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## Characterization of CD44 Induction by IL-1: A Critical Role for Egr-1

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# Characterization of CD44 Induction by IL-1: A Critical Role for Egr-1<sup>1</sup>

Katherine A. Fitzgerald and Luke A. J. O'Neill<sup>2</sup>

The adhesion molecule CD44 is a multifunctional, ubiquitously expressed glycoprotein that participates in the process of leukocyte recruitment to sites of inflammation and to their migration through lymphatic tissues. In this study, we have investigated the effect of the proinflammatory cytokine IL-1 $\alpha$  on CD44 gene expression in the human immortalized endothelial cell line ECV304. Immunoblotting of cell extracts showed constitutive expression of a 85-kDa protein corresponding to the standard form of CD44, which was potently up-regulated following IL-1 $\alpha$  treatment. Furthermore, IL-1 $\alpha$  induced expression of v3- and v6-containing isoforms of CD44, which migrated at 110 and 140–180 kDa, respectively. The effect of IL-1 $\alpha$  on CD44 standard, v3- and v6-containing isoforms was dose and time dependent and was inhibited in the presence of IL-1 receptor antagonist. To elucidate the molecular mechanisms regulating CD44 expression in response to IL-1 $\alpha$ , we investigated the effect of IL-1 $\alpha$  on CD44 mRNA expression. Reverse-transcriptase PCR and Northern analysis demonstrated an increase in CD44 mRNA expression indicating a transcriptional mechanism of control by IL-1 $\alpha$ . Furthermore, IL-1 $\alpha$  increased expression of a reporter gene under the control of the CD44 promoter (up to  $-1.75$  kb). The effect of IL-1 $\alpha$  was critically dependent on the site spanning  $-151$  to  $-701$  of the promoter. This effect required the presence of an Egr-1 motif at position  $-301$  within the CD44 promoter since mutation of this site abolished responsiveness. IL-1 $\alpha$  also induced Egr-1 expression in these cells. These studies therefore identify Egr-1 as a critical transcription factor involved in CD44 induction by IL-1 $\alpha$ . *The Journal of Immunology*, 1999, 162: 4920–4927.

The CD44 gene codes for a family of alternatively spliced, multifunctional adhesion molecules that participate in lymphocyte-endothelial cell interactions as a lymphocyte homing receptor (1–3), adhesion of cells to the extracellular matrix (4), T cell activation and adherence (5), metastasis formation (6), and more recently, in the pathogenesis of inflammatory disease (7–9). Hemopoietic cells, fibroblasts, and glial cells predominantly express an 85-kDa form of the molecule referred to as standard CD44. In addition, a number of larger m.w. variant CD44 isoforms have been reported (10, 11), which are generated by alternative usage of amino acid sequences encoded by 10 variant exons (termed v1-v10) (12). CD44 variant isoform expression was initially believed to occur as a result of aberrant splicing in tumor cells; however, variant expression has subsequently been detected on normal cells (13). CD44 variant exon 6-containing isoforms (CD44v6)<sup>3</sup> have been linked with tumor metastasis (5), and have also been shown to be transiently up-regulated on B and T cells after antigenic or mitogenic stimulation (5, 14), and also at sites of inflammation (7–9).

Recently, a number of studies have proposed a role for CD44 during the inflammatory response, in particular in the recruitment of leukocytes to sites of inflammation (1). Expression of CD44 is

elevated in inflamed tissue (7), and v3- and v6-containing CD44 molecules are up-regulated at inflamed sites in ulcerative colitis (8). Furthermore, three studies have shown that an anti-CD44 Ab, IM7, has antiinflammatory effects in several in vivo models of inflammation (15–17). Finally, binding of low m.w. fragments of hyaluronic acid (HA) to alveolar macrophages via CD44 elicits the expression of a number of proinflammatory chemokines (18). This extends an earlier observation showing that HA fragments are capable of activating the transcription factor NF- $\kappa$ B (19), thereby suggesting that CD44 also signals downstream target genes involved in orchestrating the immune and inflammatory response. Although CD44 expression has been documented in numerous cell types, little is known about the molecular regulation of the CD44 gene, particularly in the context of the inflammatory milieu. In this study, we have examined the effect of the central proinflammatory cytokine IL-1 (IL-1 $\alpha$ ) on CD44 gene expression. We chose the human immortalized endothelial cell line ECV304 as our model system. We demonstrate that IL-1 $\alpha$  potently up-regulates standard CD44 mRNA and protein expression. In addition, we provide the first evidence that IL-1 $\alpha$  up-regulates variant isoforms of CD44-containing variant exons v3 and v6. IL-1 $\alpha$  also increases the expression of a reporter gene under the control of the CD44 promoter. The effect of IL-1 $\alpha$  on the CD44 promoter is mediated via the immediate early gene product Egr-1 (also known as *NGFI-A*, *Krox-24*, and *Zif 268* (20, 21)), which is a ubiquitously expressed transcription factor. Although IL-1 has previously been shown in independent reports to induce CD44 (22, 23) and affect Egr-1 (24–26), this is the first report identifying an IL-1-responsive gene, whose expression is critically dependent on Egr-1.

## Materials and Methods

### Materials

The human endothelial cell line ECV304 was obtained from the European Collection of Animal Cell Cultures (ECACC, Salisbury, U.K.). Heat-inactivated FCS, penicillin-streptomycin (10,000 IU/ml-10,000  $\mu$ g/ml), trypsin-EDTA (10 $\times$  solution), and L-glutamine (200 mM) were from Life Technologies (Gaithersburg, MD). Human rIL-1 $\alpha$  was a gift from Dr. J.

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<sup>3</sup> Abbreviations used in this paper: CD44v6, CD44 variant exon 6-containing isoforms; CAT, chloramphenicol acetyltransferase; CD44v3, CD44 variant exon 3-containing isoforms; HA, hyaluronic acid; IL-1Ra, IL-1R antagonist.

