Biochemical and Biophysical Research Communications **289,** 264–268 (2001)
doi:10.1006/bbrc.2001.5970, available online at http://www.idealibrary.com on IDEAL®

NC1 Domain of Human Type VIII Collagen (α 1) Inhibits Bovine Aortic Endothelial Cell Proliferation and Causes Cell Apoptosis

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Received October 16, 2001

Endostatin, a natural angiogenesis inhibitor, had been identified for years. It opened a new approach for cancer therapy. Sequence analysis reveled that endostatin is the NC1 domain (non-triple-helical domain) of collagen XVIII. In this report, the cDNA of NC1 domain of type VIII collagen (α 1) was cloned and expressed as soluble form in *Escherichia coli*. The recombinant protein was purified with Ni-NTA agarose column and named as vastatin. It inhibited the proliferation of bovine aortic endothelial (BAE) cell stimulated by basic fibroblast growth factor (bFGF) in a dose-dependent manner. The ED_{50} of vastatin was 0.6 μg/ml, while the ED_{50} of endostatin was 0.5 μg/ml. Treatment of BAE cell with vastatin caused G_0–G_1 arrest and cell apoptosis. It is interesting that sequence analysis showed that there was only about 12% amino acid sequence homology between vastatin and endostatin. The structure–function relationship of these angiogenesis molecules remains to be elucidated.

Key Words: **vastatin; type VIII collagen; NC1 domain; angiogenesis inhibitor; apoptosis.**

NC domain (non-triple-helical domain) of collagen has been shown to be a functional important portion and may attribute to form supramoleculor structures in extracellular matrix (1). Recently studies demonstrated that the NC domains of several collagens might play an important role in angiogenesis as inhibitors. Endostatin, the well-defined angiogenesis inhibitor, was isolated from hemangioendothelioma medium and was revealed to be the C-terminus of collagen XVIII (2, 3). It inhibited the endothelial cell proliferation *in vitro* and inhibited the growth and metastasis of solid tumors in nude mice. Restin, the C-terminus of collagen XV, a homologous protein of endostatin, also showed the ability to inhibit the angiogenesis (4). In the recent reports, the NC1 domains of α 1 and α 2 chain of type IV collagen (arresten and canstatin) were also found to be inhibitors of angiogenesis *in vitro* and *in vivo* (5, 6).

Collagen XVIII, collagen XV, and collagen IV are all components of vascular basement membrane. Basement membrane is thin, sheet-like structures associated with epithelial and endothelial cells and provides a mechanical support for them. Basement membrane is composed of collagens, laminin, HSPGs (heparan sulfate proteoglycan), fibronectin, and lactin (1, 5, 7). These molecules self-associate and interact with other molecules to form supramoleculor networks which serve as an anchoring substrate for vascular cells. Type VIII collagen is also present within vascular basement membrane beneath endothelial cells (1, 9). It is produced by endothelial cells, keratinocytes, mast cells, microvascular endothelial cells and some tumor cells (8).

Since these three collagens, type XVIII, XV, and IV (α1 and α2), are all present in the vascular basement membrane and their NC1 domains (endostatin, restin canstatin and arresten) all showed the activity to inhibit angiogenesis (2, 4–6), we suspected that the NC1 domain of collagen VIII may also possess the same activity. In the present study, the cDNA of NC1 domain of the type VIII collagen (α 1) was cloned and expressed in *E. coli*. The purified recombinant protein was named as vastatin. We demonstrated for the first

Abbreviations used: NC domain, non-triple-helical domain; BAE cell, bovine aortic endothelial cell; FCS, fetal calf serum; DMEM, Dulbecco's modified Eagle medium; IPTG, isopropyl thio-β-D-galactopyranoside; PAGE, polyacrylamide gel electrophoresis; PI, propidium iodide; PCR, polymerase chain reaction; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetraliumbromide.

