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Immunology

Expression of membrane interleukin 1 by fibroblasts transfected with murine pro-interleukin 1α cDNA

(cytokines/gene transfer/protein processing/macrophages)

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ABSTRACT **Studies of interleukin 1 (IL-1) α and β have emphasized their functional similarities. IL-1α and -β are encoded by ancestrally related genes that have diverged dramatically in primary sequence; however, only modest differences in the regulation or biological activity of IL-1α and IL-1β have been documented. Here we show that mouse L cells transfected with murine pro-IL-1α cDNA expressed biologically active, 33-kilodalton pro-IL-1α, and that this pro molecule was neither processed to the 17-kilodalton mature form nor secreted. The transfected cells also expressed membrane-associated IL-1 biological activity, indicating that the pro-IL-1α cDNA can direct expression of membrane-associated IL-1 and that cleavage of the pro molecule is not required for membrane presentation. In contrast, transfection of pro-IL-1β cDNA did not generate biologically active material in L cells. Evidence is presented that the native murine IL-1β precursor molecule is also biologically inactive in peritoneal exudate cells stimulated with lipopolysaccharide. These differences in distribution of the bioactive forms of IL-1α and IL-1β may provide selective advantages for the maintenance of two gene products with similar functions.**

The interleukin 1 (IL-1) molecules are potent immunomodulatory cytokines produced primarily by monocytes and macrophages. Although defined as costimulators of T-cell activation, these proteins show diverse activities associated with proliferation, inflammation, and wound repair (1–3). Activated macrophages express soluble intracellular and extracellular forms (4), as well as a membrane-associated form (5), of IL-1 bioactivity. Although molecular and biochemical analyses have defined two distinct IL-1 molecules, termed IL-1α and IL-1β (6–12), their relationship to the biologically defined forms of IL-1 activity is not completely understood.

IL-1α and IL-1β are each produced as 31- to 33-kilodalton (kDa) soluble, cytosolic polypeptides in both mice and humans. With the exception of murine IL-1β, which has not been purified from a native source, the 17-kDa forms of the IL-1α and IL-1β molecules found in culture supernatants have been shown to be carboxyl-terminal fragments of the respective intracellular precursors (6–8). IL-1α and IL-1β cannot be distinguished on the basis of biological activity. Purified native and recombinant IL-1α and IL-1β show very similar, if not identical, activities as measured in a broad variety of assay systems (3).

These similarities in IL-1α and IL-1β expression and function are not, however, reflected in primary sequence. Murine IL-1α and IL-1β demonstrate only 45% nucleotide identity and only 23% amino acid identity without extended conserved domains (8, 9). Even when conservative substitutions are considered, the maximum amino acid homology is only 43%. It is also of interest to note that the IL-1 cDNAs encode neither a secretion signal peptide consensus sequence nor a hydrophobic, membrane-spanning domain (6–9). Thus, the cellular processing and transport pathways leading to the expression of membrane-associated and extracellular IL-1 remain undefined.

Genetic analysis has shown that the IL-1α and IL-1β genes are closely linked on mouse chromosome 2 (13). This finding and the conservation of intron/exon junctions (10–12) strongly suggest that the IL-1α and IL-1β genes arose by duplication of an ancestral gene and have since diverged. The observation that the murine and human IL-1 homologs are more highly conserved than IL-1α and IL-1β within either species‖ indicates that the duplication took place before human/mouse speciation. It is interesting that, although these ancestrally related genes have diverged >55% in primary nucleotide sequence, neither has become a pseudogene. Perhaps more striking is the finding that both IL-1α and IL-1β have retained the ability to bind a single IL-1 receptor and mediate all of the biological activities associated with IL-1 (14). We interpret this to indicate that there is strong genetic selection for the presence of two distinct molecules that are able to bind to the same receptor. We feel that an understanding of the nature of this genetic selection will be required for a complete understanding of the role of IL-1 in the inflammatory response.

