ASC, a Novel 22-kDa Protein, Aggregates during Apoptosis of Human Promyelocytic Leukemia HL-60 Cells*

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The cytoskeletal and/or nuclear matrix molecules responsible for morphological changes associated with apoptosis were identified using monoclonal antibodies (mAbs). We developed mAbs against Triton X-100-insoluble components of HL-60 cells pretreated with all-trans retinoic acid. In particular, one mAb recognized a 22-kDa protein that exhibited intriguing behavior by forming an aggregate and appearing as a speck during apoptosis induced by retinoic acid and other anti-tumor drugs. Cloning and sequencing of its cDNA revealed that this protein comprises 195 amino acids and that its C-terminal half has a caspase recruitment domain (CARD) motif, characteristic of numerous proteins involved in apoptotic signaling. We referred to this protein as ASC (apoptosis-associated speck-like protein containing a CARD). The ASC gene was mapped on chromosome 16p11.2–12. The antisense oligonucleotides of ASC were found to reduce the expression of ASC, and consequently, etoposide-mediated apoptosis of HL-60 cells was suppressed. Our results indicate that ASC is a novel member of the CARD-containing adaptor protein family.

First described by Kerr (1), apoptosis is an important genetically programmed process regulating the growth and development of organisms. This phenomenon also mediates normal and neoplastic tissue growth by the removal of excess cells (2). Induction of apoptosis in the promyeloleukemic cell line, HL-60, may be achieved with a variety of biological and chemical agents including retinoic acid (RA)³ (3–8).

Recently, the CARD was identified as the region with significant similarity to the RAIDD and ICH-1 N-terminal domains. ICH-1, Ced-3, ICE, and Mch6, all proteins containing the CARD, have been reported to act in apoptotic signaling (9). The CARD has been proposed to play a regulatory role in apoptosis by allowing proteins such as Apaf-1 to associate with caspase-9 (10, 11). RAIDD is a part of the TNFR1-TRADD-RIP complex and recruits ICH-1 through the CARD (12). The viral apoptosis inhibitor IAP of the two cellular homologs c-IAP1 and c-IAP2 is part of the TNFR2-TRAF complex through the CARD (13, 14). Ced-4 recruits Ced-3 via the CARD (15). The CARDs of these proteins associate with electrostatic forces, and this binding specificity between CARDs is determined by the charge distribution on the domain surfaces (16).

In this study, we identified a novel protein, ASC. This soluble protein was located in the cytosol of healthy HL-60 cells; however, in apoptotic cells, it was visualized as a speck. Closer examination of this speck revealed that the protein formed an aggregate with a hollow center. Although the C-terminal of ASC was found to contain a CARD, the N-terminal was found to be homologous to that of pyrin, the causative gene product for familial Mediterranean fever (17). The CARD domain of ASC may be involved in apoptotic signaling, thereby initiating pro-apoptotic effects.

EXPERIMENTAL PROCEDURES
Preparation of Triton X-100-insoluble Materials of HL-60 Cells—The methods described previously for preparation of the cytoskeleton and nuclear matrix (18) were used with slight modifications. Triton X-100-insoluble materials of HL-60 were used for immunization of mice and an enzyme-linked immunosorbent assay.

Pre-embedding Electron Microscopy—HL-60 cells were treated with etoposide (10 μg/ml) for 12 h and collected. Cells were fixed with 3% hydrogen peroxide in 70% ethanol and refixed with 4% paraformaldehyde. The cells were immunostained and refixed with 1% glutaraldehyde. All subsequent steps were performed as described previously (19).

Cell Fractionation—HL-60 cells were harvested and lysed in hypotonic solution. 60% sucrose solution was added to the cell lysate to result in a final concentration of 10%. The whole cell lysate was successively fractionated by centrifuging.

