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An Endothelial Cell Growth Factor from the Mouse Neuroblastoma Cell Line NB41

ANDREW P. LEVY,[1] RAFAEL TAMARGO,[2] HENRY BREM,[2] and DANIEL NATHANS[1]

[1]*Howard Hughes Medical Institute Laboratory, Department of Molecular Biology and Genetics, and* [2]*Department of Neurological Surgery, Johns Hopkins University School of Medicine, Baltimore, Maryland*

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A growth factor that stimulates the proliferation of endothelial cells from human umbilical vein but is not mitogenic for fibroblastic cells is present in medium conditioned by the mouse neuroblastoma cell line NB41. In a partially purified preparation, factor activity coeluted from a reverse-phase high-pressure liquid chromatography (HPLC) column with a reduced protein of about 24 kd. Activity recovered following electrophoresis of HPLC fractions corresponded to protein of 43–51 kd in the absence of reducing agent and to protein of 23–29 kd after reduction. Antiserum raised against a peptide corresponding to the putative N-terminal amino acid sequence of the 24-kd protein reacted with the 24-kd protein and with a protein of about 47 kd in the nonreduced preparation. After N-glycanase treatment, the immunoreactive 24-kd protein had a mobility corresponding to 19 kd. We infer that the native NB41 factor is a glycosylated dimer whose biochemical and biological properties distinguish it from other endothelial cell growth factors.

KEYWORDS: growth factor, endothelial cells, angiogenesis

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INTRODUCTION

The formation of new blood vessels is an essential feature of organogenesis, tissue repair, and the growth of tumors (Folkman and Klagsburn, 1987). Endothelial cells of preexisting capillaries penetrate the basement membrane, migrate through surrounding tissue, and proliferate to form new capillaries. This process is thought to be mediated by soluble angiogenic factors. A number of angiogenic proteins have been identified, some of which induce proliferation of cultured endothelial cells. These include transforming growth factor α (TGF-α) (Schreiber et al., 1986) and the fibroblast growth factors, acidic FGF and basic FGF (Esch et al., 1985; Gimenez-Gallego et al., 1985; Jaye et al., 1986; Abraham et al., 1986), which are mitogenic *in vitro* for endothelial cells, as well as a variety of mesodermally derived cells (Gospodarowicz et al., 1978), and platelet-derived endothelial cell growth factor (PD-ECGF) (Ishikawa et al., 1989), which is mitogenic for endothelial cells but not fibroblasts (Miyazono et al., 1987). Two hematopoietic growth factors, granulocyte colony-stimulating factor (G-CSF) (Nicola et al., 1982) and granulocyte/macrophage colony-stimulating factor (GM-CSF) (Burgess et al., 1977) have recently been shown to stimulate endothelial cell proliferation *in vitro* (Bussolino et al., 1989), and other angiogenic proteins induce blood vessel growth *in vivo* but are not mitogenic for cultured endothelial cells, e.g., TGF-β (Baird and Durkin, 1986), angiogenin (Kurachi et al., 1985), and tumor necrosis factor (TNF) (Leibovitch et al., 1987; Fräter-Schroder et al., 1987).

We set out to identify additional endothelial cell growth factors that may play a role in angiogenesis. A number of cultured cell lines were surveyed for the release of endothelial cell mitogens. The mouse neuroblastoma cell line NB41 produced the most activity. Here we report that the NB41 ECGF has properties that distinguish it from other well-characterized endothelial cell mitogens and angiogenesis factors. The NB41 factor has the properties of a glycosylated dimer of about 50 kd with potent mitogenic activity for endothelial cells from human umbilical vein, but no activity for fibroblastic cells. The factor preparation also induces angiogenesis in the avascular rabbit cornea.

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METHODS

Cell Culture

Primary human endothelial cells were obtained by perfusing the umbilical vein with 0.05% Worthington Collagenase II (Gimbrone et al., 1974a). The cells were shown to be Factor VIII-positive by direct immunofluorescence (Hoyer et al., 1973). The cells were used in experiments from passage 5–10 and were routinely propagated and expanded in Medium 199 (M199) supplemented with 20% fetal calf serum (FCS) (Hyclone), 30 μg/ml endothelial cell growth supplement (ECGS, Sigma), and 90 μg/ml heparin (Sigma) (Thornton et al., 1983). NB41A3, a clonal mouse neuroblastoma cell line derived from the transplantable mouse C1300 neuroblastoma (Jackson Laboratories) originally cloned by Augusti-Tocco and Sato (Augusti-Tocco and Sato, 1980), was obtained from the American Type Culture Collection. Cells were grown in DMEM:Ham's F-12 medium supplemented with either 10% FCS for cell attachment to the substrate or a modified form of N1 medium described by Bottenstein (1984) (insulin, 5 μg/ml; transferrin, 5 μg/ml; selenium, 3×10^{-8} M; and putrescine, 100 μm) (Collaborative Research) when collecting medium for factor purification.

