The immediate-early gene product MAD-3/EDG-3/IκBα is an endogenous modulator of fibroblast growth factor-1 (FGF-1) dependent human endothelial cell growth

Timothy Hla[a,*], Ann B. Zimrin[b], Mark Evans[b], Karin Ballas[b], Thomas Maciag[b]

[a] *Department of Physiology, MC3505, University of Connecticut School of Medicine, 263 Farmington Avenue, Farmington, CT 06030, USA*
[b] *Department of Molecular Biology, Holland Laboratory, American Red Cross, Rockville, MD 20855, USA*

Received 29 July 1997

Abstract The tumor promoter phorbol 12-myristic 13-acetate inhibits the growth of human endothelial cells and induces the formation of capillary-like, tubular structures. We report the novel growth regulatory function of the immediate-early gene, edg-3, which is identical to the IκBα/MAD-3 gene. We employed phosphothioate oligonucleotides (PTO) directed against the translation initiation site of IκBα to inhibit its expression. The antisense IκBα PTO-treated cells exhibited an exaggerated growth response to fibroblast growth factor-1 (FGF-1). In contrast, IL-1-induced growth arrest response was not modulated. These data suggest that the early response gene IκBα is an endogenous regulator of endothelial cell growth in vitro.

Key words: Angiogenesis; NFκB; IκB; Fibroblast growth factor; Endothelium

1. Introduction

The vascular endothelium is a monolayer of cells that are quiescent under normal physiological conditions and carry out a diverse array of functions such as the control of vascular tone [1], regulation of hemostasis [2], hemopoietic cell extravasation [3] and immune reactions [4]. Endothelial cell migration, proliferation and differentiation are part of the angiogenic program that occurs in normal wound healing processes as well as in pathological states such as tumor angiogenesis [5] and rheumatoid arthritis [6]. Endothelial cells grown in vitro are widely used as a model system for angiogenesis since almost all of the steps involved in angiogenesis can be recapitulated under defined conditions [7]. Furthermore, regulators of angiogenesis, namely the fibroblast growth factors [8] and vascular endothelial cell growth factor [9] as proliferative modulators and interleukin-1 (IL-1α), tumor necrosis factor (TNFα), transforming growth factor-β (TGF-β) and the tumor promoter phorbol myristic acetate (PMA) as non-proliferative modulators of angiogenesis were discovered based on the in vitro properties of endothelial cells [10]. The cytokines and PMA rapidly inhibit the FGF-1-induced mitogenesis of endothelial cells and promote a phenotypic change into a elongated, fibroblast-like shape [11]. Prolonged treatment of endothelial cells grown on type-I collagen matrix with PMA results in the formation of capillary-like structures containing lumens and anastomoses [12]. This has been defined as the phenotypic differentiation of endothelial cells in vitro [10,12]. It is, however a reversible process because proteolytic degradation of the capillary-like, tubular structures and exposure to FGF-1 results in the reversion into a cobblestone-shaped, highly proliferative phenotype [13].

Molecular mechanisms involved in the processes of angiogenesis have been the subject of intensive investigation. It has been recognized that proteolytic modifications of the extracellular matrix are involved in the phenotypic differentiation [13]. In addition, the plasminogen activator activity in endothelial cells was induced by angiogenic preparations and the active principle was shown to be FGF-2 [14]. Furthermore, collagenase inhibitors blocked the phenotypic differentiation of endothelial cells into capillary-like tubules [15]. More recently, Montesano and colleagues have proposed that the protease and protease inhibitor gene expression by the endothelial cells is modulated by extracellular cytokines and that this balance may influence the phenotype of endothelial cell differentiation [16]. That extracellular matrix modification is important in endothelial cell differentiation is illustrated by several studies which demonstrated the differential gene regulation of collagen type I [17], the proteoglycan decorin [18], and the secreted, extracellular protein SPARC [19] in differentiating endothelial cells. Indeed, rapid formation of endothelial cell networks that resemble tubules is achieved by plating the cells onto Matrigel, an extracellular matrix secreted by EHS sarcoma cells which is enriched in laminin and several growth factors and cytokines [20].

