Role of B61, the Ligand for the Eck Receptor Tyrosine Kinase, in TNF-α–Induced Angiogenesis

Akhilesh Pandey, Haining Shao, Rory M. Marks, Peter J. Polverini, Vishva M. Dixit*

B61, a cytokine-inducible endothelial gene product, is the ligand for the Eck receptor protein tyrosine kinase (RPTK). Expression of a B61-immunoglobulin chimera showed that B61 could act as an angiogenic factor in vivo and a chemoattractant for endothelial cells in vitro. The Eck RPTK was activated by tumor necrosis factor–α (TNF-α) through induction of B61, and an antibody to B61 attenuated angiogenesis induced by TNF-α but not by basic fibroblast growth factor. This finding suggests the existence of an autocrine or paracrine loop involving activation of the Eck RPTK by its inducible ligand B61 after an inflammatory stimulus, the net effect of which would be to promote angiogenesis, a hallmark of chronic inflammation.

The B61 protein is a glycosyl phosphatidylinositol (GPI)–linked TNF-α–, interleukin-1β (IL-1β)–, and lipopolysaccharide (LPS)-inducible endothelial gene product that can also exist in a soluble form (*1*, *2*). We have produced a B61-immunoglobulin (B61-Ig) chimera (*3*) that consists of the Fc region of human IgG1 fused to the COOH-terminus of B61 (minus the terminal 23 amino acids that are presumably cleaved during GPI linkage). Because B61 is a cytokine-inducible molecule on endothelial cells (*1*), we reasoned that its receptor Eck (*4*) may also be expressed by endothelial cells. A flow cytometric analysis using B61-Ig chimera showed that human umbilical vein endothelial cells (HUVECs) did express a B61 receptor (*5*). Metabolic labeling of HUVECs and immunoprecipitation analysis (*6*) showed that the B61-Ig chimera but not a control-Ig (*3*) chimera precipitated a protein of ~130 kD from these cells (Fig. 1A), which is in the same size range as the Eck RPTK. To confirm that this was Eck, we subjected the material immunoprecipitated with the B61-Ig chimera to immunoblotting (*7*) with an affinity-purified polyclonal antibody raised against the unique COOH-terminus of Eck (*8*). The 130-kD protein immunoprecipitated by the B61-Ig chimera was the Eck RPTK (Fig. 1B), indicating the presence of Eck on endothelial cells.

The B61-Ig chimera behaved as an agonist and was capable of inducing Eck autophosphorylation (*7*) (Fig. 1C). Although B61 bound and activated Eck, it was also possible that B61 could bind receptor tyrosine kinases other than Eck on endothelial cells. B61-Ig chimera failed to immunoprecipitate any in vitro kinase activity after Eck had been depleted (*6*) from HUVEC lysates (Fig. 1D). Thus, Eck was the major RPTK bound by B61 on endothelial cells.

Because the function of Eph-Eck RPTK family members is unknown, we investigated whether B61-mediated Eck activation might modulate the angiogenic response; first, because B61 is induced by the angiogenic cytokine TNF-α, and second, because B61 is up-regulated in an in vitro capillary tube differentiation assay (*9*). To address this possibility, we implanted B61-Ig or control-Ig chimera impregnated into Hydron pellets into the normally avascular rat cornea (*10*). An angiogenic response was detectable with as little as 25 ng of B61-Ig chimera (Fig. 2, A and B). No angiogenic response was observed with the control chimera (*5*). Additionally, angiogenesis induced by the B61-Ig chimera could largely be abolished by preincubation with protein A–Sepharose to deplete the test sample of Ig chimera (Fig. 2, A and B).

Angiogenesis encompasses elements of endothelial cell proliferation [on which B61 has no influence (*5*)] and migration (*11*). The effect of B61 on endothelial cell migration was examined. Chemotaxis assays on bovine adrenal capillary endothelial (BCE) cells were carried out in a modified Boyden chamber microwell assay system (*12*). The B61-Ig chimera at doses as low as 1 ng/ml was able to induce migration of these endothelial cells (Fig. 2C). On a molar basis, the response was similar to that of basic fibroblast growth factor (bFGF). No chemotactic response was observed with the control-Ig chimera (*5*). A checkerboard analysis was carried out to confirm that the response was chemotactic as opposed to chemokinetic (Table 1). This chemotactic response as expected was inhibited by preincubation of the B61-Ig chimera with protein A–Sepharose (*5*).

