The Interleukin-1β–converting Enzyme (ICE) Is Localized on the External Cell Surface Membranes and in the Cytoplasmic Ground Substance of Human Monocytes by Immuno–Electron Microscopy

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Summary

Interleukin-1β (IL-1β)–converting enzyme (ICE) is a novel cysteine protease that cleaves the 31-kD inactive cytoplasmic IL-1β precursor into active extracellular 17-kD IL-1β. The ICE gene product is a 45-kD proenzyme that requires proteolytic processing to activate ICE. Active ICE is a heterodimer consisting of equal amounts of p20 and p10 subunits. Generation of active ICE is affected by the removal of an 11-kD NH_2-terminal precursor domain (p11) and an internal 19–amino acid sequence that separates the 20- and 10-kD subunits. Immuno–electron microscopy was performed on human monocytes with immunoglobulins recognizing the active (p20) or precursor (p11) domains of ICE. Elutriated monocytes were stimulated with 50 pM lipopolysaccharide followed by heat-killed *Staphylococcus aureus* under conditions that induce maximal rates of IL-1β secretion. Ultrathin cryosections were cut from fixed frozen pellets of these monocytes and were immunogold labeled with either antibody. Active and precursor domain ICE epitopes were localized in the cytoplasmic ground substance, but they were not detected within the endoplasmic reticulum, the Golgi apparatus, and secretory granules of activated or inactive monocytes. Importantly, numerous ICE p20 epitopes were also observed on the extracellular surfaces of the cell membrane, and were concentrated on the microvilli. Very similar patterns of ICE localization were obtained with unstimulated blood monocytes. In contrast, ICE p11 epitopes were not detected on the surfaces of these monocytes. Likewise, labeling of fixed ultrathin cryosections of monocytes with a biotinylated irreversible ICE inhibitor [Ac-Tyr-Val-Lys(biotin)-Asp-(acyloxy)-methyl-ketone] showed that the compound localized on the outer cell surface as well, and to a lesser extent, within the cytoplasmic ground substance. Furthermore, antipeptide antibodies specific for either the mature or precursor domains of IL-1β were both localized upon the cell membrane after stimulation of IL-1β secretion. Lipopolysaccaride-primed monocytes that synthesized, but did not secrete IL-1β, exhibited only cytoplasmic staining. The data suggests that mature IL-1β is generated via cleavage of the 31-kD inactive cytoplasmic IL-1β precursor by ICE after association with the plasma membrane during secretion.

IL-1β is a pleiotropic proinflammatory cytokine that is primarily secreted by activated monocytes or macrophages. While the biological effects of IL-1β are well described, little is known about the detailed mechanism responsible for IL-1β processing and secretion. Synthesis, processing, and secretion of IL-1β occurs through a well-orchestrated, tightly regulated sequence of events. In monocytes, IL-1β is synthesized in response to a wide variety of stimulants, the most potent being LPS or other bacterial cell wall products. IL-1β is first synthesized in monocytes as an inactive 31-kD precursor (proIL-1β)[1] (1, 2). ProIL-1β lacks a definable signal sequence, is synthesized on cytosolic polyribosomes, and has been localized to the cytoplasmic ground substance outside of the ER by immmuno–electron microscopy (immunoEM; 3, 4).

[1] *Abbreviations used in this paper:* ER, endoplasmic reticulum; HKSA, heat-killed *Staphylococcus aureus*; ICE, interleukin-1–converting enzyme; immunoEM, immuno–electron microscopy; L-709,049, unlabeled reversible ICE inhibitor; L-742,395, biotinyl-irreversible ICE inhibitor; L-743,066, nonbiotinylated irreversible ICE inhibitor; proIL-1β, inactive 31-kD IL-1β precursor; ProICE, 45-kD proenzyme ICE gene product.

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After proIL-1β synthesis, maturation and secretion of the active 17.5-kD IL-1β occurs through an ill-defined pathway. Pulse-chase analysis suggests that the cleavage and secretion of IL-1β occurs in a cotemporal manner (5). ProIL-1β is cleaved between residues Asp^{116} and Ala^{117} by the protease IL-1–converting enzyme (ICE) (6, 7) to generate active IL-1β. Active ICE has been purified from human monocytes and identified as a novel heterodimeric thiol protease composed of two subunits, p20 and p10. Mature ICE (p20/p10) is derived from a single 45-kD proenzyme (p45 ICE) by proteolysis at ICE-like cleavage sites, resulting in the excision of an 11-kD NH_2-terminal precursor domain (p11) and a 2-kD linker separating the 20- and 10-kD subunits (8–11). Unlike IL-1β, ICE is produced constitutively by human blood monocytes. Subcellular fractionation of human monocytes has detected ICE activity and the p45 ICE proenzyme predominantly in the cytosolic fraction (7, 12).

A tetrapeptide aldehyde inhibitor of ICE that blocks production of mature IL-1β from LPS-stimulated human blood monocytes has been described (11). Treatment of LPS-stimulated monocytes with this inhibitor results in a specific dose-dependent inhibition (IC_{50} = 1 μM) of IL-1β processing. Despite inhibition of processing, IL-1β secretion remains unaffected. Instead, a quantitative shift from secretion of mature IL-1β to release of proIL-1β occurs, suggesting that proIL-1β processing is not required for, but is cotemporal with, the secretion of mature IL-1β (11). Furthermore, this observation suggests that proIL-1β is a secretion-competent form of IL-1β.

The biochemical detection of both the 45-kD proenzyme ICE gene product (proICE) and proIL-1β in the cytosol raises the question of how these two proteins are mobilized to generate mature extracellular IL-1β. One hypothesis is that events leading to apoptosis are primary mechanisms of IL-1β secretion (13, 14). Treatment of LPS-stimulated murine macrophages with agents that cause apoptosis lead to the release of large amounts of mature IL-1β. Conditions resulting in necrosis lead to the release of proIL-1β or improperly processed IL-1β (13). These observations imply that apoptosis-related processes such as plasma membrane vesiculation and blebbing may lead to release of mature IL-1β.

We have performed high resolution immunoEM with immunoprobes of precursor and mature ICE and IL-1β epitopes, as well as with a novel ICE active site detection method, to further characterize the IL-1β secretory pathway in human blood monocytes. We show that processed active ICE is localized on the external surfaces of the intact plasma membrane in stimulated and unstimulated human monocytes. Both mature and proIL-1β are also detected on external surfaces of the plasma membrane, which does not exhibit observable blebbing during conditions of rapid IL-1β secretion in stimulated monocytes. These observations indicate that proIL-1β is proteolytically processed during transport across the plasma membrane in the absence of apoptosis.

Materials and Methods

Antibodies. Rabbit antibodies (JD3) were raised against the catalytic domains (p20/p10) of mature ICE purified from THP-1 cells (a cultured human monocyte line), and to the NH_2-terminal amino acid sequence 34-47 (I34-47) of proICE (12). A rabbit antibody that specifically detects amino acids 3-21 of proIL-1β (15) was a generous gift of Dr. Mark D. Wewers (Dept. of Medicine, Ohio State University, Columbus, OH). Rabbit antipeptide antibodies specific for the COOH-terminal amino acid sequence 197-215 of mature IL-1β were used as previously described (1, 3). For immunoEM experiments, IgG fractions of the above antisera were purified by chromatography on protein A–Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ). Rabbit antipeptide antibodies recognizing the ICE-generated cleavage fragment of proIL-1β containing a COOH-terminal Asp^{116} were prepared to the peptide Cys-Nle-Asp-Thr-Asp-Asn-Glu-Ala-Tyr-Val-His-Asp-COOH as previously described (1). Antisera were characterized for their ability to recognize the immunizing peptide by direct ELISA in peptide coated 96-well microtiter plates. Positive antisera were then counterscreened for their ability to recognize proIL-1β, ICE-cleaved proIL-1β, or mature IL-1β. Antiserum 502T was chosen for its ability to react with the cleaved proIL-1β with >100-fold more sensitivity than uncleaved proIL-1β in a direct ELISA. Selectivity of the antiserum was further demonstrated by showing that 502T could not detect proIL-1β or mature IL-1β by immunoprecipitation and by Western blotting (data not shown).