Early signal transduction and transcriptional events induced in differentiating endothelial cells are not well understood. The FGF family of polypeptides are thought to transduce the mitogenic signal via the tyrosine phosphorylation of intracellular substrates [8]. The tumor promoter PMA, however, is a potent inducer of the protein kinase C family of isoenzymes, which induce the serine and threonine phosphorylation of cellular substrates [21]. We have focused our attention on the PMA-inducible immediate-early (IE) genes in human umbilical vein endothelial cells (HUVEC). In growth factor induction of mitogenesis in fibroblasts, the IE genes encode critical, regulatory proteins that are necessary for the cell-cycle traverse [22]. We and others have characterized IE genes induced in endothelial cells in response to PMA [23], TNFα [24] and IL-1α [25]. We reported the cloning and characterization of an abundant PMA-inducible IE gene termed edg-1 that encoded a novel G-protein-coupled receptor [26].

*Corresponding author. Fax: +1 (860) 679-1269.
E-mail: hla@sun.uchc.edu

Abbreviations: PMA, phorbol myristic acetate; HUVEC, human umbilical vein endothelial cells; IE, immediate-early; PTO, phosphothioate oligonucleotides; FGF, fibroblast growth factor; TNF, tumor necrosis factor; IL-1, interleukin-1; PCR-ASH, polymerase chain reaction-amplified subtractive hybridization

PII S0014-5793(97)01053-3

Dixit et al. have reported the cloning of several IE genes induced in response to TNFα, including c-jun, adhesion molecules of the selection family, a novel Zinc finger protein implicated in apoptosis [27] and the *eck* ligand B61 [28]. Recently, an IL-1-inducible IE gene encoding an amyloid-like protein was isolated from HUVEC [25]. In this report, we describe the identification of the IκBα protein, an inhibitor of the NFκB transcription factor as an IE gene product induced during the early phases of PMA-induced differentiation of HUVEC.

2. Materials and methods

2.1. Cell culture

HUVEC were grown on fibronectin-coated dishes in medium-199 containing 10% fetal bovine serum and 150 μg/ml of crude endothelial cell growth factor and 5 units/ml of heparin [13]. Cells were subcultured at the ratio of 1:5 and used between the passages of 3 and 12. At confluence, cells were maintained in medium without growth factor and heparin for 2 days to achieve quiescence. For growth assays, cells were grown in medium containing purified recombinant human FGF-1 and heparin and cell numbers were determined in a Coulter counter. The following 18mer phosphothioate oligonucleotides against IκBα were synthesized and used: sense=5′-ATG TTC CAG GCG GCC GAG-3′; antisense=5′-CTC GGC CGC CTG GAA CAT-3′. After synthesis, the PTOs were washed in ethanol and dissolved in sterile water, heated at 95°C for 5 min and frozen in aliquots. The PTOs at indicated concentrations were added directly to cell culture medium every other day.

2.2. RNA preparation and Northern blot analysis

Total RNA was purified by the guanidinium isothiocyanate procedure [30]. RNA was electrophoresed on a 1% agarose gel containing 2.2 M formaldehyde and Northern blot analysis was conducted using the buffer system described by Church and Gilbert [30]. Hybridizations and washes were done at 65° C in 20% formamide.

2.3. PCR-ASH cloning and sequencing procedures

Cloning of PMA/CHX-induced cDNAs from HUVEC were done by PCR-ASH procedures as described previously [23]. The inducibility of the cDNA cloned were confirmed by Northern blot analysis. Sequence analysis of the deletion subclones of the edg-3 cDNA were conducted using the automated DNA sequencer. Sequence homology searches were conducted against the Genbank and EMBL databases using the BLAST algorithm [31].

2.4. In vitro translation

Transcripts for IκB were prepared using the T7 RNA polymerase transcription system (Promega Biotec). Capped mRNAs were translated in [^{35}S]-methionine supplemented rabbit reticulocyte lysates, separated on a 10% SDS-PAGE and autoradiographed. In some experiments, 1 μg each of either sense or antisense PTOs were added to the translation mixes.

2.5. Metabolic labelling and immunoprecipitation

Confluent HUVEC were treated with the sense and antisense IκBα PTO for 2 days. Cells were then metabolically labelled with 100 μCi/ml of [^{35}S]-methionine/cysteine labelling mix (New England Nuclear) for 4 h. Some cultures received 1 ng/ml of IL-1α during the labelling period. Cells were then lysed with ice cold phosphate buffered saline (pH 7.4) containing 0.5% Triton X-100, 10 μM of pyrrolidine dithiocarbamate, 10 μg/ml of chymosin, 10 μg/ml of aprotinin and 1 mM PMSF. Cell lysates were cleared by centrifugation, pre-cleared with protein-A Sepharose and immunoprecipitated with the polyclonal antibody raised against the recombinant human IκBα protein. Immunoprecipitates were resolved on a 12% SDS-PAGE, incubated in a fluorographic solution and autoradiographed.