Effects of Anti-tumor Agents—Twelve h after medium exchange, HL-60 cells in exponential growing phase were suspended with fresh control medium or media-containing anti-tumor agents: agents that interact with topoisomerase, i.e. 10 μg/ml etoposide (VP-16) (Lastertm, Nippon-Kayaku) or 1 μg/ml camptothecin (CPT; Topogen); an antimitabolite; 10 μg/ml cytarabine (ara-C) (1-β-D-arabinofuranosylcytosine; Cylocidetm, Nippon-Shinyaku); a metallic agent; 1 μg/ml cisplatin (CDDP) (Randa™, Nippon-Kayaku); an antimicrotubule agent; 1 μg/ml vincristine (VCR) (Oncovirtm, Shionogi); a differentiation agent; 10 μM all-trans retinoic acid (ATRA) (Sigma) or a miscellaneous chemothera...

³ The abbreviations used are: RA, retinoic acid; CARD, caspase recruitment domain; ASC, apoptosis-associated speck-like protein containing a CARD; ICE, interleukin-1β-converting enzyme; Ced, cell death abnormal; ICH, ICE and Ced-3 homolog; CLARP, caspase-like apoptosis-regulatory protein; RIP, receptor interacting protein; RICK, RIP-like interacting CLARP kinase; RAIDD, RIP-associated ICH-1/Ced-3-homologous protein with a death domain; Mch, mammalian Ced-3 homolog; mAb, monoclonal antibody; FITC, fluorescein isothiocyanate; bp, base pair(s); Apaf, apoptotic protease activating factor; TNFR, tumor necrosis factor receptor; TRADD, TNFR1-associated death domain protein; IAP, inhibitor of apoptosis protein; RACE, rapid amplification of cDNA ends; FISH, fluorescence in situ hybridization; TUNEL, terminal deoxynucleotidyl transferase-mediated DUTP nick end labeling; ARC, apoptosis repressor with CARD; TRAF, TNFR-associated factor.
peptidase agent; 2 milliliters/ml bleomycin (BLM) (Bles™, Nippon-Kayaku). After treatment with these agents, cells were fixed and analyzed as described above.

Isolation of cDNA-coding ASC—Total RNA from HL-60 cells was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction using Liogen (Wako). To purify mRNA, pre spun-pinch oligo-dT-cellulose columns (Amersham Pharmacia Biotech) were used. A 5′UTR cDNA library was constructed using a TimeSaver™ cDNA synthesis kit with poly(A) primers and random primers, a cDNA rapid cloning module, and λ-DNA in vitro packaging module (all from Amersham Pharmacia Biotech).

The cDNA library described above was screened with the anti-ASC mAb, and positive clones were amplified by polymerase chain reaction. The 5′ noncoding region was cloned by 5′-RACE (Life Technologies, Inc.). Sequence homology searches were carried out using the BLAST computer program at the National Center for Biotechnology Information.

Construction of Expression Plasmids—The entire open reading frame of ASC was inserted into pcDNA3 (Invitrogen). The DNA construct was transfected into COS-7 cells by lipofection using Transome™ (Wako) according to the manufacturer’s instructions.

Screening of a Human PAC Library and FISH—A human PAC DNA pool (20) was screened using primers for the 3′-translated region of ASC under the following polymerase chain reaction conditions: initial denaturation at 94 °C for 30 s followed by 30 cycles of 94 °C 30 s, 55 °C 30 s, and 72 °C 30 s, and a final elongation step at 72 °C for 7 min (GeneAmp 9600 system, Perkin-Elmer Corp.). Primers were 5′-TGGAGGACCTG-GAGCCGGACG-3′ and 5′-CAAGCTTGGTCTTCTGATAGT-3′. DNA of positive clones was prepared using a Plasmid Midi kit (Qiagen) according to the manufacturer’s recommendations. Aliquots of 1 μg of PAC DNA labeled with biotin-16-dUTP (Roche Molecular Biochemicals) by nick end translation were hybridized to normal metaphase chromosomes from 10 μg of human Cot 1 DNA (Life Technologies, Inc.) to suppress repetitive sequences, as described previously (21). FISH signals were detected with FITC-conjugated avidin (Vector Laboratories), and chromosomes were counterstained with propidium iodide (magenta) to suppress repetitive sequences, as described previously (21). FISH signals were detected with FITC-conjugated avidin (Vector Laboratories), and chromosomes were counterstained with propidium iodide (magenta) to suppress repetitive sequences, as described previously (21).

