Mechanism of ASC-mediated apoptosis: Bid-dependent apoptosis in type II cells

Mechanism of ASC-mediated apoptosis: Bid-dependent apoptosis in type II cells

Mizuho Hasegawa[1,4], Kouji Kawase[1], Naohiro Inohara[2], Ryu Imamura[1], Wen-Chen Yeh[3], Takeshi Kinoshita[1], and Takashi Suda[1]

[1] Center for the Development of Molecular Target Drugs, Cancer Research Institute, Kanazawa University, 13-1 Takaramachi, Kanazawa, Ishikawa 920-0934, Japan

[2] Department of Pathology, The University of Michigan Medical School, Ann Arbor, Michigan 48109, USA

[3] Department of Medical Biophysics, University of Toronto, Toronto, Ontario, M5G 2C1, Canada

Footnotes

Correspondence: T Suda, Center for the Development of Molecular Target Drugs, Cancer Research Institute, Kanazawa University, 13-1 Takaramachi, Kanazawa, Ishikawa 920-0934, Japan. TEL: 81-76-265-2736; FAX: 81-76-234-4525; E-mail: sudat@kenroku.kanazawa-u.ac.jp

4. Current address of MH: Department of Pathology, The University of Michigan Medical School, Ann Arbor, Michigan 48109, USA

5. While we were revising this paper, a physiological stimulus for CARD12 was reported to be bacterial flagellin.

Running title: Mechanism of ASC-mediated apoptosis

Keywords: apoptosis, ASC, CARD12, caspase, Bid, Bax

Abstract

ASC is an adaptor molecule that mediates apoptotic and inflammatory signals, and implicated in tumor suppression. However, the mechanism of ASC-mediated apoptosis has not been well elucidated. Here, we investigated the molecular mechanisms of ASC-mediated apoptosis in several cell lines using a CARD12-Nod2 chimeric protein that transduces the signal from muramyl dipeptide into ASC-mediated apoptosis. Experiments using dominant-negative mutants, small-interfering RNAs, and peptide inhibitors for caspases indicated that caspase-8 was generally required for ASC-mediated apoptosis, while a requirement for caspase-9 depended on the cell type. In addition, CLARP/FLIP, a natural caspase-8 inhibitor, suppressed ASC-mediated apoptosis, and *Clarp*-/- mouse embryonic fibroblasts were highly sensitive to ASC-mediated apoptosis. Bax-deficient HCT116 cells were resistant to ASC-mediated apoptosis as reported previously, although we failed to observe colocalization of ASC and Bax in cells. Like Fas-ligand-induced apoptosis, the ASC-mediated apoptosis was inhibited by Bcl-2 and/or Bcl-XL in type-II but not type-I cell lines. Bid was cleaved upon ASC activation, and suppression of endogenous Bid expression using small-interfering RNAs in type-II cells reduced the ASC-mediated apoptosis. These results indicate that ASC, like death receptors, mediates two types of apoptosis depending on the cell type, in a manner involving caspase-8.

Introduction

ASC (also called TMS1) is a cytoplasmic adaptor protein consisting of a pyrin domain (PYD) and a caspase recruitment domain (CARD). This adaptor protein links CARD12 (also called Ipaf or CLAN), a member of the Apaf-1-like proteins, to signal transduction pathways leading to apoptosis and NF-κB activation (Masumoto et al., 2003). ASC also links several PYD-containing members of the Apaf-1-like proteins (called NALPs or PYPAFs) with caspase-1, and induces the caspase-1-mediated maturation of inflammatory cytokines, such as IL-1β (Martinon et al., 2002; Wang et al., 2002). Accordingly, ASC-deficient macrophages fail to secrete IL-1β and resist cell death upon intracellular infection with *Salmonella* (Mariathasan et al., 2004).

Another line of studies has shown that the expression of ASC is down-regulated in various malignant tumors (McConnell and Vertino, 2004). In addition, the genes for ASC and CARD12 are targets of p53, and are required for the apoptosis induced by p53 and chemotherapeutic drugs in some human cells (Ohtsuka et al., 2004; Sadasivam et al., 2005). Thus, the CARD12-ASC axis seems to play an important role in tumor suppression and chemosensitivity of cancer cells.

Several research groups have studied the molecular mechanism of ASC-mediated apoptosis, but the results have been controversial. It was first reported that a dominant-negative mutant for caspase-9 but not caspase-8 inhibits the apoptosis induced by over-expressing ASC in HEK293 cells (McConnell and Vertino, 2000). However, a dominant-negative mutant of caspase-8 but not caspase-9 inhibits the apoptosis induced by the co-expression of ASC and CARD12 or by the forced oligomerization of ASC in 293T cells (Masumoto et al., 2003). It was also reported that ASC interacts with Bax and recruits it to mitochondria, implying the caspase-9-dependent

pathway (Ohtsuka et al., 2004).

The lack of information regarding the upstream molecules that activate CARD12 or other ASC-activating Apaf-1-like proteins has prevented us from investigating the functions of ASC under physiological conditions.[5] To overcome this problem, we recently established an experimental system in which muramyl dipeptide (MDP) activates ASC-mediated signal transduction pathways through a chimeric protein (C12N2) consisting of the CARD (ASC-interacting domain) from CARD12 and the nucleotide-binding oligomerization domain and leucine-rich repeats (MDP-binding region) from Nod2, another Apaf-1-like protein (Hasegawa et al., 2005). Here, using this system, we investigated the molecular mechanisms of ASC-mediated apoptosis in several cell lines.

Results

MDP induces apoptosis through C12N2 and ASC.

