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Isolation of a cDNA for a Growth Factor of Vascular Endothelial Cells from Human Lung Cancer Cells: Its Identity with Insulin-like Growth Factor II

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We have found growth-promoting activity for vascular endothelial cells in the conditioned medium of a human lung cancer cell line, T3M-11. Purification and characterization of the growth-promoting activity have been carried out using ammonium sulfate precipitation and gel-exclusion chromatography. The activity migrated as a single peak just after ribonuclease. It did not bind to a heparin affinity column. These results suggest that the activity is not a heparin-binding growth factor (including fibroblast growth factors) or a vascular endothelial growth factor. To identify the molecule exhibiting the growth-promoting activity, a cDNA encoding the growth factor was isolated through functional expression cloning in COS-1 cells from a cDNA library prepared from T3M-11 cells. The nucleotide sequence encoded by the cDNA proved to be identical with that of insulin-like growth factor II.

Key words: Vascular endothelial cells — Growth factor — Insulin-like growth factor II — Lung cancer — Expression cloning

It is well recognized that tumor growth is dependent on the development of new blood vessels (angiogenesis). Many tumor cells produce growth factors for vascular endothelial cells. Such growth factors include basic fibroblast growth factor (FGF), acidic FGF, vascular endothelial growth factor (VEGF), and transforming growth factor-α (TGF-α).[1,2] Acidic FGF, basic FGF and VEGF have affinity for heparin and bind to heparin Sepharose affinity columns. We observed that the conditioned medium from a human lung cancer cell line, T3M-11, stimulated the growth of endothelial cells derived from porcine aorta. The activity did not bind to a heparin Sepharose affinity column, and TGF-α was not detected in the conditioned medium. These findings led us to analyze the growth-promoting activity produced by T3M-11 cells.

In this work, we isolated a cDNA encoding this growth factor for vascular endothelial cells by using a COS-1 cell expression screening system and determined the nucleotide sequence.

MATERIALS AND METHODS

Cell cultures T3M-11 cell line,[3] established in this laboratory from a human small cell lung cancer, was cultured in a mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-10 nutrient mixture (1:1) without serum. Porcine aortic endothelial cells[4] were cultured in F-10 medium with 10% fetal calf serum (FCS). COS-1 cells were maintained in DMEM with 5% FCS.

Characterization of growth-promoting activity in the conditioned medium of T3M-11 cells T3M-11 cells were cultured in DMEM and F-10 nutrient mixture (1:1) without FCS. Conditioned medium was collected, concentrated 100-fold by using a hollow-fiber apparatus equipped with an AIh 1010 hollow fiber unit (molecular weight cut-off of 6000) (Asahi Chemical Industry, Tokyo). Ammonium sulfate (40% saturation) was added to the concentrate. After equilibration for 2 h at 4°C, the sample was centrifuged at 2075g for 15 min. The precipitate was collected, suspended in 10 mM Tris HCl buffer (pH 7.4) and applied to a TSK-G3000SW gel exclusion column (0.75×6 cm), which was eluted at a flow rate of 1 ml/min. The absorbance of the column effluent was monitored at 280 nm. Fractions (1 ml) were collected and assayed for growth-promoting activity. The column was calibrated using bovine serum albumin (BSA) (Mr 67,000), ovalbumin (Mr 43,000), and ribonuclease (Mr 13,700). Active fractions from the TSK-G3000SW column were pooled and loaded on a heparin-Sepharose CL-6B column (0.8×2.2 cm) equilibrated with 10 mM Tris HCl, pH 7.4. After a wash with 2.5 ml of 10 mM Tris HCl containing 150 mM NaCl, the column was eluted sequentially with 4 ml each of 500 mM, 1000 mM, 1500 mM, and 2000 mM NaCl in 10 mM Tris HCl, pH 7.4. Eluted fractions (1 ml) were assayed for growth-promoting activity.

