Soluble Recombinant Endostatin Purified from Escherichia coli: Antiangiogenic Activity and Antitumor Effect

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Abstract

Endostatin is a potent and specific antiangiogenic protein capable of inhibiting the growth of murine and xenotransplanted human tumors. Thus far, however, recombinant endostatin prepared from Escherichia coli is insoluble after purification and therefore inappropriate for clinical settings. A soluble form of endostatin is available from a yeast system with relatively low yield and high cost, which has made it difficult to produce endostatin in quantities sufficient for extensive clinical evaluation. In this study, we developed a protocol to generate soluble recombinant murine endostatin from E. coli at a yield of 150 mg/liter-culture and 99% purity. The in vivo antiangiogenic and antitumor activities of the soluble recombinant endostatin are equally as potent as those of the previously published insoluble form. A similar protocol may be used to produce soluble human endostatin.

Introduction

Generally, tumor growth is critically dependent on blood supply (1). Folkman showed that suppression of tumor angiogenesis leads to tumor starvation and tumor regression. Therefore, the tumor vascular system has become an important target for cancer therapy. In contrast to malignant cells that are able to become resistant to conventional chemotherapy or radiotherapy, a body of data indicates that endothelial cells do not develop resistance to antiangiogenic agents (1). An increasing number of antiangiogenic agents have been discovered. Among these, endostatin was identified by O’Reilly et al. (2).

Endostatin is a Mr 20,000 protein that specifically and strongly inhibits tumor angiogenesis. It was originally isolated from the supernatant of a cultured murine hemangiendothelioma cell line and represents a COOH-terminal fragment of collagen XVIII. The endostatin gene has been cloned and expressed as a recombinant protein in Escherichia coli and yeast expression systems (2, 3). Animal studies demonstrated that recombinant endostatin strongly inhibits the growth of a variety of murine and xenotransplanted human tumors (4, 5).

Purification of bioactive recombinant endostatin from E. coli, however, has proved challenging because the endostatin is purified under denaturing condition (e.g., urea) but invariably becomes insoluble under physiological conditions. The most likely biochemical explanation for this precipitation phenomenon is that endostatin becomes misfolded during the process of renaturation. It is believed that injection of precipitated endostatin into animal results in in vivo conversion into a soluble form, thus enabling it to exert its antiangiogenic and antitumor activities (2). However, this refolding process likely depends on a variety of in vivo factors that are biologically variable, thus rendering this in vivo renaturation unpredictable. In addition, accurately assaying insoluble endostatin is problematic. For this and other reasons, the efficacy and activity of endostatin are difficult to reproduce accurately. Because of these issues, insoluble endostatin is unlikely to become clinically useful.

In recognition of the shortcomings of insoluble endostatin, a soluble form of recombinant endostatin was produced via a yeast expression system (3). However, the relatively low yield of the system has made it difficult to produce this agent in quantities sufficient for extensive clinical evaluation. In addition, its production is very expensive and represents a large step up in cost over bacterial recombinant methods. These barriers have hampered the widespread translation of endostatin research to clinical practice. There is therefore a great need to increase the yield and to reduce the cost of the production of recombinant endostatin that is suitable for clinical use. Our strategy centered on the optimization of the E. coli expression system because of its higher efficiency in expressing foreign proteins as compared with the yeast system. In this study, we demonstrate generation of soluble recombinant murine endostatin from E. coli and evaluate its antiangiogenic and antitumor activities. We have developed a purification protocol by which recombinant endostatin can be expressed with high efficiency, purified as inclusion bodies, and finally refolded into a soluble form. Our in vivo experiments showed that the SRE possesses antiangiogenic and antitumor activity comparable with the originally published insoluble form.

Materials and Methods

Plasmid, Host Bacteria, and Expression of Recombinant Murine Endostatin. Plasmid expressing murine endostatin with a histidine tag (pTB01#4) was provided by Dr. Judah Folkman (Harvard Medical School, Boston, MA; Ref. 2). The expression of endostatin is driven by IPTG-inducible T7lac promoter elements (6). The bacteria strain used for endostatin expression is the BL21(DE3)pLyS strain from Promega [Madison, WI (7, 8)].