Cells. An enriched population of PBMC was obtained by leukophoresis from healthy human volunteers. PBMC were purified by density gradient centrifugation over Ficoll-Hypaque followed by centrifugal elutriation as previously described (7, 16, 17). Approximately 5×10^8 monocytes of >95% purity as judged by size distribution analysis were obtained with this protocol. After elutriation, the purified blood monocytes were resuspended to 5×10^5 cells/ml in RPMI containing 1% fresh autologous human serum and stimulated for 3–6 h with heat-killed *Staphylococcus aureus* (HKSA; 10^6 CFU/10^5 monocytes per ml) as described previously (16). To obtain maximal rates of cytokine secretion for IL-1β–labeling experiments, elutriated human blood monocytes were primed for 4 h in medium with 50 pg/ml LPS, and IL-1β secretion was stimulated by treatment with HKSA for 2 h (16). The extent of IL-1β secretion into the culture medium was assessed by ELISA.

ImmunoEM Labeling of ICE Epitopes. After stimulation, purified monocytes were fixed in a solution of 3.5% paraformaldehyde, 0.05% glutaraldehyde, and 0.1 M sucrose in PBS, pH 7.4, in the presence of microwave irradiation, followed by overnight fixation with a mixture of paraformaldehyde, lysine, and periodate (18, 19). Pellets of fixed monocytes were infiltrated in 2% low gelling temperature agarose, cryoprotected by infiltration with 2.3 M sucrose and 50% polyvinylpyrrolidone in phosphate buffer, pH 7.2 (20), frozen by injection into liquid propane at −185°C in a KF-80 apparatus (Reichert Scientific Instruments, Buffalo, NY), and stored under liquid nitrogen. To permit access of antibodies or ICE probes (see below) to all sectioned subcellular compartments, ultrathin (~80-nm) frozen sections were cut at −105°C (21) on a Reichert Ultracut S ultramicrotome equipped with an FCS cryoattachment, and transferred to formvar-coated 200 mesh hexagonal nickel grids. Sections were treated with a clarified solution of 5% nonfat dry milk and BSA buffer (1% BSA in PBS, pH 7.8, containing 0.1% Na azide) for 30 min to block nonspecific binding, followed by overnight incubation in 5 μg/

ml solutions of various rabbit antipeptide IgGs in BSA buffer at 4°C. For specificity controls, 5 μg/ml JD3 IgG was absorbed with 250 μg/ml purified recombinant human ICE, and 5 μg/ml I34-47 IgG was absorbed with 3 mg/ml of the peptide used for immunization and was clarified by centrifugation before immunolabeling. Other controls consisted of 5 μg/ml solutions of the respective preimmune rabbit IgGs in BSA buffer. After extensive washing in PBS, the bound primary rabbit IgGs were detected with a goat anti-rabbit 5-nm colloidal gold conjugate (GARG 5; Amersham Corp., Arlington Heights, IL) diluted 1/50 in BSA buffer for 1 h at 23°C. The grids were then washed in PBS and fixed in 2% glutaraldehyde in PBS for 10 min, postfixed in 2% OsO_4 in H_2O for 20 min, stained with 2% uranyl acetate in H_2O for 30 min, and finally absorption stained with 0.002% lead citrate in 2% polyvinyl alcohol as described (21). Grids were examined and electron micrographs were taken with an electron microscope (200CX; Jeol Ltd., Tokyo, Japan) at 80 kV at an initial magnification of 20,000.

ImmunoEM Labeling of the ICE Active Site. For direct labeling of the ICE active site, intact living resting or activated monocytes were treated with 50 nM–20 μM biotinyl-irreversible ICE inhibitor L-742,395 [Ac-Tyr-Val-Lys(biotin)-Asp-(acyloxy)-methylketone] (22) for 0.5 to 6 h before fixation, washed, and prepared for immunoEM as described above. For controls, other cell cultures were incubated with 20 μM L-743,066, a nonbiotinylated but otherwise identical irreversible ICE inhibitor (22). Ultrathin frozen sections of these cells were then cut, and the incorporated biotinyl L-742,395 was detected using sequential incubations with 5 μg/ml avidin-DN (Vector Laboratories, Burlingame, CA) in BSA buffer, a biotinyl affinity-purified goat antiavidin antibody (Vector Laboratories), and a rabbit anti-goat IgG 5 nm colloidal gold conjugate (RAGG5; Amersham). In an alternative approach to enhance the access of the labeled probe to intracellular ICE sites, thin sections of fixed cells were stained directly with biotinylated irreversible ICE inhibitor. To protect the ICE active site from excessive denaturation, human monocytes were activated (6 h with HKSA) in 50 μM unlabeled reversible ICE inhibitor L-709,049, then fixed, washed, and prepared for immunoEM. Grids containing ultrathin cryosections of these cells were then incubated with serial dilutions of L-742,395 in PBS, and stained with avidin-DN, goat antiavidin antibody, and RAGG5 as described above. To monitor the specificity of this staining protocol, other grids were treated with a 400-fold molar excess of unlabeled reversible ICE inhibitor L-709,049 or the unlabeled L-743,066 before and during incubation with the biotinylated inhibitor. In another control, BSA buffer was substituted for the avidin-DN step of the biotin detection protocol.

Labeling of Mature and Prodomain IL-1β Epitopes. Monocytes were elutriated from human blood and then primed for 4 h in medium with 50 pg/ml LPS. A high rate of IL-1β secretion was initiated by stimulation with HKSA for an additional 2 h. After pelleting and processing for immunoEM, ultrathin frozen sections of either primed or stimulated monocytes were labeled with antibodies that distinguish the prodomain from the mature form of IL-1β. Grids were incubated with a rabbit IgG directed against amino acid residues 197-215 of mature IL-1β, or with another rabbit IgG recognizing amino acid residues 3-21 of IL-1β precursor, followed by a GARG5 as detailed above. As a control, the mature IL-1β IgG was absorbed with a 1.5 mg/ml solution of purified recombinant human IL-1β in BSA buffer before staining.

Results

Characterization of Antibodies. Active ICE (p20/p10) is derived by proteolytic cleavage of the 11-kD propiece (p11) and a 2-kD linker from the inactive 45-kD proenzyme (p45). A polyclonal antibody raised against purified active human ICE (JD3) recognizes epitopes located in a single p20 band in immunoblots of affinity-purified active ICE (Fig. 1). Conversely, another antipeptide polyclonal antibody raised to amino acid residues 34–47 of the ICE propiece (I34-47) does not react with affinity-purified active ICE. Both JD3 and I34-47 IgG label p45 in immunoblots of crude whole cell extracts from stimulated or unstimulated human blood monocytes and THP.1 cells, while the p20 subunit is not detectable (Fig. 1).