We previously generated HEK293 cell-derived stable cell lines (MAIL8) that express ASC and C12N2. MDP stimulation induced the interaction of C12N2 and ASC, oligomerization of ASC, and caspase-8-dependent NF-κB activation, leading to IL-8 production in these cell lines (Hasegawa et al., 2005). Furthermore, upon MDP stimulation, some MAIL8 cell lines showed clear apoptotic changes, such as plasma membrane blebbing, cell shrinkage (Figure 1a), phosphatidyl-serine externalization (Figure 1b), nuclear condensation and fragmentation, and the degradation of chromosomal DNA to nucleosome-sized units (supplementary Figure S1). MDP induced increased apoptosis only when 293T cells were transiently transfected with the combination of ASC and C12N2 cDNAs, although ASC alone induced weak apoptosis

irrespective of MDP stimulation (Figure 1c). (Hereafter, apoptosis induced by transient transfection with C12N2 and ASC followed by MDP stimulation is termed C12N2+ASC+MDP-induced apoptosis.) Similarly, C12N2+ASC+MDP induced apoptosis in the HCT116 and SW480 colon cancer cell lines, and MKN45 stomach cancer cell line (Figure 1d). Thus, C12N2 was capable of transducing MDP stimulation into ASC-mediated apoptosis.

Caspase-8 is essential for ASC-induced apoptosis, while the requirement for caspase-9 depends on the cell type

We then investigated the role of caspases in our experimental system. Consistent with a previous report (Masumoto et al., 2003), a dominant-negative mutant of caspase-8 but not caspase-9 or caspase-1 inhibited the C12N2+ASC+MDP-induced apoptosis in 293T cells and HCT116 cells (Figure 2, a and b). The dominant-negative mutant of caspase-9 but not caspase-8 inhibited the Bax-induced apoptosis in HCT116 cells (supplementary Figure S2a), confirming that the caspase-9 mutant could inhibit caspase-9-dependent apoptosis. Furthermore, down-modulation of the endogenous caspase-8 expression using three different caspase-8-targeting siRNAs inhibited the MDP-induced apoptosis in MAIL8 cells, whereas a caspase-9-targeting siRNA did not do so (Figure 2c and supplementary Figure S3a). The pan-caspase inhibitor z-VAD-FMK, caspase-8 inhibitor z-IETD-FMK, and caspase-3 inhibitor z-DEVD-FMK inhibited the MDP-induced apoptosis in MAIL8 cells, whereas little or no inhibition was observed with the caspase-9 inhibitor z-LEHD-FMK or granzyme B inhibitor z-AAD-FMK (Figure 2d). Similarly, the inhibitor for caspase-8 but not caspase-9 inhibited the C12N2+ASC+MDP-induced apoptosis in HCT116 cells (Figure 2e, left panel). However, both caspase-8 and caspase-9 inhibitors strongly inhibited the C12N2+ASC+MDP-induced apoptosis

in SW480 cells (Figure 2e, right panel). In addition, experiments using siRNAs indicated that MKN45 cells require caspase-8 but not caspase-9, while the A549 lung carcinoma cell line requires both caspase-8 and caspase-9 for this response (supplementary Figure S3b and c). The dominant negative mutant of caspse-9 also inhibited the C12N2+ASC+MDP-induced apoptosis in A549 and SW480 but not MKN45 cells (supplementary Figure S2b). These results indicate that caspase-8 plays an essential role in ASC-mediated apoptosis, but the requirement of caspase-9 in this response depends on the cell type.

CLARP (FLIP) inhibits ASC-mediated apoptosis

Because CLARP regulates death factor-induced apoptosis by inhibiting caspase-8 activation, we investigated the modulatory role of CLARP in ASC-mediated apoptosis. CLARP is mainly expressed in two isoforms, a short form (CLARP-S) and a long form (CLARP-L) (Thome and Tschopp, 2001). As expected, CLARP-L and CLARP-S inhibited the Fas- but not Bid-induced apoptosis of 293T cells (Figure 3a). Under these conditions, CLARP-L and CLARP-S inhibited the apoptosis induced by the coexpression of a constitutively active mutant of CARD12 (CARD12Δ) and ASC. Consistently, CLARP-S potently inhibited the MDP- but not staurosporine-induced apoptosis of MAIL8 cells (Figure 3b). CLARP-S also inhibited the C12N2+ASC+MDP-induced apoptosis in HCT116, SW480, and A549 cells (Figure 3c and supplementary Figure S2c). Furthermore, cell death induced by the coexpression of CARD12Δ and ASC or the overexpression of ASC alone was enhanced in *Clarp*-/- MEF compared with wild-type MEF, while the Nod1-induced apoptosis was comparable between the two lines (Figure 3d). These results indicate that CLARP inhibits ASC-mediated apoptosis.

ASC induces Bax-dependent apoptosis, but does not colocalize with Bax

Recently, Ohtsuka et al. (Ohtsuka et al., 2004) reported that ASC induces apoptosis by recruiting Bax to mitochondria. Consistent with this report, *BAX*-/- HCT116 cells, compared with *BAX*+/+ cells, were resistant against C12N2+ASC+MDP-induced apoptosis (Figure 4a). Furthermore, Bcl-2 inhibited the C12N2+ASC+MDP-induced apoptosis in *BAX*+/+ HCT116 cells (Figure 4b). However, when we coexpressed ASC and Bax in COS7 cells and examined their colocalization with mitochondria using Mitotracker, ASC formed aggregates called 'specks' as described previously (Hasegawa et al., 2005) and did not colocalize with mitochondria, while Bax showed clear localization at mitochondria (Fig 4C, upper panels). In contrast, ASC and caspase-8 coexpressed in COS7 cells colocalized at the specks (Fig 4C, lower panels). These results strongly suggest that the mitochondrial pathway was involved in the ASC-mediated apoptosis in HCT116 cells; however, ASC interacted with caspase-8 rather than Bax. Furthermore, this response did not require caspase-9 (Figure 2, b and e). A possible explanation for the latter result is given in the Discussion.

ASC induces two types of apoptosis depending on the cell type

Two types of signal transduction pathways for the death receptor-initiated, caspase-8-mediated apoptosis are operational and which one is used depends on the cell type (Scaffidi et al., 1998). In type I cells, activated caspase-8 induces apoptosis by directly activating caspase-3. In type II cells, caspase-8 cleaves the proapoptotic BH3-only protein, Bid, which in turn activates the Bcl-2- or Bcl-XL-inhibitable mitochondrial apoptotic pathway (Li et al., 1998; Luo et al., 1998). Based on this distinction, HCT116, A549, and SW480 cells were determined to be type II, whereas MKN45 and 293T cells were type I (Figure 5a).