For small-scale expression screening, pTB01#4 was introduced into competent BL21(DE3)pLyS bacterial cells as described by the manufacturer. Ten colonies were randomly picked from the obtained transformants on a Kan-LB plate (10 grams/liter tryptone, 5 grams/liter yeast extract, 10 grams/liter NaCl, and 50 mg/liter kanamycin), and each of these was inoculated into 1 ml of Luria-Bertani medium containing 50 mg/liter kanamycin. These 10 isolates were cultured in a 37°C shaker overnight at 280 rpm, and 50 μl of bacteria from each overnight culture was then transferred to a new tube containing 1 ml of the same broth, followed by continuous shaking culture at 37°C until A600nm reached 0.8. At this point, IPTG was added to the culture at a final concentration of 0.3 mM to induce the expression of endostatin. After culturing in a 37°C shaker for another 3-h period, 10 μl of bacteria sample were collected from each 1-ml culture, followed by microcentrifugation at 1000 rpm
vortexing. After a 5-min incubation at room temperature, the
buffer E [0.05 M Tris-HCl (pH 8.0), 0.01 mM oxidized glutathione, and 1 mM
previously (2). Briefly, bacteria pellet was collected with low-speed centrifu-
gation with 8 ml urea. The lysate was then applied to a
Ni$_2^+$-NTA column (Qiagen). After washing with 8 ml urea containing 10 mM
imidazole, endostatin was eluted with 8 ml urea containing 250 mM imidazole.
The dialysis product was subject to an endotoxin level determination (Lilum
Amebocyte Lysate Progent/plus; BioWhittaker, Inc., Walkersville, MD). Quan-
tification of the endostatin protein before dialysis was performed using the
Bio-Rad protein dye method as described by the manufacturer. Finally, the
endostatin product was dialyzed against 1× PBS (molecular weight cutoff,
6000–8000) at 4°C. During the dialysis, the purified protein precipitates to
form IRE.

**Purification of SRE from E. coli.** Bacteria were collected from the IPTG-
induced 1-liter scale centrifugation by centrifugation at 2500 × g for 10 min at 4°C. The
pellet was then resuspended in 100 ml of buffer A [0.1 M Tris-HCl (pH 8.0)
and 5 mM EDTA], followed by incubation at room temperature for 15 min,
with the addition of lysozyme at a final concentration of 50 µg/ml. The
suspension was then sonicated with a VibraCell VC50 sonicator (Sonic &
Materials, Inc.) in the presence of 0.1% sodium deoxycholate, followed by
centrifugation at 8000 × g for 10 min. The supernatant was discarded, and the
pellet was resuspended in 100 ml of buffer A containing 0.1% sodium
deoxycholate. The centrifugation/resuspension procedure was repeated twice.
The resultant pellet from the last centrifugation was dissolved in 30 ml of
buffer B [0.05 M Tris (pH 8.0), 1% SLS, and 1 mM DTT] and centrifuged at
8000 × g for 10 min at 4°C. The clear supernatant obtained was then
transferred to dialysis tubing with a molecular weight cutoff of 8000 and
dialyzed twice against 1500 ml of buffer C [0.05 M Tris-HCl (pH 8.0) and 0.1
mM DTT] at 4°C for 4 h. The recombinant protein was then further dialyzed
twice against 1500 ml of buffer D [0.05 M Tris-HCl (pH 8.0) and 1000 ml of
buffer E] [0.05 M Tris-HCl (pH 8.0), 0.01 mM oxidized glutathione, and 1 mM
reduced glutathione] at 4°C for 4 h/dialysis cycle, respectively. A final dialysis
(4°C, 5 h) against 0.05 M Tris-HCl (pH 8.0) was performed twice to eliminate the
glutathione redox dialysate. The dialysis product was subject to endotoxin
determination. The purified protein was finally quantitated by the Bio-Rad
protein dye method as described by the manufacturer, aliquoted, and stored at
−20°C.

**SDS-PAGE Analysis.** SDS-PAGE was performed according to a standard
procedure under reducing condition (9). Bacterial lysates from the small-scale
expression screening procedure and 10 µg of SRE obtained as the final
purification product were used as SDS-PAGE samples, respectively. Briefly,
samples suspended in 10 µl of 1× sampling buffer were loaded on a 15%
SDS-PAGE gel and run until the prestained molecular weight standard (Bio-
Rad) revealed a good resolution, followed by staining with Coomassie Blue
and destaining with methanol and acetic acid.