3. Results

3.1. Characterization of the edg-3 cDNA

In order to clone low-abundance IE transcripts that are induced during the differentiation of HUVEC, we utilized the PCR-ASH procedure [23] and isolated several cDNA probes from PMA and cycloheximide-treated (4 h) mRNA population. One such clone, initially termed edg-3, hybridized to a PMA-inducible transcript of 1.8 kb. In quiescent cells the

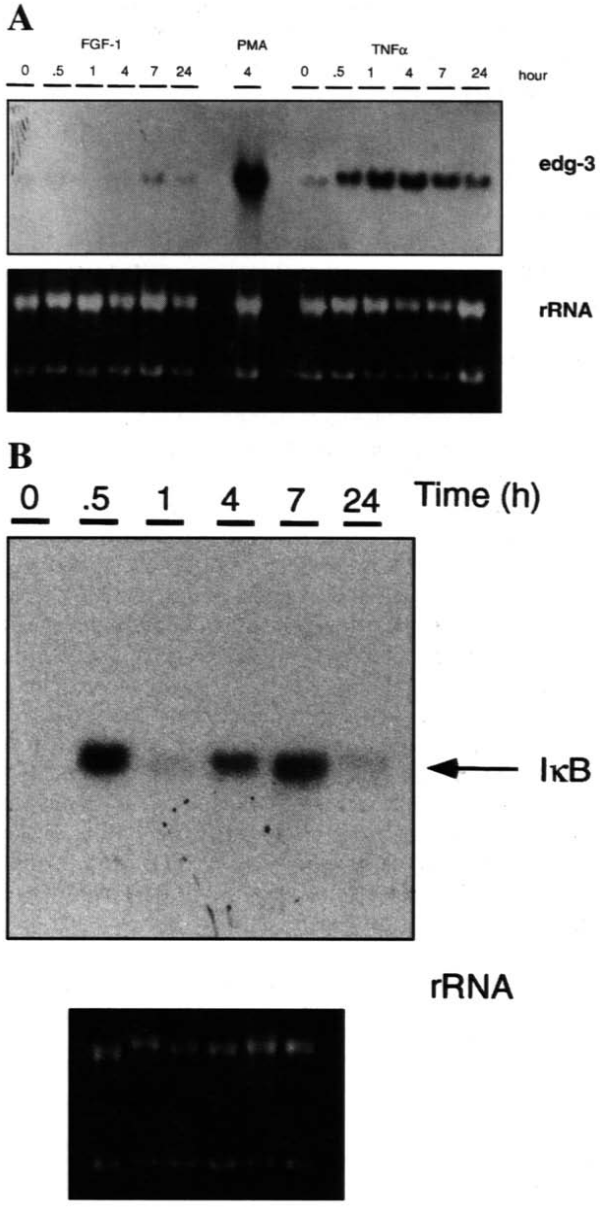


Fig. 1. A: Quiescent HUVEC were treated with either 10 ng/ml FGF-1 containing 5 units/ml heparin or 20 ng/ml PMA or 10 units/ml TNFα for indicated periods of time and Northern blot analysis was conducted using the edg-3 cDNA probe. The ethidium bromide-stained gel (rRNA) is shown to indicate the relative equivalence of RNA load. Data indicate a representative experiment which was repeated twice. B: Quiescent HUVEC were treated with 1 ng/ml IL-1α for indicated periods of time and Northern blot analysis was conducted with the edg-3 cDNA probe. The ethidium bromide-stained gel (rRNA) is shown to indicate the relative equivalence of RNA load. Data indicate a representative experiment which was repeated twice.