The results described so far led us to hypothesize that the reason why ASC activation induced the mitochondrial apoptosis pathway in HCT116 cells was because they are a type II cell line. To confirm this hypothesis, we investigated the effect of Bcl-XL on the ASC-mediated apoptosis in other cell lines (Figure 5b). Bcl-XL inhibited C12N2+ASC+MDP-induced apoptosis in the type II (A549 and SW40) cells but not in the type I (MKN45 and 293T) cells. Thus, as with FasL-induced apoptosis, cells can be divided into two types based on their mechanism of ASC-mediated apoptosis.

ASC mediates Bid-dependent apoptosis in type II cells

We then investigated the role of Bid in ASC-mediated apoptosis. Two different Bid-targeting siRNAs inhibited the C12N2+ASC+MDP- but not staurosporine-induced apoptosis in HCT116 cells (Figure 6a). In contrast, FADD-targeting siRNA, which inhibits FADD expression (Imamura et al., 2004), did not affect these responses (data not shown). Similarly, a Bid- but not a FADD-targeting siRNA inhibited the C12N2+ASC+MDP-induced apoptosis in another type II cell line, A549 (Figure 6b). In contrast, neither Bid-targeting siRNA nor RICK-targeting siRNA (as a negative control) inhibited the MDP-induced apoptosis in the MAIL8 cell line, which was derived from HEK293, a type I cell line, while caspase-8-targeting siRNA (as a positive control) inhibited this apoptosis (Figure 6c). The Bid-targeting siRNA also inhibited the C12N2+ASC+MDP-induced apoptosis in SW480, but not MKN45 cells (supplementary Figure S4). To confirm the cleavage of Bid by caspase-8 during ASC-mediated apoptosis, we generated C12N2+ASC-expressing HCT116-derived stable transfectants (HCT-MAIL). MDP induced massive apoptosis in the HCT-MAIL cell lines (supplementary Figure S5). Western blot analyses revealed that full-length Bid was decreased 9 h after the MDP addition (Figure 6d).

Furthermore, pan-caspase or caspase-8 inhibitors but not granzyme B inhibitor suppressed the Bid degradation. The caspase-9 inhibitor caused a weak inhibition of Bid degradation, possibly because of the cross-inhibition of caspase-8. Taken together, these results suggest that the ligand-dependent, Apaf-1-like protein-mediated oligomerization of ASC induces the activation of caspase-8, which in turn induces the proteolytic maturation of Bid, and activates the mitochondrial apoptotic pathway in type II cell lines. In addition, FADD was not required for the ASC-mediated apoptosis, excluding the possibility that ASC worked by inducing the production of a death factor that requires FADD to induce apoptosis.

C12N2 induces apoptosis mediated by endogenous ASC upon MDP stimulation.

The cancer cell lines used in this study so far expressed undetectable or very low levels of ASC (Figure 7a). Thus they required transfection of both C12N2 and ASC to be sensitive to MDP-induced apoptosis. Therefore, we searched for cell lines expressing endogenous ASC, and found the NUGC4 stomach cancer and COLO205 colon adenocarcinoma cell lines expressing a substantial amount of ASC (Figure 7a). Then we established a C12N2-expressing stable cell line (NUC12N2) from the NUGC4 cells. MDP induced strong apoptosis in NUC12N2 cells (Figure 7b), and this apoptosis was inhibited by ASC- and caspase-8-targeting siRNAs (Figure 7, c and d). Thus, C12N2 is capable of activating endogenous ASC to induce caspase-8 dependent apoptosis upon MDP stimulation.

Discussion

Using MAIL8 cells, we previously demonstrated that C12N2 interacts with ASC upon MDP

stimulation, and induces caspase-8-dependent NF-κB activation and IL-8 production (Hasegawa et al., 2005). In this study, we demonstrated that this interaction also induced caspase-8-dependent apoptosis in various cancer cell lines. This system is remarkably similar to the FasL-Fas system, because caspase-8 is involved in both apoptosis and NF-κB activation induced by FasL (Imamura et al., 2004). However, caspase-8 but not FADD colocalized with ASC in mammalian cells (Hasegawa et al., 2005). Experiments using FADD-targeting siRNA revealed that the ASC-mediated NF-κB activation (Hasegawa et al., 2005) and apoptosis (this study) do not require FADD. Interestingly, ASC and FADD are structurally related to each other. Both proteins consist of two homophilic protein interaction domains with characteristic structure called death domain fold. Actually, the CARD of ASC interacts with the CARD of CARD12 (Geddes et al., 2001) and the PYD of ASC interacts with caspase-8 (Masumoto et al., 2003). Therefore, it is likely that ASC itself is a FADD-like adaptor that recruits caspase-8 to CARD12. Alternatively, some other proteins may connect ASC with caspase-8.

Recently, Ohtsuka et al. (Ohtsuka et al., 2004) demonstrated that Bax is involved in ASC-mediated apoptosis, and proposed that ASC recruits Bax to the mitochondria. However, if ASC directly induces Bax-mediated apoptosis, the apoptosis would depend on caspase-9 rather than caspase-8. Therefore, these results were considered inconsistent with the previous report (Masumoto et al., 2003) showing that ASC mediated caspase-8-dependent apoptosis in 293T cells. Furthermore, in our hands, the colocalization of ASC and Bax was not observed when these proteins were coexpressed in cells. Instead, we showed here that ASC induces the cleavage of Bid by caspase-8, and induces Bax-dependent apoptosis in type II cell lines. Many of the data described by Ohtsuka et al. including those indicating that the expression of ASC induces the translocation of Bax to the mitochondria, Bax-dependent cytochrome c release from

mitochondria, and caspase-9 activation, are consistent with our conclusion. In addition, MDP induced the cleavage of Bid in MAIL8 cells (data not shown), although Bid is not essential for ASC-mediated apoptosis in this cell line. Therefore, we propose that ASC activates the mitochondrial apoptotic pathway using caspase-8 and Bid in general, although we cannot exclude an alternative and redundant mechanism in which ASC transiently interacts with Bax to activate the mitochondrial apoptotic pathway in certain cell lines.