**Measurement of Residual Detergent (SLS) in Purified SRE.** The SLS
concentration in purified SRE was determined using a turbidimetric assay (10).
Briefly, the SRE sample was serially diluted in distilled water to a final
solution of 1 ml, and then 0.1 ml of 1 M HCl was added to each dilution and mixed by
vortexing. After a 5-min incubation at room temperature, the A$_{450}$ nm values of the
mixtures were measured. The amount of SLS present in the SRE sample was
calculated from a standard curve made by using a series SLS with known
concentrations ranging from 0.0025–0.025%. In this method, the A$_{450}$ nm value is a linear function of SLS concentration between 0.005% (0.05 mg/ml) and
0.05% (0.5 mg/ml).

**Testing of the Antiangiogenic Effect of Recombinant Endostatin.** In
vivo Matrigel assay was used to test the antiangiogenic effect of soluble
endostatin as described previously (11). Phenol red-free, growth factor-
reduced Matrigel (Collaborative Biomed and Cell Products, Bedford, MA) was
mixed with 50 ng/ml recombinant human FGF-1 prepared as described pre-
viously (12) and 1 unit/ml heparin (Elkins-Sinn, Cherry Hill, NJ). C57BL/6 mice
received s.c. injection in each flank of 0.4 ml of the prepared Matrigel.
One day after injection, mice were divided into three groups (five mice/group).
A control group was treated s.c. with PBS, and the rest of the groups were
treated s.c. with either SRE or IRE (20 mg/kg, twice a day). Endostatin
was injected at sites distant from the site of the Matrigel injection. Mice were
sacrificed after 10 days of treatment. Harvested Matrigel plugs were fixed
overnight in 10% buffered formalin at 4°C and then placed into 70% ethanol
before being processed for paraffin sections. Immunohistochemical staining
for factor VIII was carried out using Veetastain ABC according to the manu-
facturer’s instructions. Primary rabbit-derived antibody to human factor VIII
was obtained from Dako. All photomicrographs were taken under identical
conditions.

Confluent cultures of human umbilical vein endothelial cells were used to
assess the ability of endostatin to inhibit endothelial cell migration and growth.
These cells were grown with DMEM/10% FCS on 60-mm Falcon tissue
culture dishes. Once the culture became confluent, half of the cell monolayer
was sharply denuded using a sterile razor, and the demarcation line was etched
onto the tissue culture vessel. The culture was washed with sterile PBS and
incubated in DMEM/10% FCS/FGF-1 (10 ng/ml) containing either 0, 5, 10, or
30 ng/ml SRE. Cells were fed daily and fixed with 5% buffered formaldehyde
96 h after monolayer denudation. A calibrated ocular micrometer measured the
distance traveled by the endothelial monolayer from the original denudation
line. Measurements were taken from a point 0.5 cm proximal and distal from the
center of the dish. Three cultures were used to test each concentration of
endostatin.

**In Vivo Evaluation of the Antitumor Effect of Recombinant Endostatin.**
The 3LL Lewis lung carcinoma model has been used extensively in preclinical
testing of endostatin (2, 4, 5, 13). In this study, we used 3LL-C75, a cell clone
isolated from 3LL Lewis lung carcinoma irradiated with UV light (14), to
assess the antitumor effect of recombinant endostatin. In a separate set of
animal experiments performed in our laboratory, this clone exhibited higher
tolerance sensitivity to the growth-inhibitory effect of endostatin than the original 3LL
luciferase.

Tumor cells were cultured in RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, and antibiotics. 3LL-C75 cells (1 × 10$^6$) were inocu-
lated into C57BL/6 mice. When tumors reached about 0.5 cm in diameter, mice
were divided into three groups (five mice/group). Two groups of mice were
inoculated s.c. with either SRE or IRE (20 mg/kg) twice a day. The control
group received PBS injections. Tumor size was monitored by measuring the longest
dimension (length) and shortest dimension three times/week with a dial caliper,
and the tumor volume was calculated as width$^2$ length × 0.52 (15). All data
are presented as mean ± SE. The experiments were terminated when tumors
in control groups reached 2.0 cm in diameter or induced undue morbidity as
per the protocol approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

**Results**

**Expression and Purification of SRE Protein.** Our small-scale
expression screening procedure identified a bacterial colony possess-
ing the high expression efficiency (data not shown), from a cohort of
10 pTB01#4-transformed colonies. This clone was subsequently used
for large-scale (1 liter) expression and purification.

