either HLA-DR- or CD40-induced cell death (Table 1). In contrast, Fas-triggered apoptosis in Raji cells was completely inhibited by YVAD-cmk, the caspase-3 protease inhibitor DEVD-CHO and the caspase inhibitor Z-Asp-DCB (43,44). Thus, the caspases, at least those affected by the applied inhibitors, do not seem to be involved in CD40- or HLA-DR-mediated B cell death. Since PARP is a well-characterized cellular substrate of caspases (45), to further confirm the above observation, Z-Asp-DCB-treated and untreated cells were incubated with anti-HLA-DR, anti-CD40 or anti-Fas mAb and then the cleavage of PARP to its apoptotic fragment of 89 kDa was determined by Western blot analysis using PARP-specific antibody. Clearly, the cleavage of PARP was only observed during Fas-induced cell death and was prevented by Z-Asp-DCB (Fig. 7). Thus, the caspases leading to PARP cleavage are not involved in HLA-DR- and CD40-induced B cell death.

Table 1. Effects of various inhibitors on HLA-DR- and CD40-mediated cell death

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>HLA-DR%</th>
<th>CD40%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actinomycin (5 µg/ml)</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Cycloheximide (20 µg/ml)</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Cytochalasin B (20 µM)</td>
<td>85–100</td>
<td>90–100</td>
</tr>
<tr>
<td>EDTA (2 mM)</td>
<td>25–31</td>
<td>75–84</td>
</tr>
<tr>
<td>DTT (1 mM)</td>
<td>50–82</td>
<td>95–100</td>
</tr>
<tr>
<td>TLCK (100 µM)</td>
<td>40–73</td>
<td>39–50</td>
</tr>
<tr>
<td>TPCK (50 µM)</td>
<td>44–74</td>
<td>60–75</td>
</tr>
<tr>
<td>DCI (25 µM)</td>
<td>15–30</td>
<td>60–75</td>
</tr>
<tr>
<td>IA acid (80 µM)</td>
<td>45–65</td>
<td>55–75</td>
</tr>
<tr>
<td>E-64 (100 µM)</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>CI-II (100 µM)</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>PMSF (1 mM)</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>NEM (10 µM)</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Leupeptin (100 µM)</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Pepstatin (50 µM)</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>YVAC-cmk (600 µM)</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>DEVD-CHO (600 µM)</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Z-Asp-DCB (200 µM)</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>3ABA (20 mM)</td>
<td>60–77</td>
<td>NS</td>
</tr>
<tr>
<td>NAM (40 mM)</td>
<td>50–78</td>
<td>NS</td>
</tr>
</tbody>
</table>

*aResults are expressed as percentage of inhibition of specific cell death from at least three experiments.

bMaximal non-toxic concentrations.

NS: non-significant inhibition.

Fig. 7. HLA-DR- and CD40-mediated death do not induce PARP cleavage. Raji cells were either treated with caspase inhibitor Z-Asp-DCB (100 µM) or left without treatment for 5 h. Cell death was then triggered with anti-HLA-DR mAb L243 (10 µg/ml), anti-CD40 mAb G28.5 (1 µg/ml) or anti-Fas CH-11 (100 ng/ml) for adequate time periods. Cells were then lysed and immunoblotting was performed with specific anti-PARP mAb as described in Methods.
Differential inhibition of HLA-DR- and CD40-mediated cell death by 3ABA and MIBG

TNF-α- and Fas-mediated apoptosis is governed by the activation of caspases that leads to PARP cleavage (41,46). Inhibition of PARP activity by 3ABA, without affecting its cleavage, completely blocks TNF-α-induced apoptosis but failed to affect Fas-mediated response (47). Given this, and since PARP is not cleaved upon CD40 or HLA-DR ligation, we determined whether PARP activity is required for CD40- or HLA-DR-mediated cell death, using two different inhibitors, 3ABA and nicotinamide (NAM). Table 1 shows that both drugs had a potent inhibitory effect on HLA-DR- but were without any effect on CD40-mediated cell death. Increasing the concentration of 3ABA (40 mM) or decreasing the concentration of anti-CD40 mAb did not alter the resistance of the CD40-mediated cell death response to the inhibitory effect of these drugs (data not shown). Since 3ABA, in addition to its inhibition of the nuclear PARP activity, has an inhibitory effect on some mono-ADP-ribosyltransferase activity (48,49), the observed effect on HLA-DR-mediated cell death can be due to the inhibition of mono-ADP-ribosyltransferase. To clarify this issue, cells were pretreated with a specific inhibitor of mono-ADP-ribose transferase, MIBG, that inhibits the activity of arginine-specific mono-ADP-ribosyltransferase in intact cells (50) but does not affect PARP activity (51). The results presented in Fig. 8(A) show that 200 μM MIBG partially inhibited (43%) HLA-DR-mediated cell death in Raji cells. Increasing the concentration of MIBG to 400 μM resulted in 65% inhibition of HLA-DR-mediated cell death in Raji cells (data not shown) and 80% inhibition in LAD cells (Fig. 8B). Unexpectedly, MIBG also inhibited CD40-mediated cell death in Raji cells (Fig 8A) and in LAD cells (Fig. 8B). The ADP-ribosyltransferase inhibitors 3ABA or MIBG did not affect the viability of Raji or LAD cells, or the sensitivity of Raji cells to Fas-induced cell death (Fig. 8A). Thus, the common sensitivity of the CD40 and HLA-DR response to the mono-ADP-ribosyltransferase inhibitor, and their differential sensitivity to 3ABA suggests that different mono-ADP-ribosyltransferase activities are associated with HLA-DR- and CD40-mediated cell death.