We showed here that caspase-9 was not essential for ASC-mediated apoptosis in HCT116 cells. Nonetheless, Bax and Bid were required for and Bcl-2 inhibited the ASC-mediated apoptosis in these cells, indicating that activation of the mitochondrial apoptotic pathway is required. These results can be explained by the fact that Bcl-2 controls the mitochondrial release of not only cytochrome c, the activator of caspase-9, but also other pro-apoptotic proteins, such as Smac/DIABLO, Omi/HtrA2, and AIF (Du et al., 2000; van Loo et al., 2002). In this context, it is noteworthy that Sun et al (Sun et al., 2002) previously suggested that Smac promotes caspase-9-independent apoptosis when sufficient caspase-8 activation occurs but XIAP interferes with apoptosis at the level of caspase-3 activation. In contrast, in a different cell type in which the caspase-8 activity is not high enough to fully activate caspase-3 even in the presence of Smac, cytochrome c-dependent caspase-9 activation would be also required to achieve full-blown apoptosis. It is likely that the HCT116 cell line represents the former case and the SW480 and A549 cell lines represent the latter.

The CARD12-ASC axis seems to play an important role in tumor suppression and chemosensitivity as described in the Introduction. On the other hand, CLARP is expressed at high levels in tumor cells, including classic Hodgkin's lymphoma, melanomas, and pancreatic cancers (Bullani et al., 2001; Elnemr et al., 2001; Mathas et al., 2004), and postulated that the

overexpression of CLARP is advantageous to tumor cells, because it confers a resistance to death factors. Here, we demonstrated that CLARP renders cells resistant to ASC-mediated apoptosis. Thus, another reason for tumor cells to overexpress CLARP may be to resist the effects of ASC. ASC has also been connected to autoinflammatory syndromes (Gumucio et al., 2002). Therefore, the role of CLARP in ASC-mediated apoptosis may have important implications for this type of diseases.

ASC- or CARD12-deficient macrophages are resistant to the cell death induced by *Salmonella* infection (Mariathasan et al., 2004), and rapid apoptosis of *Salmonella*-infected macrophages depends on caspase-1, indicating that caspase-1 plays an important role in CARD12 and/or ASC-mediated apoptosis in a macrophage (Jesenberger et al., 2000). This is not surprising because each of CARD12 and ASC is capable of inducing caspase-1 activation (Poyet et al., 2001; Wang et al., 2002), and macrophages express a large amount of pro-caspase-1. However, caspase-1-deficient macrophages succumbed to *Salmonella*-induced apoptosis after a 4-h infection (Jesenberger et al., 2000). Consistently, a monocytic cell line THP-1 stably transfected with CARD12 was reported to be highly sensitive to the caspase-dependent but caspase-1-independent cell death induced by *Salmonella* (Damiano et al., 2004). Similarly, infection of *Francisella tularensis* induces apoptosis in caspase-1- but not ASC-deficient macrophages after a 24-h infection (Mariathasan et al., 2005). It is noteworthy that caspase-8 is activated in *Salmonella*-infected macrophages after a 3-h infection (Jesenberger et al., 2000). Taken together, it is possible that the ASC-caspase-8 axis is operational as a backup mechanism for apoptosis of bacteria-invaded macrophages.

Based on the discussions above, an implication of our conclusion that caspase-8 plays an important role in the CARD12- and ASC-mediated apoptosis is that the inhibition of caspase-8

may reduce the sensitivity of cancer cells to chemotherapy and affect the resistance of animals to certain types of microbes. These may be important considerations, especially given that caspase-8 has been considered as a drugable target for hepatitis therapy (Zender et al., 2003). Conversely, the strategy of using an artificial protein like C12N2 to activate ASC may be useful in tumor therapy in the future.

Materials and methods

Plasmids

The expression plasmid for a dominant-negative mutant of caspase-1 (pEF-Casp1C285S) was generated by PCR-based site-directed mutagenesis. The expression plasmid for BAX was kindly provided by Dr. Tsujimoto (Osaka University Medical School, Osaka, Japan). Other plasmids were described previously (Hasegawa et al., 2005; Imamura et al., 2004; Inohara et al., 1999; Ogura et al., 2001; Wang et al., 2004).

Cell lines

The MAIL8 cell lines (Hasegawa et al., 2005) and the apoptosis-sensitive 293T cell line (Wang et al., 2004) were described previously. The HCT116 cell line and its Bax-deficient mutant (Dr. Bert Vogelstein, Johns Hopkins University School of Medicine), and the A549 cell line (Dr. Tsukasa Matsunaga, Kanazawa University Graduate School of Natural Science and Technology) were kindly provided by the researchers named. The SW480, MKN45, NUGC4 and COLO205 cell lines were obtained from the Cell Resource Center for Biomedical Research, Tohoku University. *Clarp* (-/-) mouse embryonic fibroblasts (MEF) were described previously

(Yeh et al., 2000).

Small interfering RNAs (siRNAs)

The caspase-8-targeting siRNAs #2 and #3, and ASC-targeting siRNA #1 and #2 were purchased from Dharmacon (Chicago, IL). A caspase-9-targeting siRNA (sense, 5'-GAGUUUGAGGGGAAAUGCAGAUUUGAG-3'; antisense, 5'-CAAAUCUGCAUUUCCCCUCAAACUCAU-3', iGENE, Tsukuba, Japan), and Bid-targeting siRNA #1 (sense, 5'-GGCAGAUUCUGAAAGUCAAGAAGACAG-3'; antisense, 5'- GUCUUCUUGACUUUCAGAAUCUGCCAU -3', iGENE) and Bid-targeting siRNA #2 (sense, 5'-GAAGACAUCAUCCGGAAUATT; antisense, 5'-UAUUCCGGAUGAUGUCUUCTT-3', Dharmacon) were custom-synthesized. Other siRNAs were described previously (Imamura et al., 2004).

Apoptosis assays

The proportion of apoptotic cells was determined by flow cytometry after staining the cells with propidium iodide and Cy5-annexin V as described previously (Wang et al., 2004), unless otherwise described. In experiments involving transient transfection of plasmids, cells were transfected with plasmid DNA including pEGFP-C1, and apoptosis of GFP^+ cells were analyzed. The survival of MEF was assessed as described previously (Inohara et al., 1997).