Tumor necrosis factor–α, a pleiotropic cytokine, has powerful catabolic and proinflammatory effects (*13*). Low doses (0.01 to 10 ng) of TNF-α induce angiogenesis in vivo in the absence of an inflammatory infiltrate (*14*, *15*). The angiogenic action of TNF-α may arise from the direct stimulation of endothelial or stromal cells resulting in the release of secondary mediators. In particular, prostaglandins (*15*) and platelet-activating factor (*16*) have been proposed to mediate

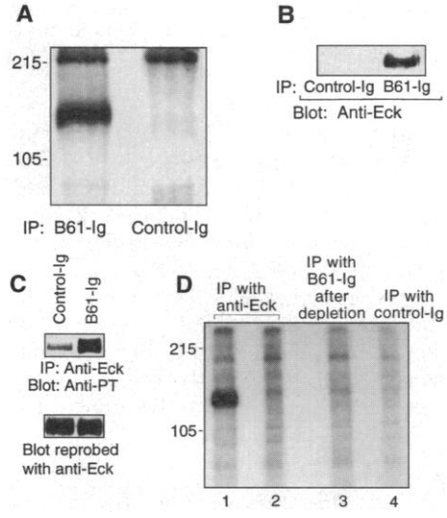
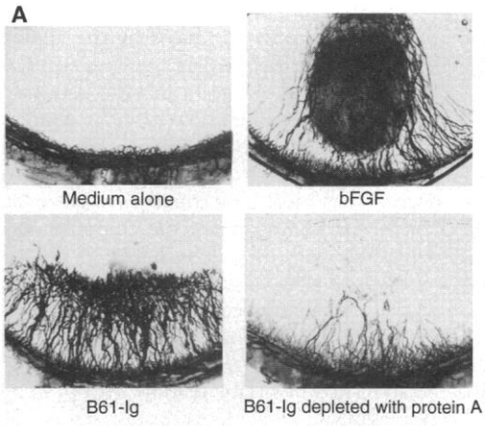


Fig. 1. Characterization of Eck RPTK on HUVECs. (**A**) Metabolically labeled HUVEC lysates immunoprecipitated (IP) with B61-Ig or control-Ig chimera (*6*) as indicated. (**B**) Immunoprecipitation with B61-Ig or control-Ig chimera as indicated followed by protein immunoblotting (Blot) (*7*) with affinity-purified antibody to Eck (anti-Eck) (*8*). (**C**) Treatment of HUVECs with B61-Ig or control-Ig chimera (each 1 μg/ml) as indicated. The cell lysates were immunoprecipitated with anti-Eck followed by immunoblotting with 4G10 anti-phosphotyrosine monoclonal antibody (anti-PT) (*7*). (**D**) In vitro kinase assays (*6*) on lysates immunoprecipitated with anti-Eck before (lane 1) or after Eck (lane 2) depletion; supernatant from lane 2 was immunoprecipitated with B61-Ig (lane 3). Lysates were directly immunoprecipitated with control-Ig (lane 4).

Table 1. Checkerboard analysis of the response of BCE cells to B61 (*12*). Data are presented as the number of cells that migrated through the filter in 10 high-power fields (magnification, ×400) after 2 hours of incubation. Each point represents the mean of values derived from three independent experiments.

B61-Ig (ng/ml) in lower chamber	B61-Ig (ng/ml) in upper chamber			
	0	50	100	250
0	18	21	22	18
50	28	21	41	31
100	87	74	32	24
250	59	92	72	21

A. Pandey, H. Shao, V. M. Dixit, Department of Pathology, University of Michigan Medical School, Ann Arbor, MI 48109, USA.
R. M. Marks, Department of Internal Medicine, University of Michigan Medical School, Ann Arbor, MI 48109, USA.
P. J. Polverini, School of Dentistry, University of Michigan, Ann Arbor, MI 48109, USA.
*To whom correspondence should be addressed.