Assay of Cell Growth

The [^{3}H]thymidine incorporation assay (Glaser et al., 1980) was as follows: Endothelial cells that had not been given fresh medium for 3 days were seeded at 2000 cells/microwell in M199–20% FCS in a 96-well plate (Costar) pretreated with 1% gelatin. After 24 hr, test substances made up in 0.20 ml of M199–20% FCS with 90 μg/ml heparin were added; 42–48 hr later, the cells were pulsed with [^{3}H]thymidine (77 Ci/mmol) at 10 μCi/ml in MEM–0.5% FCS for 2 hr and incorporation into DNA measured by TCA precipitation. A unit of activity is the amount of factor that gives 50% maximal incorporation in this assay. Confluent BALB/c 3T3 cells were maintained in MEM–0.5% FCS for 3 days. Test substances made up in MEM–0.5% FCS were added to the cells, and 18 hr later, the cells were pulsed with [^{3}H]thymidine for 2 hr and [^{3}H]thymidine incorporation measured as described above.

Cell proliferation was assayed as follows: 50,000 endothelial cells that had not been given fresh medium for 3 days were seeded in M199–20% FCS in 35-mm-diameter petri dishes that had been pre-coated with 1% gelatin. After 24 hr, the medium was aspirated, and test substances made up in M199–20% FCS with 90 μg/ml heparin were added. At this time approximately 10,000 cells were present. The medium was aspirated on day 4 after plating and replaced with fresh medium (M199–20% FCS, 90 μg/ml heparin) and test substance. On day 6 the medium was aspirated, the cells washed twice with PBS-EDTA, and then once with PBS-EDTA supplemented with 1% trypsin. The cells were counted in duplicate in a hemocytometer.

Large-Scale Cell Culture

After allowing the NB41 cells to attach to roller bottles in DMEM:Ham's F12 10% FCS, the medium was changed to DMEM:Ham's F12 with modified N1 supplements. This serum-free conditioned medium was collected and replaced every 24 hr for 3–4 days. Immediately after collection, the medium was clarified by centrifugation and filtered through a 0.45-μm membrane and stored at 4°C.

Purification Procedure

Conditioned medium, 20 liters, was adjusted to pH 6.2 with 1 M acetic acid and batch-adsorbed to 50 g Bio-Rex 70 overnight in a microcarrier spinner carboy. The resin was collected on a sintered glass filter, washed three times with 100 mM sodium phosphate, pH 6.2, and then made into a slurry and poured into a column. Elution was with 10 column volumes of 0.5 M NaCl in PBS directly onto a 10 ml conconavalin A (Con A)–Sepharose column (Pharmacia) at 10 cm/hr. The Con A column was washed with 25 ml of 0.5 M NaCl in PBS and protein eluted with 60 ml of 0.2 M α-methyl mannose, 0.2 M α-methyl-glucose, 0.5 M NaCl–PBS. The eluate was dialyzed overnight against 4 liters of distilled water, filtered through a 0.2-μm filter, and pumped onto a 30 × 2.1 mm reverse-phase C4 column (Brownlee Laboratories). For elution of protein, the gradient was 100% solution A, 0–5 min; 0–50% solution B, 5–70 min; 50–100% B, 70–75 min; and 100% B, 75–85 min at a flow rate of 0.5 ml/min. (Buffer A was 0.4% *N*-ethyl-morphilinium acetate, pH 6.5 (Aldrich); solution B, 100% acetonitrile). The Con A eluate was pumped on at 0.5 ml/min, and 2-min fractions were collected from the gradient. Fractions were assayed for stimulation of DNA synthesis in human umbilical vein endothelial cells (HUV-EC).

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Electrophoresis and Recovery of Factor

HPLC fractions were analyzed by SDS-PAGE, as described (Dreyfuss et al., 1984; Spanos and Hübscher, 1983). For recovery of active factor after electrophoresis of pooled fractions (Hunkapiller et al., 1983), gels were cut into 0.5-cm slices and electroeluted in buffer containing 48 mM glycine, 6.25 mM Tris HCl of pH 6.8, and 50 μg/ml bovine serum albumin (BSA).

Antipeptide Sera and Western Blotting

For partial amino acid sequencing of the HPLC-purified protein, a high-pressure liquid chromatography (HPLC) fraction or reduced 24-kd protein blotted onto Immobilon (Matsudaira, 1987) after electrophoresis was analyzed in the Applied Biosystems microsequencer (Hewick et al., 1981). Rabbits were immunized with synthetic peptide conjugated to hemocyanin (Wand and Eipper, 1987). Protein samples to be tested for antigen were subjected to SDS-PAGE, transferred to nitrocellulose (Towbin et al., 1979) or Immobilon (Matsudaira, 1987), and incubated with immune or preimmune serum and, finally, with alkaline phosphatase-conjugated goat anti-rabbit antibody (Stratagene).