Construction of a cDNA library Total cellular RNA was isolated from T3M-11 cells by means of the acid

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guanidinium-phenol-chloroform extraction method[5] and was subjected to an oligo (dT) cellulose column to isolate polyadenylated RNA. Polyadenylated RNA (3 mg) was primed with *Not*I primer-adapter (Promega Corporation, Madison, WI) and reverse-transcribed with M-MLV reverse transcriptase (Superscript, Life Technologies, Inc., Gaithersburg, MD). Second-strand cDNA was synthesized using a modification of the method of Gubler and Hoffman.[6] The cDNA was ligated to *Eco*RI adapter (Promega) and inserted into the pSSRa II eukaryotic expression vector.[7] *Escherichia coli* (XL1-Blue, Stratagene Cloning Systems, La Jolla, CA) was transformed by electroporation. Individual recombinant clones were inoculated into 96-well tissue culture plates and cultured overnight in LB medium with 100 mg/ml ampicillin. Culture medium from 12 wells was pooled, then plasmids were prepared from each pool by the boiling method[8] and used to transfect COS-1 cells.

Transfection of COS-1 cell Nonconfluent COS-1 cells were harvested from tissue culture flasks with 0.05% trypsin and 0.53 m*M* EDTA. The cells were plated into 6-well tissue culture plates at 3×10^5 cells/well in 3 ml of DMEM with 5% FCS and cultured overnight. The cells were washed 3 times with PBS (−), and incubated in 0.3 ml of Dulbecco's phosphate-buffered saline (PBS (−)) with 500 mg/ml of DEAE-dextran and 1.5 mg/ml of the plasmids at 37°C for 30 min, then in 3 ml of DMEM with FCS and 80 mg/ml of chloroquine for 2.5 h. The cells were again washed with PBS (−), incubated in DMEM with FCS and 10% dimethyl sulfoxide for 2 min, then incubated in 3 ml of DMEM with 5% FCS. After 12 h the cells were washed with PBS (−) and incubated in 1 ml of HEPES-DMEM with 0.5% FCS for 48 h. The conditioned medium was collected and subjected to bioassay for growth-promoting activity against porcine aortic endothelial cells. Efficiency of transfection was determined by transfecting plasmids which had β-lactamase gene from *E. coli* as the insert and staining with X-gal.[9] The conditioned medium from these cells was also used as a negative control in the bioassay.

[^{3}H]Thymidine incorporation into porcine aortic endothelial cells Growth-promoting activity of the conditioned medium from transfected COS-1 cells was monitored in terms of [^{3}H]thymidine incorporation into porcine aortic endothelial cells. The nonconfluent endothelial cells were harvested from tissue culture flasks with 0.05% trypsin and 0.53 m*M* EDTA. The cells were plated into 24-well tissue culture plates at 1×10^4 cells/well in 0.5 ml of F-10 with 0.5% FCS. At 24 h later, 0.1 ml of the conditioned medium was added to each well and the cells were incubated for 16 h. After 10 h of labeling with 0.1 mCi of [^{3}H]thymidine (6.7 Ci/mM, NEN Research Products, Boston, MA), the cells were fixed with 10% trichloroacetic acid. Unincorporated [^{3}H]thymidine was removed by repeated washing with PBS (−), then the cells were lysed with 0.1 *N* NaOH, and incorporated radioactivity was counted in a liquid scintillation counter.

Nucleotide sequence determination The cDNA from the plasmid with positive activity was inserted into M13 filamentous phage vector. Sequential deletion mutants were made and the nucleotide sequence was determined by the dideoxynucleotide chain-termination procedure.[10] Both strands were completely sequenced.

Growth-promoting activity of IGF-II on endothelial cells The endothelial cells were seeded as described above and recombinant human insulin-like growth factor II (IGF-II), (Wakunaga Pharmaceutical Co., Ltd., Hiroshima) was added to each well at various concentrations. [^{3}H]Thymidine incorporation was measured as described. Cell numbers were also counted each day.

RESULTS

Characterization of growth-promoting activity Ammonium sulfate precipitation was utilized for the first purification step. At neutral pH, 40% saturation of ammonium sulfate precipitated about 90% of the activity. The sample from ammonium sulfate precipitation was applied to a column of TSK-G3000SW equilibrated with 10 m*M* Tris HCl, pH 7.4 (Fig. 1). This procedure yielded a single peak of bioactivity that eluted just after ribo-