Immunofluorescence confocal microscopy

Immunofluorescence confocal microscopy was performed as described previously (Hasegawa et al., 2005), except that anti-human Bax mouse IgG2b monoclonal antibody (MBL, Nagoya,

Japan) was used in this study.

Supplementary information is available at the Oncogene's website.

Acknowledgements

This work was supported in part by Grants-in-Aid for Scientific Research on Priority Areas (Cancer) from the Ministry of Education, Culture, Sports, Science and Technology, the Japanese Government, and a grant from the Novartis Foundation (Japan) for the Promotion of Science.

References

Bullani RR, Huard B, Viard-Leveugle I, Byers HR, Irmler M, Saurat JH, et al. (2001). Selective expression of FLIP in malignant melanocytic skin lesions. *J Invest Dermatol*, **117:** 360-364.

Damiano JS, Newman RM,Reed JC. (2004). Multiple roles of CLAN (caspase-associated recruitment domain, leucine-rich repeat, and NAIP CIIA HET-E, and TP1-containing protein) in the mammalian innate immune response. *J Immunol*, **173:** 6338-6345.

Du C, Fang M, Li Y, Li L,Wang X. (2000). Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. *Cell*, **102:** 33-42.

Elnemr A, Ohta T, Yachie A, Kayahara M, Kitagawa H, Fujimura T, et al. (2001). Human pancreatic cancer cells disable function of Fas receptors at several levels in Fas signal transduction pathway. *Int J Oncol*, **18:** 311-316.

Geddes BJ, Wang L, Huang WJ, Lavellee M, Manji GA, Brown M, et al. (2001). Human

CARD12 is a novel CED4/Apaf-1 family member that induces apoptosis. *Biochem Biophys Res Commun*, **284:** 77-82.

Gumucio DL, Diaz A, Schaner P, Richards N, Babcock C, Schaller M, et al. (2002). Fire and ICE: the role of pyrin domain-containing proteins in inflammation and apoptosis. *Clin Exp Rheumatol*, **20:** S45-53.

Hasegawa M, Imamura R, Kinoshita T, Matsumoto N, Masumoto J, Inohara N, et al. (2005). ASC-mediated NF-kappa B activation leading to IL-8 production requires caspase-8 and is inhibited by CLARP. *J Biol Chem*, **280:** 15122-15130.

Imamura R, Konaka K, Matsumoto N, Hasegawa M, Fukui M, Mukaida N, et al. (2004). Fas ligand induces cell-autonomous NF-kappa B activation and IL-8 production by a mechanism distinct from that of TNF-alpha. *J Biol Chem*, **279:** 46415-46423.

Inohara N, Koseki T, del Peso L, Hu Y, Yee C, Chen S, et al. (1999). Nod1, an Apaf-1-like activator of caspase-9 and nuclear factor-kappaB. *J Biol Chem*, **274:** 14560-14567.

Inohara N, Koseki T, Hu Y, Chen S,Nunez G. (1997). CLARP, a death effector domain-containing protein interacts with caspase-8 and regulates apoptosis. *Proc Natl Acad Sci USA*, **94:** 10717-10722.

Jesenberger V, Procyk KJ, Yuan J, Reipert S,Baccarini M. (2000). Salmonella-induced caspase-2 activation in macrophages: a novel mechanism in pathogen-mediated apoptosis. *J Exp Med*, **192:** 1035-1046.

Li H, Zhu H, Xu CJ,Yuan J. (1998). Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell*, **94:** 491-501.

Luo X, Budihardjo I, Zou H, Slaughter C,Wang X. (1998). Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface

death receptors. *Cell*, **94:** 481-490.

Mariathasan S, Newton K, Monack DM, Vucic D, French DM, Lee WP, et al. (2004). Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. *Nature*, **430:** 213-218.

Mariathasan S, Weiss DS, Dixit VM,Monack DM. (2005). Innate immunity against Francisella tularensis is dependent on the ASC/caspase-1 axis. *J Exp Med*, **202:** 1043-1049.

Martinon F, Burns K,Tschopp J. (2002). The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Mol Cell*, **10:** 417-426.

Masumoto J, Dowds TA, Schaner P, Chen FF, Ogura Y, Li M, et al. (2003). ASC is an activating adaptor for NF-kappa B and caspase-8-dependent apoptosis. *Biochem Biophys Res Commun*, **303:** 69-73.

Mathas S, Lietz A, Anagnostopoulos I, Hummel F, Wiesner B, Janz M, et al. (2004). c-FLIP mediates resistance of Hodgkin/Reed-Sternberg cells to death receptor-induced apoptosis. *J Exp Med*, **199:** 1041-1052.

McConnell BB,Vertino PM. (2000). Activation of a caspase-9-mediated apoptotic pathway by subcellular redistribution of the novel caspase recruitment domain protein TMS1. *Cancer Res*, **60:** 6243-6247.

McConnell BB,Vertino PM. (2004). TMS1/ASC: the cancer connection. *Apoptosis*, **9:** 5-18.

Ogura Y, Inohara N, Benito A, Chen FF, Yamaoka S,Nunez G. (2001). Nod2, a Nod1/Apaf-1 family member that is restricted to monocytes and activates NF-kappaB. *J Biol Chem*, **276:** 4812-4818.

Ohtsuka T, Ryu H, Minamishima YA, Macip S, Sagara J, Nakayama KI, et al. (2004). ASC is a Bax adaptor and regulates the p53-Bax mitochondrial apoptosis pathway. *Nat Cell Biol*, **6:**

121-128.

Poyet JL, Srinivasula SM, Tnani M, Razmara M, Fernandes-Alnemri T,Alnemri ES. (2001). Identification of Ipaf, a human caspase-1-activating protein related to Apaf-1. *J Biol Chem*, **276:** 28309-28313.

Sadasivam S, Gupta S, Radha V, Batta K, Kundu TK,Swarup G. (2005). Caspase-1 activator Ipaf is a p53-inducible gene involved in apoptosis. *Oncogene*, **24:** 627-636.

Scaffidi C, Fulda S, Srinivasan A, Friesen C, Li F, Tomaselli KJ, et al. (1998). Two CD95 (APO-1/Fas) signaling pathways. *Embo J*, **17:** 1675-1687.