Angiogenesis Assay in the Rabbit Cornea

The assay followed the procedure of Gimbrone et al. (1974b). Briefly, pooled peak fractions from the HPLC column plus 9 mg of BSA were lyophilized and then put through a 200-μm mesh with a glass pestle. Elvax (Langer and Folkman, 1976; Langer et al., 1981), an ethylene-vinyl acetate copolymer, was added so that protein was 20% by mass of the final polymer. The sample was processed and implanted as described (Langer and Folkman, 1976). Control polymers were made up from BSA and equivalent fractions from a blank HPLC column eluate. The length of the blood vessels extending from the corneal–scleral junction toward the polymer was measured by means of a Zeiss split-beam stereomicroscope 7 and 14 days after implantation.

RESULTS

Preliminary Characterization of an ECGF from NB41 Cells

To detect previously undescribed ECGFs, media conditioned by a variety of cells were tested for their ability to stimulate DNA synthesis in cultures of HUV-EC using the assay described in Methods. Conditioned medium from the mouse neuroblastoma cell line NB41 (Augusti-Tocco and Sato, 1980), previously shown to be mitogenic for endothelial cells (Suddith et al., 1975), was the most active, showing about six times greater activity per unit volume than conditioned medium from the next most active cell line tested (C6 glioblastoma).

Preliminary experiments indicated that the activity of NB41 conditioned medium was not due to a low-molecular-weight dialyzable factor and that it differed from acidic and basic FGF and other well-characterized ECGFs (Folkman and Klagsburn, 1987). Serum-free conditioned medium from NB41 did not stimulate DNA synthesis in BALB/c 3T3 cells, primary human placental fibroblasts, or rat embryo fibroblasts, whereas FGF is a potent mitogen for fibroblasts (Gospodarowicz et al., 1978). Compared with the FGFs (Shing et al., 1984), NB41 factor had a lower affinity for heparin–Sepharose with elution of activity at 0.5 M NaCl versus >1 M NaCl for the FGFs (Shing et al., 1984). NB41 factor bound to Con A and lentil lectin–Sepharose and was eluted from Con A–Sepharose with a mixture of α-methyl-mannose and α-methyl-glucose, indicating that it is glycosylated or associated with a glycoprotein. Treatment with reducing agents (β-mercaptoethanol or dithiothreitol) followed by dialysis eliminated more than 80% of the NB41 factor activity, suggesting that the factor has one or more essential disulfide bonds, and treatment with 1% SDS followed by dialysis led to little or no loss in activity.

Partial Purification of the NB41 Factor

To prepare NB41-conditioned medium for purification of factor, cells were grown in roller bottles and in serum-free conditioned medium collected as described in Methods. After testing different fractionation procedures, the purification scheme summarized in Table 1 was developed. Figure 1 shows the protein content and activity of HPLC fractions from two different preparations of factor. SDS-PAGE analysis under reducing conditions of the active fractions from RP-HPLC (84% of total activity between 22.5–31.5% acetonitrile) showed several bands on silver stain (Fig. 1). As illustrated, different preparations showed somewhat variable protein profiles. However, in each case the protein

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TABLE 1
Purification of NB41 Factor (see Methods)

Purification Step	Total activity (Units $\times 10^{-5}$)	Recovery of biological activity (%)	Total protein (μg)	Specific activity (units/μg protein	Fold purification
Conditioned medium	7.8	100	370,000	2.1	1
BioRex 70	3.7	47	18,000	21	10
Con A-Sepharose	1.7	22	1,500	110	53
RP-HPLC	1.1	14	44[a]	2,500[a]	1,200[a]

A unit of activity is defined as the amount needed for half-maximal stimulation in the [^{3}H]thymidine incorporation assay.
[a]Because of the small amount of sample available, protein concentration of the RP-HPLC material was estimated from the A230 value (serum albumin reference) rather than the BioRad protein assay as for the other steps.

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band at approximately 24 kd tracked with the activity. In the purest preparation (see Fig. 1B), the 24-kd species is the predominant protein in the fractions with the highest activity. The final specific activity in the most active preparation calculated from the DNA synthesis assay and the A230 was 2500 units/μg protein. However, judging from the amount of protein that appeared in stained PAGE gels, there appeared to be considerably less protein in the peak fractions than estimated from the A230 value.

Evidence the Factor Is a Disulfide-Linked Dimer

Because the factor was stable in SDS, the approximate molecular mass of the active factor could be determined by SDS-PAGE. Peak HPLC fractions (Fig. 1A) were combined, and samples containing 20,000 units of factor were treated with SDS electrophoresis buffer with or without β-mercaptoethanol. The samples were then subjected to electrophoresis in a 10% SDS-polyacrylamide gel with prestained molecular-weight standards placed on either side of the sample lanes. Slices from the sample lanes were subjected to electroelution, as described in Methods, and assayed for activity. Recoveries were 18% of the initial activity for the sample not treated with β-mercaptoethanol and 5% for the β-mercaptoethanol-treated sample. Under nonreducing conditions the estimated molecular mass of the recovered activity was 43–51 kd, while under reducing conditions, the estimated molecular mass of the recovered activity was 23–29 kd (Fig. 2), the latter corresponding to the 24-kd protein that tracked with the activity during HPLC fractionation (Fig. 1). Similar results were obtained when the experiment was repeated with a different factor preparation. We conclude that the native NB41 factor probably consists of two subunits of about 24 kd joined by disulfide linkage. The lower overall recovery of activity from the reduced samples is presumably due to inefficient reformation of active dimer. Since the reduced form has approximately one-half the estimated molecular mass of the native form, the subunits may be identical.