Discussion

The regulation of B cell death plays an important role in the selection of antigen-specific B cells in the course of the humoral immune response, controlling B cells homeostasis and limiting their transformation. Multiple mechanisms, mediated by different cell surface molecules, are now described as regulators of B cell death (42,53). The data obtained in the course of the present investigation show that engagement of HLA-DR or CD40 molecules on several transformed B cell lines leads to rapid cell death that is mediated by the activation of a preformed death program as evidenced by its independence from de novo protein synthesis. The kinetics, the additive cell death response triggered by HLA-DR and CD40 mAb, and the protease inhibitor sensitivity profiles suggest that triggering of both molecules activates a common cell death system. This cell death system does not involve Fas–FasL, TNF–TNFR or LFA-1–ICAM interactions, but requires LFA-1-independent cell–cell attachment that is mediated by yet undefined receptor–ligand system(s). Although the activation of the caspases leading to PARP cleavage is not involved in CD40- and HLA-DR-mediated death, the activity of different mono-ADP ribosyltransferase is necessary for triggering these responses. Despite these similarities, CD40- and HLA-DR-mediated cell death are not dependent on the presence or the absence of one another. The HLA-DR-mediated response seems to target broader B cell populations than that of CD40 and the activation status seems to be the only limiting factor for HLA-DR-mediated cell death. The susceptibility of normal activated B cells and activated Ramos cells to the HLA-DR signal indicates that the cell death system and its signaling pathway are in place and functional, while the resistance of these cells to CD40-triggered death suggests that CD40 is not linked to the HLA-DR cell death program. The CD40-mediated response was mainly observed in EBV-transformed B cell...
lines and the EBV+ Burkitt’s lymphoma Raji; however, whether this response is influenced by the transformation status of the cells and/or the presence of EBV-encoded proteins is not yet clear. Recent observations demonstrating that the engagement of CD40 on transformed cells of mesenchymal and epithelial origin (26), on myelomas (21), and on B cells hybridomas (generated by fusing activated B cells with SPAZ-4) (20) leads to cell death support the implication of the transformation status in this response. However, the second possibility cannot be ruled out, because it is well established that LMP1, a EBV-encoded membrane protein, binds to the intracellular TNFR-associated factors (54–56), that are associated with CD40 and other members of TNFR family (57,58), and were implicated in certain cell death systems. Also, LMP2A, another EBV-encoded protein, negatively regulates certain signaling pathways in B cell lines by binding and constitutively activating certain kinases (59,60), some of which are also involved in CD40 signaling (61). Thus, the constitutive recruitment of signaling molecules to LMP may alter the signaling pathways coupled to CD40 leading to the activation of a cell death system rather than signaling for survival.

One of the most striking findings to emerge from this study is the rapid response observed upon ligation of CD40 or HLA-DR, which is somehow different from that previously reported for Fas- and TNF-induced cell death in various systems. In normal B cells, it has been reported that ligation of CD40 for 72 h increases the surface expression of Fas and promotes cell susceptibility to Fas-induced apoptosis following interaction with FasL+ T cells (7,9,23). On the other hand, stimulation of normal resting B cells via HLA-DR for 72 h or more induces the expression of FasL, does not change the Fas expression but increases cells sensitivity to Fas-mediated cell death (25). However, the results presented in this report clearly indicate that CD40- and HLA-DR-mediated cell death in B cells is independent from Fas–FasL interactions. The involvement of TNFR in this response is unlikely as exogenous TNF did not cause Raji cell death (data not shown) and blocking with anti-TNF mAb did not affect CD40- or HLA-DR-mediated cell death. These results suggest that HLA-DR and CD40 can also activate a novel cell death system in human transformed B cells that is independent from Fas–FasL and TNF–TNFR interactions.

The cell death system triggered via CD40 or HLA-DR requires cell–cell interactions that are clearly independent from the LFA-1–ICAM system. This conclusion is supported by the ability of HLA-DR and CD40 to trigger cell death in the LAD cell line (derived from a LFA-1-deficient patient), and by the failure of anti-CD18 mAb to inhibit CD40- and HLA-DR-mediated responses. The molecules mediating the LFA-1-independent homotypic adhesion can be either directly involved in cell death or indirectly by activating a cell death mediator(s). Indeed, they can be novel and/or known molecules but with an undefined role in either cell–cell adhesion or cell death. Further studies are in progress to determine the nature of these molecules and their possible involvement in cell death.