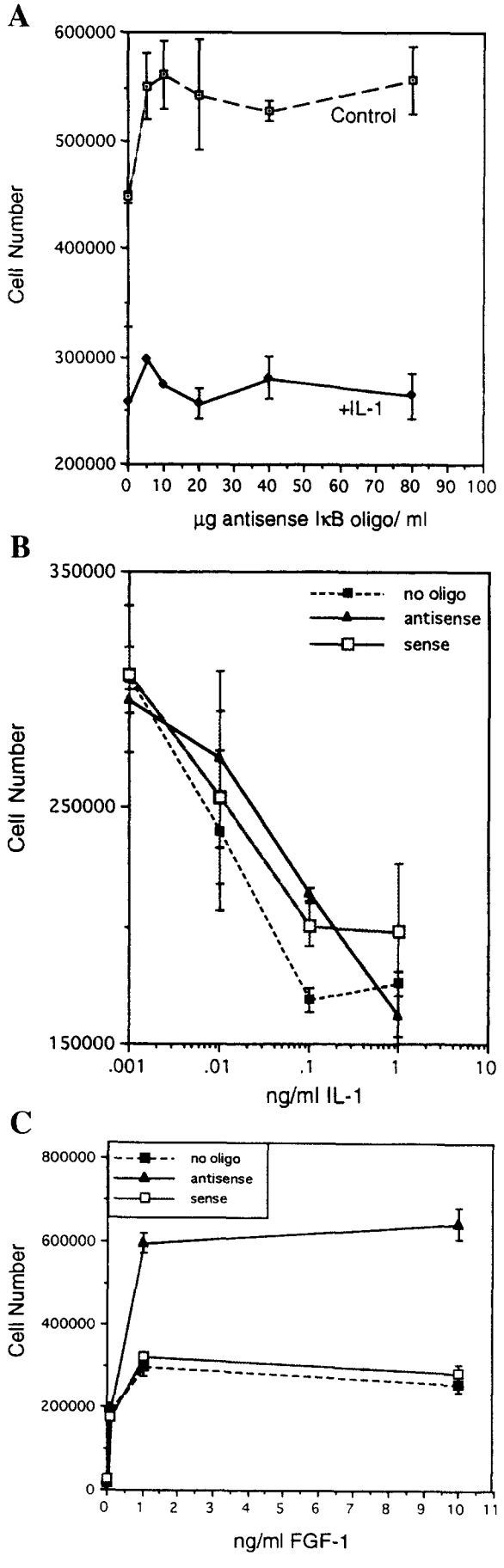
edg-3 mRNA is expressed at very low levels; however, upon treatment with PMA for 2 h, elevated steady-state levels of transcript were observed followed by a decline at 24 h (data not shown) [34]. Cycloheximide alone induced the edg-3 mRNA and 'super-induced' the kinetics of induction (data not shown). These data indicated that edg-3 is a PMA-inducible IE gene in HUVEC. Both TNFα and IL-1α induced the edg-3 mRNA (Fig. 1). While FGF-1 induced the steady-state mRNA level of edg-3 in a transient manner, the kinetics of induction was different (Fig. 1).

The structure of the edg-3 cDNA was determined by DNA sequence analysis of the 900 bp cDNA fragment. Sequence analysis followed by database homology search indicated that edg-3 is identical to the human monocyte adherence-induced IE gene MAD-3/IκBα [28]. Thus, the edg-3 gene is hereinafter referred to as the IκBα gene.

3.2. *Effect of IκBα antisense PTO on basal and IL-1α-treated HUVEC growth*

In order to assess the role of the IκB protein on the FGF-stimulated and cytokine-inhibited growth of HUVEC, sense and antisense phosphothioate oligonucleotides (PTO) synthesized against the translation initiation site of the IκBα transcript were utilized to block its expression. HUVEC were grown in complete growth media in the presence of increasing concentrations of the antisense IκBα PTO which were added to the medium every two days. As shown in Fig. 2A, the ability of the cytokine IL-1α to block the growth of HUVEC was not affected by the IκBα antisense PTO; however, increased cell numbers were obtained in the presence of the IκBα antisense PTO and FGF-1. The ability of both sense and antisense IκBα PTO to modulate the growth-inhibitory effect of IL-1α was investigated in detail. As shown in Fig. 2B, dose-dependent inhibition of HUVEC growth by IL-1α was not modulated by either sense or antisense IκBα PTO. In contrast, the effect of FGF-1 to increase HUVEC cell number was greatly potentiated in the presence of antisense IκBα PTO but not the sense counterpart (Fig. 2C).

The effect of cell density on the potentiation of the IκBα PTO was investigated next. Different number of HUVEC were seeded at day 1 and the cells were grown in complete growth medium containing either sense or antisense IκBα PTO. The cell number was then determined at day 7. When cells were seeded at low density (5×10^4 cells/well), normal proliferation occurred and yielded 8×10^5 cells per well at