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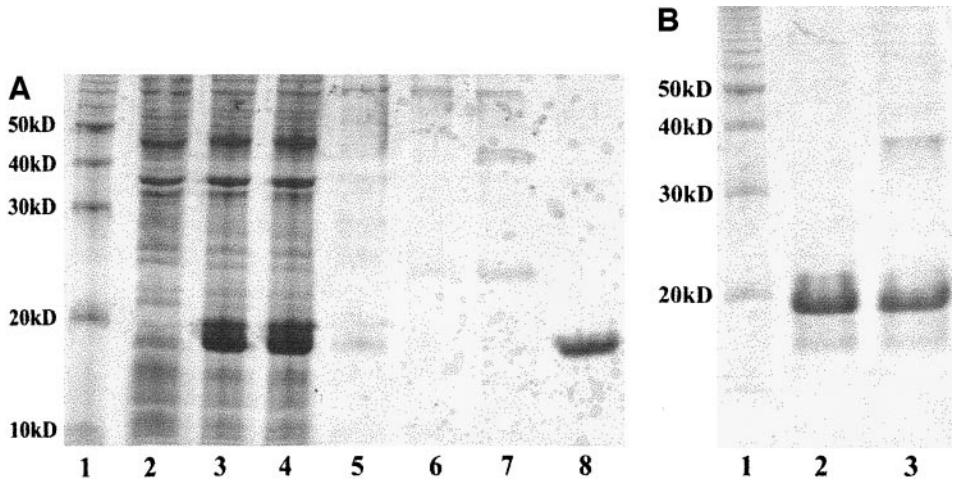

FIG. 1. Protein analysis by SDS–PAGE. Samples were loaded onto a 15% gel followed by staining with Coomassie blue. (A) Lane 1, protein marker; lane 2, total protein of bacterial before induction; lane 3, total protein of bacterial after induction; lane 4, soluble protein of bacterial; lane 5, eluted fraction of 10 mM imidazole from column; lane 6, eluted fraction of 20 mM imidazole; lane 7, eluted fraction of 40 mM imidazole; lane 8, purified vastatin. (B) Lane 1, protein marker; lane 2, purified vastatin under reducing condition; lane 2, purified vastatin under nonreducing condition.

time that vastatin was a potent angiogenesis inhibitor and showed an apoptosis inducing activity on bovine aortic endothelial cell.

MATERIALS AND METHODS

Vastatin cDNA cloning and expression vector construction. The total RNA was purified from human umbilical cord using TRIZOL reagent (GIBCO BRL) as protocol manual. Total RNA was used as the template for cDNA synthesis using Superscript RNase H^- transcriptase (GIBCO BRL) according to the manufacturer's instruction. PCR was performed with Ex-Taq DNA polymerase (TaKaRa). The synthetic oligonucleotides were obtained from Shanghai Sangon. The primers were designed as follows: RT primer, 5′CAGGGAGAGTATCTGCCAGAT 3′; sense primer, 5′AGATATA**CATATG**CAGGGAGAGTATCTGCCAGAT 3′; antisense primer, 5′GTG**CTCGAG**CATGGGATACAATAAATATCCTGA 3′; the *Nde*I site and *Xho*I sites were introduced into the sense and antisense primer, respectively. PCR was performed in PE480 thermal cycler (Perkin–Elmer, NJ) for 35 cycles: denaturation 30 s, 94°C; annealing 30 s, 55°C; extension 30 s, 72°C. PCR product was run on 1% agarose gel in TBE (10 mM Tris–borate, 1 mM EDTA, pH 8.0), and visualized by ethidium bromide staining.

The amplified cDNA fragment was digested with *Nde*I and *Xho*I and ligated into predigested pET24a(+) (Novagen), resulting in the expression plasmid pETVIII. The 3′ end of the sequence was ligated in-frame with a polyhistidine tag sequence.