Saklatvala (Kennedy Institute of Rheumatology, London, U.K.), while TNF- $\alpha$  was a gift from Dr. S. Foster (Zeneca, U.K.). The human rIL-1 receptor antagonist (IL-1Ra) was a gift from Dr. R. Thompson (Synergen, Boulder, CO). mAbs against human CD44 standard (anti-human CD44H), CD44v3, and CD44v6 were from R&D Systems (Abingdon, U.K.). Mouse mAb Brick 238 was a gift from Dr. Dermot Kelleher (St. James's Hospital, Dublin, Ireland). Digoxigenin high prime DNA labeling and chemoluminescent detection kit and Titan reverse-transcriptase PCR kit were from Boehringer Mannheim (East Sussex, U.K.). The 22-bp oligonucleotide, 5'-AGT TGA GGG GAC TTT CCC AGG C-3', containing the NF- $\kappa$ B consensus sequence (underlined), T4 polynucleotide kinase, RNase inhibitor, *Taq* DNA polymerase, and PCR m.w. markers were from Promega (Madison, WI). CD44 promoter plasmid pRb chloramphenicol acetyltransferase (CAT) was a gift from Dr. Dermot Walls (Dublin City University, Dublin, Ireland). pBLCD44 and pBLMCD44 constructs were kindly donated by Dr. John Monroe (Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, PA). The 27-bp oligonucleotide, 5'-GGA TCC AGC GGG GGG GAG CGG GGG CGA-3', containing the Egr-1 consensus site and antisera to human Egr-1 were from Santa Cruz Biotechnology (Santa Cruz, CA). [ $\gamma$ -<sup>32</sup>P]ATP (3000 Ci/mmol), D-THREO [dichloroacetyl-<sup>14</sup>C] chloramphenicol (56 mCi/mmol), and enhanced chemiluminescence (ECL) reagent were from Amersham International (Aylesbury, U.K.). Poly(dI-dC) was from Pharmacia Biosystems (Milton Keynes, U.K.). All other chemicals were from Sigma (Poole, Dorset, U.K.).

#### Cell culture and treatments

ECV304 cells were cultured in medium 199 (HEPES modification) supplemented with 10% (v/v) FCS, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 2 mM L-glutamine, and maintained at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. Cells were seeded at  $1 \times 10^5$  ml<sup>-1</sup> for experiments and pretreated with test compounds or left untreated before the addition of IL-1 $\alpha$ , as described in figure legends. All experiments were conducted in complete medium at 37°C, unless otherwise stated.

#### Preparation of whole cell lysates

Confluent ECV304 cells in six-well plates (3-ml vol) were treated as described in figure legends. Treatment was terminated with ice-cold PBS, and total cell lysate from each well was extracted in ice-cold radioimmune precipitation buffer (27). Protein estimations of cell extracts were determined by the dye-binding assay of Bradford (28).

#### Immunoblot analysis

Equivalent amounts of protein (4–15  $\mu$ g) were resolved by SDS-PAGE, according to the method of Laemmli (29). Proteins were electrotransferred onto nitrocellulose membranes (0.45  $\mu$ m). Nonspecific sites were blocked and blots were incubated with primary Abs (see figure legends) for 1 h at room temperature. Blots were then incubated with the relevant peroxidase-conjugated secondary Ab (1/1000–2000 dilution) for 45 min at room temperature. Visualization was by chemoluminescence according to the manufacturer's recommendations (Amersham International).

#### Reverse-transcriptase PCR

Total cellular RNA was isolated from ECV304 cells in 100-mm petri dishes, which had been left untreated or stimulated with IL-1 $\alpha$  (10 ng/ml) for 4 h, using TRI-reagent, according to the manufacturer's instructions (Sigma). Reverse transcription and PCR amplification were performed in a one-step reaction, according to the manufacturer's recommendations (Titan, Boehringer Mannheim, East Sussex, U.K.). Primers for PCR amplification were chosen at the 5' and 3' ends of exon 5 and variant exon 6 (exon 10) for detection of CD44 standard and v6 using the following forward and reverse primers: CD44 standard forward, 5'-AAGACATCTACCCC AGCA-3'; CD44 standard reverse, 5'-GGTAGCAGGGATTCTGTC-3'; CD44v6 forward, 5'-CAGGCACTCCTAGTAGT-3'; and CD44v6 reverse, 5'-GGGTAGCAGGGATTCTGTC-3'. The constitutively expressed gene  $\beta$ -actin was also reverse transcribed in a separate reaction as a qualitative and quantitative control using the following forward and reverse primers: exon 3 forward, 5'-CGTAACTGGCATCGTG-3'; exon 4 reverse, 5'-GTTTCGTGGATGCCACAG-3'.

#### Northern blot analysis

A 400-bp  $\beta$ -actin and 179-bp CD44 standard exon 5-specific probe were generated by PCR from human genomic DNA as template using the forward and reverse primer pairs described above. PCR reactions were performed under mineral oil in a total volume of 50  $\mu$ l containing 500 mM KCl, 100 mM Tris-HCl, pH 9 (25°C), 1% Triton X-100, 200  $\mu$ M of each

dNTP, 1.5 mM MgCl<sub>2</sub>, 200 pmol of each primer, and 1.75 U *Taq* polymerase. The reaction mixture was brought to a temperature of 95°C for 5 min, followed by amplification for 30 cycles, 0.5-min denaturation at 95°C, 1-min annealing at 55°C, 1-min extension at 72°C, followed by final extension at 72°C for 10 min using a MJ Research (Cambridge, MA) minicycler. DNA was nonradioactively labeled with digoxigenin-11-dUTP using the random primed method according to the manufacturer's recommendations (Boehringer Mannheim). Northern blot hybridization was performed at 50°C overnight with 25 ng labeled probe/ml hybridization solution. Nylon membranes were washed twice for 15 min at room temperature in 2 $\times$  wash buffer (2 $\times$  SSC, 0.1% SDS), twice for 15 min at 68°C in 0.5 $\times$  wash buffer (0.5 $\times$  SSC, 0.1% SDS), and once at 68°C for 30 min. The blot was then equilibrated for 1 min in washing buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5, 0.3% (v/v) Tween-20). Blots were blocked for 60 min in blocking solution (Boehringer Mannheim) and then incubated for 30 min with antidigoxigenin-alkaline phosphate conjugate (1/10,000) in blocking solution at room temperature. Blots were washed twice for 15 min in wash buffer, followed by equilibration for 2 min in detection buffer (100 mM Tris-HCl, 100 mM NaCl, pH 9.5). The blot was then incubated for 5 min with the chemoluminescent substrate CSPD (disodium 3-(4-methoxy-xyspiro (1, 2-dioxetane-3, 2'-(5'-chloro)tricyclo[3.3.1.1<sup>3,7</sup>]decan-4-yl)phenyl phosphate), according to the manufacturer's instructions, followed by a 15-min incubation at 37°C, and exposed to x-ray film.