B

Sample	ng/pellet	Proportion of positive responses (%)	
Medium alone		0/3	(0)
bFGF	50	5/5	(100)
B61-Ig	5	2/3	(66)
	25	4/4	(100)
	50	4/4	(100)
Control-Ig	5	0/4	(0)
	25	0/3	(0)
	50	0/3	(0)
B61-Ig–depleted sample	5	0/3	(0)
	25	1/5	(20)
	50	1/5	(20)

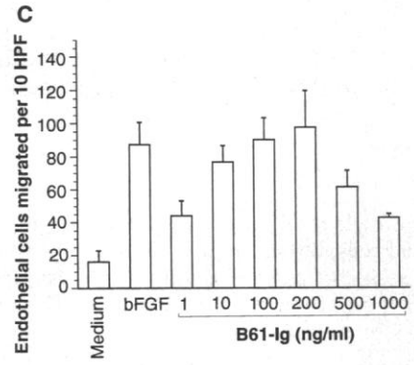


Fig. 2. (**A**) B61 induces corneal neovascularization. Angiogenic activity was assayed in the rat corneas as described (*10*). Colloidal carbon–perfused corneas are shown 7 days after implantation of a Hydron pellet containing medium alone, bFGF (50 ng), B61-Ig (25 ng), or B61-Ig sample depleted with protein A–Sepharose. (**B**) Summary of corneal neovascularization (*10*). Corneas implanted with Hydron pellets containing the indicated factors were observed for 7 days. A positive neovascularization was recorded only if sustained directional ingrowth of capillary sprouts and hairpin loops toward the implant was observed. Negative responses were recorded either when no growth was observed or when only an occasional sprout or hairpin loop displaying no evidence of sustained growth was detected. (**C**) Chemotaxis of endothelial cells. Chemotactic response of BCE cells to medium alone, bFGF (50 ng), or B61-Ig as indicated. Data are presented as the number of cells that migrated through the filter in 10 high-power (magnification, ×400) fields (HPF) after 2.5 hours of incubation. Endothelial cell chemotaxis was performed in 48-well, blind well chemotaxis chambers (Nucleopore Corp.) as described (*12*). Four replicates, 10 fields per replicate, were tested for each sample, and experiments were repeated at least twice.

TNF-α–induced angiogenesis. Because B61 is angiogenic, this result suggests that the induction of B61 and subsequent activation of the Eck RPTK could be responsible for the angiogenic effects of TNF-α. To address this possibility, we first asked whether TNF-α could induce Eck autophosphorylation. As shown in Fig. 3, exposure of HUVECs to TNF-α resulted in activation of Eck RPTK as shown by its autophosphorylation. This activation was due to the induction of B61 because treatment of HUVECs with TNF-α in the presence of an antibody to B61 (anti-B61) (*17*) resulted in inhibition of Eck autophosphorylation (Fig. 3). The TNF-α–induced expression of B61 was restored to baseline levels by the addition of anti-B61 (Fig. 3). Addition of a control antibody had no effect on Eck autophosphorylation or B61 expression (*5*). Thus, the level of expression of B61 correlated directly with the extent of autophosphorylation of the Eck RPTK, whereas the absolute amount of Eck remained unchanged (*18*) (Fig. 3).

To address directly the possibility that B61 was responsible for the angiogenic potential of TNF-α in vivo, we implanted into rat corneas Hydron pellets impregnated with TNF-α or bFGF with or without anti-B61. TNF-α alone elicited an angiogenic response, but simultaneous administration of TNF-α and anti-B61 resulted in a greatly attenuated angiogenic response (Fig. 4). Anti-B61 administered alone had no effect on vascularization in the cornea (*5*). Basic FGF is a potent inducer of angiogenesis but does not induce B61 (*9*). Consistent with this, anti-B61 did not abolish bFGF-induced angiogenesis (Fig. 4). Thus, B61 specifically mediated TNF-α– but not bFGF-induced angiogenesis. The angiogenesis induced by B61, like that induced by TNF-α, was not accompanied by an inflammatory infiltrate (Fig. 4).

