Intracellular Precursor Interleukin (IL)-1α, but Not Mature IL-1α, Is Able to Regulate Human Endothelial Cell Migration in Vitro*

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The human umbilical vein endothelial cell (HUVEC) has a finite lifespan in vitro, and senescent HUVEC contain elevated levels of the negative growth regulator interleukin (IL)-1α. IL-1α is translated as a signal peptide sequence-less cytosolic 31-kDa precursor (IL-1α p), which undergoes proteolytic activation to release the mature carboxyl terminus 17-kDa protein (IL-1α m). Both the IL-1α p and IL-1α m proteins are biologically active as exogenous cytokines. Interestingly, only IL-1α p contains a nuclear localization sequence between residues 79 and 85. To further study the role of intracellular IL-1α in the regulation of human endothelial cell function, a spontaneous HUVEC transformant was stably transfected with IL-1α p, IL-1α m, and the IL-1α p K82N mutant, which attenuates the nuclear traffic of IL-1α p. Interestingly, the IL-1α p transfectants were found to have a lower migratory potential than either IL-1α m or IL-1α p K82N transfectants, and the addition of the IL-1 receptor antagonist did not alter the migration of these cells. Immunofluorescence microscopy demonstrated that only the IL-1α p transfectants exhibited prominent staining for β-catenin-associated cell-to-cell contacts, as well as pronounced vimentin intermediate filaments and actin cytoskeleton staining. These data suggest that IL-1α p, and not IL-1α m, may function as an intracellular regulator of the migratory capacity of the human endothelial cell and that the nuclear localization sequence present within IL-1α p may be involved in regulating this function.

Interleukin (IL)-1 consists of a family of cytokines which play an important role in inflammation and the response to injury (1). The family consists of three members, IL-1α, IL-1β, and the IL-1 receptor antagonist protein (IRAP). Signal transduction is initiated by the binding of either IL-1α or IL-1β to its receptor; conversely, as its name suggests, the binding of IRAP to the IL-1 receptor does not activate any downstream effector molecules, but rather, acts as a competitive inhibitor of ligand-receptor binding (2).

The IL-1 family is expressed as precursor proteins; the precursors of IL-1α and IL-1β are translated as 31-kDa proteins, which are processed to the mature carboxyl terminus 17-kDa forms (3). A calpain-like protease cleaves IL-1α between residues 112 and 113 (4); however, expression of either the precursor (p) IL-1α p or mature (m) IL-1α m forms of the protein in a rabbit reticulocyte system has shown that both IL-1α p and IL-1α m bind to the IL-1 receptor and are biologically active (5, 6). Conversely, IL-1β is only functional as the 17-kDa protein, with proteolytic activation by the IL-1β-converting enzyme being necessary for its activity (7). Interestingly, the 16-kDa amino-terminal domain of the IL-1α p contains a nuclear localization signal (NLS) (8) and is translocated to the nucleus and produces a transformed phenotype when expressed in rat mesangial cells (9).

Activation of monocytes by lipopolysaccharide or phorbol myristic acid leads to the release of IL-1α p and IL-1α m (10, 11); however, like the structurally related fibroblast growth factor (FGF) prototypes (12), IL-1α lacks a classical signal sequence for secretion (3). Although receptor-mediated endocytosis and nuclear association of secreted IL-1α m has been demonstrated (13), there is increasing evidence to suggest that the intracellular form of IL-1α p is biologically active, and may be directed to the nucleus via a nuclear translocation signal (8, 14, 15), in a manner similar to the FGF prototypes (16). Similarly, while IRAP contains a functional signal sequence (1), an alternatively transcribed IRAP mRNA is expressed as an intracellular signal peptide sequence-less protein (17).

Human umbilical vein endothelial cells (HUVECs) have a limited proliferative capacity in vitro (18) and senescent HUVEC populations are refractory to the chemotactic and mitogenic (19) response of FGF, which is required for HUVEC propagation (20). The senescent HUVEC population contains elevated steady state levels of the IL-1α transcript, as well as increased levels of IL-1 response genes (21). Furthermore, the addition of an IL-1α-specific antisense oligomer to the senescent cells extends their proliferative capacity in vitro (22).