#### Transient transfection and reporter gene assays

Transient transfection studies were conducted using plasmids, which contained regions of the CD44 upstream regulatory region upstream of the reporter gene, CAT. Confluent ECV304 cell monolayers were resuspended after trypsinization in PBS in 0.4-cm electroporation cuvettes (Invitrogen, Groningen, The Netherlands). A total of 10  $\mu$ g plasmid DNA was added to cells. After mixing, cells were left on ice for 10 min before transfection by electroporation using an Invitrogen Electroporator II (Invitrogen), with the following settings: capacitance @ max, resistance @ infinity. Following a brief pulse at 250 V, 25 mA, and 25 W, cells were cooled on ice for 10 min and then resuspended in medium prewarmed to 37°C. Cells were allowed to recover for 24 h, medium removed, cells washed with prewarmed PBS, and fresh medium replaced. The cells were then maintained in a humidified atmosphere of 5% CO<sub>2</sub> for another 24 h before treatment with cytokines for 24 h, as described in figure legends. Cell extracts were prepared by repeated freeze/thaw cycles, and protein concentrations were determined. Equivalent amounts of protein from each sample were incubated with 1 mM acetyl coenzyme A and 0.3  $\mu$ Ci D-THREO [dichloroacetyl-<sup>14</sup>C]chloramphenicol (56 mCi/mmol) in a final volume of 91.5  $\mu$ l overnight at 37°C. The reaction was terminated by the addition of 350  $\mu$ l ethylacetate and samples vortexed for 30 s. The samples were then centrifuged at 12,000  $\times$  g for 1 min in a bench top centrifuge. The upper phase was removed and dried under vacuum. The pellet was resuspended in 12  $\mu$ l ethylacetate and resolved on silica-TLC plate (0.2 mm thickness) in chloroform:methanol (19:1, v/v). The plate was dried, autoradiographed to locate the acetylated and nonacetylated species of [<sup>14</sup>C]chloramphenicol, and then analyzed by electronic autoradiographic Instant Imaging (Packard Instrumentation, Meriden, CT).

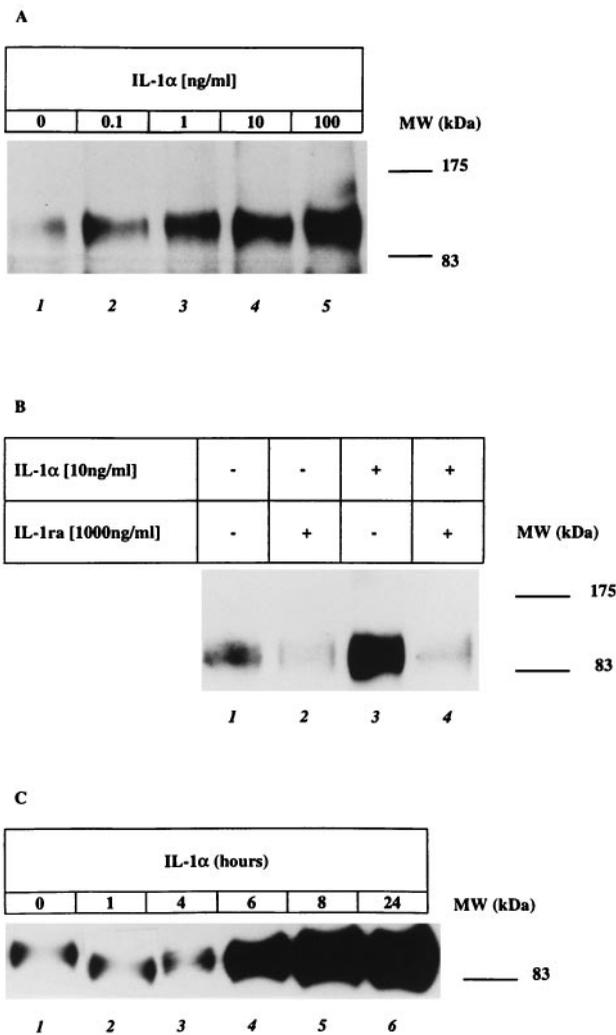
#### Electrophoretic mobility shift assay

Nuclear extracts were prepared, as described by Osborn et al. (30), from confluent ECV304 cells in six-well plates (3 ml vol) treated as described in figure legends. Nuclear extracts (4–8  $\mu$ g protein) were incubated with 10,000 cpm of a 22-bp oligonucleotide containing the NF- $\kappa$ B consensus site (5'-AGT TGA GGG GAC TTT CCC AGG C-3'), or a 27-bp oligonucleotide containing the Egr-1 consensus site (5'-GGA TCC AGC GGG GGG GAG CGG GGG GAG CGA-3') that had previously been labeled with [ $\gamma$ -<sup>32</sup>P]ATP (10 mCi/mmol) by T4 polynucleotide kinase. Incubations were performed for 30 min at room temperature, in the presence of 2  $\mu$ g poly(dI-dC) as nonspecific competitor, and 10 mM Tris-HCl, pH 7.5, containing 100 mM NaCl, 1 mM EDTA, 5 mM DTT, 4% glycerol, and 100  $\mu$ g/ml nuclease-free BSA. Incubation mixtures were subjected to electrophoresis on native 5% (w/v) polyacrylamide gels, which were subsequently dried and autoradiographed.

## Results

#### Induction of CD44 by IL-1 $\alpha$ in ECV304 cells

We first examined the effect of IL-1 $\alpha$  on standard CD44 expression in ECV304 cells, by performing immunoblotting on cell extracts. Fig. 1A shows the detection of constitutive CD44, which migrates at the predicted molecular mass of 85 kDa (Fig. 1A, lane



**FIGURE 1.** IL-1 $\alpha$  up-regulates standard CD44 expression in ECV304 cells. **A**, ECV304 cells were incubated with IL-1 $\alpha$  over the concentration range 0.1–100 ng/ml for 24 h, as indicated (*lanes 2–5*). **B**, Cells were preincubated with IL-1Ra (1000 ng/ml) (*lanes 2 and 4*) for 1 h before stimulation with IL-1 $\alpha$  (10 ng/ml) for 24 h (*lanes 3 and 4*). **C**, Cells were stimulated with IL-1 $\alpha$  (10 ng/ml) from 0–24 h. Whole cell lysates were extracted, and equivalent amounts of total protein were fractionated on 10% SDS-PAGE, transferred to nitrocellulose, and probed with anti-human CD44-specific mAb CD44H (500 ng/ml). A CD44-specific band of 85 kDa was detected. No other protein complexes except those shown were detected. Results are representative of three separate experiments.