To assess further the relationships between IL-1α and IL-1β and the biologically defined forms of IL-1, we have produced monoclonal antibodies specific for at least two independent epitopes of murine IL-1α (unpublished work). These antibodies bound to, and inhibited the biological activity of, recombinant murine IL-1α but not recombinant murine IL-1β. They only partially inhibited the IL-1 activity found in macrophage culture supernatants (presumably a mixture of IL-1α, IL-1β, and perhaps other costimulator molecules) but completely inhibited the membrane-associated IL-1 bioactivity present on fixed lipopolysaccharide (LPS)-stimulated murine peritoneal exudate cells (PECs). These results indicate that membrane-associated IL-1 is structurally related to IL-1α and may be a product of the same gene. Other investigators have reported similar results with monoclonal antibodies directed against human IL-1α peptides (15) and a polyclonal anti-murine IL-1α antiserum (16). We have now transfected murine fibroblasts with pro-IL-1α or pro-IL-1β cDNA to assess whether these cells could produce intracellular, membrane-associated, and/or secreted IL-1.

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Abbreviations: IL-1, interleukin 1; LPS, lipopolysaccharide; PEC, peritoneal exudate cell; SFFV-LTR, Friend spleen focus-forming virus 5′ long terminal repeat; SV40, simian virus 40.

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‖Murine IL-1α vs. human IL-1α, 62% amino acid identity; murine IL-1β vs. human IL-1β, 68%; murine IL-1α vs. murine IL-1β, 23%; human IL-1α vs. human IL-1β, 26%.

MATERIALS AND METHODS

Materials. Except as indicated, all reagents were purchased from Sigma. CBA/J retired breeder mice were from The Jackson Laboratory. Restriction enzymes were purchased from New England Biolabs, and radioisotopes from New England Nuclear and Amersham. Except where specifically noted, all media and tissue culture reagents were obtained from GIBCO. LPS (*Salmonella typhimurium*) was from Ribi ImmunoChem Research (Hamilton, MT). Fetal bovine serum was purchased from HyClone, and plastic tissue culture products were from Corning.

Construction and Transfection of IL-1 Expression Plasmids. Plasmid pFP502, containing the Friend spleen focus-forming virus 5′ long terminal repeat (SFFV-LTR), was kindly provided by Pamela Ohashi (University of Toronto) (17). A 700-base-pair (bp) *Pst* I–*Pvu* I fragment of pFP502, containing the SFFV-LTR, was modified by the addition of synthetic oligonucleotide linkers and ligated to Bluescript plasmid (Stratagene Cloning Systems, La Jolla, CA) that had been digested with *Sal* I and *Eco*RI. The *Eco*RI site 3′ to the SFFV-LTR was maintained for the insertion of cDNA fragments to be transcribed under the control of this promoter element. The simian virus 40 (SV40) early splice region and late polyadenylylation signals were obtained on a 912-bp *Ban* I–*Bam*HI fragment of pSV2cat and ligated 3′ to the *Eco*RI site to *Sma* I- and *Bam*HI-digested SFFV-LTR/Bluescript. A 2661-bp *Pvu* II–*Bam*HI fragment of pSV2neo (18), containing the neomycin-resistance gene (whose expression makes mammalian cells resistant to antibiotic G418) with the SV40 early region promoter and splicing and polyadenylylation signals, was ligated to *Bam*HI-digested SFFV-LTR/SV40/Bluescript. The final construct (SFFV.neo; Fig. 1) was confirmed by restriction endonuclease digestion and nucleotide sequencing of appropriate regions. Full-length pro-IL-1α cDNA (pmIL1 1–270) was a gift of Peter Lomedico (Hoffmann–La Roche). The *Hin*dIII–*Xba* I insert was ligated to *Hin*dIII- and *Xba* I-digested pUC19, and a 5′ *Eco*RI site was introduced by ligation of a synthetic oligonucleotide adapter into the *Hin*dIII site at the 5′ end of the IL-1α cDNA. The IL-1α cDNA was recovered on a single *Eco*RI fragment and was then ligated to *Eco*RI-digested SFFV.neo. The pro-IL-1β cDNA (pMuIL-1β, ref. 9) was kindly provided by Patrick Gray (Genentech, South San Francisco, CA). The 1300-bp *Eco*RI insert was ligated to *Eco*RI-digested SFFV.neo. SFFV.neo plasmids were stably introduced into mouse fibroblasts (19) by transfection using calcium phosphate precipitation (20).