In our protocol, the yield of purified SRE is approximately 150
mg/liter culture. This yield is similar to the IRE production made in
our laboratory with the published insoluble protocol (2). The final
dialysate in the soluble protocol did not contain any visible precipi-
tants or fine particles and remained solubilized either at 4°C or after
melting from −20°C storage. The levels of endotoxin in purified SRE
and IRE were lower than 8 endotoxin unit/ml. When 10 µg of protein
were analyzed by reducing SDS-PAGE, a single band of $M_t$ 20,000
responding to endostatin was seen (Fig. 1). The identity of this
homogeneous preparation was further confirmed by an endostatin-
specific ELISA test (Cytimmune Science, College Park, MD). Almost

$^4$ Unpublished data.
all of the protein is reactive to the antiendostatin antibody provided in the kit (data not shown). Turbidimetric assessment of SLS showed that after serial dialysis, the amount of residual detergent in our final product was less than 0.005% (0.05 mg/ml), indicating that more than 99.5% of SLS used to solubilize the inclusion bodies was removed. The solubility remains stable even after the redox pair is eliminated from the system, indicating that this approach is effective in making soluble recombinant murine endostatin.

**Antiangiogenic Activities of SRE.** Matrigel-FGF from mice treated with saline shows a plethora of vascular endothelial cells, as indicated by factor VIII positivity and by the ability of these cells to form vessel-like or lumenal structures, within the Matrigel (Fig. 2, A and B). In marked contrast, there is a paucity of cells within Matrigel harvested from mice treated with insoluble (Fig. 2C) or soluble (Fig. 2D) endostatin (20 mg/kg/day, 9 days). No inflammation was evident in any of these sections. These results indicate that at this dose level, the antiangiogenic effects of SRE and IRE were equivalently potent.

The ability of the SRE to inhibit monolayer migration of human umbilical vein endothelial cells after partial denudation was tested. Our result showed that 5 and 10 ng/ml endostatin resulted in a 42% and 55% inhibition, respectively. This is significantly different from control cultures (P = 0.037 and 0.030, respectively). This effect was particularly dramatic at 30 ng/ml endostatin because none of these endothelial monolayers was able to traverse the original denudation line. Thus, no migration occurred at this concentration of endostatin. This result indicates that the *E. coli*-derived SRE has inhibitory activity on endothelial cell migration similar to that of the endostatin purified from the recombinant yeast system (4).

**In Vivo Antitumor Effect of SRE.** To assess the antitumor activity of the obtained SRE, we used the 3LL-C75 lung carcinoma model in this study. C57BL/6 mice bearing a tumor of about 0.5 cm in diameter were divided into three groups (five mice/group). Two groups of mice were treated twice with 20 mg/kg/day of either SRE or IRE. Control mice received injection of PBS in parallel. A substantial inhibitory effect was observed in mice treated with either IRE or SRE, and the degree of inhibition appeared to be similar (Fig. 3). These results suggest that at this dose, the bioactivity of the soluble form of recombinant endostatin is similar to that of insoluble endostatin. Thus, an expression and purification system for SRE protein from *E. coli* has been successfully established in a laboratory setting.

**Discussion**

Here we report a protocol for purification of murine endostatin protein from *E. coli* as a soluble form. As a result, the bioactivity and solubility of the endostatin are very well maintained, whereas a high yield (150 mg/liter culture) and a high purity (>99%) have been achieved. The *in vivo* antiangiogenic activity and antitumor effect of the purified protein are comparable to those of the IRE prepared under denaturing condition. The yield and purity of this antiangiogenic protein produced from the reported procedure allow its virtual application at different laboratory levels. The established protocol also has the potential to be adapted to a larger scale production.