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MATERIALS AND METHODS

Plasmid Construction—The IL-1α p cDNA in the plasmid Cfla was digested with Asel and HincII to release a 935-base pair fragment containing the full-length IL-1α open reading frame. The fragment was blunt-ended with Klenow enzyme (Boehringer Mannheim) and inserted into the pMEXneo vector (24) previously digested with BamHI, blunt-ended with Klenow enzyme, and treated with calf intestine alkaline phosphatase (Boehringer Mannheim). To generate the IL-1α cDNA, the polymerase chain reaction (PCR) was used with a 5'-primer containing a Smal site and the Kozak sequence, encompassing an ATG site, upstream of the serine residue at position 113 of the IL-1α sequence. The 3'-primer was downstream of the TAG stop codon and contained a HincII site. The primers were: sense, 5'-GCTAGCCCGGGCACTTGTAGGAAGG-3' and antisense, 5'-CATTCTGGCACTTTGGACAGC-3'. The PCR product was ligated into the TA cloning vector (Invitrogen), the IL-1α p sequence excised with Smal and HincII and ligated into pMEXneo as described above. The PCR product was confirmed by sequencing.

Cell Culture, Transfection, and Biologic Assays—ECV cells were propagated in M199 media (JHR Biosciences) containing 10% (v/v) fetal bovine serum (FBS) and were stably transfected with 10 μg of either the insert-less pMEXneo vector or the vector containing IL-1α p, IL-1α m, or IL-1α p K82N (8) using the calcium phosphate procedure (Stratagene) and selection with 1.2 mg/ml G418 (Life Technologies, Inc.). The human melanoma cell line, A375, was propagated in Dulbecco's modified Eagle's medium (JHR Biosciences) supplemented with 10% (v/v) FBS and used to assess the function of IL-1α in lysates derived from the various ECV cell transfectants (26). Cell growth was measured by staining the cells with 0.1% (w/v) crystal violet, and the absorbance of each sample was measured at 570 nm using a microplate reader (Molecular Devices). The migration was performed using a wound repair model (27).

Cell Lysis and Immunoblot Analysis—Confuent monolayers of vector control, IL-1α p or IL-1α m ECV transfectants were lysed as described (8). Aliquots were resolved by 12.5% (w/v) SDS-PAGE as described (25). The proteins were transferred to 0.2-μm nitrocellulose membranes, and membranes were probed for IL-1α-specific proteins using a 1:1200 dilution of a goat anti-human IL-1α antibody (1.21 mg/ml), in 50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl and 5% (w/v) milk. Bands were visualized by enhanced chemiluminescence as described (19). The antibodies used were: a monoclonal anti-β-catenin antibody (Transduction Laboratories, C19220L3), rabbit anti-mouse cortactin antisera 2719 (28), and a monoclonal anti-vimentin antibody (Dako, M725). For staining of filamentous actin, coverslips were incubated in blocking buffer (19) for 1 h, incubated with fluorescein-conjugated phalloidin (1 μg/ml) (Sigma) for 30 min, embedded and photographed as described (19).

RESULTS AND DISCUSSION

The proliferative and migratory responses of HUVEC in vitro are dependent upon the addition of FGF (20, 29), and addition of IL-1α attenuates these effects (19). Certain strains of senescent HUVEC have an increased steady state level of intracellular IL-1α p (21), and studies from our laboratory have shown that the elevated levels of IL-1α correlates with an attenuation of the migratory and growth response of senescent HUVEC to FGF-1 (19). To further investigate the role of intracellular IL-1α p in the human endothelial cell, a spontaneous HUVEC transformant, the ECV cell, was stably transfected with the pMEXneo vector alone or with this plasmid containing either IL-1α p, IL-1α m, or the IL-1α p K82N NLS point mutant, which attenuates nuclear traffic of intracellular IL-1α p (8). The ECV cell line was chosen, since (i) it has similar properties to normal endothelial cells, (ii) it expresses extremely low levels of endogenous IL-1α, (iii) this cell line is inhibitory to resting IL-1α transfectants expressing high levels of IL-1α protein would not be selected due to an impaired proliferative phenotype, it should still be possible to select stable IL-1α transfectants with low levels of IL-1α expression, which would permit ECV cell growth and enhanced studies on the intracellular function of IL-1α. Individual clones were selected and expanded, and cell lysates were analyzed for IL-1α by immunoblot analysis (Fig. 1A). While no IL-1α-specific proteins were detected in samples from vector control-transfected cells, a single 31-kDa band was detected in the IL-1α p and IL-1α p K82N transfectants. Similarly, a band of approximately 17 kDa was detected in clones transfected with the IL-1α m-containing plasmid.