→

Fig. 2. A: Effect of antisense PTO against IκBα on basal and IL-1-inhibited HUVEC cell growth. HUVEC were seeded on fibronectin coated dishes at the density of 10^4 cells/well at day 1. Control cells received the complete growth medium (10% FBS+endothelial cell growth supplement in M199+heparin). Some wells were treated with 10 ng/ml of human IL-1 alpha in complete growth medium. Indicated amounts of the antisense PTO were added every two days and the cell number at day 10 was determined. B: Effect of IκBα PTO on the IL-1α inhibition of HUVEC cell growth. HUVEC were allowed to grow as above with 10 ng/ml of FGF-1 for 10 days. Some cultures were treated with either sense or antisense IκBα PTO in the presence of indicated concentrations of IL-1α. Cell number was determined at day 10. C: Effect of sense and antisense IκBα PTO on FGF-1-induced HUVEC growth. HUVEC were seeded at 10^4 cells/well at day 1 in medium 199+10% FBS+5 units/ml of heparin+indicated concentrations of purified FGF-1. Sense and antisense IκBα PTO (40 mg/ml; 6.7 mM) were added every 2 days. Cell number at day 10 was determined.

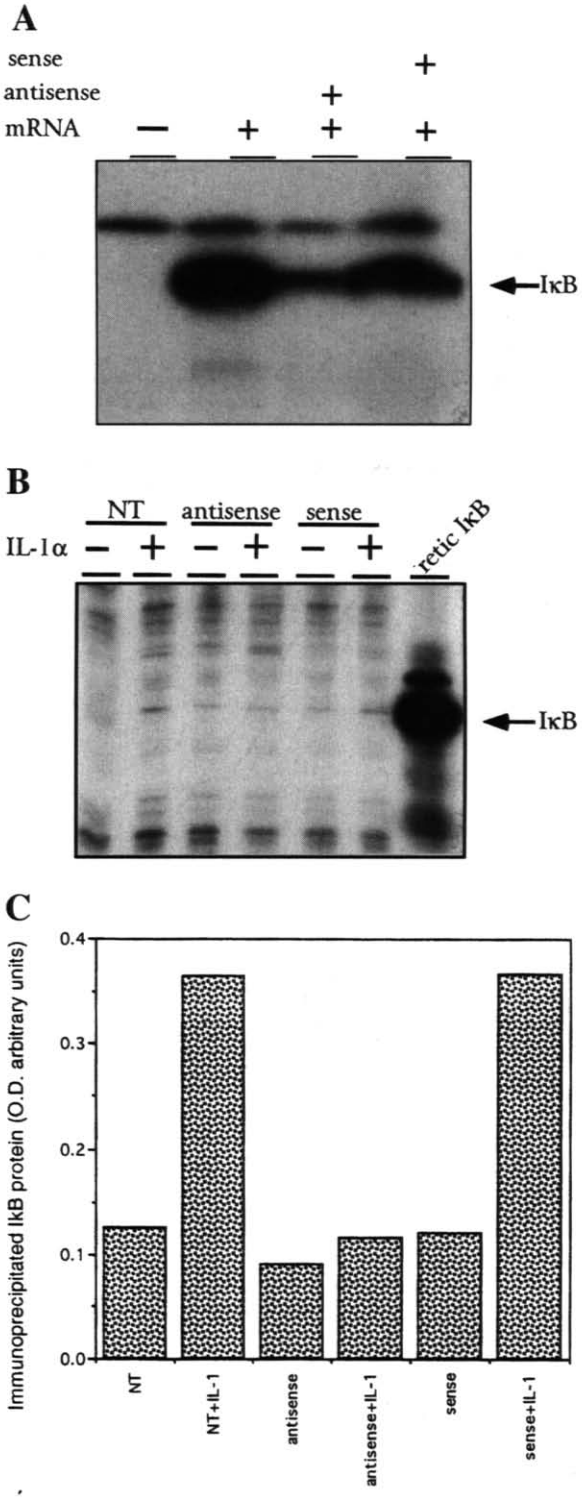
day 7. Antisense but not sense IκBα PTO-treated cells however, reached a higher saturation density of 1.25×10^6 cells per well. Independent of initial inoculum, the antisense IκBα PTO-treated cells achieved significantly higher saturation density than the sense-treated or control cells. The morphology of antisense-treated cells confirmed that inhibition of IκBα resulted in higher saturation density without grossly altering cell shape or phenotypic differentiation.

We next determined the effect of the PTO synthesized against the translation initiation site of the RelA polypeptide which constitutes the p65 subunit of the NFκB protein on HUVEC growth. The RelA PTOs were used previously to show the requirement of RelA in the adhesion molecule expression of a variety of cells [32]. In addition, we have previously shown that this PTO blocks the NFκB-dependent induction of cyclooxygenase-2 in synovial fibroblasts [47]. Neither the sense nor antisense RelA PTO modulated FGF-1-induced HUVEC cell growth (data not shown). This suggests that the function of IκB independent of RelA is involved in the growth regulation phenomenon in endothelial cells.