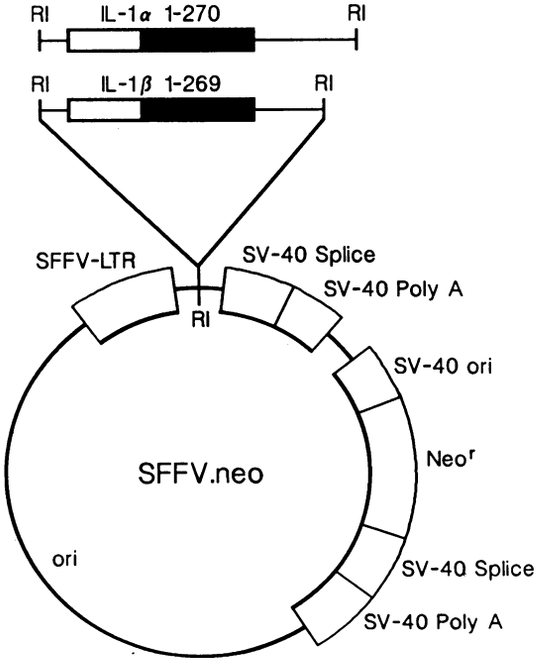

FIG. 1. Expression plasmids. Schematic of the SFFV.neo expression plasmid and the insertion of pro-IL-1 cDNAs. The shaded portions of the cDNAs represent the sequences found in the mature, extracellular forms. RI, *Eco*RI, restriction enzyme cleavage site; ori, origin of replication; Neo^r, neomycin-resistance gene.

Isolation and Analysis of RNA. Total cellular RNA was isolated (21) from transfected fibroblasts or LPS-stimulated P388D1 cells (1 μg/ml for 4 hr), electrophoresed, transferred to charged nylon membranes, and hybridized with nick-translated IL-1α and -β cDNA inserts as described (22). Hybridizing species were detected by autoradiography using Kodak XAR film with an intensifying screen.

IL-1 Costimulator Assay. IL-1 activity was assessed as described (5) by using the D10.G4.1 T-cell clone as an indicator line (23, 24). IL-1 activity was determined in culture supernatants (extracellular IL-1), on paraformaldehyde-fixed cells (membrane-associated IL-1) (5), in freeze–thaw extracts of washed cells (intracellular IL-1), and in dilutions of purified recombinant murine IL-1α (unpublished work).

Metabolic Labeling of Fibroblasts with [^{35}S]Methionine. Fibroblasts were plated at 10^6 cells per 30-mm dish 24 hr before labeling. Immediately prior to labeling, the cells were washed and incubated 1 hr at 37°C in RPMI-1640 medium (without methionine) supplemented with 10% (vol/vol) dialyzed fetal bovine serum, 10 mM Hepes buffer (pH 7.2), 100 μg of penicillin per ml, 100 μg of streptomycin per ml, 2 mM L-glutamine, and 20 μM 2-mercaptoethanol. After washing, the cells were reconstituted with 1 ml of the same medium plus 0.5 mCi of [^{35}S]methionine (1000 Ci/mmol; 1 Ci = 37 GBq) and incubated at 37°C. After 4 hr, culture supernatants were collected and cells were harvested by scraping into phosphate-buffered saline (PBS: 0.14 M, NaCl/0.01 M sodium phosphate, pH 7.4) containing 1% Nonidet P-40, 0.5% $NaDodSO_4$, 1 μg of leupeptin per ml, 1 μg of pepstatin per ml, 1 mM EDTA, 10 mM iodoacetamide, and 0.5 mM phenylmethylsulfonyl fluoride (lysis buffer). Duplicate cultures were washed and reconstituted in medium with unlabeled methionine to provide a "chase." After an additional 20 hr at 37°C, cells and supernatants were harvested as above.