Evidence that the 24-kd protein was derived by reduction from a protein of approximately twice its molecular weight came from the use of an antiserum raised against a synthetic peptide corresponding to the putative N-terminal sequence of the 24-kd protein. The N-terminal sequence determined by Edman degradation of the protein in HPLC fraction 46 (Fig. 1A) in one case and of the reduced 24-kd protein electroblotted onto Immobilon in a second analysis were (A,N,Y,)(P,G)TLEGEQK and APTTEG, respectively. Rabbit antiserum raised against the synthetic peptide APTLEGEQK (conjugated to hemocyanin) reacted by Western blotting with the 24-kd protein (and presumed proteolytic fragments) when the factor preparation was reduced and with a protein of about 47 kd when the factor preparation was not reduced (Fig. 3). Prior incubation of the antiserum with peptide inhibited the reaction. However, the antiserum did not immunoprecipitate ^{125}I-labeled 24-kd protein, nor did it precipitate or inactivate the factor (data not shown). We conclude that the 24 kd protein is derived from the 47-kd protein by reduction of one or more disulfide bonds.

We also used the anti-peptide serum to demonstrate that the immunoreactive 24-kd protein is glycosylated (Fig. 4). *N*-Glycanase treatment of

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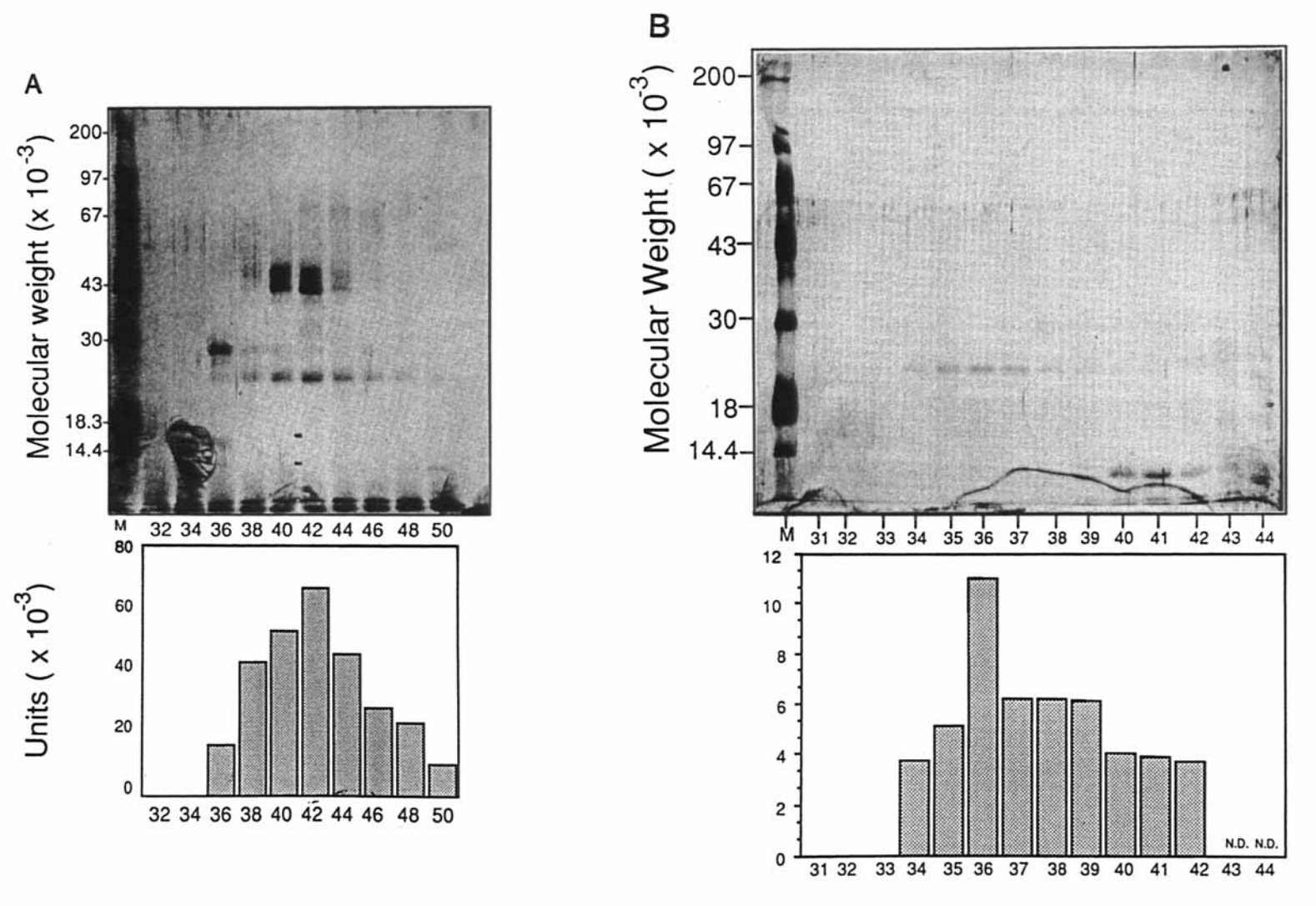