1). Treatment of cells with IL-1 $\alpha$  from 0.1–100 ng/ml induced a dose-dependent increase in expression of this 85-kDa band specific for standard CD44 (*lanes 2–5*). When a nonimmune Ab (purified mouse IgG1) was used in place of the anti-CD44 mAb (Brick 238), no bands were detected demonstrating the specificity of the Brick 238 antiserum (*not shown*). Pretreatment of cells with 1000 ng/ml of the IL-1 receptor antagonist (IL-1Ra) inhibited IL-1-induced expression of standard CD44 (Fig. 1B, *lane 4* compared with *lane 3*). In addition, basal CD44 standard expression was also reduced in the presence of IL-1Ra (Fig. 1B, *lane 2* compared with *lane 1*), indicating that constitutive expression was likely to be due to endogenous expression of IL-1. Fig. 1C shows the time course of IL-1 action. CD44 standard expression was increased in a time-dependent manner and reached maximal levels between 6 and 8 h, which were sustained at 24 h, and even up to 48 and 72 h (not

shown). Two other anti-human CD44 Abs showed an identical pattern of expression by immunoblotting (not shown).

#### Induction of CD44 variant expression by IL-1 $\alpha$ in ECV304 cells

We next examined variant isoform expression, focusing on v3- and v6-containing isoforms, which have been shown to be up-regulated during inflammation. Fig. 2A demonstrates that treatment of cells with IL-1 $\alpha$  from 0.1–100 ng/ml for 24 h induced CD44v6 in a dose-dependent manner, which migrated with an apparent molecular mass range of 140–180 kDa (Fig. 2A, *lanes 2–5*). When cells were preincubated for 1 h with IL-1Ra, there was no increase of CD44v6 following IL-1 $\alpha$  treatment (Fig. 2B, *lane 3* compared with *lane 2*). The effect of IL-1 $\alpha$  on CD44v6 expression was also time dependent, reaching maximal levels between 6 and 24 h (Fig. 2C). The complex of CD44v6 induced by IL-1 $\alpha$  was of a broad molecular mass distribution. Analysis of CD44v6 expression by 6% SDS-PAGE to further resolve protein complexes showed that the 140–180-kDa band induced by IL-1 $\alpha$  was composed of two bands, an upper band of 180 kDa and another lower more smeared band of 140–170 kDa (Fig. 2D).

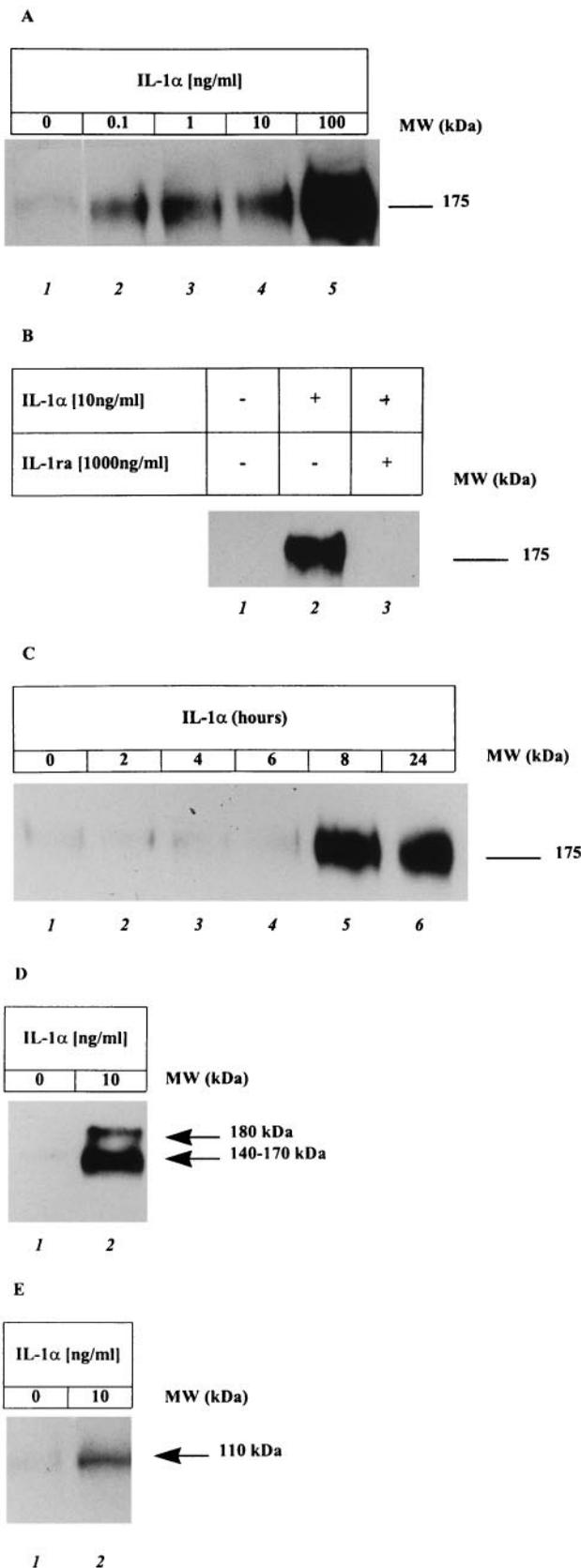
CD44v3 expression was also detected at a constitutive level (Fig. 2E) on resting cells and migrated with an apparent molecular mass of 110 kDa. This was strongly induced in response to IL-1 (compare *lane 2* with *lane 1*). Analysis of extracts with anti-human CD44v4/5 mAbs showed no detectable expression either before or after IL-1 $\alpha$  treatment over the dose range 0.01–100 ng/ml (not shown).