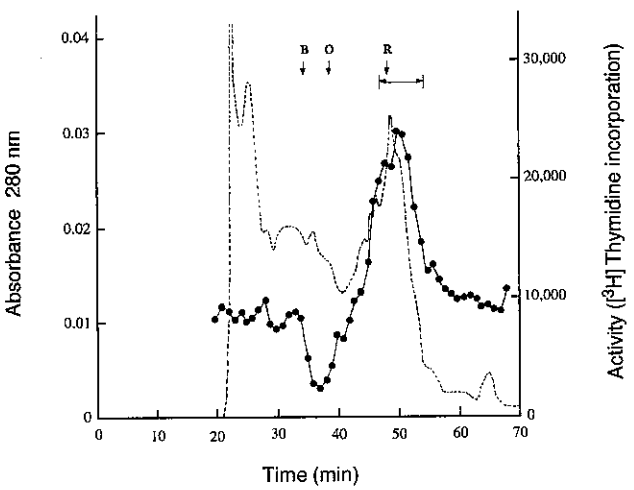

Fig. 1. Gel chromatography on a TSK-G3000SW column. For experimental details, see "Materials and Methods." For growth-promoting assay on endothelial cells, 10 ml aliquots from each fraction were added to the cells. The fractions under the horizontal bar were pooled for heparin-Sepharose chromatography. The Mr markers used (B, bovine serum albumin; O, ovalbumin; R, ribonuclease; Pharmacia P-L Biochemicals) are indicated by the arrows.

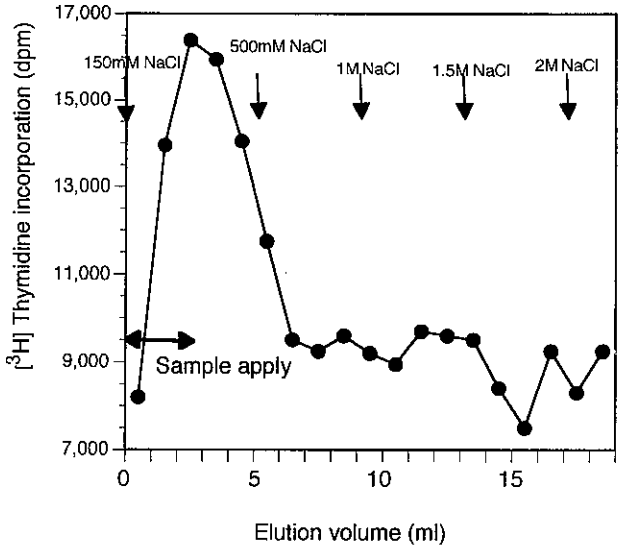

Fig. 2. Chromatography on heparin-Sepharose. For the experimental datails, see "Materials and Methods." Growth-promoting activity on endothelial cells was assayed by the addition of 10 ml aliquots of each fraction to the cells. Elutions with 500, 1000, 1500, 2000 m*M* NaCl are indicated by the arrows.

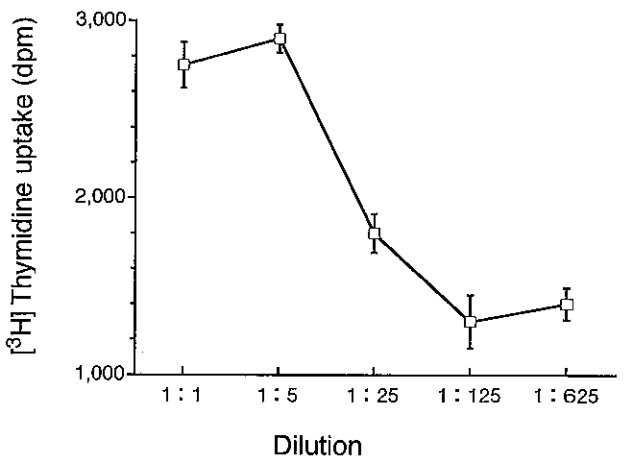

Fig. 3. Effect of the conditioned medium from the isolated positive clone on [^{3}H]thymidine uptake in vascular endothelial cells. The assay was performed as described in "Materials and Methods." The final dilutions of the conditioned medium are as indicated. Each point represents mean value±SD from four wells.

nuclease. The active fraction from the TSK-G3000SW column was loaded on a heparin Sepharose column (Fig. 2). The activity was not adsorbed on this column.