FIGURE 1. Protein content and activity of RP-HPLC fractions. (A) and (B) Results with two different factor preparations. Figures at the top are silver-stained polyacrylamide gels following electrophoresis of 3% (A) or 5% (B) of each β-mercaptoethanol-treated fraction. Figures at the bottom present the results of assaying each fraction for stimulation of DNA synthesis in HUV-EC (expressed as units per fraction). In preparation B, the Con A–Sepharose column step was omitted and a 100 ×4.6 mm C4 column was used for the RP-HPLC step. The invariant bands at 50–60 kd are staining artifacts. Numbers beneath the gels and histograms are the retention times of successive fractions collected from the RP-HPLC column.

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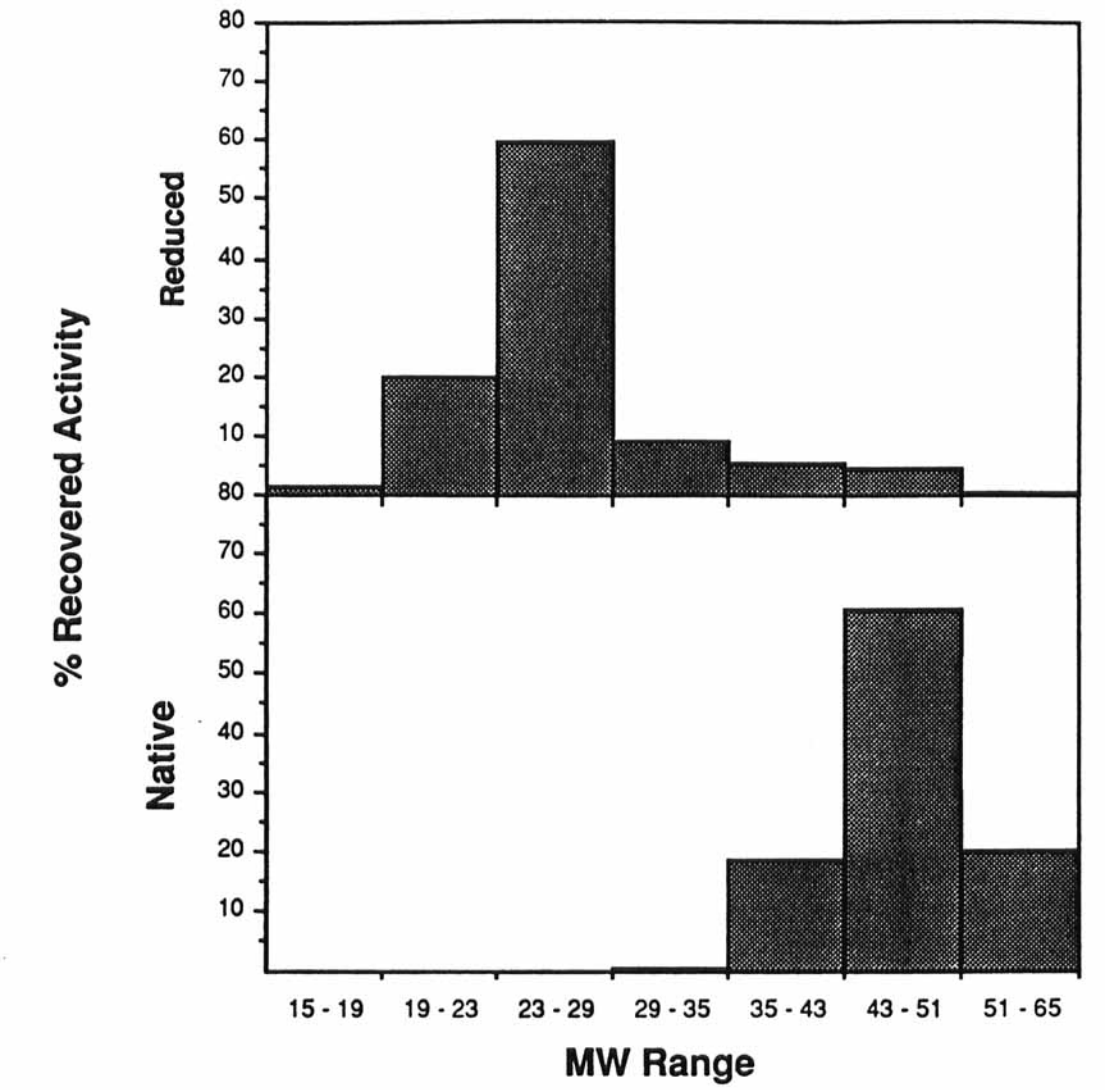

FIGURE 2. Molecular mass of the NB41 factor estimated by SDS-PAGE. Peak HPLC fractions were pooled and aliquots were treated with SDS sample buffer ± β-mercaptoethanol and then loaded in lanes bounded by prestained molecular-weight markers. The gel was sliced and protein was electroeluted from each slice to determine its mitogenic activity. "% Recovered Activity" refers to the percentage of total recovered activity present in each fraction. The overall recovery of the 20,000 units of starting material was 5% for the reduced preparation and 18% for the nonreduced preparation.

the reduced HPLC-purified factor prior to electrophoresis resulted in increased mobility of the immunoreactive protein (and a presumed breakdown product). The molecular mass of the *N*-glycanase-treated protein was approximately 19 kd.