#### IL-1 $\alpha$ increases CD44 standard and CD44v6 mRNA expression in ECV304 cells

We next tested the effect of IL-1 $\alpha$  on CD44 mRNA expression. Fig. 3A demonstrates that treatment of cells with IL-1 $\alpha$  for 4 h showed an increase in CD44 standard and v6-specific mRNA compared with untreated samples, as determined by reverse-transcriptase PCR using CD44 standard and exon v6-specific primers. The level of mRNA for the housekeeping gene  $\beta$ -actin remained constant in the different culture conditions (Fig. 3A). Northern blot analysis also demonstrated an increase in CD44 mRNA levels following treatment of cells with IL-1 $\alpha$  for 4 h as compared with untreated cultures. Fig. 3B illustrates the data obtained when RNA from cultures treated with or without IL-1 $\alpha$  were hybridized with a CD44-specific digoxigenin-labeled probe (Fig. 3B). IL-1 $\alpha$  increased levels of a 5.5-kb CD44-specific transcript above control levels.  $\beta$ -actin mRNA levels did not vary significantly between untreated and IL-1 $\alpha$ -treated cells. We had difficulty in detecting any v6-containing mRNA by Northern blotting (not shown). This was probably due to a limit of sensitivity in the assay, as mRNAs containing variants are present in low copy numbers.

#### IL-1 $\alpha$ induces CD44 promoter activity in ECV304 cells, and the Egr-1-binding motif at bp -301 of the CD44 promoter is critical for transcriptional induction

We next probed the transcriptional control of CD44 expression by examining the effect of IL-1 $\alpha$  on the CD44 promoter. Following transfection of ECV304 cells with a reporter gene construct containing 1.75 kb of the CD44 promoter linked upstream of the CAT reporter gene (31), the effect of IL-1 $\alpha$  was investigated. IL-1 $\alpha$  induced expression of CAT activity in a dose-dependent fashion (Fig. 4A). Concentrations of IL-1 $\alpha$  employed correlated to those previously shown to induce CD44 protein expression. The transcription factor Egr-1 has previously been implicated in the induction of CD44 in B cells (31), and since IL-1 $\alpha$  has been shown to induce Egr-1 expression in some cell types (24–26), we addressed whether this transcription factor was involved in the induction of



**FIGURE 2.** IL-1 $\alpha$  up-regulates CD44 variant isoform expression in ECV304 cells. *A*, ECV304 cells were incubated with IL-1 $\alpha$  over the concentration range 0.1–100 ng/ml for 24 h, as indicated (*lanes 2–5*). *B*, Cells were preincubated with IL-1Ra (1000 ng/ml) for 1 h before stimulation with IL-1 $\alpha$  (10 ng/ml) for 24 h, as indicated. *C*, Cells were stimulated with IL-1 $\alpha$  (10 ng/ml) from 0–24 h. Whole cell lysates were extracted, and equivalent amounts of total protein were fractionated on 10% SDS-PAGE,

CD44 by IL-1 $\alpha$  in this system. This was assessed using two CD44 promoter constructs. pBLCD44 contains a 550-bp region of the CD44 promoter (spanning  $-151$  to  $-701$ ), which includes the Egr-1 motif at position  $-301$  bp, shown to be important for PMA responsiveness in B cells. pBLmCD44 differs by a 3-bp mutation, which abolishes Egr-1 binding. Fig. 4*B* illustrates the CD44 promoter spanning regions  $-151$  to  $-701$  and in particular the difference between pBLCD44 CAT and pBLmCD44 CAT. Following transfection of both reporters into ECV304 cells and treatment of cultures with IL-1 $\alpha$  for 24 h, CAT activity was dose dependently increased in response to IL-1 $\alpha$  in those cells that contained pBLCD44 (Fig. 4*C*). In contrast, stimulation of pBLmCD44-transfected cells with IL-1 $\alpha$  had no effect on CAT activity. These results implicate Egr-1 as a critical factor involved in the regulation of CD44 expression by IL-1 $\alpha$ .

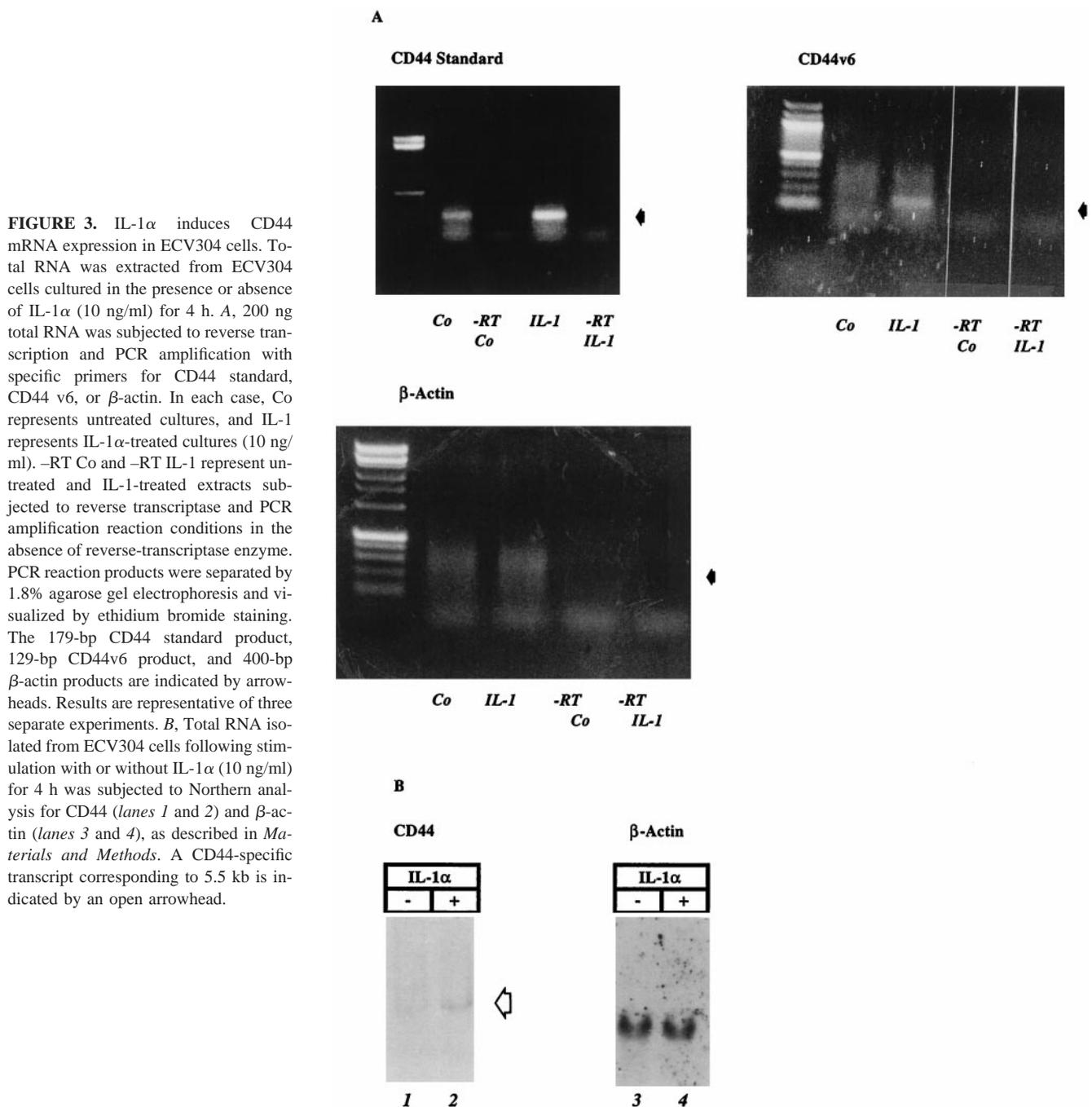
The relevance of Egr-1 as a transcriptional activator of the CD44 gene depends on the kinetics of Egr-1 protein production. To address whether Egr-1 protein is induced with appropriate kinetics to regulate CD44 transcription (i.e., within 1–2 h), ECV304 cells were stimulated with IL-1 $\alpha$  (10 ng/ml) for varying time periods (from 0–48 h), and total cellular extracts were analyzed for Egr-1 by immunoblotting. Fig. 4*D* illustrates how Egr-1 protein accumulated after 30 min, reaching maximum levels of expression at 1 h and declining thereafter. By 8 h, there was no detectable expression of Egr-1 protein. Nuclear extracts prepared from cells treated with IL-1 $\alpha$  (10 ng/ml) for 1 h also contained DNA-binding activity specific for the Egr-1 consensus site, as shown by performing an electrophoretic mobility shift assay with a probe containing the Egr-1 binding site (Fig. 4*D*, *right panel*). Taken together, these results strongly implicate Egr-1 in the induction of CD44 by IL-1 $\alpha$ .