We have shown that B61 is an angiogenic factor and an endothelial chemotaxin. Addition of TNF-α induces activation of the Eck RPTK through the induction of its cognate ligand, B61. Because B61 can exist in both cell surface–associated and soluble forms, activation of Eck could potentially occur in an autocrine or paracrine manner. This scenario is supported by the finding that an antibody to B61 abolished TNF-α– but not bFGF-induced angiogenesis, implying that activation of the Eck RPTK by its inducible ligand, B61, is intimately involved in mediating TNF-α–induced angiogenesis. It is

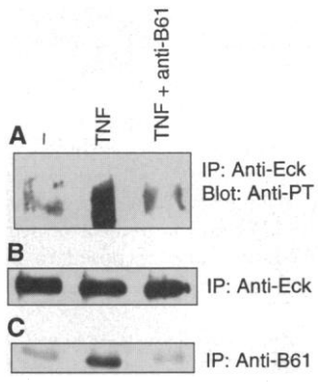
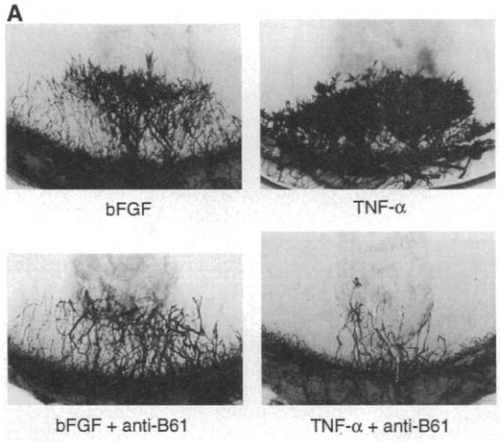


Fig. 3. TNF-α–induced Eck autophosphorylation is inhibited by anti-B61. Results shown are representative of three independent experiments. (**A**) Quiescent HUVECs were either untreated (−), or treated with TNF-α alone or TNF-α plus anti-B61 as indicated (*17*). Cell lysates were immunoprecipitated with anti-Eck followed by protein immunoblotting with 4G10 antibody to phosphotyrosine (anti-PT) (*17*). (**B**) Samples were treated as in (A) and were metabolically labeled and immunoprecipitated with anti-Eck (*18*). (**C**) Samples were treated as in (A) and (B) and were metabolically labeled and immunoprecipitated with anti-B61 (*18*).



B

Sample	Proportion of positive responses (%)	
Medium alone	0/3	(0)
bFGF	4/4	(100)
bFGF + anti-bFGF	0/3	(0)
bFGF + anti-B61	3/3	(100)
TNF-α	3/3	(100)
TNF-α + anti-TNF-α	0/3	(0)
TNF-α + anti-B61	1/6	(17)

Fig. 4. TNF-α–induced angiogenesis is inhibited by anti-B61. (**A**) Colloidal carbon–perfused corneas 7 days after implantation of a Hydron pellet containing bFGF (50 ng), TNF-α (25 ng), anti-B61 (10 μg) plus bFGF (50 ng), or anti-B61 (10 μg) plus TNF-α (25 ng). Magnification, ×35. (**B**) Summary of angiogenesis assays performed as described (*10*).

possible that B61 may also be responsible in part for the angiogenic activities of other proinflammatory factors.