Biological Properties of the HPLC-Purified Factor

The HPLC-purified factor showed the same cell specificity as the NB41 conditioned medium, i.e., it stimulated DNA synthesis in HUV-EC but not in BALB/c 3T3 cells, rat embryo fibroblasts (REF 52), and primary human placental fibroblasts (Fig. 5). It also stimulated the proliferation of HUV-EC (Fig. 5). Half-maximal stimulation in each assay was seen with 2 ng/ml of factor (based on the A230 estimate of protein concentration). With serial addition of excess factor over a period of 6 days, the cell number exceeded that of control cultures by 6-fold (Fig. 5, upper panel). In a separate experiment, serial addition of excess factor was shown to increase cell number 7-fold over 6 days without further increase

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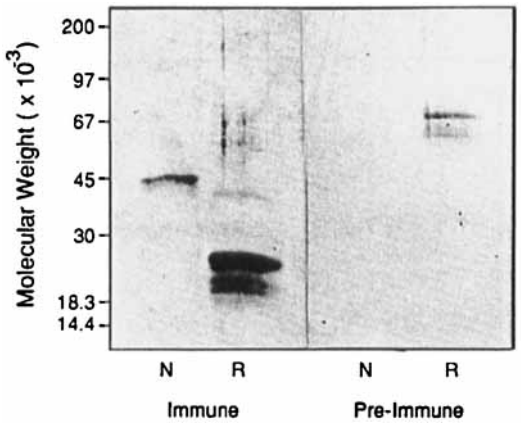

FIGURE 3. Immunoreactive proteins in the RP-HPLC-purified factor. Pooled active fractions from the HPLC column ± β-mercaptoethanol were fractioned by SDS-PAGE, and the proteins were reacted with anti-peptide serum or preimmune serum by Western blotting. N, nonreduced; R, reduced.

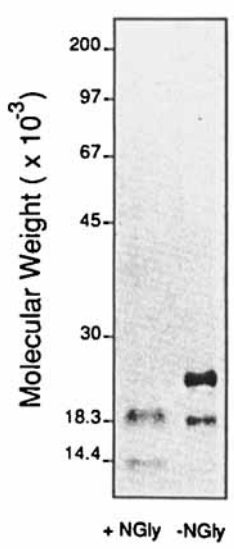

FIGURE 4. Effect of *N*-glycanase on the electrophoretic mobility of the immunoreactive 24-kd protein. HPLC-purified factor was treated with β-mercaptoethanol, and one sample was incubated with *N*-glycanase prior to fractionation by electrophoresis in an SDS polyacrylamide gel. Immunoreactive protein was detected by Western blotting with the anti-peptide serum.

thereafter, whereas in the presence of ECGS the cells continued to proliferate (Fig. 5, inset). To demonstrate that the measured DNA synthesis occurs in endothelial cells rather than contaminating fibroblasts, HUV-EC grown in the presence of [^{3}H]thymidine from days 4–6 after plating were assessed for DNA synthesis by autoradiography and for the presence of the endothelial cell marker Factor VIII antigen by direct immunofluorescence. Nearly all cells contained Factor VIII, and about 20% of these showed DNA synthesis following the 48-hr incorporation period (data not shown). The low fraction of DNA-synthesizing cells is presumably due to the late addition of [^{3}H]thymidine. These results indicate that the partially purified factor stimulates endothelial cell proliferation, but to a lesser extent than ECGS.

To determine whether the NB41 factor preparation also has angiogenic activity, Elvax polymer containing about 1 μg HPLC-purified factor (estimated from the A230) plus 9 mg BSA was implanted in the corneal pocket of rabbits, and the formation of blood vessels was assessed. A strong angiogenic reaction was seen within 7 days in each of six eyes tested. New vessel growth, measured from the corneal–scleral junction toward the polymer, averaged 1.2 mm on day 7 and 2.3 mm on day 14 when factor was present versus <0.1 mm on day 7 and 0.7 mm on day 14 when serum albumin alone was present in the polymer (Table 2). We conclude that the HPLC-purified material has potent angiogenic activity.