## Discussion

Engagement of the proinflammatory cytokine IL-1 $\alpha$  with its cell surface receptor initiates a set of signaling pathways leading to profound alterations in gene expression during the inflammatory and immune responses (for review, see Ref. 32). In this study, we have found that IL-1 $\alpha$  up-regulates the expression of the adhesion molecule CD44 in the transformed human endothelial cell line ECV304. This is in agreement with other reports that have demonstrated that IL-1 can up-regulate standard CD44 in both vascular smooth muscle cells and bovine articular chondrocytes (22, 23), although a mechanism was not elucidated in these studies. Furthermore, we provide the first demonstration that IL-1 $\alpha$  can also induce v3- and v6-containing variant isoforms of CD44. This effect was particularly interesting, given the recent observation of their up-regulation during inflammatory disease states (8).

The cell line that we used in this study, ECV304, is a transformed HUVEC (33). From the karyologic, immunocytochemical, and ultrastructural characteristics, ECV304 cells were shown to be

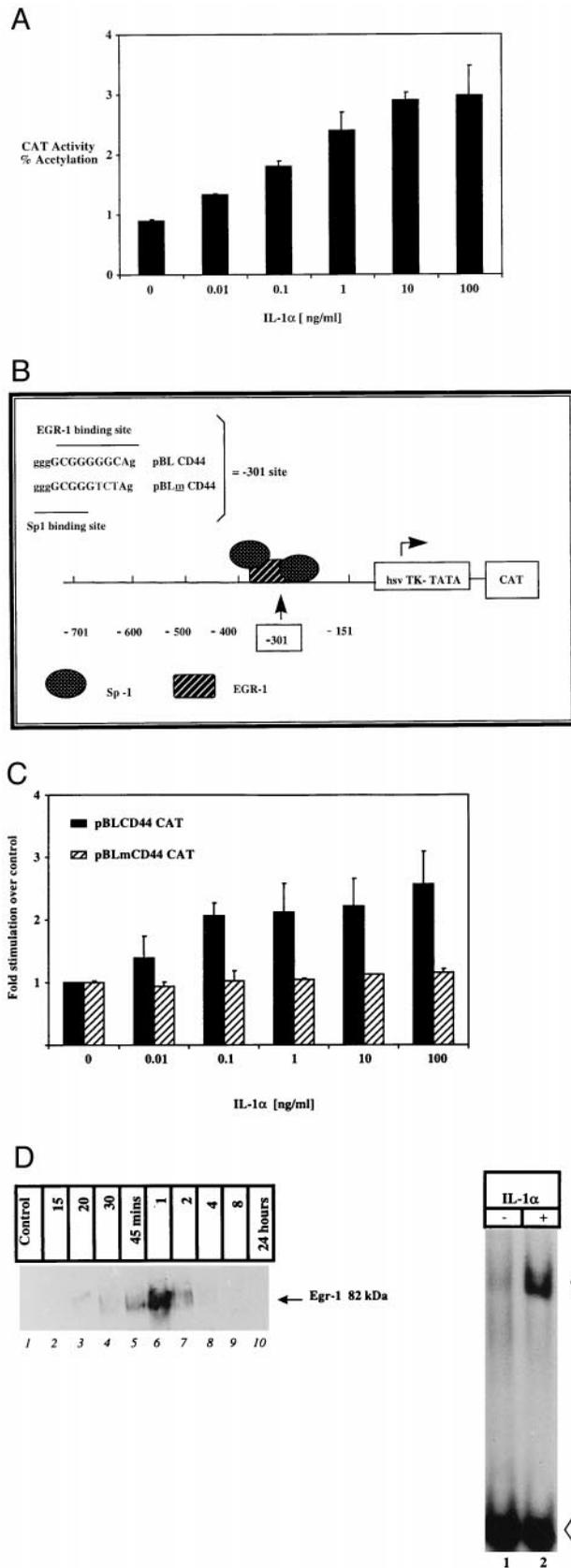
transferred to nitrocellulose, and probed with anti-human CD44v6-specific mAb (500 ng/ml). A 140–180-kDa diffuse band was detected in IL-1 $\alpha$ -treated extracts. *D*, Protein extracts from cells treated with or without IL-1 $\alpha$  (10 ng/ml) were further fractionated on 6% SDS-PAGE and transferred to nitrocellulose, and CD44v6 expression was analyzed (*lanes 1–2*). *E*, Whole cell lysates were extracted from cells cultured in the presence or absence of IL-1 $\alpha$  (10 ng/ml) for 24 h. Equivalent amounts of total protein were fractionated on 10% SDS-PAGE, transferred to nitrocellulose, and probed with anti-human CD44v3-specific mAb (500 ng/ml). A CD44v3-specific band of 110 kDa was detected as shown. Molecular mass markers in kDa are shown. No other protein complexes except those shown were detected. Results are representative of three separate experiments.