ImmunoEM Localization of Active ICE and ProICE Epitopes. When ultrathin cryosections of HKSA-stimulated monocytes were labeled with JD3 IgG, ICE epitopes were localized at the extracellular surfaces of the cell membrane surrounding numerous elongate microvilli, ruffling lamellae, and large (~0.2–1 μm diameter) pinocytotic vesicles present at the cell surface (Fig. 2 *A*). ICE was also observed in the cytoplasmic ground substance, while organelles such as the ER, secretory granules, the nuclear envelope, mitochondria, and the nucleus proper were not labeled (Fig. 2 *B*). ICE labeling was completely inhibited by preincubating 5 μg/ml JD3 IgG with 250 μg/ml purified rhICE (not shown), and preimmune rabbit IgG produced little background (Fig. 2 *C*). Thus, ICE epitopes, representing both precursor (p45) and active (p20) ICE, were localized specifically on the cell surface and in the cytoplasmic matrix exclusive of the conventional secretory organelles in activated monocytes. Cell culture supernatants from these stimulated cells contained significant amounts of IL-1β as detected by ELISA (Table I), indicating that active ICE was present in these cells. It is also noteworthy that these IL-1β secreting monocytes exhibited completely intact cell mem-

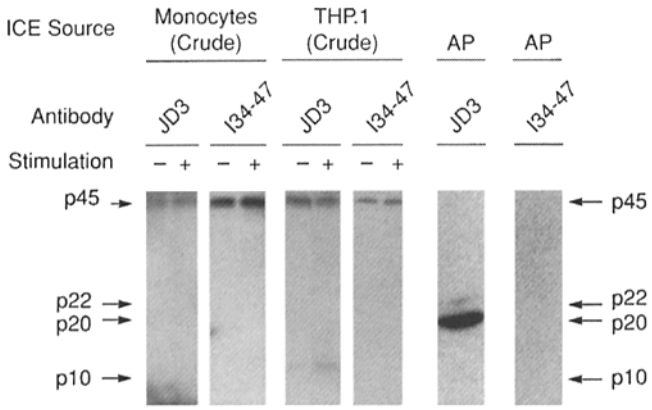

Figure 1. Immunoblots demonstrating the specificity of the JD3 and I34-47 antibodies for the ICE domains expressed by human monocytic cells. Crude whole-cell extracts of stimulated or unstimulated human monocytes, PMA-differentiated THP.1 cells, and affinity-purified human ICE (*AP*) were immunoblotted and stained with JD3 and I34-47 IgGs. Arrows indicate the positions of inactive proICE (*p45*), and the components of active ICE (*p22*, *p20*, and *p10*). JD3 and I34-47 antibodies recognize inactive proICE found in all crude cell extracts. Only JD3 IgG stains the active p20 subunit of affinity-purified ICE, which is not detectable in the cell extracts.

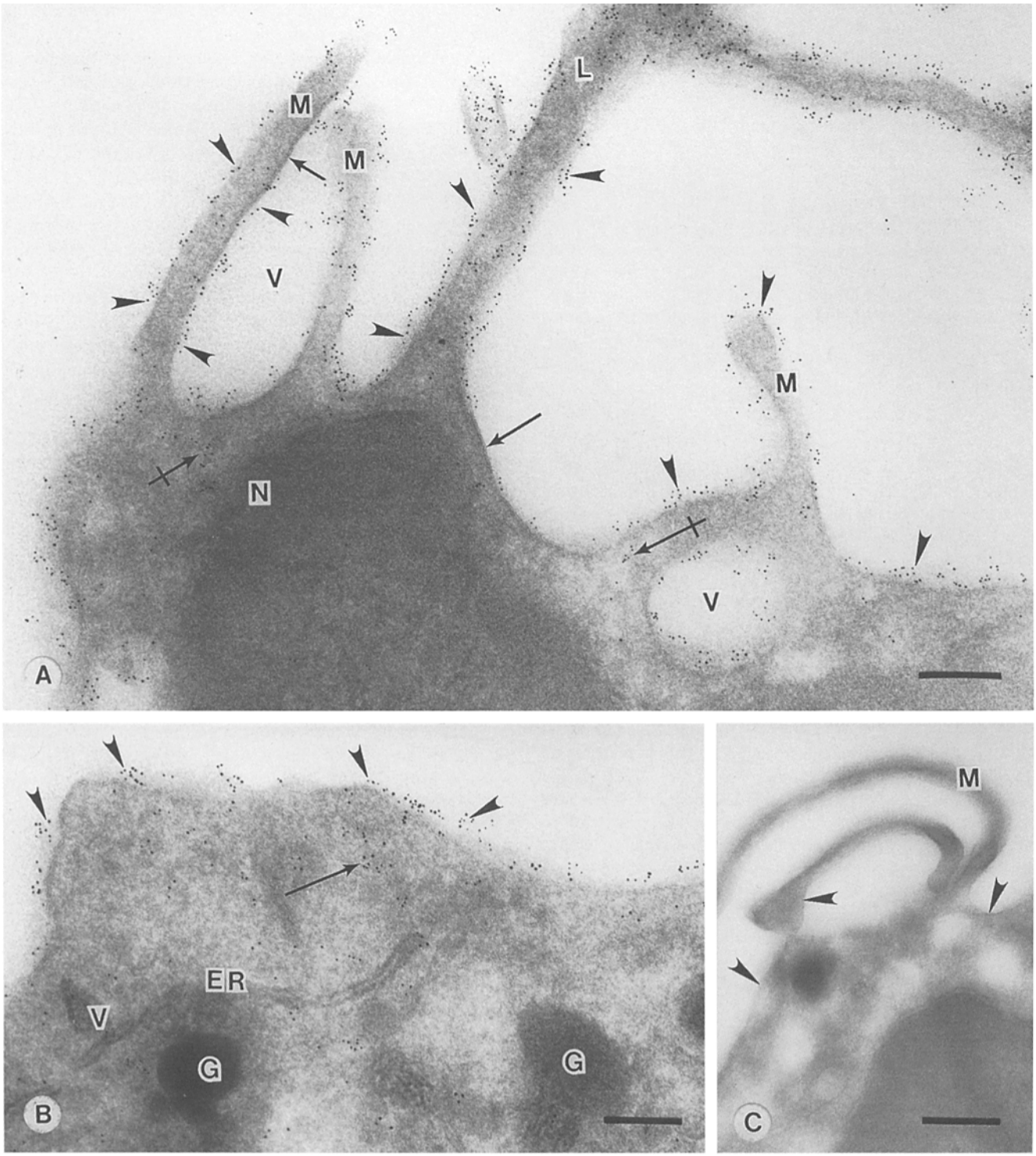

Figure 2. Immunoelectron micrographs of ultrathin cryosections of HKSA-stimulated human monocytes labeled with JD3 IgG. (*A*) ICE labeling is present on the external surfaces of the cell membrane (*arrowheads*; arrows depict the intact continuous lipid bilayer), and it is concentrated particularly on microvilli (*M*) and lamellae (*L*). Microvilli at left appear to be engaged in the formation of an intracytoplasmic vacuole with ICE-positive internal membranes (*V*). ICE epitopes are also found in the ground substance of the cytoplasm (*crossed arrows*), but they are excluded from the nucleus (*N*). No signs of apoptosis (cell surface blebbing and nuclear condensation) are evident. (*B*) Preparation similar to *A* depicting ICE JD3 epitopes localized on the external surfaces of the cell membrane (*arrowheads*) and within the cytoplasmic ground substance (*arrow*). Secretory organelles such as the ER, vacuoles (*V*), and dense granules (*G*) are not labeled. (*C*) Ultrathin cryosection of an HKSA-stimulated monocyte stained with preimmune IgG. No immunostaining is observed on the microvilli (*M*), the cell membrane (*arrowheads*), or within the cytoplasm. Bars for *A* and *B*, 0.2 μm; *C*, 0.4 μm.

Table 1. *Effect of ICE Inhibitors on Il-1β Secretion in Human Monocytes*

Stimulus*	ICE inhibitor‡	IL-Iβ secretion§	Percent inhibition
		ng/ml	
HKSA	RPMI	5.87	0
HKSA	L-709,049	1.63	72
HKSA	L-743,066	1.39	76
HKSA	L-742,395	1.88	68
HKSA‖	50 nM L-742,395	2.96	0
HKSA‖	18 μM L-742,395	0.75	75

*Elutriated human monocytes (5×10^5/ml) were stimulated with 10^6 CFU/ml of HKSA for 6 h. No IL-1β secretion was detected in companion cultures that were mock-stimulated with RPMI in the presence of 15 μM L-709,049, L-743,066, L-742,395, or medium alone.