DISCUSSION

We report here the identification and partial purification of an apparently novel ECGF from the conditioned medium of the NB41 mouse neuroblastoma cell line that stimulates the proliferation of endothelial cells from human umbilical vein and capillaries in the rabbit cornea. The active factor had an electrophoretic mobility corresponding to a protein of 43–51 kd, but after reduction with mercaptoethanol, the factor had an electrophoretic mobility corresponding to a protein of 23–29 kd. In the final HPLC purification step, activity of the eluate consistently coeluted with a 24-kd protein seen after SDS-PAGE of β-mercaptoethanol-treated fractions, and in the purest preparation, the 24-kd protein was the predominant protein present. Moreover, antiserum raised against a peptide corresponding to the putative N-terminal sequence of the 24-kd protein reacted by Western blotting with both the 24-kd reduced protein and a 47-kd protein present in nonreduced preparations of factor. (Since the anti-peptide serum did not inhibit mitogenic activity nor precipitate 24-kd protein or active factor, it was not possible to use it to relate activity to the 24- and 47-kd proteins.) Putting these various observations

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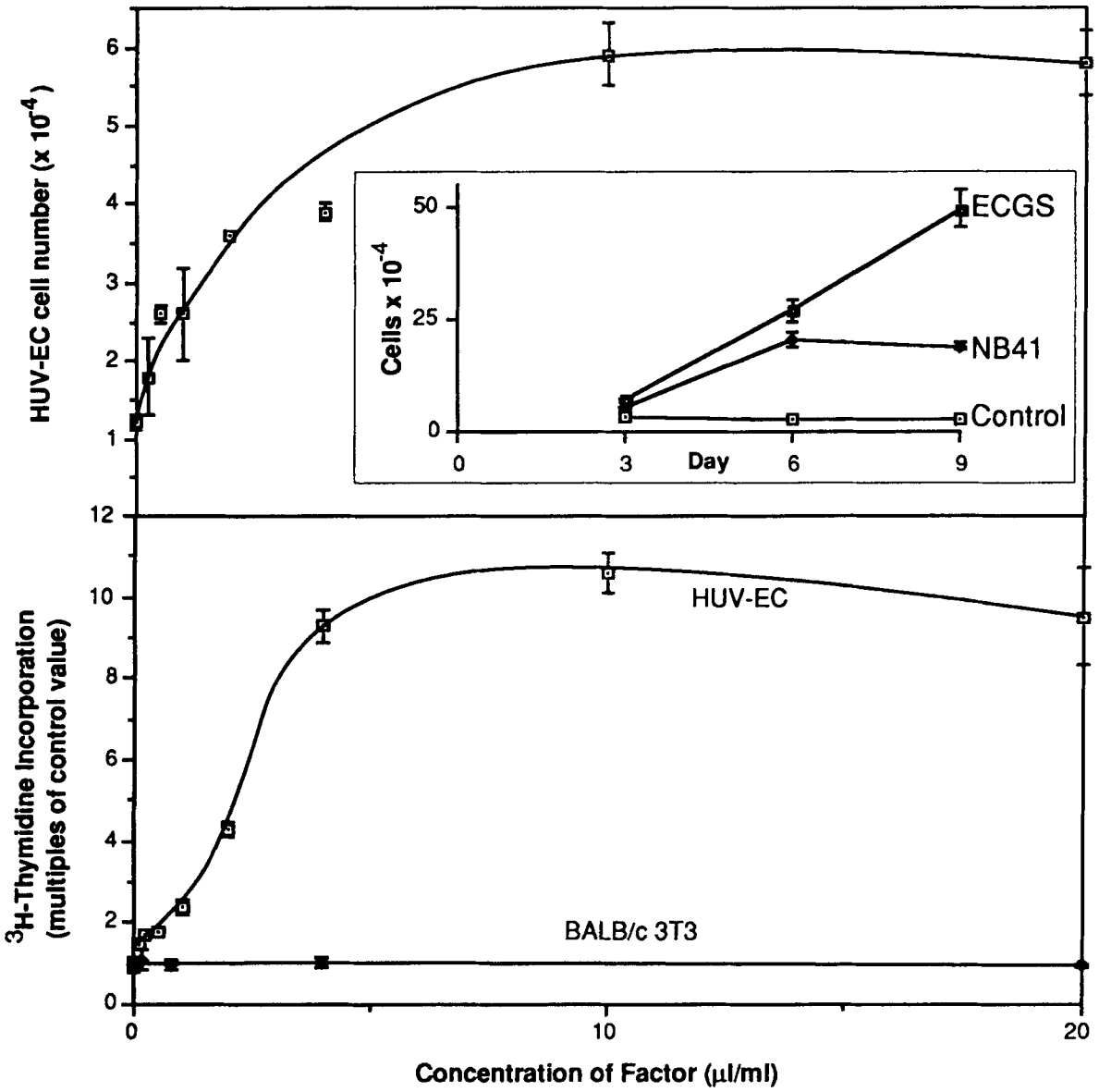