Expression and purification of vastatin. Expression plasmid pETVIII was transformed into *E. coli* BL21 (DE3) cells (Novagen) for expression. 4 ml of overnight bacterial culture was used to inoculate a 400-ml culture in LB medium. The culture was incubated at 37°C for approximately 3.5 h until the cell density reached an OD_{600} of 0.5. Then, IPTG was added to a final concentration of 0.2 mM. After a 5-h induction at 30°C, cells were harvested by centrifugation at 6000*g*. After resuspending cells in lysis buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 10 mM imidazole), lysozyme was added to the final concentration of 0.5 mg/ml and the cells were incubated at 0°C for 30 min. Cells were disrupted by sonic homogenizor. Centrifugation was carried out at 4°C for 20 min and the supernatant was loaded on a Ni-NTA agarose column. The His-tagged protein was eluted from column according to the manufacturer's instruction. The total protein of *E. coli* and each eluted fraction from the column were analyzed by 15% SDS–PAGE.

The purified protein was concentrated and further purified on HPLC (Shodex OHpak KB 803). The level of lipopolysaccharide (LPS) in the purified protein was detected using *Tachypleus* amebocyte lysate for endotoxin detection (Chinese horseshoe crab reagent manufacturer) as protocol manual.

Inhibition of bovine aortic endothelial cell proliferation. Bovine aortic endothelial cells were isolated as previous report (10) and maintained in DMEM supplemented with 10% heat-inactivated FCS. A monolayer of BAE cells growing in a 60-mm dish was dispersed in 0.05% trypsin solution. Cells were resuspended with DMEM containing 2% FCS. Approximately 2×10^3 cells in 100 μl were added in triplicate to each well of a 96-well tissue culture plate and incubated at 37°C (in 5% CO_2). Cells adhered to the plate within 24 h. The medium was replaced with 100 μl fresh DMEM containing 2% FCS, and samples of vastatin or endostatin were added to each well supplement with 5 ng/ml bFGF. 20 μg/ml polymixin B (Amresco) was added to each well to inhibit the activity of LPS. After 72 h incubation, 10 μl MTT (100 mg/ml) was added to each well and incubated for another 4 h at 37°C. 80 μl medium was pipetted out from each well and 100 μl DMSO was added and vortexed gently to dissolve the pellet (11). The absorbance of A_{570nm}, which correlates to the number of cells, was measured with a microplate reader (Model 450, Bio-Rad). The effect of vastatin on the proliferation of fibroblast cell line Balb/3T3 and hepatoma cell line Bel7404 was measured with the same method.

Cell cycle analysis. All the procedures were carried out as reported (12). Briefly, BAE cells were maintained in DMEM supplemented with 10% FCS until 50-60% confluence. The medium was changed with DMEM supplemented with 10% FCS containing 2 μg/ml recombinant vastatin and 20 μg/ml polymixin B. After 24 h incubation, the cells were trypsinized and washed gently with PBS, and then were fixed in 70% ice-cold ethanol for 30 min, cells were collected by centrifugation, 200 μl 1 mg/ml RNase was added and incubated at 37°C for 30 min, followed by staining with propidium iodide at 5 μg/ml. Cells were assessed by FACStar plus flow cytometer (Becton-Dickinson).