Preparation of [^{35}S]Methionine-Labeled PEC Supernatant, Cytosolic, and Particulate Fractions. Murine PECs were harvested as described (5), washed, and resuspended in polypropylene tubes at 10^7 cells/ml in medium without methionine, as described above. After 1 hr at 37°C, the cells were washed, resuspended in methionine-free medium supplemented with [^{35}S]methionine (0.5 mCi/ml) and LPS (1 μg/ml) and incubated at 37°C. After 1 hr, a 1-ml sample (10^7 PECs) was removed. The remaining cells were washed, resuspended in medium with unlabeled methionine and LPS (1 μg/ml), and incubated as above. Additional 1-ml samples were collected at chase times of 1, 3, 8, and 18 hr. Culture supernatants were harvested by centrifugation and the cells were washed with medium. Washed cells were disrupted by 10 strokes with a Dounce homogenizer, depleted of nuclei by centrifugation for 10 min at 500 $\times$ g in an Eppendorf microcentrifuge, and separated into cytosolic and particulate fractions by centrifugation for 12 min at 95,000 $\times$ g in a Beckman Airfuge (A-100 rotor). The particulate fraction was resuspended by Dounce homogenization in PBS with protease inhibitors and centrifuged at 95,000 $\times$ g. The washed pellet was solubilized by 10 strokes with a Dounce homogenizer in lysis buffer. After 30 min on ice, the samples were centrifuged for 10 min at 16,000 $\times$ g in an Eppendorf microcentrifuge. The resulting supernatant contained solubilized total cellular membranes.

Immunoprecipitation. Aliquots of [^{35}S]methionine-labeled samples were incubated 16–24 hr at 4°C with 1 μg of

anti-IL-1α monoclonal antibody 161.1 (to be described elsewhere) in 1 ml of PBS containing 1% Nonidet P-40 and 0.5% $NaDodSO_4$. Immunoprecipitates were harvested by incubation with 5 μl of protein A-Sepharose for 2 hr at 4°C. The precipitates were washed extensively with PBS containing 1% Nonidet P-40 and 0.5% $NaDodSO_4$, suspended in PBS containing 10% (vol/vol) glycerol, 5% (vol/vol) 2-mercaptoethanol, 2.3% $NaDodSO_4$, and 62 mM Tris·HCl (pH 6.8), heated for 2 min at 100°C, and fractionated by $NaDodSO_4$/PAGE. The gels were fixed, stained, treated with EN^{3}HANCE (New England Nuclear), dried, and exposed to Kodak XAR film at −70°C.

RESULTS

Transfection and Expression of IL-1 in Fibroblasts. An efficient mammalian cDNA expression vector (SFFV.neo) was generated that uses the SFFV-LTR as a promoter. cDNA fragments containing the full coding region of murine IL-1α or IL-1β were introduced immediately 3′ to the SFFV-LTR (Fig. 1), and the plasmids were transfected into murine fibroblasts (L cells) by calcium phosphate precipitation. Colonies of stable integrants were selected by growth in medium containing antibiotic G418, and expression of the transfected cDNA was assessed in total cellular RNA. RNA blot hybridization analysis demonstrated the production of IL-1α and IL-1β mRNA only in those cells transfected with the appropriate IL-1 cDNA (Fig. 2). Murine IL-1α and IL-1β mRNA migrate at ≈2.0 kilobases (kb) and ≈1.6 kb, respectively, as indicated by hybridization to RNA from LPS-stimulated P388D1 cells. Transcripts originating from the SFFV-LTR and incorporating the SV40 splice sequences and polyadenylylation signals were ≈0.8 kb longer than the native mRNA species. In the IL-1α transfectants, a prominent 2-kb transcript was also observed, presumably representing polyadenylylation directed by the natural IL-1α polyadenylylation signal. A minor 1.6-kb RNA species was observed in the IL-1β transfectants, again presumably as the result of alternative polyadenylylation.

Expression of IL-1 Biological Activity in Transfected Fibroblasts. The cloned transfectant cells were tested for the production of IL-1 bioactivity in a T-cell proliferation assay. Mock-transfected cells demonstrated no IL-1 bioactivity, whereas fibroblasts transfected with IL-1α cDNA elaborated both intracellular and membrane-associated IL-1 (Fig. 3 *A* and *B*). This bioactivity was completely inhibited by monoclonal antibodies to murine IL-1α. Fibroblasts transfected with pro-IL-1β cDNA, in contrast, expressed IL-1β mRNA but did not produce detectable intracellular or membrane-associated IL-1 bioactivity [in three experiments, [^{3}H]thymidine incorporation induced by fixed or disrupted IL-1β-transfected fibroblasts never exceeded that induced by mock-transfected fibroblast controls (data not shown).] In addition, none of the transfectants released detectable IL-1 into the culture medium [IL-1α transfectants, Fig. 3*C*; with IL-1β transfectants, in three experiments, no IL-1 activity was detected in supernatants collected 24–48 hr after plating (data not shown)].