‡Cells were treated with 15 μM ICE inhibitor added to their media at the beginning of stimulation.

§IL-1β was detected in culture supernatants by ELISA after 6 h of stimulation.

‖In a separate experiment, elutriated monocytes were stimulated with HKSA for 3 h and then treated with the indicated concentrations of biotinyl-ICE inhibitor for 30 min.

branes and lacked evidence of apoptosis such as cell-surface blebbing and pycnotic nuclei (Fig. 2, *A* and *B*).

In unstimulated peripheral blood monocytes, JD3 ICE epitopes were similarly localized on external surfaces of the cell membrane (especially on microvilli and in pinocytotic vesicles; Fig. 3, *A* and *B*) and in the cytoplasmic ground substance outside of the ER (Fig. 3 *B*). Since their culture media lacked detectable IL-1β (Table 1), these cells were not inadvertently activated. Very similar immunoEM patterns of ICE localization were also observed in the unstimulated human monocytic line THP.1 (data not shown). To ensure that significant numbers of unstimulated monocytes were positive for ICE, labeling was also assessed using immunofluorescent microscopy. Positive ICE staining was observed in most monocytes (>95%) of either unstimulated or stimulated cultures (Table 2). While most of the cells in the unstimulated cultures were IL-1β negative, 98% of the stimulated cells stained positive for IL-1β. Collectively, these results strongly suggest that ICE is expressed constituitively upon the cell surface and within the cytoplasm of both unstimulated and activated human monocytes.

Since the JD3 antibody detects both p20 and p45 ICE, parallel experiments were conducted with the anti-proICE antibody (I34-47) to distinguish proICE from mature ICE in activated and unstimulated monocytes. Anti–I34-47 IgG stained the cytoplasmic ground substance outside of the ER and nuclear envelope, but in striking contrast to JD3, did not label the external surfaces of the cell membrane, microvilli, and pinocytotic vesicles (Fig. 3 *C*). These data show that proICE epitopes are not detected on the cell membrane and imply that the ICE propiece is lost after ICE maturation (formation of p20/p10 ICE) at the cell surface.

Active-site EM Labeling of ICE with a Biotinylated Inhibitor. To confirm the immunoEM localization of ICE epitopes described above, we developed a highly sensitive method to label the active site of ICE in human monocytes. Intact HKSA-stimulated or resting monocytes were incubated with 15–18 μM of a biotinylated acyloxymethyl ketone (an irreversible inhibitor of ICE, L-742,395 [22]), resulting in 68–75% inhibition of IL-1β secretion by the activated monocytes (Table 1). Similar levels of inhibition were observed with non–biotinyl-acyloxymethyl ketone (L-743,066) or with the reversible aldehyde ICE inhibitor L-709,049 (Table 1), confirming the observation that biotinylation does not diminish the potency of the ICE inhibitor. Compound-treated monocytes were washed, fixed, cryosectioned, and L-742,395 detected with the avidin-immunogold amplification method. Using intact monocytes prelabeled with micromolar concentrations of L-742,395, biotinylated compound was concentrated on the extracellular surfaces of the cell membrane, microvilli, and pinocytotic vesicles, and was also observed in the cytoplasmic ground substance (Fig. 4 *A*). Similar results were obtained with nonstimulated monocytes incubated with L-742,395 (not shown). These patterns are strikingly similar to the distribution of ICE protein, as determined with antibody JD3. This suggests that the biotinylated compound is labeling the active site of ICE in intact monocytes. No signal was detected when HKSA-stimulated monocytes were preincubated with 50 nM L-742,395, a concentration which did not inhibit production of mature IL-1β (Table 1), or when monocytes were treated with micromolar quantities of nonbiotinylated ICE inhibitors (data not shown).

In striking contrast to the intact cell, active site labeling was observed at ~1,000-fold lower inhibitor concentrations if the compound was applied to ultrathin cryosections of stimulated monocytes. When 5–500 nM quantities of L-742,395 were applied to the sectioned monocytes, ICE active-site staining was again observed on the external surfaces of the cell membrane and within the cytoplasmic ground substance, but was absent from the ER and nuclear envelope (Fig. 4, *B* and *C*). This labeling was blocked by pretreatment with either the reversible ICE inhibitor L-709,049 (Fig. 4 *D*) or with the nonbiotinylated acyloxymethyl ketone (L-743,066); very little background was obtained when the sections were incubated with L-743,066 followed by the avidin-amplification reagents (not shown). These results are therefore highly specific for localization of active ICE. Since nanomolar quantities of L-742,395 failed to label monocytes if applied to intact cells, but did label ICE active sites if applied to ultrathin frozen sections of the monocytes, it appears that the ultrathin sectioning process permits the peptidyl ICE inhibitor to label subcellular sites normally not accessible in intact monocytes.

IL-1β Is Localized on the Cell Membrane during Cytokine Secretion. The localization of both p20 ICE epitopes and ICE active sites on the cell surface strongly suggests that IL-1β might interact with the monocyte cell membrane