FIGURE 5. Stimulation of HUV-EC proliferation by NB41 factor. For both the cell proliferation and [^{3}H]thymidine assay, concentration is given in microliters of RP-HPLC-purified material/ml of medium. (Upper panel) HUV-EC were seeded at 50,000 cells/35-mm dish in M199–20% FCS on day 0 and incubated with the indicated amounts of factor (made up in M199–20% FCS, 90 µg/ml heparin) added on days 1 and 4. Cells were harvested on day 6 with trypsin–EDTA and counted in a hemocytometer. Each point represents the mean of duplicate or triplicate dishes ± SEM. (Inset) Increase in cell number with time (mean ± SEM of duplicate dishes) in the presence of excess NB41 factor or excess ECGS added with fresh medium on days 0, 2, 5, 7. 80,000 cells were plated on day −1. (Lower panel) Open symbols, HUV-EC were seeded at 2000 cells/microwell in M199–20% FCS on day 0 and incubated on day 1 with different concentrations of factor. After 42–48 hr, the cells were pulsed with [^{3}H]thymidine and TCA-precipitated counts were measured as described in Methods. Each point represents the mean ± SEM of triplicate cultures. Closed symbols, [^{3}H]thymidine incorporation into DNA of BALB/c 3T3 cells, as described in Methods; a different preparation of factor with similar potency was used in this experiment. With ECGS, DNA synthesis in BALB/c 3T3 cells was stimulated 35-fold.

together, we infer that the native NB41 factor is a dimer, possibly consisting of disulfide-linked, identical subunits of about 24 kd. The results of *N*-glycanase treatment indicate that the 24-kd subunit is glycosylated and that the deglycosylated protein has a molecular weight of about 19 kd.

The biological activities of the partially purified NB41 factor include the induction of proliferation of

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TABLE 2
Vessel Length from the Corneal-Scleral Junction to the Elvax Polymer

Rabbit	Vessel length (mm)			
	7 Days		14 Days	
	NB41 factor	Control	NB41 factor	Control
1	1.0	<0.1		
2	1.0	<0.1	1.5	0.3
3	1.1	<0.1	1.6	0.7
4	1.6	<0.1	2.3	0.8
5	1.2	<0.1	3.0	0.9
6	1.1		2.9	
Mean	1.2	<0.1	2.3	0.7
SEM	0.1		0.3	0.1

Vessel length was measured as described in Methods. All eyes were examined without knowledge of the contents of the polymer. Rabbit 1 died before the 14 day value could be determined. Control eye of rabbit 6 had gross inflammation around the corneal incision.

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HUV-EC and angiogenesis. Because of the impurity of the factor preparations, its true potency is not known, nor can we be confident that the proliferation-inducing activity is due to the same molecular species as the angiogenic activity. Nevertheless, the minimal potencies (2500 units/μg of protein in the cell proliferation assay and $<1\ \mu$g protein in the angiogenesis assay) indicate that the factor is as active as many known growth factors.

The properties of the NB41 factor distinguish it from other well-characterized endothelial cell growth factors. It differs from the FGFs in its molecular properties and in its lack of mitogenic activity for fibroblastic cells. Moreover, NB41 factor is found in the medium of cultured cells, whereas FGF is cell-associated (Folkman and Klagsburn, 1987). Nor is NB41 factor antigenically related to FGF, since anti-FGF serum failed to react with proteins in an HPLC-purified NB41 factor preparation (data not shown). G-CSF and GM-CSF are glycoproteins of about 23–25 kd (Burgess *et al.*, 1977; Nicola *et al.*, 1982) recently shown to be endothelial cell mitogens (Bussolino *et al.*, 1989). In contrast to NB41 factor, neither G-CSF nor GM-CSF has the properties of a dimeric protein. Moreover, the presumed N-terminal sequence of NB41 factor (and the sequence of a recently isolated internal peptide of the 24-kd protein) are not present in the murine CSFs (Tsuchiya *et al.*, 1986; Gough *et al.*, 1984) nor other sequenced growth factors. PD-ECGF has a molecular mass (45 kd) similar to that of unreduced NB41 factor (Miyazono *et al.*, 1987). However, PD-ECGF is a single-subunit protein (Miyazono *et al.*, 1987; Ishikawa *et al.*, 1989), its Con A- and heparin-binding properties are different from those of NB41 factor, and its amino acid sequence does not include the limited sequences we have obtained for the 24-kd protin (Ishikawa *et al.*, 1989). More complete characterization of the NB41 factor will be needed to determine if it is structurally related to the CSFs or PD-ECGF.

The fact that conditioned medium of a neuroblastoma cell line is a source of growth factor for endothelial cells is of interest. Many tumors, including neuroblastomas, produce angiogenic and/or endothelial cell growth factors, the activity of which may be essential for tumor growth (Brem and Folkman, 1975; Brem *et al.*, 1988). Presumably this role of angiogenic factors reflects a similar function in organogenesis and tissue repair. Our studies have been severely limited by the small amounts of factor protein present in NB41-conditioned medium. To characterize the NB41 factor further and to explore its range of biological activities it will be essential to have adequate amounts of homogeneous factor. The partial amino acid sequences we have obtained may be useful in the isolation of cDNA encoding the factor, which can then be used to produce the 24-kd protein.

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