3.3. *Effect of IκBα PTO on IκBα polypeptide expression*

In order to confirm the effect of the antisense and sense IκBα PTO on the synthesis of the IκBα protein, we tested the ability of the PTO to influence the in vitro translation of the IκBα mRNA in [^{35}S]-methionine supplemented rabbit reticulocyte lysates. As shown in Fig. 3, addition of 1 μg of the IκBα transcript directed the synthesis of the radiolabelled polypeptide of approximately 40 kd molecular weight. Immunoprecipitation of the reticulocyte lysates with the polyclonal IκBα antiserum specifically precipitated the polypeptide band. Addition of the antisense PTO strongly inhibited (14% of control) the translation of the 40 kd IκB polypeptide. While the sense PTO also suppressed the translation somewhat (57% of control), the efficacy of the antisense PTO was quantitatively stronger. The addition of irrelevant PTOs at similar concentrations also inhibited the IκBα synthesis approximately 40% (data not shown). This may be related to the non-specific inhibition of translation induced by high concentrations of PTOs in the reticulocyte translation system. Nevertheless, these data demonstrate that the antisense IκBα PTO is able to potently suppress the translation of the IκBα polypeptide.

We next tested the effect of the IκBα PTO on the synthesis of basal and stimulated IκBα polypeptide synthesis in HUVEC. Cells were treated with either sense or antisense IκBα PTO for 2 days and stimulated with 1 ng/ml of IL-1α for 5 h. During the stimulation period, the cells were metabolically labelled with [^{35}S]-methionine and -cysteine. Cell lysates were then immunoprecipitated with the polyclonal IκBα antiserum and analyzed by polyacrylamide gel electrophoresis and autoradiography. As shown in Fig. 3B, the 40 kd polypeptide which is of similar molecular weight as the in vitro translated



←

Fig. 3. A: Effect of IκBα PTO on in vitro translation of the IκBα transcript. One μg of capped IκBα transcript was translated in the rabbit reticulocyte lysates supplemented with [^{35}S]-methionine either in the presence or absence of 1 μg of PTO. Samples were separated on 12% SDS-PAGE and autoradiographed. Data indicate a representative experiment which was repeated twice. B: Effect of IκB PTO on de novo synthesis of IκBα protein. HUVEC were treated with PTO for 2 days and stimulated with IL-1α for 4 h during which time the cells were metabolically labelled with [^{35}S]-methionine/cysteine mixture. Cell lysates were immunoprecipitated with a polyclonal antibody raised against the recombinant human IκBα protein, separated on a 12% SDS-PAGE and autoradiographed. Note that the IL-1α induction of IκBα is selectively blocked by the antisense PTO. The densitometric scan of this autoradiograph is shown in (C).

IκBα protein was immunoprecipitated. The radiolabelling of the 40 kd band was induced approximately 3-fold by IL-1 treatment in control and sense IκBα PTO-treated cells but not in the antisense IκBα PTO-treated cells. This further provides evidence that the antisense IκBα PTO is able to repress the expression of the IκB polypeptide in HUVEC.

4. Discussion

Using differential hybridization [26] as well as the PCR-ASH techniques [23], we have isolated from HUVEC, several PMA-inducible IE genes such as an orphan G-protein-coupled receptor edg-1 [26], collagenase type I [23] and cyclo-oxygenase-2 [33]. In this communication, we describe the characterization of edg-3 which is identical to the IκBα gene product. The edg-3 cDNA is strongly and transiently induced by PMA in HUVEC. Recent studies on the regulation of the IκBα gene indicate that the tandem repeats of the cis-acting NFκB enhancer elements control the transcription of the IκBα gene itself [35]. Thus, the transcription of the inhibitor is under tight regulation by the transcription factor itself, constituting a mutual autoregulatory loop [36]. Because of the extreme lability of the IκBα protein [37] and the transcriptional autoregulatory loop, the IκBα protein is being recognized as a major conduit of extracellular signals in inflammatory gene transcription [29,36,46].