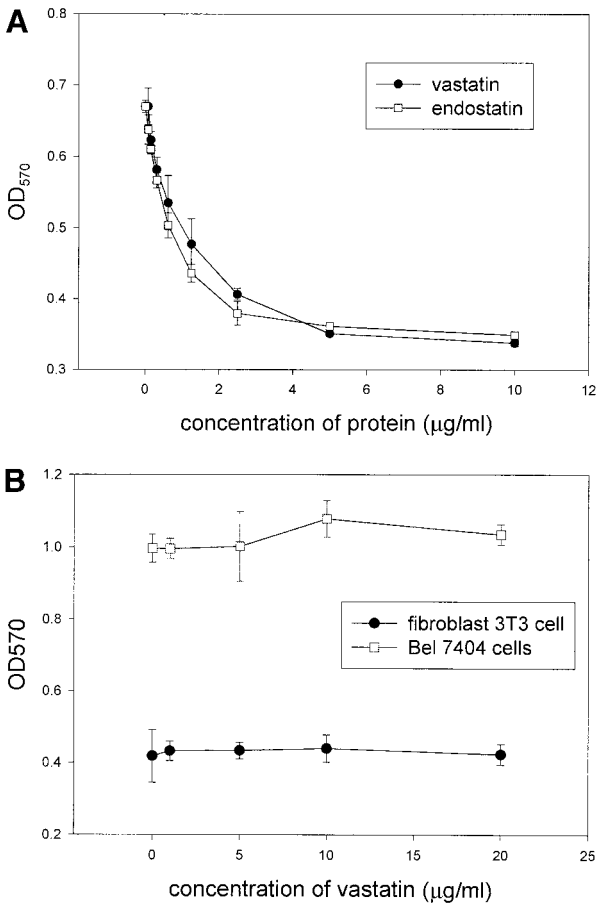

FIG. 2. (A) Vastatin and endostatin inhibit the proliferation of BAE cells stimulated by bFGF. Values represent the mean of three determinations ($n = 3$) by MTT assay. (B) Vastatin has no effect on the proliferation of fibroblast cell line Balb/3T3 cells and hepatoma cell line Bel7404. Values represent the mean of three determinations ($n = 3$) by MTT assay.

Annexin V-FITC binding assay. Annexin V-FITC (Clontech, Palo Alto, CA) binding assay was performed according to manufacturer's instruction. 2.5×10^5 cells were plated onto a 60 mm dish in DMEM containing 10% FCS. After 24 h incubation at 37°C, 5% CO_2, the medium was changed with DMEM supplement with 10% FCS, 20 μg/ml polymixin B and 5 μg/ml recombinant vastatin. After 24 h incubation, cells were trypsinized and were washed in PBS and resuspended in binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). Annexin V-FITC was added to a final concentration of 100 ng/ml, and the cells were incubated in the dark for 10 min. 10 μl of propidium iodide (PI) was added to each sample before flow cytometric analysis. For each sample, minimums of 10,000 cells were counted. Data analysis was performed with standard Cell Quest software.

RESULTS

Cloning and expression of vastatin. Vastatin cDNA fragment was obtained and amplified from the total RNA of human umbilical cord by RT-PCR. It encodes a polypeptide of 164 amino acid residues corresponding to the amino acid positions 580 to 744 of the collagen VIII (α 1). The amplified vastatin cDNA was cloned into the *E. coli* expression vector pET24a(+).

Recombinant vastatin plus six histidine was expressed in *E. coli* and purified by Ni^{2+}-nitrilotriacetic acid-agarose column. In case the *E. coli* was induced by IPTG at 37°C, most of the recombinant protein was expressed in inclusion body (data not shown). Downshift to 30°C, vastatin was expressed predominantly as soluble protein, and a band at 18 kDa was revealed by reducing SDS–PAGE (Fig. 1A). Under nonreducing condition, the purified recombinant vastatin showed two bands in PAGE, the major one was 18 kDa, and the other one was 36 kDa (Fig. 1B). According to these results, we deduced that most of the purified recombinant vastatin existed as monomer, with part of them as dimmer. The level of LPS in the purified protein was less than 160 EU/ml.

Inhibition of BAE cell proliferation. Recombinant vastatin and endostatin were assayed for their inhibitory activities on the growth of bovine aortic endothelial cells stimulated by bFGF. As shown in Fig. 2A, the proliferation of BAE cells was inhibited by endostatin or vastatin in a dose-dependent manner. The ED_{50} of vastatin was 0.6 μg/ml, while ED_{50} of endostatin was 0.5 μg/ml, close to data reported previously (2, 4). No inhibitory activities on fibroblast cell line Balb/3T3 and hepatoma cell line Bel7404 can be detected by vastatin (Fig. 2B). These results suggested that its inhibitory activity is specific to endothelial cells.