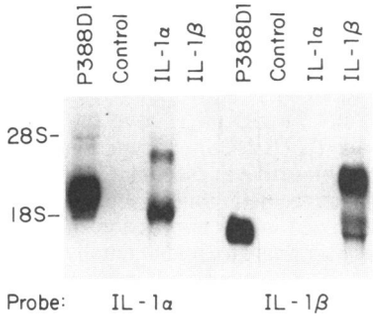

FIG. 2. RNA blot analysis of transfected fibroblasts. Total cellular RNA was isolated from LPS-stimulated P388D1 cells (P388D1 is a macrophage-like cell line), SFFV.neo-transfected control fibroblasts, and IL-1α- and IL-1β-transfected fibroblasts. After electrophoresis and transfer to charged nylon, the RNA was hybridized with radiolabeled IL-1α cDNA (*Left*) and then stripped of radioactive probe and hybridized with radiolabeled IL-1β cDNA (*Right*). Positions of 18S and 28S rRNA are indicated.

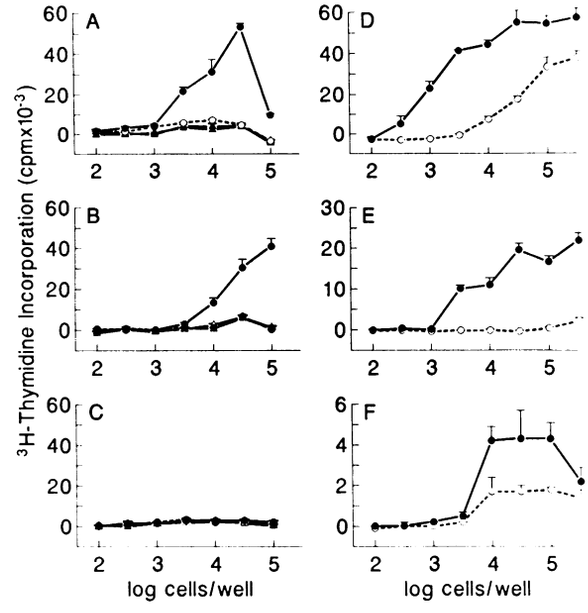

FIG. 3. Costimulator activity of transfected fibroblasts and murine PECs. Duplicate plates of transfected fibroblasts and LPS-stimulated PECs were subjected to five cycles of freezing and thawing or were fixed with 1% paraformaldehyde. Freeze–thaw lysates (*A* and *D*), fixed cells (*B* and *E*), and culture supernatants (*C* and *F*) were assayed for costimulator activity without (filled symbols), or with (open symbols) anti-IL-1α monoclonal antibody 161.1 at 10 μg/ml. (*A–C*) SFFV.neo control (△, ▲) and IL-1α transfectants (○, ●). (*D–F*) LPS-stimulated murine PECs. The abscissa for each panel is scaled to the maximum incorporation measured in each individual assay. Background incorporation never exceeded 8% of maximum incorporation and has been subtracted from each value.

Immunoprecipitation of IL-1α from Transfected Fibroblasts. Since release of IL-1 from macrophages is associated with cleavage, it was of interest to examine the molecular form of IL-1 produced in fibroblasts. Analysis of cell lysates of ^{35}S-labeled IL-1α transfectants demonstrated the production of only the 33-kDa form of IL-1α (Fig. 4*B*). No IL-1α was detected in the culture supernatants (Fig. 4*A*). These results, and the lack of detectable supernatant IL-1 bioactivity, indicate that fibroblasts are unable to process and release IL-1 in the same manner as macrophages.