**FIGURE 3.** IL-1 $\alpha$  induces CD44 mRNA expression in ECV304 cells. Total RNA was extracted from ECV304 cells cultured in the presence or absence of IL-1 $\alpha$  (10 ng/ml) for 4 h. A, 200 ng total RNA was subjected to reverse transcription and PCR amplification with specific primers for CD44 standard, CD44 v6, or  $\beta$ -actin. In each case, Co represents untreated cultures, and IL-1 represents IL-1 $\alpha$ -treated cultures (10 ng/ml). -RT Co and -RT IL-1 represent untreated and IL-1-treated extracts subjected to reverse transcriptase and PCR amplification reaction conditions in the absence of reverse-transcriptase enzyme. PCR reaction products were separated by 1.8% agarose gel electrophoresis and visualized by ethidium bromide staining. The 179-bp CD44 standard product, 129-bp CD44v6 product, and 400-bp  $\beta$ -actin products are indicated by arrowheads. Results are representative of three separate experiments. B, Total RNA isolated from ECV304 cells following stimulation with or without IL-1 $\alpha$  (10 ng/ml) for 4 h was subjected to Northern analysis for CD44 (lanes 1 and 2) and  $\beta$ -actin (lanes 3 and 4), as described in *Materials and Methods*. A CD44-specific transcript corresponding to 5.5 kb is indicated by an open arrowhead.

an immortal endothelial cell line derived from HUVEC. They express endothelial cell markers (Weibel-Palade bodies, Lectin Ulex europeus I (UEA-I), pro-urokinase type plasminogen activator (PA), and its inhibitor angiotensin-converting enzyme, tissue factor, endothelin, endothelin-converting enzyme, prostaglandin I<sub>2</sub>, and thromboxane A<sub>2</sub>) and are negative for epithelial markers and monocyte markers (33, 34). The cell adhesion molecules, ICAM-1, VCAM-1, and LFA-3, are expressed in response to inflammation or injury on endothelial cells (35–37) and have also been detected on ECV304 cells, (33, 34). We have previously characterized their responsiveness to IL-1 in terms of NF- $\kappa$ B activation (38). In addition, they have been used in studies on endothelial cell function in angiogenesis (39). ECV304 cells can therefore be considered a good model for primary endothelial cells, and it is likely that the results we have observed will be relevant in vivo.

ECV304 cells showed constitutive expression of standard CD44 as judged by immunoblotting. Incubation of cells with IL-1Ra decreased this expression, suggesting that ECV304 cells may constitutively produce IL-1 $\alpha$ , which could explain the expression of CD44 standard in this cell line. Adding IL-1 $\alpha$  caused a clear induction of standard CD44. CD44 v3- and v6-containing isoforms were also potently up-regulated in response to IL-1 $\alpha$ . CD44 v4/5 were absent from these cells either before or after stimulation with IL-1 $\alpha$ . IL-1 $\alpha$  significantly increased expression of a 140–180-kDa CD44v6- and a 110-kDa CD44v3-specific band. Since inclusion of variant exon sequences in CD44 mRNA is confined to certain cell types, there must be cell type-specific regulation of alternative splicing of CD44. Stringent regulation could be mediated by the presence of positive or negative *trans*-acting factors. Indeed, the large variability of CD44 protein isoforms implies the existence of



**FIGURE 4.** Induction of the human CD44 promoter by IL-1 $\alpha$  is critically dependent on the transcription factor Egr-1. ECV304 cells were transiently transfected by electroporation with a plasmid encoding 1.75 kb of the CD44 promoter region (pRb) linked upstream of the CAT gene (10  $\mu$ g), as described in *Materials and Methods*. Twenty-four hours following transfection, cells were incubated with IL-1 $\alpha$  for 24 h (concentrations indicated). Cell lysates

specific regulators capable of selecting certain variant exons. The sequences, factors, and mechanisms regulating alternative splicing are as yet unknown. Konig and coworkers have shown that CD44 splicing is controlled by dominant *trans*-acting factors (40), which we would hypothesize are regulated by IL-1 $\alpha$  in ECV304 cells.

CD44 has been reported to play an essential role in the recruitment of leukocytes to sites of inflammation (1), and through its interaction with its principal ligand HA has been implicated in the rolling and extravasation of leukocytes at inflammatory sites. It is currently unclear how endothelial cell CD44 might contribute to this process. Although HA is the principal ligand for CD44, additional currently unknown ligands on extravasating leukocytes may utilize CD44 or its variant isoforms on endothelial cells to facilitate extravasation. CD44 on the endothelium may also bind homotypically to CD44 on extravasating leukocytes, since it has been shown that leukocytes can adhere to each other in such a manner (41). An up-regulation of CD44 by IL-1 on endothelial cells could also function to allow activation of endothelial cells. Binding of low m.w. fragments of HA to macrophages via CD44 elicits the expression of a number of proinflammatory chemokines (18) and inducible nitric oxide synthase (42). This extends earlier observations showing that HA fragments are capable of activating the transcription factor NF- $\kappa$ B (19). Induction of NF- $\kappa$ B and nitric oxide synthase by HA fragments has also been shown in endothelial cells of the liver (43). It is important to note, however, that not all CD44-positive cells are capable of binding HA. Recent evidence suggests that binding of HA fragments by CD44 is strictly regulated (44, 45) and can be activated by stimulation with Ag, cytokines, LPS, or phorbol esters (46–49). Three different binding states of CD44 have been defined: inactive, inducible (by certain mAbs), and constitutively active (46). It is unknown whether the Abs used in this study for CD44 standard, v3, or v6 recognize active or inactive CD44, but we would hypothesize that IL-1 is capable of inducing active CD44 given the reports on other cytokines (45, 48, 50). It is therefore likely that an up-regulation of active CD44, capable of binding HA fragments on the endothelium, plays a role in signaling downstream target genes involved in orchestrating the immune and inflammatory response.