Sun XM, Bratton SB, Butterworth M, MacFarlane M,Cohen GM. (2002). Bcl-2 and Bcl-xL inhibit CD95-mediated apoptosis by preventing mitochondrial release of Smac/DIABLO and subsequent inactivation of X-linked inhibitor-of-apoptosis protein. *J Biol Chem*, **277:** 11345-11351.

Thome M,Tschopp J. (2001). Regulation of lymphocyte proliferation and death by FLIP. *Nat Rev Immunol*, **1:** 50-58.

van Loo G, Saelens X, van Gurp M, MacFarlane M, Martin SJ,Vandenabeele P. (2002). The role of mitochondrial factors in apoptosis: a Russian roulette with more than one bullet. *Cell Death Differ*, **9:** 1031-1042.

Wang L, Manji GA, Grenier JM, Al-Garawi A, Merriam S, Lora JM, et al. (2002). PYPAF7, a novel PYRIN-containing Apaf1-like protein that regulates activation of NF-kappa B and caspase-1-dependent cytokine processing. *J Biol Chem*, **277:** 29874-29880.

Wang Y, Hasegawa M, Imamura R, Kinoshita T, Kondo C, Konaka K, et al. (2004). PYNOD, a novel Apaf-1/CED4-like protein is an inhibitor of ASC and caspase-1. *Int Immunol*, **16:** 777-786.

Yeh WC, Itie A, Elia AJ, Ng M, Shu HB, Wakeham A, et al. (2000). Requirement for Casper (c-FLIP) in regulation of death receptor-induced apoptosis and embryonic development. *Immunity*, **12:** 633-642.

Zender L, Hutker S, Liedtke C, Tillmann HL, Zender S, Mundt B, et al. (2003). Caspase 8 small interfering RNA prevents acute liver failure in mice. *Proc Natl Acad Sci USA*, **100:** 7797-7802.

Titles and legends to Figures

Figure 1. MDP induces apoptosis in cells expressing C12N2 and ASC. (**a**) MAIL8-3 cells were cultured with (lower panel) or without MDP (upper panel). (**b**) MAIL8-19 cells were cultured as in (**a**), stained with propidium iodide and Cy5-annexin V, and analyzed using flow cytometry. The percentages of cells in the quadrants are shown. (**c**, **d**) 293T, HCT116, SW480, and MKN45 cells were transfected with empty vector or expression plasmids for C12N2 and/or ASC as indicated. Cells were cultured with or without MDP for 16 h.

Figure 2. General requirement of caspase-8 but not caspase-9 for the ASC-mediated apoptosis (**a**, **b**) 293T or HCT116 cells were transfected with empty vector or the expression plasmid for a dominant-negative mutant of caspase-8, caspase-9, or caspase-1, as indicated, together with expression plasmids for C12N2 and ASC. Cells were cultured with or without MDP for 16 h. (**c**) MAIL8 cells were transfected with the indicated concentrations of the caspase-8-targeting siRNA #1 or a caspase-9-targeting siRNA and stimulated with MDP for 16 h. The expression levels of endogenous caspase-8 and caspase-9 were examined by Western blotting. (**d**) MAIL8

cells were pretreated with the indicated inhibitors for 1 h and then cultured with or without MDP for 16 h. (**e**) HCT116 and SW480 cells were transfected with expression plasmids for C12N2 and ASC. Cells were then treated as in (**d**).

Figure 3. CLARP inhibits ASC-mediated apoptosis. (**a**) HEK293T cells were transfected with empty vector or expression plasmids for Fas, tBid, or CARD12Δ plus ASC with or without an expression plasmid for CLARP-L or CLARP-S and cultured for 24 h. (**b**) MAIL8 cells were transfected with the indicated amounts of CLARP-S expression plasmid. Cells were cultured with or without MDP for 16 h, or with staurosporine (STS) for 9 h. (**c**) HCT116 cells were transfected with expression plasmids for C12N2 and ASC, and the indicated amounts of CLARP-S expression plasmid. Cells were cultured with or without MDP for 16 h. (**d**) Wild-type or Clarp (-/-) MEF were transfected with empty vector or expression plasmids for Nod1ΔLRR, CARD12ΔLRR plus ASC, or ASC-Fpk3 in the presence of pEF1BOS-β-gal. Three hours post-transfection, cells were treated with AP1510. The percentage of surviving β-gal-positive cells was determined at 24 h post-transfection.

Figure 4. Bax is involved in the ASC-mediated apoptosis, but it does not interact with ASC. (**a**) Wild-type or BAX-/- HCT116 cells were transfected with empty vector or expression plasmids for C12N2 and ASC. Cells were cultured with or without MDP for 16 h. (**b**) HCT116 cells were transfected with expression plasmids for C12N2 and ASC together with the indicated amounts of Bcl-2 expression plasmid. Cells were cultured with or without MDP for 16 h. (**c**) COS7 cells were transfected with expression plasmids for Bax and ASC (upper panels) or caspase-8 and ASC (lower panels). Cells were stained with MitoTracker Green FM (Molecular Probes, Eugene,

OR), and anti-Bax (blue) and anti-ASC (red) mAbs (upper panels), or with anti-caspase-8 (blue) and anti-ASC (red) mAbs (lower panels) at 24 h post-transfection.

Figure 5. Bcl-2 family proteins regulate the ASC-mediated apoptosis in type II cell lines. (**a**, **b**) The indicated cell lines were transfected with an empty vector or Bcl-XL expression plasmid in the presence (**b**) or absence (**a**) of expression plasmids for C12N2 and ASC. Cells were stimulated with FasL (**a**) or MDP (**b**) for 16 h.

Figure 6. Bid is involved in the ASC-mediated apoptosis in type II cell lines. (**a**) HCT116 cells were transfected with or without Bid-targeting siRNAs. The cells were transfected again with expression plasmids for C12N2 and ASC or left untreated. The cells transfected with the C12N2 and ASC expression plasmids were cultured with or without MDP, while the cells transfected with siRNA alone were treated with staurosporine (STS) for 16 h. In the right panels, the expression levels of endogenous Bid and GAPDH were examined by Western blotting. (**b**) A549 cells were transfected with or without Bid-or FADD-targeting siRNA. The cells were transfected with expression plasmids for C12N2 and ASC. Cells were cultured with or without MDP for 16 h. (**c**) MAIL8 cells were transfected with or without Bid-, caspase-8-, or RICK-targeting siRNA. Cells were cultured with or without MDP for 16 h. (**d**) HCT116-derived stable transfectants expressing C12N2 and ASC were pretreated with the indicated inhibitors for 1 h and then stimulated with MDP for 9 h. The expression levels of endogenous full-length Bid and β-actin were examined by Western blotting.