Cell cycle analysis. As shown in Fig. 3, the BAE cell cycle showed a great change after treatment with vas-

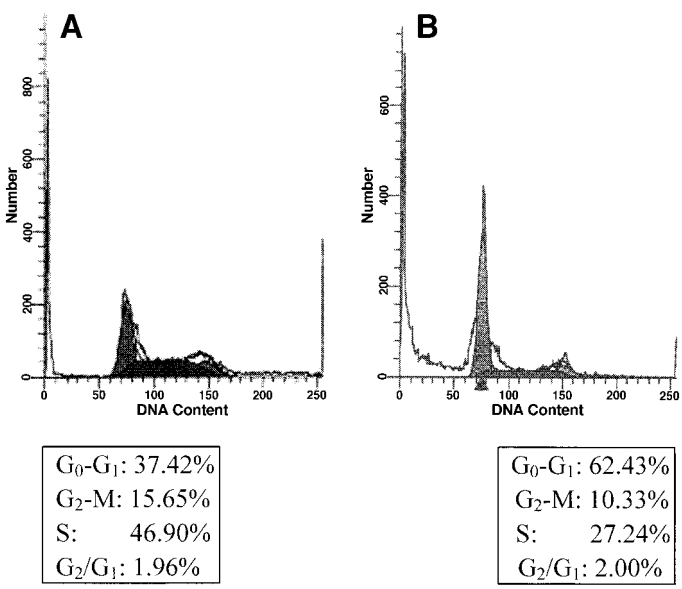

FIG. 3. Flow cytometry of BAE cells. BAE cell monolayers were exposed 24 h to vastatin in DMEM supplement with 10% FCS, and cells were assessed by FACStar plus flow cytometer (Becton–Dickinson). (A) Control without vastatin. (B) Cells were treated with vastatin (2 μg/ml).

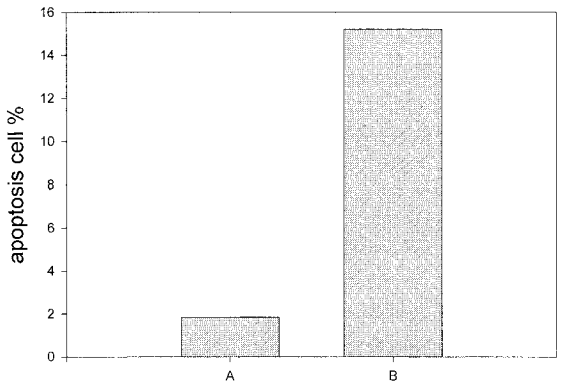

FIG. 4. Vastatin induced apoptosis in BAE cells. BAE cells were treated with vastatin for 24 h in DMEM supplement with 10% FCS, and apoptosis was assessed by Annexin V-FITC. Column A, control group; column B, cells were treated with vastatin (5 μg/ml).

tatin for 24 h. 62.43% of the cells were block in G_0/G_1 phase, compared to 37.4% of the control. The percentage of the cells in S phase was 27.24%, compared to 46.9% of the control. These results may explain partly the inhibitory effect of vastatin on the proliferation of BAE cell described above.

Cell apoptosis detection. Annexin V, a calcium-dependent phospholipid-binding protein with a high affinity to phosphatidylserine (PS), was used to detect the early stage apoptosis (13). As shown in Fig. 4, BAE cells were treated with 5 μg/ml recombinant vastatin in 10% FCS, about 15% of the cells underwent apoptosis, compared with 2% of untreated cells.

DISCUSSION

The type VIII collagen is composed of 744 amino acids. A triple-helical domain (COL) of 454 amino acids lies between a short amino-terminal non-triple-helical domain (NC2, 117 residues) and a longer carboxyl-terminal NC1 domain (173 residues) (14). It was expressed in endothelial cells, keratinocytes, mast cells and microvascular endothelials. It was reported that after vascular injury, there was an increased expression of type VIII collagen at days 2 to 14. The increased expression of the collagen VIII may contribute to vascular remodeling through the promotion of vascular smooth muscle cells (VSMC) (15).