IL-1 Bioactivity in Murine PECs. The human IL-1β precursor protein is biologically inactive by virtue of its inability to bind to the IL-1 receptor (25). Because L cells appear unable to cleave IL-1α to the mature 17-kDa form, the inability of IL-1β transfectants to produce biologically active material may indicate production of a similarly nonfunctional molecule. To assess which forms of IL-1 might be bioactive in murine macrophages, we measured the IL-1 activity present in culture supernatants, on fixed cells, and in freeze–thaw lysates of LPS-stimulated PECs. Anti-IL-1α monoclonal antibodies completely inhibited the biological activity of both intracellular and membrane-associated IL-1 (Fig. 3*D* and *E*) but only partially inhibited the activity found in culture supernatants (Fig. 3*F*). Although the addition of anti-IL-1α monoclonal antibody 161.1 at 10 μg/ml failed to

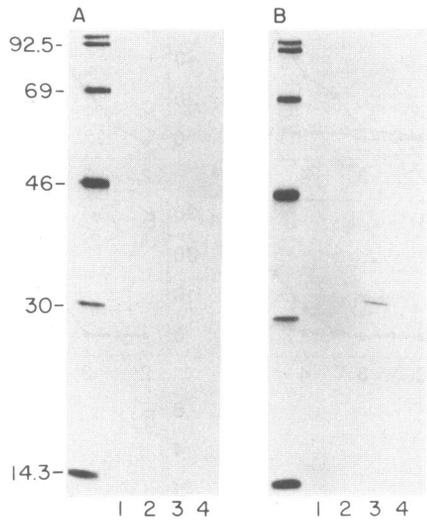

FIG. 4. Immunoprecipitation of ^{35}S-labeled fibroblasts. Transfected fibroblasts were labeled for 4 hr with [^{35}S]methionine and then chased for 0 or 20 hr by incubation in medium with unlabeled methionine, and samples were immunoprecipitated with anti-IL-1α monoclonal antibody 161.1. Left lane of each panel represents [^{14}C]methylated molecular mass markers (values given in kDa). (*A*) Culture supernatants. (*B*) Cell lysates. Lanes 1 and 2, SFFV.neo control; lanes 3 and 4, IL-1α transfectant. Lanes 1 and 3, 0-hr chase; lanes 2 and 4, 20-hr chase.

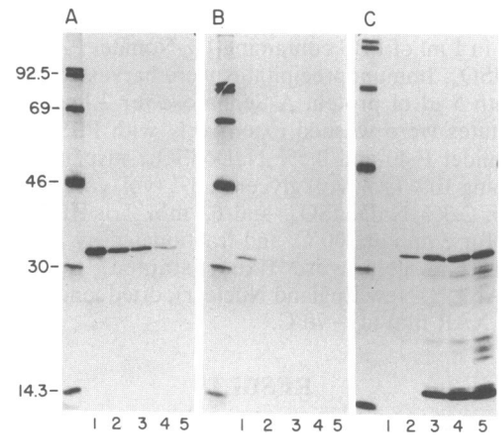

FIG. 5. Immunoprecipitation of ^{35}S-labeled murine PECs. PECs were labeled for 1 hr with [^{35}S]methionine and then chased for 0–18 hr with nonradioactive medium, and samples were immunoprecipitated with anti-IL-1α monoclonal antibody 161.1. Left lane of each panel represents [^{14}C]methylated molecular mass markers. (*A*) Cytosol. (*B*) Membranes. (*C*) Culture supernatants. Lanes 1, 0-hr chase; lanes 2, 1-hr chase; lanes 3, 3-hr chase; lanes 4, 8-hr chase; lanes 5, 18-hr chase.

inhibit all of the intracellular IL-1 activity at high PEC concentrations (Fig. 3*D*), complete inhibition was achieved with higher antibody concentrations (data not shown). In contrast, the remaining IL-1 activity in culture supernatants (Fig. 3*F*) was resistant to inhibition by higher antibody concentrations. The residual costimulator activity in these culture supernatants was presumably IL-1β, although other macrophage molecules with costimulator activity (e.g., IL-6) may have contributed to this activity. The complete inhibition of intracellular IL-1, presumably a mixture of pro-IL-1α and pro-IL-1β, by murine IL-1α-specific monoclonal antibodies strongly suggests that the murine IL-1β precursor protein is not biologically active. The data do not exclude the possibility that active murine IL-1β may be rapidly released from the cell.