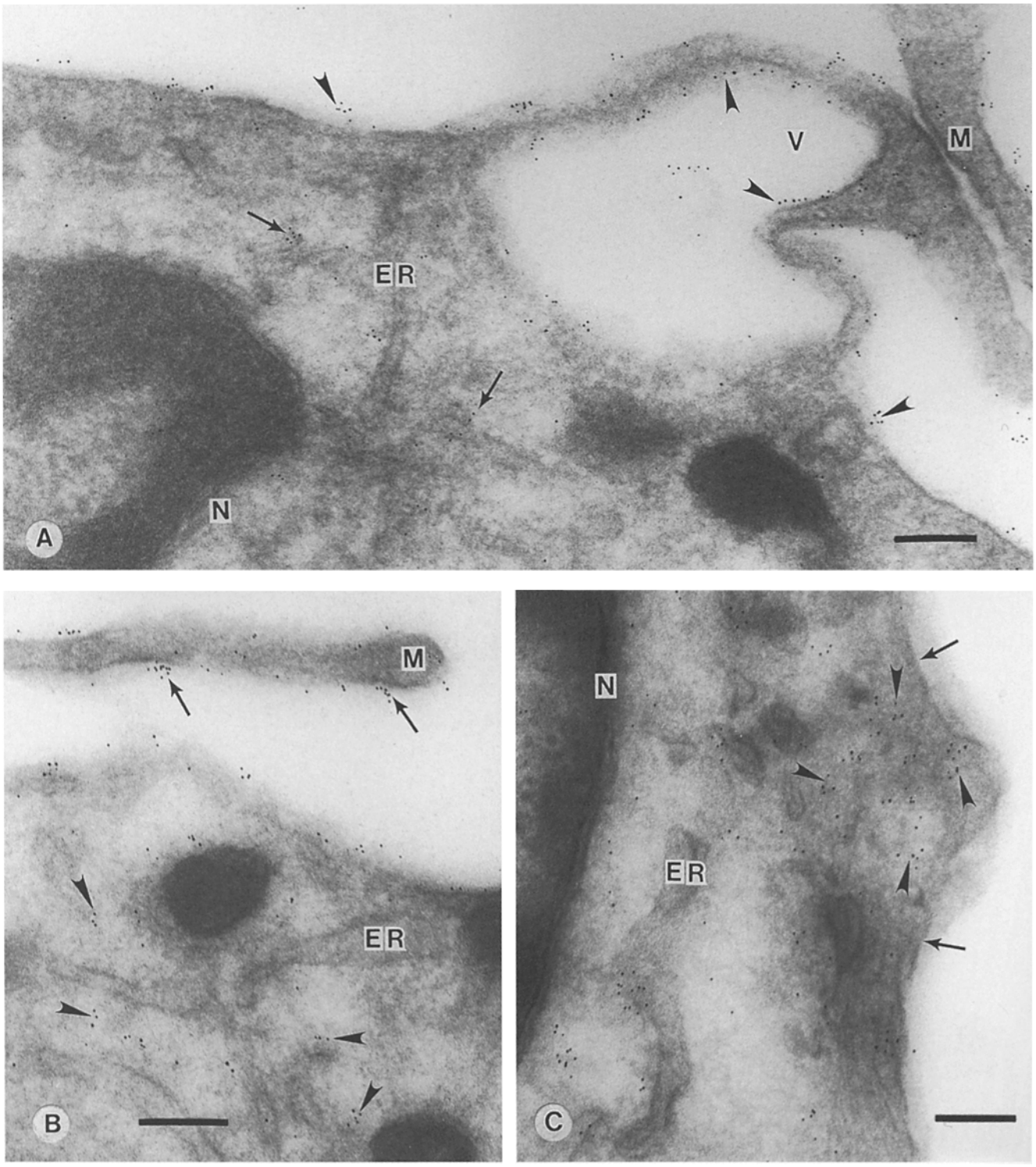

Figure 3. ImmunoEM localization of ICE JD3 and propiece (p11) epitopes in cryosections of unstimulated (*A* and *B*) or stimulated (*C*) human monocytes. (*A*) As with HKSA-activated monocytes, JD3 epitopes are localized on the external surfaces of the cell membrane (*arrowheads*), on microvilli (*M*), within pinocytic vacuoles (*V*), and in the cytoplasmic ground substance (*arrows*). The nucleus (*N*) and ER are unlabeled. (*B*) ImmunoEM micrograph of a cell similar to that in *A* illustrating the staining of JD3 epitopes on the surface (*arrows*) of a microvillus (*M*), and in the cytoplasmic ground substance (*arrowheads*) exclusive of the ER. (*C*) Immunolocalization of ICE propiece (p11) epitopes by I34-47 IgG is confined to the cytoplasmic ground substance (*arrowheads*) in HKSA-stimulated human monocytes. Unlike JD3, proICE labeling is not detectable on the outer surface of the cell membrane (*arrows*). Bars for *A–C*, 0.2 μm.

Table 2. *Immunofluorescent Microscopic Detection of ICE and IL-1β in Stimulated Versus Unstimulated Monocytes*

Antibody	Percent positive unstimulated monocytes	Percent positive stimulated monocytes
Anti-ICE (JD3)	98	95
Anti-IL-1β (197-215)	2	98

Coverslip cultures of HKSA-stimulated or unstimulated human monocytes were fixed as described (18), permeabilized with 0.1% Triton X-100 in PBS, immunostained with JD3 anti-ICE IgG or with an antibody against mature IL-1β, and bound IgG detected with FITC-conjugated goat anti-rabbit IgG. Data shown are the percentage of immunofluorescent-positive monocytes observed (sample sizes ranged from 500–800 cells per group).

during cytokine secretion. Since IL-1β was not detected at the monocyte cell membrane after conventional endotoxin stimulation (3), we sought to determine the location of the native ICE substrate proIL-1β and its cleavage product, mature IL-1β, under conditions in which the rate of IL-1β secretion is maximal (Table 3). Monocytes respond to very low LPS concentrations by synthesizing, but not secreting, proIL-1β (16, 23). Rapid processing and secretion of IL-1β can then be initiated with a high dose of LPS or HKSA, even in the presence of cycloheximide, indicating that the release of mature IL-1β occurs by conversion of proIL-1β from a preexisting pool (16). This protocol thus allows a direct comparison of the distribution of mature IL-1β and proIL-1β in nonsecreting and secreting monocytes. Human monocytes were fixed after both the priming and secretory phases of stimulation, and immunoEM was performed on ultrathin cryosections with an antibody against mature IL-1β (amino acid residues 197–215). Significant IL-1β staining was observed on the cell surface during the secretory phase of stimulation (Fig. 5 *A*), while labeling was absent from the cell surface but present in the cytoplasmic matrix during the priming phase when no IL-1β is released (Fig. 5 *B* and Table 3). Furthermore, another antibody directed against proIL-1β NH_2-terminal amino acid residues 3–21 (15) detected proIL-1β epitopes on the cell surface of secreting (Fig. 5 *C*) but not primed (Fig. 5 *D*) monocytes. The observation that proIL-1β or the propiece can be colocalized to the same subcellular site as active ICE itself lends further credence to our data concerning localization of ICE on the cell membrane. These data, however, do not indicate whether such cell surface–associated (presumably secreted) proIL-1β epitopes represent intact or fragmented IL-1β precursor.

To determine if the cleaved propiece of proIL-1β could be secreted from monocytes, polyclonal antisera were prepared to the COOH-terminal neo-epitope generated upon cleavage of proIL-1β by ICE. The specificity of the antibody was confirmed by immunoprecipitation and immunoblotting of purified proIL-1β, ICE-cleaved proIL-1β, and mature IL-1β. Antibody 502T did not detect intact proIL-1β or mature IL-1β after immunoprecipitation and Western blotting. Upon cleavage of proIL-1β with ICE, however, 502T quantitatively immunoprecipitated a 14.5-kD protein corresponding to the expected size of the proIL-1β propiece. Secretion of the propiece was confirmed by demonstrating the presence of this protein in culture supernatants of LPS stimulated monocytes along with mature IL-1β; 502T did not detect the 14.5-kD propiece in the corresponding cells (not shown). Treatment with the ICE inhibitor L-709,049 abolished the production of both the propiece and the mature IL-1β with a concomitant elevation in proIL-1β secretion (Fig. 6). The amount of propiece that was secreted appeared to be similar to the amount of secreted mature IL-1β. This is based on a spike recovery experiment that used an amount of ICE-generated IL-1β propiece equivalent to the amount of mature IL-1β found in the culture supernatants. The data suggest that proIL-1β is proteolytically processed as it is transported across the monocyte cell membrane during IL-1β secretion with a near quantitative release of propiece and mature IL-1β.

Discussion

We have used immunoEM to localize both ICE and IL-1β in human peripheral blood monocytes to further understand the role that ICE plays in the process of IL-1β secretion. Immunolocalization of ICE was accomplished using a polyclonal antibody recognizing epitopes common to the p20 subunit of active ICE and inactive p45 ICE (12), as well as an antipeptide antibody directed towards a sequence unique to the p45 ICE propiece. Immunolabeling was observed on the cell surface membrane using the antibody that recognized p20 and p45 ICE. Plasma membrane staining, however, was not detected with the ICE propiece–specific antibody. Since the propiece is absent from active ICE, this data suggests active ICE, but not proICE, is associated with the monocyte plasma membrane. In contrast, both immunoprobes detected ICE epitopes in the ground substance of the cytoplasm, outside of the ER and Golgi apparatus. This result is consistent with previous biochemical observations that ICE is found primarily in the monocyte cytosol (12). The specificity of the technique was confirmed by demonstrating that labeling was blocked by absorption of the primary antibody with purified human rICE for anti-p20 or with a specific peptide for antipropiece IgG. Staining was also abolished by substituting nonimmune IgG for primary antibody in each case.