Endostatin, restin, arresten and canstatin, C-terminal fragments of collagen XVIII, XV, and IV (α 1 and α 2) had been identified recently as inhibitors of angiogenesis. As type VIII collagen is also an essential component of basement membrane. It should be of interest to detect the possible angiogenesis inhibition activity of NC1 domain of type VIII collagen. In the present study, the NC1 domain of the type VIII collagen was cloned and expressed in *E. coli.* The soluble recombinant protein, named as vastatin, was purified with Ni-NTA column. The purified vastatin inhibited the proliferation of the BAE cell specifically with dose-dependent manner. The ED_{50} of vastatin was about 0.6 μg/ml, the same level of endostatin. Furthermore, flow cytometric measurement of BAE cells which were treated with vastatin, demonstrated a cell cycle arrest mainly in G_0/G_1 phase and a decreased S phase. Annexin V-FITC

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endostatin : -HSHRDFQPVLHLVALNSP-LSGGMRGIR-G---ADFQCFQQA---RAVGLAGT-FRAF--  :  49
arresten   : SVDH-GFLVTRHSQTIDDPQCPSGTKILYHGYSLLYVQGNERAHGQDLGTAGSCLRKFST  :  59
vastatin   : -QGE-YLPDMGLGIDGVKPPHATGAKKGKNG-----GPAYEMP---AFTAELT--APF--  :  46
               h  f    h      P    G      G      q    a   a g ag    r F

endostatin : LSSRLQDLYSIVRRADRAAVP-IVNLKDEL---LFP-SWEAL--FSGSEGPLKPGARIFS : 102
arresten   : MPFLFCNINNVCNFASRNDYSYWLSTPEPMPMSMAPITGENIRPFISRCAVCEAPAMVMA : 119
vastatin   : PPVGGPVKFNKLLYNGRQNYNPQTGIFTCE----VPGVYYFA--YHVHCKGGNVWVALFK : 100
               p       n    a R  y               P   e        c      a  f

endostatin : FDGKDVLRHPTWPQ--KSVW-------H---GSDPNGRRLT-ESYCETWRTEAPSAT--G : 147
arresten   : VHS-QTIQIPPCPSGWSSLWIGYSFVMHTSAGAEGSGQALASPGSCLEEFRSAPFIECHG : 178
vastatin   : NNE-PVMYTYDEYK--KGFL------------DQASGSAVLLLRPGDRVFLQMPSEQ--- : 142
                    v   p   p   ks w       h   g   sG a      c   f  aPs   g

endostatin : QASS-LLGGRLLGQSAASCHHAYIVLCIENSFMTASK-------------- : 183
arresten   : RGTCNYYANAYSFWLATIERSEMFKKPTPSTLKAGELRTHVSRCQVCMRRT : 229
vastatin   : ------AAGLYAGQYVHSSFSGYLLYPM---------------------- : 164
                      ag y gq a s  s y   p
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FIG. 5. The sequence alignment of endostatin, arresten, and vastatin. The same amino acids residues are marked with capital letter. Less conserved residues are marked with small letter.

binding assay showed that vastatin could induce the apoptosis of the BAE cells dramatically.

Some recent studies showed that the antiangiogenesis activity of endostatin was dependent on tyrosine kinase signaling and the heparin-binding site of endostatin. Solid-phase assay demonstrated that endostatin could bind to the fibulins and nidogen-2 (16, 17). The inhibition of endothelial cell proliferation by canstatin or arresten may be mediated by the $\alpha_1\beta_1$ integrin (9, 10). It is possible that the NC domain of different collagens in the basement membrane may bind to different receptors on the endothelial cell or in extracellular matrix. These binding may be important for endothelial cell to adhesive and migrate on basement membrane. No significant sequence homology is evident between vastatin and endostatin or canstatin as shown in Fig. 5. So their inhibition effect on the proliferation of endothelial cells may be realized via different cell surface proteins/receptors. Specific receptors binding to vastatin might be identified in the future studies.

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