Isolation of a cDNA for the growth factor To isolate cDNA encoding for the growth-promoting activity of the endothelial cells, we used a COS cell expression system. Double-stranded cDNA was synthesized using mRNA prepared from human lung cancer cells T3M-11, which produced the growth factor in serum-free medium. A cDNA library was constructed in expression vector pSSRa-P2 as 3000 pools each consisting of 12 clones. Plasmid DNA from each pool was prepared by the boiling method and introduced into COS-1 cells in 6-well tissue culture plates using DEAE-dextran. After 48 h the conditioned medium was examined for growth-promoting activity. Plasmid DNA from 1 pool yielded a [^{3}H]thymidine uptake of 2850±200 (SD) dpm. The background level of [^{3}H]thymidine uptake by the endothelial cells was 1300±150 dpm. The positive pool was partitioned into single clones and the positive clone was identified. [^{3}H]Thymidine uptake by the conditioned medium of the isolated clone is shown in Fig. 3. The cDNA of the identified positive clone was inserted into M13 vector to determine the nucleotide sequence (Fig. 4). The whole nucleotide sequence completely matched the reported IGF-II sequence[11] except for CCCCC (from nucleotide No. 649). The reported sequence was CCCC, while other authors found CCCCC.[12]

Growth-promoting activity of human IGF-II on vascular endothelial cells We examined the growth-promoting activity of recombinant human IGF-II on endothelial cells (Figs. 5 and 6) in terms of [^{3}H]thymidine incorporation and cell number. Maximum stimulation of [^{3}H]-thymidine uptake was obtained at 1×10^{-8} *M* (75 ng/ml) IGF-II. At this concentration, the cell number also increased significantly.

DISCUSSION

We demonstrated that T3M-11 cells produce a growth factor for vascular endothelial cells, which did not bind to a heparin Sepharose affinity column. A cDNA encoding a growth factor for vascular endothelial cells was isolated through functional expression cloning in COS-1 cells from a cDNA library prepared from T3M-11 cells. The nucleotide sequence of the cDNA was identical to that of IGF-II. The gene encoding human IGF-II comprises 8 exons, which are differentially expressed in various tissues and developmental stages.[13] There are 3 promoters, which generate an mRNA family. These mRNAs contain identical coding regions (exons 5, 6, and 7), but the 5′-terminal noncoding regions are different. IGF-II mRNAs isolated from tumor cells are reported to arise from the promoter associated with exon 4 or 4B that is used in fetal tissues. The mRNA isolated from T3M-11 cells appears to use either of the promoters on the basis of the 5′-terminal noncoding region. These observations indicate that T3M-11 cells express IGF-II. However, it remains unsettled whether or not the growth-promoting

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                                                GTT CACTCTGTCT CTCCCACTAT CTCTGCCCCC

-300
CTCTATCCTT GATACAACAG CTGACCTCAT TTCCCGATAC CTTTTCCCCC CCGAAAAGTA

-240
CAACATCTGG CCCGCCCCAG CCCGAAGACA GCCCGTCCTC CCTGGACAAT CAGACGAATT

-180
CTCCCCCCCC CCCCAAAAAA AAGCCATCCC CCCGCTCTGC CCCGTCGCAC ATTCGGCCCC

-120
CGCGACTCGG CCAGAGCGGC GCTGGCAGAG GAGTGTCCGG CAGGAGGGCC AACGCCCGCT

-60
GTTCGGTTTG CGACACGCAG CAGGGAGGTG GGCGGCAGCG TCGCCGGCTT CCAGACACCA

          10          20          30          40          50          60
ATG GGA ATC CCA ATG GGG AAG TCG ATG CTG GTG CTT CTC ACC TTC TTG GCC TTC GCC TCG
M   G   I   P   M   G   K   S   M   L   V   L   L   T   F   L   A   F   A   S

          70          80          90          100         110         120
TGC TGC ATT GCT GCT TAC CGC CCC AGT GAG ACC CTG TGC GGC GGG GAG CTG GTG GAC ACC
C   C   I   A   A   Y   R   P   S   E   T   L   C   G   G   E   L   V   D   T

          130         140         150         160         170         180
CTC CAG TTC GTC TGT GGG GAC CGC GGC TTC TAC TTC AGC AGG CCC GCA AGC CGT GTG AGC
L   Q   F   V   C   G   D   R   G   F   Y   F   S   R   P   A   S   R   V   S

          190         200         210         220         230         240
CGT CGC AGC CGT GGC ATC GTT GAG GAG TGC TGT TTC CGC AGC TGT GAC CTG GCC CTC CTG
R   R   S   R   G   I   V   E   E   C   C   F   R   S   C   D   L   A   L   L