Figure 7. C12N2 activates endogenous ASC to induce caspase-8 dependent apoptosis. (**a**) The

expression levels of ASC and β-actin in the indicated cell lines were examined by Western blotting. (**b**) NUC12N2 cells were cultured with or without MDP for 24 hrs. Cells were then stained with propidium iodide and Cy5-annexin V, and analyzed using flow cytometry. The percentages of annexin^{+} propidium iodide^{-} apoptotic cells were indicted. (**c** and **d**) NUC12N2 cells were transfected with control siRNA or ASC- or caspase-8-targeting siRNA. Cells were cultured with or without MDP for 16 h. In the lower panels, the expression levels of endogenous ASC, caspase-8 and β-actin were examined by Western blotting.

Fig. 1

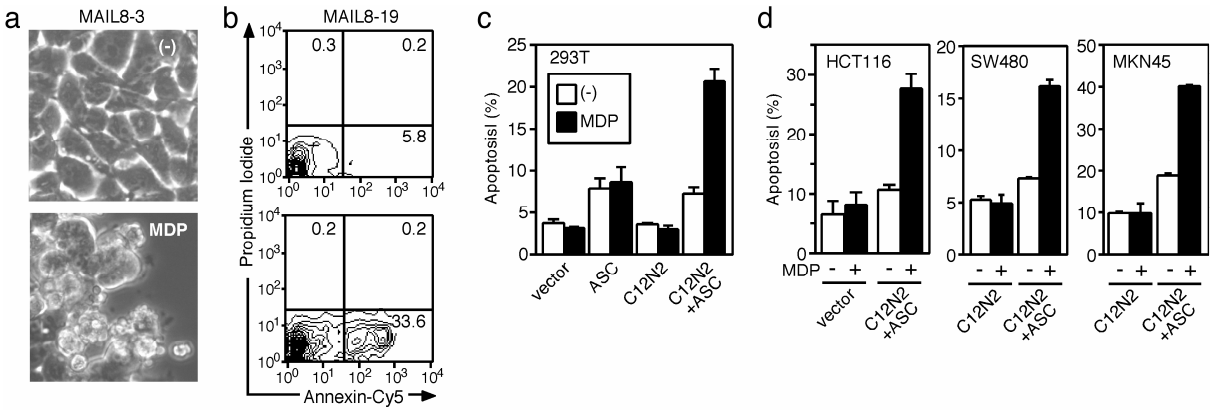


Fig. 2

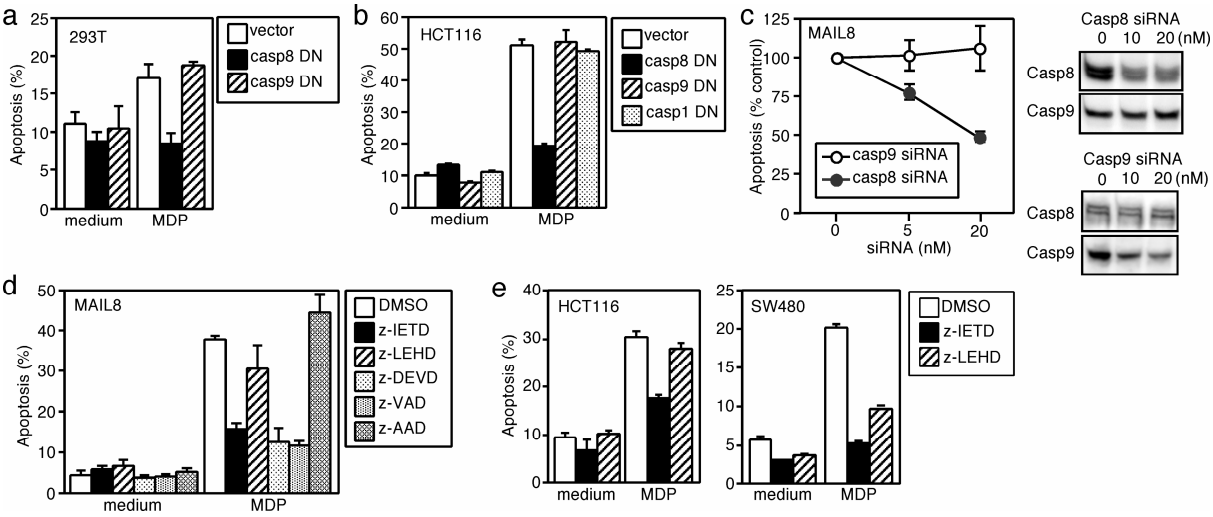


Fig. 3

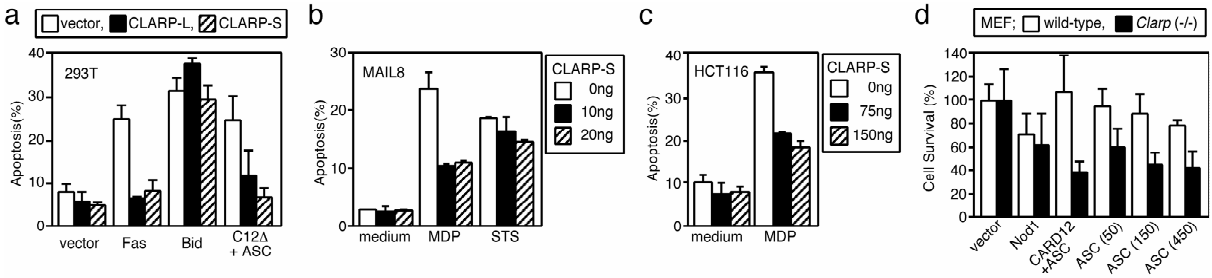


Fig. 4

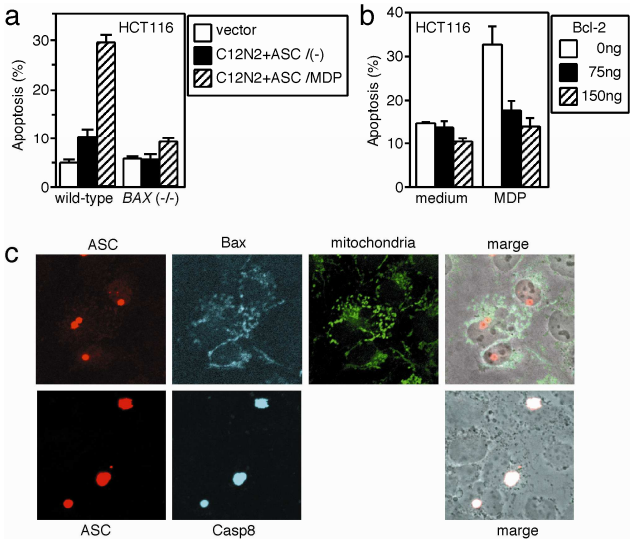

a
HCT116
vector
C12N2+ASC /(-)