To determine whether the proteins detected by immunoblot analysis were biologically active, lysates of the transfected cells were prepared and analyzed for functional IL-1α activity using inhibition of A375 human melanoma cell growth (26) (Fig. 1B). The highest concentration of vector control ECV cell lysates inhibited the growth of the A375 cells by approximately 50%. However, addition of lysate from IL-1α p-, IL-1α p K82N-, and IL-1α m-transfected ECV cells inhibited the growth of the cells significantly more than equivalent volumes of vector control lysate. Based on a standard titration, where 100 pg/ml of human recombinant IL-1α inhibited the growth of the A375 cells by 50% (data not shown), the growth inhibition assay detected 2960 pg of IL-1αp10cells for IL-1α m transfectants, 2108 pg of IL-1αp10cells for the IL-1α p transfectants, and 5244 pg/10 7 cells for the IL-1α p K82N transfectants. Only 158 pg/10 7 cells of IL-1α growth inhibitory activity was detected from lysates of vector control-transfected cells. Further, addition of 100 ng/ml IRAP reversed the growth inhibitory effect of the IL-1α p and IL-1α m lysates (data not shown), suggesting that the specific growth inhibitory factor in these lysates is IL-1α.

To determine whether expression of the IL-1α-specific proteins in the ECV cells had any effect on their migration, vector control, IL-1α p and IL-1α m ECV cell transfectants were examined in a wound assay, and the results shown in Fig. 2A represent the mean of four separate experiments. The migration of vector control ECV transfectants increased rapidly as a function of the concentration of serum and reached a maximum at 2% (v/v) FBS. Conversely, IL-1α p ECV cell transfectants cells exhibited a significantly reduced level of migration; the number of cells migrating into the denuded area was approximately 60% of the vector control ECV population. Interestingly, the IL-1α m ECV transfectants exhibited an increased migratory response relative to vector control-transfected cells. Further, the migration of additional IL-1α clones obtained from a second transfection study produced similar results as described in the legend to Fig. 2A.

To assess whether the modification of ECV cell migration by
IL-1α p and IL-1α m was a result of their intracellular biological activity, we examined the ability of the various ECV cell transfectants to respond to the addition of exogenous IRAP. We anticipated a reversal of the migratory responses in the presence of IRAP if the observed effects were due to the release of either of the two forms of IL-1α. As shown in Fig. 2B, the migration of the IL-1α p and the IL-1α m ECV cell transfectant were similar to the results in Fig. 2A, and thus, these cells were not sensitive to the addition of IRAP. The ratio of IL-1α p transfected ECV cells to vector control-transfected cells migrating into the denuded area remained constant, such that a ratio of 63.4% was determined in the absence of IRAP, while a ratio of 68.9% was found upon IRAP addition. In addition, exogenous IL-1α was able to increase the steady state mRNA levels of the IL-1 response gene, plasminogen activator inhibitor (PAI)-1 in vector control, IL-1α m and IL-1α p ECV cell transfectants (data not shown), suggesting the presence of functional IL-1 receptors at the cell surface. Thus, it is likely that the modulation of ECV cell migration by IL-1α p may be the result of the polypeptide acting as an intracellular modifier in vitro.

The FGF and IL-1 prototypes contain a nuclear localization signal, and the nuclear traffic of these proteins has been studied in detail (8, 15, 16, 30). To determine whether nuclear localization of IL-1α p affected the migratory response, the ECV cell was stably transduced with an IL-1α p affected by the release of IL-1α p point mutant in which residue 82 in the NLs was changed from lysine to glutamic acid. This residue is critical for nuclear traffic, since IL-1α p is associated with the nucleus, while the IL-1α p K82N remains cytosolic in transfected NIH 3T3 cells (8) and transfected ECV cells (data not shown) as β-galactosidase fusion proteins. Analysis of the migratory ability of the IL-1α p K82N ECV cell transfectants (Fig. 2C) demonstrated an increased level of migration relative to vector control and IL-1α p ECV cell transfectants (Fig. 2, A and B), which also was refractory to the addition of IRAP. These data suggest that nuclear localization of intracellular IL-1α p may be important for its ability to repress ECV cell migration.

Because the low migratory potential of the IL-1α p ECV cell transfectant may be related to an abundance of focal adhesion sites (FAS), to a bulky and stiff cytoskeleton or to the exaggeration of cell-to-cell contacts (31, 32), the ECV cell transfectants were examined for differences in their intracellular architecture using immunofluorescence microscopy (Fig. 3). The ECV cell transfectants were stained with an antibody against vinculin, the ubiquitous component of FAS, and no significant differences in the distribution or density of FAS between vector control, IL-1α m, and IL-1α p ECV cell transfectants were detected (data not shown). However, unlike the vector control transfectants (Fig. 3A), the IL-1α p transfectants (Fig. 3B) demonstrated a stronger intensity of staining for cell-to-cell contacts as shown by staining with an antibody against β-catenin, an essential component in the formation of adherence junctions of the human endothelial cell (32). In contrast, IL-1α m transfectants exhibited a similar staining intensity of cell-to-cell contacts as the vector control-transfected cells upon staining for β-catenin (data not shown). The staining of ECV cell transfectants with an antibody against vimentin, a major component of intermediate filaments of mesoderm-derived cells, revealed a more developed and complex network in the IL-1α p transfectants (Fig. 3D) than the vector control (Fig. 3C) transfectants. Further, ECV cells stained with fluorescein-conjugated phalloidin revealed a very small number of actin stress fibers in vector control ECV transfectants (Fig. 3E), where the filamentous actin was most associated with the cell membrane. In contrast, phalloidin staining of the IL-1α p ECV transfectants demonstrated numerous highly ordered stress fibers (Fig. 3F). Interestingly, immunofluorescence microscopy of IL-1α p ECV cell transfectants with an antibody against cortactin, a Src substrate (33) and F-actin-binding protein (34), revealed cortactin-positive cytoplasmic patches (Fig. 3H), which were not observed in either the vector control or IL-1α m ECV cell transfectants (Fig. 3G and data not shown).