          250         260         270         280         290         300
GAG ACG TAC TGT GCT ACC CCC GCC AAG TCC GAG AGG GAC GTG TCG ACC CCT CCG ACC GTG
E   T   Y   C   A   T   P   A   K   S   E   R   D   V   S   T   P   P   T   V

          310         320         330         340         350         360
CTT CCG GAC AAC TTC CCC AGA TAC CCC GTG GGC AAG TTC TTC CAA TAT GAC ACC TGG AAG
L   P   D   N   F   P   R   Y   P   V   G   K   F   F   Q   Y   D   T   W   K

          370         380         390         400         410         420
CAG TCC ACC CAG CGC CTG CGC AGG GGC CTG CCT GCC CTC CTG CGT GCC CGC CGG GGT CAC
Q   S   T   Q   R   L   R   R   G   L   P   A   L   L   R   A   R   R   G   H

          430         440         450         460         470         480
GTG CTC GCC AAG GAG CTC GAG GCG TTC AGG GAG GCC AAA CGT CAC CGT CCC CTG ATT GCT
V   L   A   K   E   L   E   A   F   R   E   A   K   R   H   R   P   L   I   A

          490         500         510         520         530         540
CTA CCC ACC CAA GAC CCC GCC CAC GGG GGC GCC CCC CCA GAG ATG GCC AGC AAT CGG AAG
L   P   T   Q   D   P   A   H   G   G   A   P   P   E   M   A   S   N   R   K

                                                                      600
TGAGCAAAAC TGCCGCAAGT CTGCAGCCCG GCGCCACCAT CCTGCAGCCT CCTCCTGACC
***
                                                                      660
ACGGACGTTT CCATCAGGTT CCATCCCGAA AATCTCTCGG TTCCACGTCC CCCTGGGGCT

                                                                      720
TCTCCTGACC CAGTCCCCGT GCCCCGCCTC CCCGAAACAG GCTACTCTCC TCGGCCCCCT

                                                                      780
CCATCGGGCT GAGGAAGCAC AGCAGCATCT TCAAACATGT ACAAAATCGA TTGGCTTTAA

                                                                      840
ACACCCTTCA CATACCCTCC CCCCAAATTA TCCCCAATTA TCCCCACACA TAAAAAATCA

                                                                      900
AAACATTAAA CTAACCCCCT TCCCCCCCCC CCACAACAAC CCTCTTAAAA CTAATTGGCT

                                                                      960
TTTTAGAAAC ACCCCACAAA AGCTCAGAAA TTGGCTTTAA AAAAAACAAC CACCAAAAAA

AATCAATTGG CTAAAAAAAA AAAAAAAAA
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Fig. 4. Nucleotide sequence and predicted amino acid sequence of IGF-II cDNA isolated from T3M-11 cells. The first nucleotide of IGF-II in the putative open reading frame is referred to as nucleotide No. 1. The putative stop codon is indicated by asterisks (***).

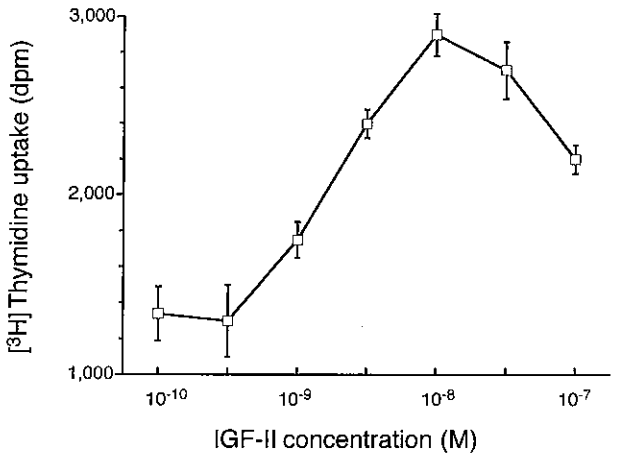

Fig. 5. Effect of IGF-II on [^{3}H]thymidine uptake in vascular endothelial cells. The assay was performed as described in "Materials and Methods" with recombinant human IGF-II at various concentrations.