REFERENCES AND NOTES

1. L. B. Holzman, R. M. Marks, V. M. Dixit, *Mol. Cell. Biol.* **10**, 5830 (1990).
2. H. Shao, A. Pandey, M. Seldin, K. S. O'Shea, V. M. Dixit, *J. Biol. Chem.* **270**, 5636 (1995).
3. The B61-Ig chimera was made with the following primers generated by polymerase chain reaction: 5′ primer with a custom Nhe I site (underlined), CCG CGG CTA GCT GAT CGC CAC ACC GTC TTC TGG AAC AGT, and a 3′ primer with a Bam HI site (underlined), CTC GGG ATC CCT GTG ACC GAT GCT ATG TAG AAC CCG CAC. The control-Ig chimera was made as described (*19*). The amplified fragments were digested and cloned into Nhe I– and Bam HI–cut CD5-IgG1 vector [A. Aruffo, I. Stamenkovic, M. Melnick, C. B. Underhill, B. Seed, *Cell* **61**, 1303 (1990)]. The Ig chimeras were purified from pooled supernatants of transfected 293T cells as described (*19*).
4. T. D. Bartley *et al.*, *Nature* **368**, 558 (1994).
5. A. Pandey, R. M. Marks, P. J. Polverini, V. M. Dixit, unpublished data.
6. HUVECs were grown in 2% fetal bovine serum (FBS) without any exogenous growth factors for 48 hours prior to all of the following assays. The cells were metabolically labeled for 8 hours with ^{35}S-cysteine and ^{35}S-methionine as described [A. W. Opipari, M. S. Boguski, V. M. Dixit, *J. Biol. Chem.* **267**, 12424 (1992)]. Cells on 100-mm dishes were lysed on ice in lysis buffer containing 1% NP-40, 50 mM tris, and 150 mM NaCl in the presence of protease inhibitors [leupeptin (5 μg/ml), aprotinin (5 μg/ml), soybean trypsin inhibitor (50 μg/ml), and pepstatin (5 μg/ml)] for 30 min. The cells were then scraped, clarified by centrifugation, and the supernatants incubated overnight with the indicated antibody or chimera (10 μg per immunoprecipitation); 50 μl of a 50% slurry of protein A–Sepharose were added and the samples were incubated for 1 hour and then washed three times in lysis buffer. Sample buffer containing 2% β-mercaptoethanol was added, the samples were boiled for 5 min, and the eluted proteins were resolved on 10% SDS-polyacrylamide gels. To deplete Eck, we incubated the samples with 20 μg of anti-Eck followed by addition of protein A/G-Sepharose. In vitro kinase assays were done as described (*8*).
7. For immunoblotting, the cells were lysed in lysis buffer containing 1% NP-40, 50 mM tris, and 150 mM NaCl in the presence of protease inhibitors. Orthovanadate (1 mM) was included for Figs. 1C and 3A. After blocking overnight in 1% bovine serum albumin (BSA) in tris-buffered saline containing 0.1% Tween (TBS-T) at 4°C, the filter was incubated with anti-Eck (*8*) or 4G10 antibody to phosphotyrosine (UBI) at a concentration of 1 μg/ml. Bound primary antibody was visualized with the ECL kit (Amersham). Reprobing to detect Eck protein was done as described [A. Pandey, D. F. Lazar, A. R. Saltiel, V. M. Dixit, *J. Biol. Chem.* **269**, 30154 (1994)].
8. R. A. Lindberg and T. Hunter, *Mol. Cell. Biol.* **10**, 6316 (1990).
9. V. Sarma, F. W. Wolf, R. M. Marks, T. B. Shows, V. M. Dixit, *J. Immunol.* **148**, 3302 (1992).
10. Angiogenic activity was assayed in the avascular cornea of F344 female rat eyes (Harlan Laboratories, Madison, WI) as described (*20*). Briefly, each sample was combined with an equal volume of sterile Hydron casting solution (Interferon Sciences, New Brunswick, NJ), and 5-μl aliquots were pipetted onto the surface of 1-mm diameter Teflon rods (Dupont Co.) glued to the surface of a glass petri dish. The resulting pellets were air-dried in a laminar hood and refrigerated overnight. Just before implantation, the pellets were rehydrated with a drop of lactated Ringers solution and then placed in a surgically created intracorneal pocket ~1.5 mm from the limbus. Corneas were observed for a period of 7 days; the animals were then perfused with a colloid carbon solution and the corneas removed, flattened, and photographed.
11. J. Folkman and M. Klagsbrun, *Science* **235**, 442 (1987).
12. Chemotaxis was assayed as described (*14, 21*). Briefly, we prepared chemotaxis membranes (Nucleopore, 5-μm pore size) by soaking them sequentially in 3% acetic acid overnight and for 2 hours in gelatin (0.1 mg/ml). Membranes were rinsed in sterile water, dried under sterile air, and stored at room temperature for up to 1 month. Bovine adrenal gland capillary endothelial (BCE) cells, maintained in gelatin-coated flasks in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS were used as target cells. Twenty-four hours before use, BCE were starved in DMEM with 0.1% BSA. Twenty-five microliters of cells suspended at a concentration of 1 × 10^6 cells/ml in DMEM with 0.1% BSA were dispensed into each of the bottom wells. A chemotaxis membrane was positioned on top of the bottom wells, and the chambers sealed, inverted, and incubated for 2 hours to allow cells to adhere to the membrane. Chambers were then reinverted, and 50 μl of test medium were dispensed into the top wells and reincubated for an additional 2 hours. Membranes were fixed and stained with Diff-Quick staining kit (Baxter Diagnostics Inc., McGraw Park, IL) to enumerate membrane-bound cells and cells that had migrated through the membrane to the opposite surface.
13. C. Baglioni, in *Tumor Necrosis Factors: The Molecules and Their Emerging Role in Medicine*, B. Beutler, Ed. (Raven, New York, 1992), pp. 425–438.
14. S. J. Leibovich *et al.*, *Nature* **329**, 630 (1987).
15. L. F. Fajardo, H. H. Kwan, J. Kowalski, S. D. Prionas, A. C. Wilson, *Am. J. Pathol.* **140**, 539 (1992).
16. G. Montrucchio *et al.*, *J. Exp. Med.* **180**, 377 (1994).
17. Quiescent HUVECs were then treated with TNF-α (500 U/ml) or TNF-α plus anti-B61 (20 μg/ml, 30 min before addition of TNF-α and 3 and 5 hours after addition of TNF-α) and grown for 8 hours in 1% BSA (Fig. 3A). Eck was immunoprecipitated with anti-Eck (*8*) and antiphosphotyrosine immunoblotting was done as described (*7*). Polyclonal antibody to B61 was raised against recombinantly expressed human B61 and then affinity purified.
18. Quiescent HUVECs were treated as in (*17*) and then metabolically labeled for 8 hours in the presence of 1% BSA. Cell lysates were incubated with anti-B61 (3E6) (*2*) or anti-Eck (*8*) for 2 hours at 4°C. Immune complexes were precipitated by the addition of protein A/G-Sepharose, washed three times in lysis buffer, dissolved in SDS sample buffer, resolved by SDS–polyacrylamide gel electrophoresis under reducing conditions, and subjected to autoradiography.
19. H. Shao, L. Lou, A. Pandey, E. B. Pasquale, V. M. Dixit, *J. Biol. Chem.* **269**, 26606 (1994).
20. R. M. Streiter *et al.*, *Am. J. Pathol.* **141**, 1279 (1992).
21. A. E. Koch *et al.*, *Science* **258**, 1798 (1992).