To confirm and extend the immunoEM localization of p20 ICE protein in monocytes, we also developed a method to label the active site of ICE with a specific biotinylated probe. Intact human monocytes were activated with HKSA and then treated with L-742,395 (22). After fixation and cryosectioning of the cells, a significant amount of label was found to be associated with the plasma membrane, as well as with the cytosolic ground substance. This labeling appeared specific and significant since staining did not occur in the presence of the nonbiotinylated but otherwise iden-

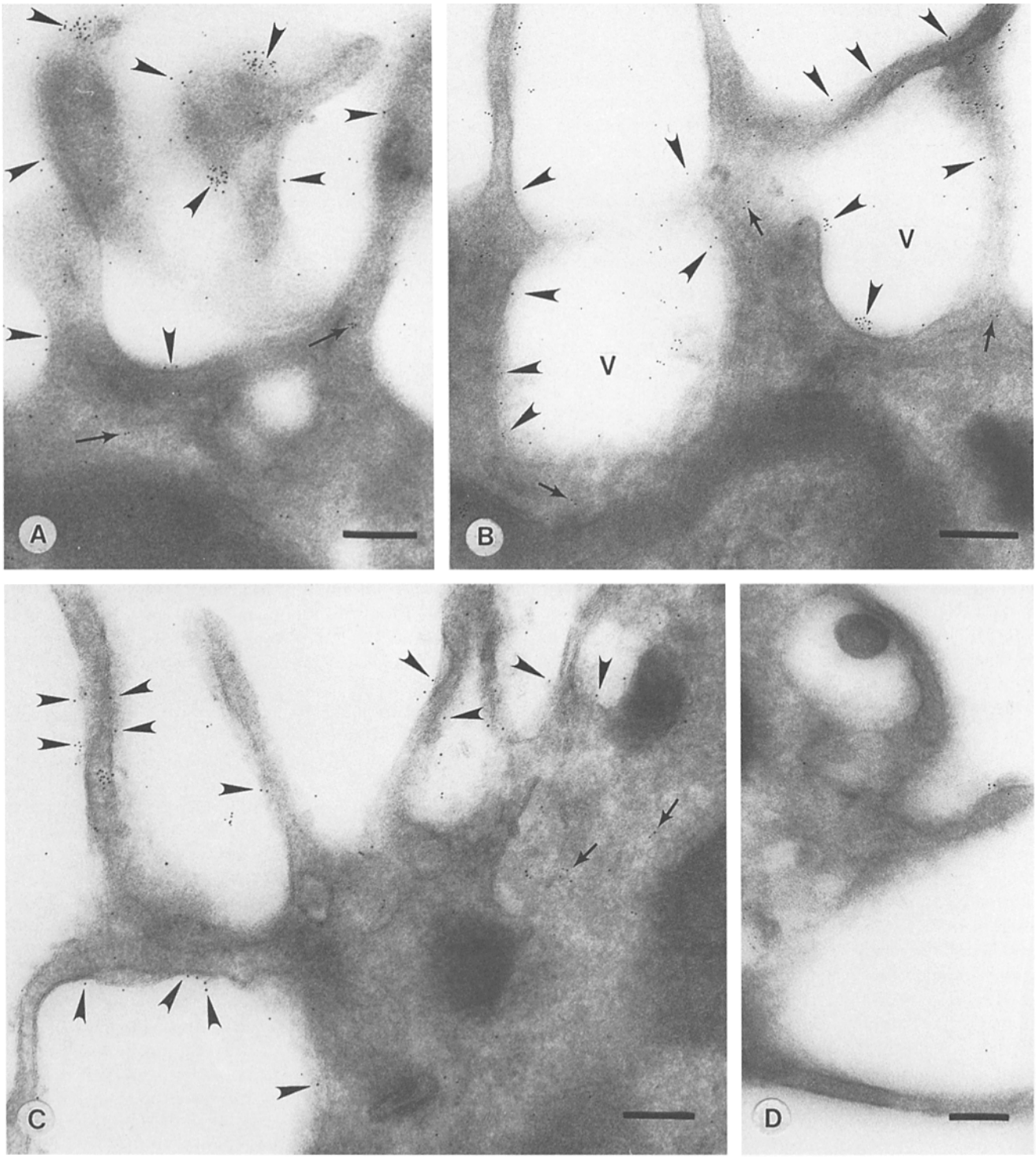

Figure 4. Immunoelectron micrographs illustrating ICE active site labeling in HKSA-stimulated human monocytes. (*A*) Monocyte that was treated with 18 μM L-742,395 before fixation and cryosectioning exhibits ICE active site labeling on the outer surface of the cell membrane (*arrowheads*) and within the cytoplasmic ground substance (*arrows*). This cell is from a culture that secreted 0.75 ng IL-1β/10^5 cells/ml into the medium (representing a 75% inhibition of IL-1β secretion). (*B*) Staining of active ICE with 500 nM L-742,395 after fixation and cryosectioning. ICE active sites are localized on the outer surfaces of the cell membrane (*arrowheads*), in pinocytic vacuoles (*V*), and within the cytoplasmic ground substance (*arrows*). (*C*) Active-site labeling of an activated monocyte cryosection with 5 nM biotinyl-irreversible ICE inhibitor. Although there is reduced staining, the patterns of active ICE labeling are similar to those observed in (*B*). (*D*) ICE active site labeling of a cryosection with 500 nM biotinyl-irreversible ICE inhibitor in the presence of a 400-fold molar excess of unlabeled L-709,049 added as a competitor. Most ICE active site labeling as illustrated in *B* is absent under these conditions. Bars for *A–D*, 0.2 μm.

Table 3. *IL-1β Secretion after the Staged Stimulation of Human Monocytes*

Stimulus	IL-1β secretion[§]
	ng/ml
LPS*	0.18
LPS + HKSA[‡]	2.60
RPMI	0.0

*Elutriated human monocytes (5×10^5/ml) were stimulated with LPS (50 pg/ml) for 6 h.
‡Cells (5×10^5/ml) were activated with LPS (50 pg/ml) for 4 h, followed by 10^6 CFU HKSA for an additional 2 h.
§IL-1β was detected in culture supernatants by ELISA after a total of 6 h stimulation.

tical ICE inhibitor L-743,066. Furthermore, L-742,395 labeled the cell surface membrane at micromolar concentrations but not at nanomolar concentrations when applied to whole cells. These results correlate directly with the quantities of L-742,395 required for inhibition of IL-1β secretion from these same cells. The correlation between labeling of the cell membrane with an ICE inhibitor and concomitant inhibition of IL-1β secretion strongly suggests that active ICE is localized to the plasma membrane.

Although the results of the whole-cell labeling experiment were highly significant, they were limited because micromolar quantities of L-742,395 were required to label the monocytes, suggesting that a portion of the intracellular ICE pool might not be accessible to this compound. A postsectioning method was therefore developed for labeling the ICE active site. This procedure involved detection of the biotinyated compound on ultrathin cryosections of fixed monocytes with an avidin-based amplification system. Labeling was performed directly on the sections to allow access of the ICE probes to all sectioned intracellular compartments. Intact monocytes were initially treated with the reversible ICE inhibitor L-709,049 in vitro to protect the active site before fixation, and they were then washed to deprotect the enzyme and remove any potential competitor. Subcellular sites of active ICE that were rendered accessible by the ultrathin sectioning process were then detected by labeling the cryosections with the biotinylated ICE inhibitor. Under these conditions, active ICE was localized to both the cell surface membrane and the cytoplasmic ground substance using nanomolar quantities of ICE inhibitor. Similar staining patterns were observed in stimulated or unstimulated monocytes. This labeling could be inhibited with an excess of the nonbiotinylated parent compound (a peptidyl acyl-oxy-methyl-ketone) or with the reversible ICE inhibitor L-709,049 (a peptidyl aldehyde). The latter result is particularly significant since these inhibitors share a tetrapeptide sequence (YVAD) required for ICE recognition, but they inhibit the enzyme by distinct chemical mechanisms (11, 22). The tetrapeptide sequence is a key determinant of inhibitor selectivity for ICE, and it suggests that the labeling occurs through specific recognition as opposed to nonspecific alkylating properties of this compound. Similarly, the specificity of these inhibitors for ICE should preclude labeling of ICE homologues since the selectivity of YVAD is 10^4 greater for ICE than for CPP32, an ICE-like protease recently linked to apoptosis (23a). Thus, the L-742,395 localized on monocyte sections most likely represents labeling of ICE.