Pulse–Chase Labeling of IL-1α in PECs. IL-1α processing in PECs was further investigated by [^{35}S]methionine pulse–chase labeling and immunoprecipitation of murine PEC lysates, membranes, and culture supernatants. Pulse-labeled intracellular 33-kDa IL-1α was chased through PECs during an 18-hr incubation (Fig. 5*A*) and appeared as both 33-kDa and 17-kDa IL-1α in the culture supernatant (Fig. 5*C*). In this experiment, 33-kDa extracellular IL-1α was detected prior to the appearance of the 17-kDa form. Immunoprecipitated material migrating between 33 and 17 kDa likely represented nonspecific degradation of the pro molecule. Of particular interest, 33-kDa IL-1α was immunoprecipitated from the detergent-soluble particulate fraction of PECs over the same time course of IL-1α movement from inside the cells to outside (Fig. 5*B*).

DISCUSSION

These data provide important insights into the molecular nature of membrane-associated IL-1. First, pro-IL-1α cDNA can direct the production of a membrane-associated IL-1 bioactivity by murine fibroblasts. This indicates that additional IL-1 genes, or the use or cryptic genomic transmembrane exons as a result of alternative mRNA splicing, are not required to generate membrane-associated IL-1. Second, murine fibroblasts are unable to process and release IL-1 in the same manner as PECs, suggesting that cleavage of IL-1 is mediated by a tissue-specific proteolytic pathway. The production of membrane-associated IL-1 in the absence of IL-1 proteolytic processing indicates that cleavage is not required for membrane presentation.

In these experiments, fibroblasts transfected with murine pro-IL-1β cDNA did not produce biological activity. This may reflect a lack of IL-1β protein production or may reflect the generation of a nonfunctional IL-1β product. Since there are no murine IL-1β-specific antibodies available, we were unable to demonstrate directly the presence of IL-1β protein in these transfected cells. Studies in which a portion (amino acids 23–269) of the IL-1β cDNA was expressed in *Escherichia coli* (unpublished work) and studies in which the full-length IL-1β cDNA was expressed in COS cells (9) have shown that the IL-1β cDNA encodes a functional molecule. Thus, the absence of costimulator activity in IL-1β-transfected L cells appears specific. An explanation for this lack of expression of bioactivity may be provided by our experiments (Fig. 3 *D–F*) in which the intracellular and membrane-associated IL-1 activities of LPS-stimulated murine PECs were completely inhibited by anti-IL-1α antibodies, whereas the culture supernatant activity was only partially inhibited. The residual costimulator activity in culture supernatants probably represents IL-1β, although other macrophage molecules with costimulator activity (e.g., IL-6) may contribute. The complete inhibition of intracellular IL-1 (presumably a mixture of pro-IL-1α and pro-IL-1β) by murine IL-1α-specific monoclonal antibodies suggests that the murine IL-1β precursor protein is not biologically active or, alternatively, that bioactive murine IL-1β does not accumulate inside cells. The accumulation of bioactive intracellular IL-1α, and the presentation of a bioactive membrane-associated IL-1α, defines a functional difference in the expression of IL-1α and IL-1β. This difference may relate to fundamentally different functions of the two IL-1 molecules and may provide a selective advantage for the maintenance of two distinct IL-1 genes.

We suggest that the primary function of IL-1α may be presentation as a membrane-associated molecule, crucial in cognate interactions between antigen-presenting cells and responding T cells. The restriction of IL-1 to the membranes of stimulated antigen-presenting cells would naturally limit the effects of IL-1 to only those cells directly involved in the immune response. Given the potent, often damaging, effects of IL-1 on a broad spectrum of tissues, it may be advanta-

geous to limit the activity of IL-1 under conditions of low stimulation (e.g., antigen presentation). IL-1β, in contrast, may be the primary extracellular mediator of the hormonal effects of IL-1. Recent publications suggest that IL-1β is the primary extracellular IL-1 measured in the culture supernatants of LPS-stimulated human monocytes and in the serum of septic patients (26, 27). In addition, another recent report indicated that human IL-1α is retained as a cell-associated molecule for up to 12 hr after LPS stimulation, whereas IL-1β is released and found in the supernatant after only 2 hr (28). Conditions of maximal or systemic stimulation (e.g., sepsis) may drive the production of large amounts of both IL-1α and IL-1β. If IL-1β is released from the cells with greater efficiency, it may function as the major effector molecule of the distal effects of IL-1.

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