Apoptosis Assessment by the TUNEL Method—Intriguing Behavior of the New 22-kDa Protein, ASC, during Apoptosis

Intriguing Behavior of the New 22-kDa Protein, ASC, during Apoptosis—Changes in cellular architecture during apoptosis and/or differentiation of leukemic cells were determined by developing mAbs against the Triton X-100-insoluble fraction of RA-treated or -untreated HL-60 cells. One mAb recognized an antigen with a molecular mass of 22-kDa. Western blotting analysis revealed that, unlike control cells, a portion of this protein in RA-treated cells was resistant to extraction with Triton X-100/cytoskeleton buffer (Fig. 1A). DNA condensation in apoptotic cells was detected by immunofluorescence after extracting DNA fragments with 70% ethanol at −20 °C for 30 min (22). A bright speck-like signal was observed in RA-treated (4 days) HL-60 cells undergoing apoptosis (Fig. 1, Ba and Bb, arrowheads) and this protein was thus termed ASC. Over 50% of the RA-treated HL-60 cells were found to be apoptotic. In most HL-60 cells bearing an ASC speck, chromatin condensation was visualized by Hoechst 33258, thus implying that in HL-60 cells, ASC is concentrated into a speck and is associated with the apoptotic process.

By using pre-embedding electron microscopy, it was possible to obtain a more precise location for the ASC speck in apoptotic HL-60 cells. The cells were pretreated with etoposide (10 μg/ml) for 12 h, and the histochemical staining of apoptotic HL-60 with the ASC mAb was examined. Our findings revealed that the speck was detected in the periphery of the apoptotic HL-60 cells and appeared to be an aggregate with a hollow interior.

![FIG. 1. Clustering of ASC in apoptotic HL-60 cells.](image)

**TABLE I**

| Drug | Control | Etoposide | Camptothecin | Cytarabine | Cisplatin | Vincaistine | Bleomycin | All-trans retinoic acid |
|------|---------|-----------|-------------|-----------|-----------|------------|----------|------------------------|
| Concentration (μg/ml) | 10 | 1 | 10 | 1 | 1 | 2 | 1 | 2 |
| Incubation time (h) | 24 | 24 | 24 | 24 | 24 | 24 | 24 | 24 |
| Apoptotic cells (%) | 10.3 ± 3.1 | 54.3 ± 7.1* | 44.7 ± 10.3* | 50.6 ± 6.0* | 40.0 ± 8.5* | 42.7 ± 6.3* | 37.4 ± 4.9* | 33.7 ± 4.8* |
| Cells with speck-like signal (%) | 6.4 ± 2.2 | 37.9 ± 6.5* | 31.9 ± 7.0* | 38.4 ± 6.9* | 26.9 ± 3.4* | 33.4 ± 6.8* | 29.1 ± 3.3* | 24.5 ± 4.3* |
| Cells with speck-like signal (%) of apoptotic cells (%) | 0.62 ± 0.09 | 0.70 ± 0.11 | 0.72 ± 0.07 | 0.76 ± 0.07 | 0.69 ± 0.13 | 0.78 ± 0.08 | 0.79 ± 0.10 | 0.73 ± 0.06 |

* Significantly different from control values using Student’s t test (p < 0.05).

HL-60 cells were cultured with etoposide or without 1 μM RA for 4 days. The cells were suspended in 0.5% Triton X-100/cytoskeleton buffer and separated into soluble and insoluble fractions by centrifugation at 1,500 × g for 10 min. Both fractions of the whole cell lysate were subjected to Western blotting using anti-ASC mAb. The size of the protein standard in kilodaltons is shown on the left. B. immunofluorescence microscopy of RA-treated cells revealed the localization of ASC. The fluorescence signal of ASC appeared as a speck (Ba). HL-60 cells were cultured with 1 μM RA for 4 days, fixed with 70% ethanol at −20 °C for 30 min, and immunostained in anti-ASC mAb. DNA was stained by blue Hoechst 33258, and apoptotic cells were visualized as a speck of ASC (Bb, arrowheads). Scale bar is 10 μm. C, pre-embedding electron microscopy showed that ASC was localized in the periphery of apoptotic HL-60 cells and was found to be an aggregate with a hollow center. Scale bar is 1 μm. D, cell fractionation determined the location of ASC. Aliquots of 20 μg of the pellet (p.p.) and supernatant (sup.) were analyzed by Western blotting with anti-ASC mAb. The 1,000 × g ppt., 10,000 × g ppt., 100,000 × g ppt., and 100,000 × g sup. fractions contained approximately 35, 13, 17, and 35% of whole cell lysate, respectively. The sizes of protein standards in kilodaltons are shown on the left.
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FIG. 2. Correlation between the number of drug-induced apoptotic cells and the number of cells with the speck-like aggregate of ASC. A, HL-60 cells were plated at 3 × 10^5 cells/ml in the absence (control) or presence of camptothecin (●), bleomycin (▲), vincristine (○), cytarabine (▲), or etoposide (▲) at the concentrations and for the incubation times indicated in Table I. Pearson’s correlation coefficient R was 0.929, and R^2 was 0.862. B, speck-like fluorescence signals (rhodamine, red) of ASC (arrowheads) were detected in TUNEL-positive (FITC, green) apoptotic HL-60 cells pretreated with etoposide (10 μg/ml).