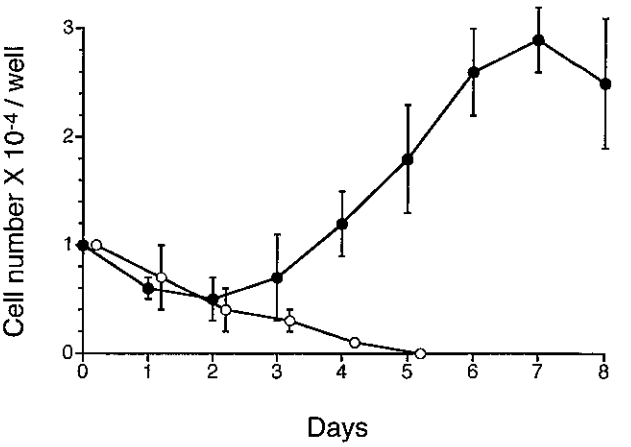

Fig. 6. Growth curve of vascular endothelial cells in the presence of IGF-II. The endothelial cells were seeded as described in "Materials and Methods" with 1×10^{-8} *M* (75 ng/ml) recombinant human IGF-II (●) or 10% PBS (−) (○). The cell numbers were counted each day.

activity found in the conditioned medium is attributable to IGF-II itself. We found that a monoclonal antibody to IGF-II abolished the growth-promoting activity in the conditioned medium (Table I). These results suggest that T3M-11 expressed and secreted IGF-II, and the growth-promoting activity on porcine endothelial cells is attributable to IGF-II itself.

Many tumor cells, including lung cancers, have been shown to produce IGF-II.[13] The clinical implications of tumor-derived IGF-II are hypoglycemia and promotion of growth of the tumors themselves (autocrine growth). Although IGF-II has been reported to stimulate the growth of vascular endothelial cells, the major interest has been limited to its metabolic effect on endothelial cells, including stimulation of glucose transport, amino acid transport, and glucose oxidation.[14] Human recombinant IGF-II stimulated the growth of endothelial cells in a dose-related manner (Fig. 5). Maximum response was obtained at 75 ng/ml. A possible explanation for the relatively high concentration required for the maximum stimulation is the presence of an IGF-binding protein in the culture medium, which could inhibit the stimulatory effect of IGF-II.[15] Our result suggests a possible role of IGF-II as a mitogenic factor for vascular endothelial cells.

IGF-II has been shown to stimulate the proliferation of lung cancer cells.[16] Our observations suggest that IGF-II stimulates the growth of not only lung cancer cells, but also vascular endothelial cells *in vivo*.

In this assay, maximum stimulation of [^{3}H]thymidine uptake by the positive clone was only twice as high as the negative control. In addition, the sensitivity was not high.

Table I. Effect of a Monoclonal Antibody to IGF-II on Growth-promoting Activity

	Growth-promoting activity (%)
T3M-11 CM	100
T3M-11 CM+mAb to IGF-II	0
T3M-11 CM+mouse IgG	54
mAb to IGF-II	0
Mouse IgG	6.9
IGF-II (1 μg/ml)	69.4

Conditioned medium (CM) of T3M-11 cells and a monoclonal antibody (mAb) to rat IGF-II (10 μg/ml), which shows cross reactivity with human IGF-II were mixed and incubated at 4°C for 2 h. After the addition of Protein G Sepharose (50 μl/ml) and bovine serum albumin (10 μg/ml), the mixture was incubated at 4°C for another 2 h. Then the mixture was centrifuged at 10,000*g* for 1 min and the residual growth-promoting activity in the supernatant was determined as described in "Materials and Methods." Mouse IgG was used for reference.

At least 10^{-9} *M* (nanogram per milliliter order) IGF-II was needed for detection of the growth activity towards endothelial cells, as shown in Fig. 5. Expression cloning under these conditions was very difficult. To increase expression of the growth activity in the conditioned medium, we (1) used SRa promoter, which is one of the most potent promoters in eukaryotic cells and worked in a wide range of cell types,[17] (2) made plasmid groups consisting of only 12 clones to increase the final concentration of the product from each clone, and (3) used HEPES-DMEM. Since HEPES-DMEM has a higher

buffering activity than DMEM, only one-third volume of HEPES-DMEM was needed for the incubation of COS-1 cells during the expression period as compared with DMEM, and higher concentrations of expressed products were anticipated.

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