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How Baseball Outfielders Determine Where to Run to Catch Fly Balls

Michael K. McBeath,* Dennis M. Shaffer, Mary K. Kaiser

Current theory proposes that baseball outfielders catch fly balls by selecting a running path to achieve optical acceleration cancellation of the ball. Yet people appear to lack the ability to discriminate accelerations accurately. This study supports the idea that outfielders convert the temporal problem to a spatial one by selecting a running path that maintains a linear optical trajectory (LOT) for the ball. The LOT model is a strategy of maintaining "control" over the relative direction of optical ball movement in a manner that is similar to simple predator tracking behavior.

Even recreational baseball outfielders appear to know virtually from the moment of bat contact where to run to catch a fly ball. In this task, the ball's approach pattern renders essentially all major spatial location and depth cues unusable until the final portion of the trajectory. Cues such as stereo disparity, accommodation, image expansion rates, and occlusion help to guide final adjustments in the interception path (*1*, *2*). During most of the task, the only usable information appears to be the optical trajectory of the ball (the changing position of the ball image relative to the background scenery). Conceivably, outfielders could derive the destination from an assumed projected parabolic trajectory, but research indicates that observers are very poor at using such a purely computational approach (*3*). In addition, factors such as air resistance, ball spin, and wind can cause trajectories to deviate from the parabolic ideal (*1*, *4*).

One proposed model is that outfielders run along a path that simultaneously maintains horizontal alignment with the ball and maintains a constant change in the tangent of the vertical optical angle of the ball, tan α (Fig. 1) (*5*–*9*). As the ball rises, tan α increases, but at a rate that is a function of the running path selected. If the fielder runs too far in (so that the ball will land behind him), $\partial(\tan \alpha)/\partial t$ will increase. If he runs too far out (so that the ball will land in front of him), $\partial(\tan \alpha)/\partial t$ will decrease. The fielder can arrive at the correct desti-

M. D. McBeath and D. M. Shaffer, Department of Psychology, Kent State University, Kent, OH 44242–0001, USA.
M. K. Kaiser, Human and Systems Technologies Branch, NASA Ames Research Center, Moffett Field, CA 94035–1000, USA.

*To whom correspondence should be addressed.