FIG. 3. Structure of ASC and alignment with related proteins. A, schematic structure of ASC. The domain homologous to the N terminus of pyrin (Pyrin-like domain) and CARD are shown as black (left) and dark gray (right) boxes, respectively. B, cDNA and deduced amino acid sequences of ASC. C, alignment of the CARDs of ASC (GenBank™ accession number AF013263) and related proteins containing the CARD motif have been cloned: ARC (L49431), RICK/RIP2 (L49432), caspase-2, RAIDD, Apaf-1 (U13021), and cIAP1 and cIAP2 (U79115). D, alignment of the N-terminal ends of ASC and pyrin (accession number AF018080). Shading indicates identical residues.

The location of ASC in HL-60 cells was also determined by cell fractionation. ASC was found predominantly in the supernatant after centrifugation at 100,000 × g for 60 min, but a small portion was also found in the pellet after centrifugation at 10,000 × g for 20 min (Fig. 1D). It was deduced, therefore, that ASC is essentially a soluble protein in the cytosol.

This phenomenon was also seen in HL-60 cells pretreated with various anti-tumor agents for the induction of apoptosis (Table I). A significant correlation was observed between the number of apoptotic cells and the number of cells with a speck-like fluorescence signals of ASC (Fig. 2, A and B).

The cDNA of ASC encodes a predicted peptide of 195 amino acids with a predicted isoelectric point of 6.4 and a mass of 21.7 kDa. The schematic structure of human ASC (Fig. 3A), nucleotide, and deduced protein sequences are shown (Fig. 3B). A PSI-BLAST search of the NIH database revealed that the 87 amino acid residues in the C terminus of ASC had significant homology (expect-value < 0.001) to the CARD of both mamma-}

FIG. 4. Analysis of ASC expression in human tissues by Northern blotting and of tumor cell lines by Northern and Western blotting. A, 10 μg of total RNAs from various tumor cell lines. The membrane fraction of tumor cell lines was rehybridized with a radio-labeled human glyceraldehyde-3-phosphate dehydrogenase probe (CLONTECH). Blots were hybridized with an ASC cDNA probe. C, 40 μg of protein from various tumor cell lines were detected with anti-ASC mAb. Size markers in kilobases (A and B) or kilodaltons (C) are on the left.

FIG. 5. Effect of etoposide for the HL-60 cell transfectant using antisense oligonucleotides. A, HL-60 cells plated at 2 × 10^5 cells/ml were incubated with antisense-(76–95) oligonucleotides (●) or with the controls, sense-(76–95) oligonucleotides (○) and sense-(71–90) oligonucleotides (□). After incubation for 24 h, 10 μg/ml etoposide was added to each well and incubated for the times indicated. The HL-60 cells treated with antisense-(76–95) oligonucleotides were significantly resistant to etoposide-induced apoptotic stimuli in comparison to both controls. Error bars are the means ± S.E. for four independent experiments. *, Significantly different from the control values using Student’s t-test (p < 0.05). B, the expression level of ASC was reduced by antisense-(76–95) oligonucleotides. Using NIH Image software, the HL-60 cells with antisense-(76–95) oligonucleotides expressed 57% less ASC than both controls.
CLARP and regulates apoptosis induced by the CD95/Fas receptor pathway (25). RICK/RIP2 is also a component of the TNFR1, cIAP1, and TRAF family signaling complexes (26). Similarly, ASC may be involved in apoptotic signaling through the CARD.