In addition to its well-established role as a regulator of transcription of inducible genes, the Rel family of proteins also regulate cell growth, oncogenesis and apoptosis [38]. In that respect, the IκBα polypeptide can interact with multiple members of the Rel family, including the c-Rel, Rel-A and Rel-B polypeptides [39,40]. It was recently shown that the protooncogene c-Rel is found associated with IκBα in the cytoplasm and in contrast to the Rel-A/IκBα complex, inflammatory cytokine treatment did not result in the nuclear translocation of the c-Rel polypeptide in HUVEC [41]. This indicated that there may be additional IκBα-mediated signalling pathways distinct from the well-known transcriptional regulatory function of NFκB. Indeed, ankyrin repeat-containing polypeptides that are structurally related to IκB are known regulators of mating-type switching in *S. cerevesiae* (SWI4 and SWI6) [42] and leukemogenesis in humans (bcl3) [43]. While the c-Rel polypeptide was originally characterized as a protooncogene involved in growth regulation [44], recent studies have indicated that c-Rel may also regulate the process of apoptosis during development [45]. It is generally thought that different combinatorial Rel complexes are involved in the mediation of diverse nuclear signalling events; however, specific biological role of distinct Rel complexes are not well understood. In addition, cytoplasmic regulation (retention) of distinct complexes by the IκB family members and the corresponding signalling pathways are not well defined.

Our data indicate that the antisense IκBα PTO specifically enhances the fibroblast growth factor-dependent cell growth of HUVEC. In contrast, the antisense IκBα PTO does not modulate the IL-1-induced growth arrest in HUVEC. The sense IκBα PTO, however, did not have appreciable effects on protein synthesis or HUVEC growth. Because the antisense IκBα PTO inhibited the in vitro translation of the IκBα transcript and suppressed the de novo synthesis of the polypeptide in IL-1α-treated HUVEC, perturbation of the normal turnover of the IκBα protein results in the enhanced endothelial cell density upon reaching confluence.

Data presented in this report suggest that the IκBα protein is involved in a signalling pathway that limits the growth of HUVEC. The increase in saturation density of HUVEC can result from either increased cell growth due to the loss of contact inhibition or decreased cell death due to apoptosis. Alternatively, both of these mechanisms may be operative. Further understanding of the IκBα-mediated growth regulatory pathway in vascular endothelial cells may yield insights into mechanisms of angiogenesis.

Acknowledgements: This work is supported by NIH grants DK45659, HL49094 to T.H. and HL35627 to T.M.

References

[1] Furchgott, R.F. and Vanhoutte, P.M. (1989) FASEB J. 3, 2007–2018.
[2] Weksler, B.B. (1987) in: Hemostasis and Thrombosis, J.B. Lippincott Co., Philadelphia, PA, pp. 804–815.
[3] Albelda, S.M., Smith, C.W. and Ward, P. (1994) FASEB J. 8, 504–512.
[4] Pober, J.S., Gimbrone, M.A., Cotran, R.S., Reiss, C.S., Burakoff, S.J., Fiers, W. and Ault, K.A. (1983) J. Exp. Med. 157, 1339–1353.
[5] Folkman, J. (1985) Adv. Cancer Res. 43, 175–203.
[6] Harris, E.D. (1990) New Eng. J. Med. 322, 1277–1289.
[7] Folkman, J. and Haudenschild, C.C. (1980) Nature 288, 551–556.
[8] Burgess, W.H. and Maciag, T. (1989) Ann. Rev. Biochem. 58, 575–606.
[9] Leung, D.W., Cachianes, G., Kuang, W.J., Goeddel, D.V. and Ferrara, N. (1989) Science 246, 1306–1309.
[10] Maciag, T. (1990) in: V. DeVita and S. Rosenberg (Eds.), Advances in Oncology, J.B. Lippincott Co., Philadelphia, PA, pp. 83–96.
[11] Doctrow, S.R. and Folkman, J. (1987) J. Cell Biol. 104, 679–687.
[12] Montesano, R. and Orci, L. (1987) J. Cell. Physiol. 130, 284–291.
[13] Maciag, T., Kadish, J., Wilkins, L., Stemerman, M.B. and Weinstein, R. (1982) J. Cell Biol. 94, 511–520.
[14] Moscatelli, D., Presta, M. and Rifkin, D.B. (1986) Proc. Natl. Acad. Sci. USA 83, 7537–7541.
[15] Moscatelli, D., Jaffe, E. and Rifkin, D.B. (1980) Cell 20, 343–351.
[16] Montesano, R., Pepper, M.S., Mohle-Steinlein, U., Risau, W., Wagner, E.F. and Orci, L. (1990) Cell 62, 435–445.
[17] Iruela-Arispe, M.L., Hasselaar, P. and Sage, E.H. (1991) Lab. Invest. 64, 174–186.
[18] Järveläinen, H., Iruela-Arispe, M.L., Kinsella, M.G., Sandell, L.J., Sage, E.H. and Wight, T.N. (1992) Exp. Cell. Res. 203, 395–401.
[19] Iruela-Arispe, M.L., Bornstein, P. and Sage, E.H. (1991) Proc. Natl. Acad. Sci. USA 88, 5026–5030.
[20] Grant, D.S., Kleinman, H.K. and Martin, G.R. (1990) Ann. N.Y. Acad. Sci. 588, 61–72.
[21] Nishizuka, Y. (1988) Nature 334, 661–665.
[22] Herschman, H. (1991) Ann. Rev. Biochem. 60, 281–319.
[23] Hla, T. and Maciag, T. (1990) Biochem. Biophys. Res. Comm. 167, 637–643.
[24] Dixit, V., Green, J., Sharma, V., Holzman, L.B., Wolf, F.W., O'Rourke, K., Ward, P.A., Prochownik, E.V. and Marks, R.M. (1990) J. Biol. Chem. 265, 2973–2978.
[25] Breviario, F., d'Aniello, E.M., Golay, J., Peri, G., Bottazzi, B., Bairoch, A., Saccone, S., Marzella, R., Predazzi, V., Rocchi, M., Della Valle, G., Dejana, E., Montovani, A. and Introna, M. (1992) J. Biol. Chem. 267, 22190–22197.
[26] Hla, T. and Maciag, T. (1990) J. Biol. Chem. 265, 9308–9313.
[27] Opipari, A.W., Hu, H.M., Yabkowitz, R. and Dixit, V.M. (1992) J. Biol. Chem. 267, 12424–12427.
[28] Holzman, L.B., Marks, R.M. and Dixit, V.M. (1990) Mol. Cell. Biol. 10, 5830–5838.
[29] Haskill, S., Beg, A.A., Tompkins, S.M., Morris, J.S., Yurochko,