The ability of HLA-DR and CD40 signals to trigger a rapid cell death that reaches its maximum after 1 h of stimulation suggests that de novo protein synthesis is not required for this cell death. A solid confirmation of this hypothesis is the failure of CHX to inhibit this response, indicating that ligation of HLA-DR or CD40 molecules leads to the activation of pre-existing apoptotic effector molecules.

Similar to many receptor–ligand interactions, dimerization of HLA-DR or CD40 molecules on the cell surface is required for various cellular events induced via these molecules (62,63). Data presented in this report demonstrate that pretreatment with cytochalasin B completely blocks HLA-DR- and CD40-mediated cell death, indicating that the cytoskeleton integrity plays an important role in this response. The cytoskeleton, which plays an important role in cell–cell interactions, can also be necessary for either HLA-DR and CD40 dimerization and/or their association with other signaling molecules.

The sensitivity of CD40- and HLA-DR-mediated cell death to various proteases inhibitors is similar to that of TNF- and Fas-induced apoptosis, with the exception of the applied caspase inhibitors (38). The possible involvement in HLA-DR- and CD40-mediated cell death of an uncharacterized caspase(s) that is less sensitive to the caspase inhibitors used in our study cannot be ruled out completely. However, although caspase(s) activation leading to PARP cleavage is a sign of apoptosis in many cellular systems (41,46), our results indicate that this is not always the case and this is supported by previous observations (64–68). Recently, it has been shown that inhibition of caspases by peptides inhibitors, although preventing DNA fragmentation and PARP cleavage, does not always prevent the ultimate death of the cells (69–73). It has been proposed that death inducers activate caspase-dependent as well as caspase-independent pathways and that the caspase-dependent pathway was not always required for cell death (69). Alternatively, the availability of ATP or the kinetics of the cell death response may dictate the activation or not of the caspase-dependent pathway(s) where in conditions of low ATP levels or during a fast cell death response, such as the one described here, the caspase-independent pathway(s) is the chef executor (74,75).

Mono-ADP-ribosylation modulates the function of various proteins including the cytoskeleton (76), integrins (77,78) and those involved in signal transduction (79,80). Although, TNF- and Fas-mediated apoptosis is governed by the activation of caspases that lead to PARP cleavage, TNF-mediated cell death involves mono-ADP-ribosyltransferase activity that is inhibitable by 3ABA and MIBG (81), whereas Fas-mediated response does not appear to require ADP-ribosyltransferase activity (this study and 47). The inhibitory effect of MIBG suggests the implication of mono-ADP-ribosyltransferase in the activation of the cell death system by MHC class II and CD40. However, the differential sensitivity of HLA-DR- and CD40-mediated cell death to 3ABA suggests that different mono-ADP-ribosyltransferase activities are associated with these receptors. It is possible that one mono-ADP-ribosyltransferase activity, associated with MHC class II, can be inhibited by 3ABA and the other, associated with CD40, is resistant to 3ABA. The CD40-associated ADP-ribosyltransferase activity may be similar to the 3ABA-resistant and the MIBG-sensitive ADP-ribosyltransferase activity, recently described in murine T cell (80). It is also possible that HLA-DR-induced signals involve two different mono-ADP-
ribosyltransferase activities where the MIBG-sensitive mono-ADP-ribosyltransferase activity is shared with CD40. These findings suggest that the cell death pathway triggered through CD40 overlaps but is not identical with the cell death signaling pathway linked to HLA-DR.

The fact that engagement of HLA-DR molecules triggers cell death only in human activated B cells and B cell lines suggests an important role for this form of B cell death in the elimination of immune effector cells that have already executed their functions in the course of the immune response. This may also serve to eliminate dangerous cells, such as those of an autoimmune nature, upon their activation. On the other hand, for CD40-mediated cell death, one can speculate that tumor-infiltrating lymphocytes may use this mechanism to eliminate some tumor cells in vivo (26). Thus, the above proposed correlation between the B cell-transformed phenotype and the CD40 signaling leading to the activation of cell death program is possible, and indeed may highlight a biological significance for this form of B cell death.

Acknowledgements

We would like to thanks Drs F. Rousset, P. Cresswell, J. Thibodeau, G. G. Poirier and R. Bazin for their generous gifts. This work was supported by grants to W. Mourad from the Medical Research Council of Canada (MRC) and Arthritis Society of Canada. R. Al-D. is a member of laboratoire d’immunogenetique humaine, INSERM, U396, Paris, France

Abbreviations

3ABA 3-aminobenzamide  
CD40L CD40 ligand  
CHX cycloheximide  
EBV Epstein–Barr virus  
FasL Fas ligand  
NAM nicotinamide  
MIBG m-iodobenzylguanidine  
PARP poly(ADP-ribose) polymerase  
TNFR tumor necrosis factor receptor

References

28 Mehndate, K., al-Daccak, R., Damdoumi, F. and Mourad, W. 1996. Synergistic effect between CD40 and class II signals overcome the requirement for class II dimerization in
CD40- and HLA-DR-mediated B cell death