The N terminus of ASC displayed a high degree of homology to N-terminal pyrin (Fig. 3D), the causative gene product of familial Mediterranean fever (17).

Transient Expression of ASC from Plasmid Constructs—The characteristics of ASC were ascertained by a transient expression experiment. Immunofluorescence and Western blotting enabled the detection of ASC after transient expression in COS-7 cells. The mAb against ASC was used to detect the subcellular localization of ASC in COS-7 cells. Additionally, this mAb probed whole cell lysate from COS-7 cells transfected with pcDNA3 (empty vector) and pcDNA3-ASC. A ring-like fluorescent signal was detected in some COS-7 cells transfected with pcDNA3-ASC, but not in those transfected with pcDNA3 (data not shown). Western blots revealed a 22-kDa band in COS-7 cells transfected with pcDNA3-ASC. This was the same size as that of ASC in HL-60 cells (data not shown).

Chromosomal Localization of the ASC Gene—The chromosomal location of the ASC gene was determined by FISH, wherein we screened the human PAC library to isolate two independent PAC clones containing the ASC gene. The clones were shown to overlap with each other by restriction analysis and were mapped to 16p11.2-p12 by FISH (data not shown). For further refined mapping, we used the Stanford G3 radiation hybrid mapping panel (Research Genetics). Two-point maximum likelihood analysis indicated a linkage to markers SHGC-64124 and SHGC-35326 with log of odds (lod) scores of 9.54 and 8.93, respectively, as calculated by the Stanford Human Genome Center radiation hybrid web server. The marker SHGC-35326 was located between D16S3093 and D16S409 at 16p11.2. Based on these findings, the ASC gene was located at 16p11.2–12.

Tissue and Cellular Distributions of ASC—We examined the expression of ASC in several tumor cell lines using Northern and Western blotting (Fig. 4, A and C). It was found to be restricted to two leukemia cell lines, HL-60 and U937, and a melanoma cell line, WM35. In addition, traces of ASC mRNA expression were detected in the chronic myelogeneous leukemia cell line, K562. Interestingly, ASC expression was not detected in other leukemic cell lines such as Jurkat T-cell lymphoma and Daudi Burkitt's lymphoma. Furthermore, although ASC was expressed in the early stage melanoma cell line, WM35, its presence was not detected in another melanoma cell line, WM793. Multiple-tissue Northern blot showed an 0.8-kilobase transcript in various human normal tissues (Fig. 4B). With the exception of K562, the expression of ASC in various tumor cell lines was confirmed by Western blotting using our anti-ASC mAb. Our data showed that ASC expression occurred in HL-60, U937, and WM35 (Fig. 4C). These observations suggested that the level of ASC expression may vary according to the cell lineage, maturation stage, or cell transformation.

ASC Promotes Etosiposide-induced Apoptosis—Fig. 5A shows that in etoposide-induced apoptosis of HL-60 transfectant by antisense oligonucleotides, a significant decrease in the percentage of apoptosis was observed in comparison with control cells. Western blotting and NtM Image software showed that the HL-60 transfectant by antisense oligonucleotides-(76–95) expressed 57% less ASC than the controls by sense oligonucleotides-(76–95) or sense oligonucleotides-(71–90) (Fig. 5B). Similar findings were also observed in vincristine-induced apoptosis (data not shown). These results showed that ASC may have pro-apoptotic activity by increasing the susceptibility of HL-60 cells to apoptotic stimuli by anti-cancer drugs such as etoposide or vincristine. It is possible that just as Apaf-1 has sensitizing effects for etosiposide-induced apoptosis (27) so ASC has pro-apoptotic effects on some apoptotic pathways.

Although at present it is not clear in which apoptotic pathway the CARD of ASC is involved, this study showed that low expression levels of a new CARD-containing molecule, ASC, decreases etosiposide-induced apoptosis in HL-60 cells. The soluble nature of ASC appeared to change to an insoluble one, and ASC appeared as a speck in apoptotic HL-60 cells.

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