We have reported that HUVEC senescence in vitro may be mediated by the intracellular function of the signal peptide-less
cokine, IL-1α (21, 22). Since our data suggest that the expression of IL-1α p, but not IL-1α m, in the ECV cell results in an impaired migratory phenotype that is (i) serum-dependent, (ii) refractory to the presence of exogenous IRAP, (iii) sensitive to point mutagenesis of the IL-1α p NLS, and (iv) correlates with an apparent increase in stress fibers, it is likely that these changes are due to the functional properties of intracellular precursor IL-1α as a nuclear protein. Although our data demonstrate that intracellular IL-1α p is able to repress ECV cell migration, we were unable to convincingly correlate this event with a decrease in ECV cell division. While it was possible to obtain IL-1α p ECV cell transfectants whose proliferative capacity appeared to be diminished in comparison with vector control ECV cell transfectants (data not shown), this phenotype was not consistently observed in four different IL-1α p transfectants analyzed.

It is interesting that, unlike the IL-1α p ECV cell transfectants, the IL-1α m and IL-1α p K82N ECV cell transfectants were not able to repress cell migration; however, the IL-1α translation product of all forms of the IL-1α protein appears to be functional as exogenous proteins in the inhibition of A375 cell growth. In addition, the steady state levels of the IL-1α responsive gene PAI-1 is elevated in the IL-1α p, IL-1α p K82N, as well as the IL-1α m ECV cell transfectants (data not shown). Thus it appears that although both forms of IL-1α are functional and can modify steady state gene expression, only the precursor form of IL-1α appears to be involved in the regulation of human endothelial cell migration. Further, the induction of IL-1α-dependent genes does not correlate with the regulation of endothelial cell migration, and this is consistent with our results, which show that the FGF-dependent induction of cell cycle-specific gene expression in senescent HUVEC populations may not be sufficient to promote a proliferative or migratory phenotype (19).

The mechanism utilized by IL-1α p to attenuate endothelial cell migration in vitro is not known, although it does appear likely that intracellular IL-1α p may be able to alter the cytoskeleton of the human endothelial cell. Interestingly, the appearance of prominent actin stress fibers in the IL-1α p ECV cell transfectants resembles a similar morphologic feature observed in the senescent HUVEC and human diploid fibroblasts (35). These stress fibers were distributed throughout the cytoplasm, unlike stress fibers of migrating cells, which are known to exhibit polarity, being localized at the leading edge (36). Because it is well established that the lamellipodium is formed by depolymerization and repolymerization of the actin cytoskeleton, and this process is regulated by phosphoinositide turnover (36), it is possible that intracellular IL-1α p may be able to modify these events.

The bulky actin cytoskeleton and prominent network of vimentin filaments could contribute to the low motility of IL-1α p ECV transfectants. In addition, the prominence of intracellular adhesion sites as observed by the presence of β-catenin in cell-to-cell contacts may also contribute to the decrease in the motility of the IL-1α p transfectants. Indeed, the redistribution of β-catenin to the region of cell-to-cell contacts has been shown to be regulated by the Src signaling pathway (37). The observations that (i) FGF-1 is known to regulate Src activity in human endothelial cells (38), (ii) the phosphorylation of Src and its translocation to focal adhesions are involved in cell migration (39, 40), and (iii) the kinase activity of the Src protein is decreased in senescent HUVECs, which contain elevated levels of IL-1α p, with a corresponding decrease in the phosphorylation of the Src substrate, cortactin (19), are consistent with this premise. However, the high level of endogenous Src kinase activity in the ECV cell precluded a study of the role of Src in the migration of the transfectants. While the functional significance of the cortactin-positive patches observed in the IL-1α p transfectants is not known, the ability of the Src substrate, cortactin, to associate with F-actin (34) could represent a novel type of adhesion structure that may impair cell motility.

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