The fact that ICE can be specifically detected on the monocyte plasma membrane by these methods is an indicator of the high resolution and sensitivity of our immunoEM technique. The stability of the interaction between ICE and the plasma membrane, as well as the quantity of cell membrane-associated ICE observed, however, cannot be ascertained from the present data. In addition, ICE activity has not been unequivocally demonstrated in association with membrane or particulate fractions from human monocytes by subcellular fractionation and biochemical methods. Indeed active ICE, as defined by the presence of p20/p10 subunits, has not been detectable in monocytes, where >99% of the observable ICE is the p45 species of ICE (12). There are several possible reasons for this apparent discrepancy. First, immunoEM localization was performed on monocytes cross-linked under conditions known to preserve native cell structure and antigenicity as much as possible, thus avoiding possible damage and/or loss of active ICE that might occur during cellular lysis and fractionation (3, 21). Second, the high resolution of immunoEM enables specific labeling of individual ICE active sites on relatively small amounts of membrane directly within the complex intracellular ultrastructure. This allows one to readily distinguish between active-ICE label localized on the cell membrane from ICE epitopes present in the cytosolic matrix. Third, the tremendous amplification potential of the biotin/avidin/antiavidin detection system permits detection of small quantities of a given epitope that is ordinarily not detectable by more conventional immunolabeling methods (24, 25). Given the sensitivity of the techniques that were used, the evidence suggests that ICE activity levels are low and are under strict regulation in monocytes.

The localization of ICE to the outer surface of the plasmalemma by two distinct methods strongly suggests that IL-1β secretion occurs through the plasma membrane, and that processing occurs on or in close association with the lipid bilayer. To extend this hypothesis, we sought to determine the location of the native ICE substrate, proIL-1β, and its cleavage product, mature IL-1β. To accomplish this, we compared the subcellular localization of IL-1β between monocytes that had synthesized proIL-1β, but either were actively secreting mature IL-1β or not (16). Localization of proIL-1β was accomplished by the use of an antipeptide antibody directed towards an NH_2-terminal prodomain epitope (1, 3, 15). A second antibody specific for the COOH-terminal domain was used to localize both mature and proIL-1β (1). In primed monocytes that were not secreting IL-1β, both prodomain and COOH-terminal IL-1β epitopes were located exclusively in the cytosolic ground substance. Subsequent treatment of the cells with

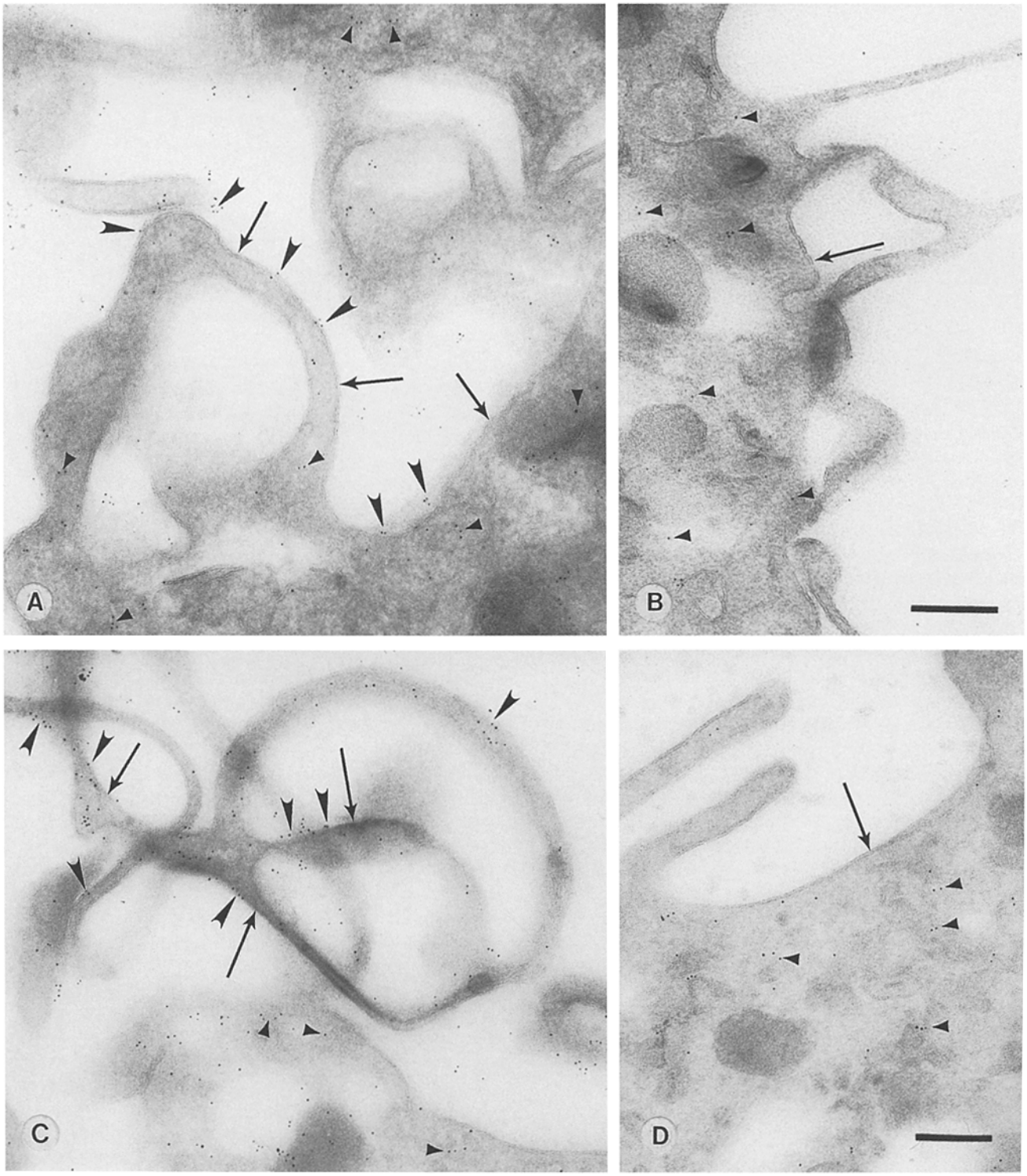

Figure 5. Mature (*A* and *B*) and precursor (*C* and *D*) IL-1β epitopes are localized on intact cell surface membranes of activated human blood monocytes during the secretory (*A* and *C*), but not in the priming phases (*B* and *D*) of the staged secretory process. Arrows in all panels delineate intact continuous cell surface membranes that show no evidence of apoptosis. (*A* and *B*) Mature IL-1β (amino acids 197–215) is localized on the cell surface membrane (*large arrowheads*) and in the cytoplasmic matrix (*small arrowheads*) of IL-1β–secreting cells (*A*), and is only observed in the cytoplasm (*small arrowheads*) of primed nonsecreting cells, exclusive of the cell membrane (*B*). (*C* and *D*) Similarly, IL-1β precursor (amino acids 3–21) is detected on the cell surface membrane (*large arrowheads*) and in the cytoplasmic ground substance (*small arrowheads*) of cells secreting IL-1β (*C*), but is restricted to the cytoplasm (*small arrowheads*) in primed cells (*D*). Bars for *A–D*, 200 nm.