C12N2+ASC /MDP
Apoptosis (%)
wild-type
BAX (-/-)
b
HCT116
Bcl-2
0ng
75ng
150ng
Apoptosis (%)
medium
MDP
c
ASC
Bax
mitochondria
marge
ASC
Casp8
marge

Fig. 5

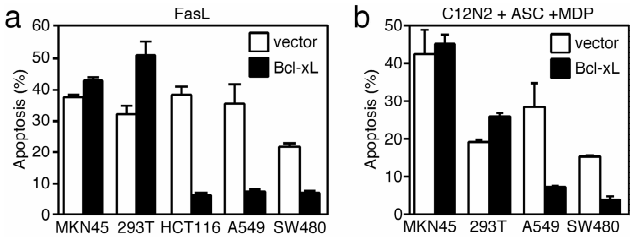

a
FasL
vector
Bcl-xL
Apoptosis (%)
MKN45
293T
HCT116
A549
SW480
b
C12N2 + ASC +MDP
vector
Bcl-xL
Apoptosis (%)
MKN45
293T
A549
SW480

Fig. 6

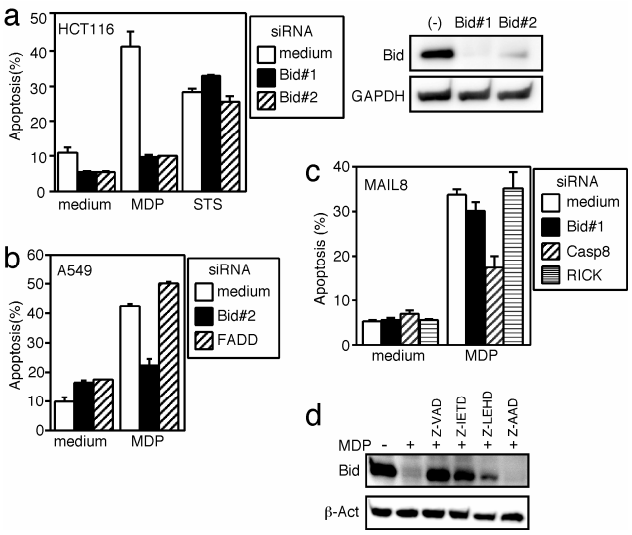
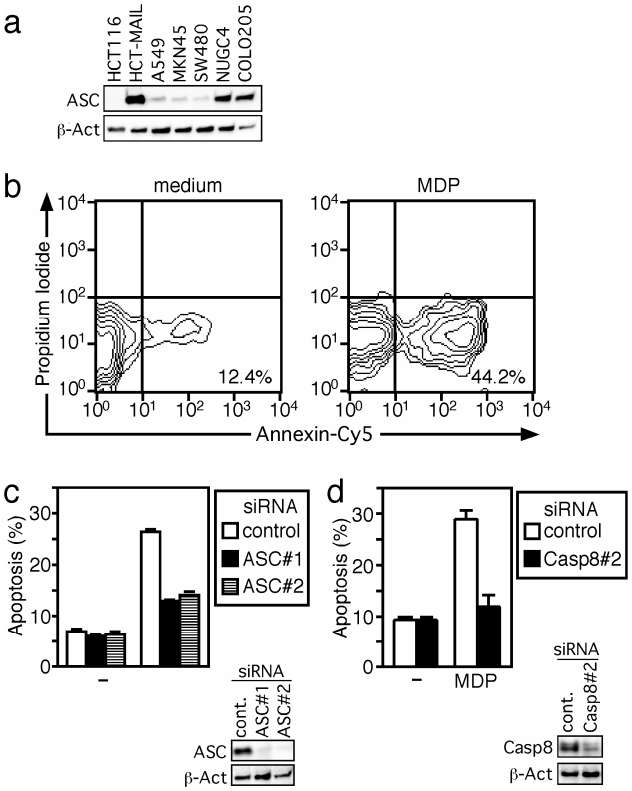

a
HCT116
Apoptosis(%)
medium
MDP
STS
siRNA
medium
Bid#1
Bid#2
(-) Bid#1 Bid#2
Bid
GAPDH
b
A549
Apoptosis(%)
medium
MDP
siRNA
medium
Bid#2
FADD
c
MAIL8
Apoptosis (%)
medium
MDP
siRNA
medium
Bid#1
Casp8
RICK
d
MDP - + + Z-VAD + Z-IETD + Z-LEHD + Z-AAD
Bid
β-Act

Fig. 7


a
HCT116
HCT-MAIL
A549
MKN45
SW480
NUGC4
COLO205
ASC
β-Act
b
medium
MDP
Propidium Iodide
12.4%
44.2%
Annexin-Cy5
c
Apoptosis (%)
siRNA
control
ASC#1
ASC#2
–
siRNA
cont.
ASC#1
ASC#2
ASC
β-Act
d
Apoptosis (%)
siRNA
control
Casp8#2
–
MDP
siRNA
cont.
Casp8#2
Casp8
β-Act