A.D., Sampson-Johannes, A., Mondal, K., Ralph, P. and Baldwin, A.S. (1991) Cell 65, 1281–1289.
[30] Church, G.M. and Gilbert, W. (1986) Proc. Natl. Acad. Sci. USA 81, 1991–1995.
[31] Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) J. Mol. Biol. 215, 403–410.
[32] Narayanan, R., Higgins, K.A., Perez, J.R., Coleman, T.A. and Rosen, C.A. (1993) Mol. Cell. Biol. 13, 3802–3810.
[33] Hla, T. and Neilson, K.M. (1992) Proc. Natl. Acad. Sci. USA 89, 7384–7388.
[34] Shaw, G. and Kamen, R. (1986) Cell 46, 659–667.
[35] de Martin, R., Vanhove, B., Cheng, Q., Hofer, E., Csizmadia, V., Winkler, H. and Bach, F.H. (1993) EMBO J. 12, 2773–2779.
[36] Collins, T. (1993) Lab. Invest. 68, 499–507.
[37] Schreck, R. and Baeuerle, P.A. (1991) Trends Cell Biol. 1, 39–42.
[38] Liou, H. and Baltimore, D. (1993) Curr. Biol. 5, 477–487.
[39] Beg, A.A. and Baldwin, A.S. (1993) Genes Dev. 7, 2064–2070.
[40] Rice, N.R. and Ernst, M. (1993) EMBO J. 12, 4685–4695.
[41] Read, M.A., Whitley, M.Z., Williams, A.J. and Collins, T. (1994) J. Exp. Med. 179, 503–512.
[42] Nasmyth, K. and Dirick, L. (1991) Cell 66, 995–1013.
[43] Ohno, H., Takimoto, G. and McKeithan, T.W. (1990) Cell 60, 991–997.
[44] Gilmore, T.D. and Temin, H. (1986) Cell 44, 791–800.
[45] Abbadie, C., Kabrun, N., Bouali, F., Smardova, J., Stéhelin, D., Vandenbunder, B. and Enrietto, P. (1993) Cell 75, 899–912.
[46] Muegge, K., Williams, T.M., Kant, J., Karin, M., Chiu, R., Schmidt, A., Siebenlist, U., Young, H.A. and Durum, S.K. (1989) Science 246, 249–251.
[47] Crofford, L.J., Tan, B., McCarthy, C.J. and Hla, T. (1997) Arthritis Rheum. 40, 226–236.