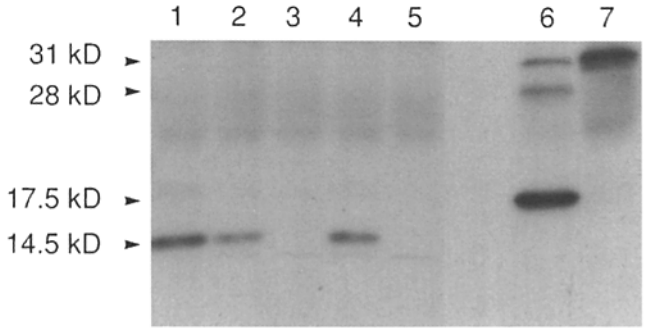

Figure 6. Identification of ICE-dependent proIL-1β cleavage products in supernatants of HKSA-stimulated human monocytes. IL-1β secretion products were identified by sequential immunoprecipitation and Western blotting with antibody 502T (lanes *1–5*) or with a rabbit anti–human IL-1β polyclonal antibody (lanes *6* and *7*) (16). Lane *1*, 60 ng of ICE-digested proIL-1β standard, no immunoprecipitation; lane *2*, spike recovery of 60 ng of ICE-digested proIL-1β in unstimulated monocyte cell culture supernatant; lane *3*, unstimulated monocyte cell culture supernatant; lanes *4* and *6*, cell culture supernatant from LPS-stimulated monocytes; lanes *5* and *7*, cell culture supernatant from LPS-treated monocytes in the presence of 100 μM L-709,049. The amount of IL-1β immunoprecipitated from the culture was 60 ng, based on determination of secreted IL-1β by ELISA.

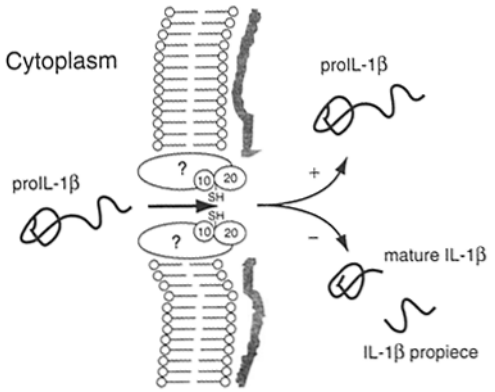

Figure 7. Transmembrane model of directional processing and transport of proIL-1β by ICE. ProICE present in the cytoplasm is proteolytically activated, assembled, and inserted into the cell membrane as a hypothetical multimeric ICE pore complex in association with as yet unknown transmembrane accessory proteins(?=unknown proteins). ICE pores consist of active ICE p20/p10 heterodimers arranged with their active sites (*SH*) protruding through the glycocalyx coating the outer membrane surface. Properly oriented proIL-1β may only enter the ICE pore through the cytoplasmic face of the cell membrane. ProIL-1β is proteolytically activated in the absence (−) of an ICE inhibitor, resulting in the secretion of mature IL-1β plus the IL-1β propiece. Intact proIL-1β is secreted in the presence (+) of ICE inhibitor. Like proIL-1β, ICE inhibitors may gain access to the ICE pore complex solely through openings on the cytoplasmic face of the plasmalemma. This hypothesis suggests that to inhibit ICE, sufficient cytoplasmic concentrations of peptidyl ICE inhibitors are required to interact with membrane-associated ICE in intact monocytes.

HKSA to induce high rates of IL-1β secretion resulted in a mobilization of IL-1β to the plasma membrane detectable with prodomain or mature domain IL-1β antibodies. In this system, the bulk of secreted IL-1β is the mature processed form (16). That the prodomain of IL-1β is also found associated with the plasma membrane suggests that this domain is also secreted.

Previous experiments have suggested that the propiece of proIL-1β is both cell-associated and secreted from stimulated monocytes (26). This study, however, did not ascertain whether the release of the propiece was related to ICE activity and/or IL-1β secretion. We have now detected a processed proIL-1β propiece in the medium using antibodies directed against the Asp-COOH–containing neo-epitope generated by ICE cleavage at the Asp^{116}–Ala^{117} position of proIL-1β. Treatment of monocytes with the ICE inhibitor L-709,049 resulted in inhibition of mature IL-1β (11) and IL-1β propiece secretion. Taken together with our immunoEM data, these results indicate that proIL-1β is probably processed close to the external surface of the plasma membrane during secretion, resulting in the release of mature IL-1β and the IL-1β propiece (Fig. 7).

Our observations raise the possibility that IL-1β is secreted via a nonclassical mechanism. We propose a model of directional IL-1β processing and transport directly through the plasma membrane (Fig. 7). There is some evidence to suggest that ICE is necessary and sufficient for secretion of IL-1β: coexpression of proIL-1β and ICE is required for the release of processed IL-1β in both transfected COS cells and baculovirus-infected insect cells (27, 28). The requirement for ICE catalytic activity in IL-1β secretion is not clear. In LPS-stimulated human monocytes, inhibition of IL-1β processing with low molecular weight ICE inhbitors such as L-709,049 does not block secretion; there is a quantitative release of proIL-1β instead. In Sf9 cells infected with baculovirus coexpressing proIL-1β, *crm* A, ICE, and IL-1β, however, secretion is completely blocked (28). This suggests that the interaction of ICE with a high affinity, large molecular weight inhibitor is capable of sterically blocking IL-1β secretion. Similarly, the observation that both IL-1β and IL-1α secretion are significantly reduced in mice that have had the ICE gene knocked out supports the hypothesis that ICE may be required for secretion, but not processing, of IL-1α since proIL-1α is not cleaved by ICE (29). This data further supports the hypothesis that ICE may be associated with IL-1 secretion that is independent of its proteolytic activity. Our data suggest that cytoplasmic proICE is proteolytically cleaved to form active p20/p10 heterodimer in association with the cell membrane. Because ICE has no known structural motifs associated with membrane localization, a mechanism must exist to localize the protein to the plasma membrane. We propose that ICE multimers are arranged into heteromeric pore complexes perhaps associated with as yet unknown transmembrane proteins. ICE would be oriented such that its p20/p10 heterodimers are arranged with their active sites protruding through the glycocalyx coating the outer membrane surface. Access of enzyme by substrate would occur through the cytoplasmic face of the cell membrane. Since both proIL-1β and the natural inhibitor crmA (30) do not serve as ICE substrates or inhibitors, respectively, when supplied extracellularly to cultured monocytes (data not shown), we think that the active site of ICE is functionally masked to extracellular macromolecules. The ABC family

of transporters may provide a paradigm for the association of ICE with transmembrane pore complexes. Similar proteins are involved in the secretion of both small hormones like yeast A factor (31, 32) or very large proteins such as hemolysin A in *Escherichia coli* (reviewed in reference 33).

There is a growing current literature documenting that ICE is a member of a family of cysteine proteases that cleave substrates at Asp P1, which may be associated with apoptosis in several systems (34–40). Overexpression of ICE has been shown to result in apoptosis, a process that may ultimately lead to the release of intracellular components in cell culture (37, 39, 40). Similarly, several reports have associated the onset of apoptosis with IL-1β secretion from monocytes (13, 14). Induction of apoptosis in LPS-treated murine macrophages by ATP treatment resulted in the release of processed IL-1β, and intracellular enzymes such as lactate dehydrogenase (13, 14). It is important to note, however, that apoptosis was observed in ATP-treated murine macrophages derived from ICE-deficient mice. Interestingly, the expected release of proIL-1β from these cells failed to occur after apoptosis (41). The data suggest that ICE itself is not necessary for the onset of apoptosis, and that IL-1β secretion is an active process that requires ICE. Apoptosis itself is probably driven by other distinct ICE-like proteases under tight regulatory control, whose activity is unrelated to IL-1β secretion (34, 36, 39, 42). The ultrastructural characteristics of actively secreting monocytes discerned from the high resolution frozen sections used in this study support this point as well. Secretion of IL-1β occurs from monocytes with no demonstrable evidence of the membrane and nuclear perturbations that occur during apoptosis (reviewed in references 43 and 44). In particular, the lipid bilayer of the cell membrane appeared totally intact and continuous. Microvilli and lamellipodia exhibited a normal morphology and did not exhibit any signs of blebbing. The nuclear chromatin was dispersed, and it lacked signs of margination and condensation. We conclude that IL-1β secretion may occur in stimulated human PBMC by a novel secretory mechanism that does not involve apoptosis, cell death, or surface membrane lysis.

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