were prepared and analyzed for CAT activity, as described under *Materials and Methods*. Results are mean  $\pm$  SD for a single experiment (triplicate samples), which is representative of three separate experiments. *B*, Schematic representation of constructs used: pBLCD44 and pBLmCD44 differ by the indicated 3-bp mutation that abolishes Egr-1 binding at position -301 of the CD44 promoter. *C*, 10  $\mu$ g of pBLCD44 or the mutant reporter pBLmCD44 was transiently transfected as described in *Materials and Methods*. Twenty-four hours following transfection, the cultures were evenly divided and either stimulated with IL-1 $\alpha$  (concentrations indicated) or left unstimulated. Following an additional 24 h of incubation, the cells were harvested and assayed for CAT activity. The results depict the mean fold induction (compared with unstimulated cells) from six independent experiments  $\pm$  SEM. *D*, Whole cell lysates were extracted from human endothelial cells cultured in the presence or absence of IL-1 $\alpha$  (10 ng/ml) for the indicated times. Equivalent amounts of total protein were fractionated on 15% SDS-PAGE, transferred to nitrocellulose, and probed with anti-human Egr-1-specific polyclonal Ab. An 82-kDa Egr-1 band was detected as indicated. In the *right panel*, ECV304 cells were cultured until confluent before being exposed to IL-1 $\alpha$  (10 ng/ml) or an equivalent volume of medium control for 1 h. Nuclear extracts were prepared following stimulation and analyzed for Egr-1-binding activity, as described under *Materials and Methods*. Retarded protein-DNA complexes are indicated by a closed arrowhead, and free probe by an open arrowhead. Representative of three separate experiments.

The precise function of variant CD44 isoforms is also as yet unclear. The insertion of variant exons could modulate HA-binding specificities of the molecule or create additional binding sites for as yet unidentified ligands. CD44-containing exon v3, which can be modified by the addition of heparin sulfate, has been shown to bind the chemokine macrophage-inflammatory protein-1 $\beta$  (51), raising the possibility that glycosaminoglycan-modified CD44 might function in the binding and presentation of growth factors. CD44v3 on endothelial cells could therefore facilitate the formation of a reservoir of chemokines or growth factors, sequestering them from the circulation. An up-regulation of endothelial cell CD44v3 by IL-1 could enhance this process during inflammation, facilitating an enhancement of the extravasation process.

CD44v6 expression has been postulated to play a role in tumor metastasis, enhancing the ability of nonmetastatic cells to disseminate and metastasize (6). It has also been shown to be transiently up-regulated after antigenic or mitogenic stimulation of B and T cells (5, 14). Indeed, CD44 has been shown to be costimulatory for T cells (14, 52), thereby providing further evidence to suggest a role in enhancing the immune and inflammatory response. IL-1 $\alpha$  has costimulatory effects on T cells (53, 54), and it is possible that this may involve CD44 expression. As mentioned above, an up-regulation of CD44v6 on the endothelial cell surface could also participate in cell activation associated with lymphocyte extravasation. CD44 and its v3- and v6-containing variants can therefore be added to the list of adhesion molecules induced in response to IL-1 $\alpha$  during inflammation and immunity.

Having established CD44 expression at the protein level in ECV304 cells, we next focused on the mechanisms, which regulate its expression. We found that IL-1 $\alpha$  increased CD44 gene transcription, giving rise to an increase in mRNA for CD44 standard and v6-containing variants. Sequence analysis of the CD44 upstream regulatory region reveals the absence of TATA and CCAAT elements classically found in eukaryotic promoters (55). In addition, unlike other adhesion molecules regulated by IL-1 $\alpha$ , no  $\kappa$ B consensus sequences exist in the upstream regulatory region of CD44 (56). However, sequence analysis of the CD44 5' flanking region by Maltzman and coworkers (31) identified the presence of a potential binding site for the transcription factor Egr-1 at position -301 upstream of the transcription start site of the gene, which overlaps binding sites for the constitutive transcription factor Sp1. Overlapping Egr-1/Sp1 sites have previously been shown to be important in regulating transcription from other promoters (57, 58). The Egr-1 site within the CD44 promoter was shown to be essential for CD44 induction in B lymphocytes in response to B cell receptor cross-linking or PMA stimulation (31). Since IL-1 $\alpha$  has been shown to induce Egr-1 in other cell types (22, 23), we tested whether it was involved in activation of the CD44 promoter in ECV304 cells. We found that mutation of the Egr-1 site at position -301 in a CD44 promoter construct spanning -151 to -701 abolished its responsiveness to IL-1 $\alpha$ . We further found Egr-1 to be induced in the cells by IL-1 $\alpha$ . Egr-1 can therefore be implicated as a key transcription factor regulating CD44 expression by IL-1 $\alpha$  in ECV304 cells. Although IL-1 has been shown to induce Egr-1 in other cell types, this study represents the first characterization of a gene whose induction by IL-1 is dependent on Egr-1.

Another possible participant in the regulation of CD44 gene expression by IL-1 $\alpha$  is the transcription factor AP-1, since IL-1 $\alpha$  has been shown to induce AP-1 in several cell types (53, 59, 60). A presumptive AP-1 binding site was located at position -110 of the CD44 promoter, which was suggested as the target for CD44 induction by the proto-oncogene products *ras* or *src* (61, 62). More recently, fibroblast cells transformed with *c-fos* also showed an

up-regulation of CD44 expression that correlated with enhanced invasiveness (63), although whether this was a direct effect on the CD44 promoter was not determined. Our study does not rule out the possibility that AP-1 or other transcription factors may play a role in CD44 induction by IL-1 $\alpha$ , although clearly Egr-1 plays a key role.

In conclusion, we have demonstrated that standard, v3- and v6-containing isoforms of CD44 are potently up-regulated by the proinflammatory cytokine IL-1 by a mechanism involving Egr-1. These results suggest a mechanism whereby CD44 is induced during inflammation and immunity.

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