The IPTG-inducible T7lac promoter used in our system has previously been shown to be highly efficient in expressing heterologous
proteins, including endostatin (2, 16). With the conventional purification protocol described previously (2), the cultured bacteria are lysed under denaturing condition (8 M urea), and the dissolved endostatin is then subject to affinity chromatography for His tag-specific purification. However, the purified protein misfolds and precipitates during the dialysis that eliminates urea from the solvent system (2). In this study, we used a different purification approach that takes the advantages of inclusion body formation in the endostatin-expressing E. coli cells. Many proteins expressed in the described system aggregate and form intrabacterial inclusion bodies. The inclusion bodies can be easily collected by simple procedures such as centrifugation so that the target protein can be highly enriched at early stages of purification. The enrichment, followed by a rinsing procedure, could virtually be optimized to achieve high purity without further purification procedures such as affinity chromatography. In addition, inclusion bodies also provide the aggregated protein molecules with protection against degradation by intrabacterial proteases, which is not an uncommon posttranslational event for proteins heterologously expressed in bacteria. In most cases, however, protein molecules aggregated in inclusion bodies are not correctly folded, resulting in the generation of insoluble and/or biologically inactive molecules. Therefore, recovery of a recombinant protein from inclusion bodies requires an effective refolding process with optimized conditions that might vary from protein to protein. In our approach, detergent SLS and reducing agent DTT were used to dissolve the inclusion bodies that are partly but highly purified by repeated rinsing with a mild detergent-containing buffer. The dissolved endostatin molecules are then subject to a refolding procedure using a redox pair and oxidized and reduced glutathiones to facilitate formation of a stable conformation that makes and keeps the endostatin soluble.

This study outlines a strategy for the isolation of a soluble form of endostatin. We have focused this strategy on exhibiting that SRE is equivalent to IRE at a typical dose level (20 mg/kg/12 h) previously shown to be active in a large number of studies (4, 13). The true potency of SRE will require more extensive dose ranging studies. However, our preliminary data show that the same dose of SRE given once daily gives a similar angiogenesis inhibition as shown here (data not shown). Thus, we anticipate that this soluble material will have potency over a large dose range.

Endostatin is a potent antiangiogenic protein and an antitumor factor. However, the insoluble nature of the published version of E. coli recombinant endostatin hampered its clinical application. Although soluble endostatin prepared from a yeast system is being used in ongoing Phase I clinical trials, the low yield (approximately 20 mg/liter culture) and high cost of the system have made it difficult to produce in quantities that are realistic for comprehensive clinical evaluation and application. Our results presented in this report offer an alternative method that will prove valuable in helping to determine the clinical activity of endostatin. Obviously, it will be of great interest and importance to adopt a similar method in preparation of soluble human endostatin. This subject is being explored in our laboratory.

Acknowledgments

We thank Drs. Judah Folkman, Micheal O’Reilly, Jie Lin, and Tomas Boehm for providing the endostatin expression plasmid and extensive discussion on this work and other relevant subjects.

References

FUTURE ANNUAL MEETINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH

2002 April 6–10, San Francisco, CA
2003 April 3–9, Toronto, Ontario, Canada
2004 March 27–31, Orlando, FL

AACR SPECIAL CONFERENCES IN CANCER RESEARCH

A number of meetings are now being organized in the AACR’s series of smaller scientific meetings. Following are the topics, dates, locations, and program committees for these meetings. When full details of each meeting are available, AACR members will be the first to receive complete brochures and application forms for participation in these important conferences. Nonmembers may receive this information by sending their names and addresses to Meetings Mailing List, American Association for Cancer Research, Public Ledger Building, 150 South Independence Mall West, Suite 826, Philadelphia, PA 19106-3483. Up-to-date program information is also available via the Internet at the AACR’s website (http://www.aacr.org/confrnc.html).

CALENDAR OF EVENTS

FASEB Summer Research Conference Growth Factor Receptor Tyrosine Kinases in Mitogenesis, Morphogenesis and Tumorigenesis, August 4–9, 2001, Snowmass Village, CO. 30 Category I CME credit hours. Contact: Julie Levin. Phone: 301.530.7094; Fax: 301.571.0650; Email: jlevin@faseb.org; Website: www.faseb.org/meetings/src.

Nanotechnology in Early Detection of Cancer Workshop, Gaithersburg, MD, August 30–31, 2001. Contact: Pothur R. Srinivas, National Cancer Institute, 6130 Executive Plaza North, Room 309, Rockville, MD 20852. Phone: 301.594.1044; Fax: 301.402.8990; E-mail: srinivap@mail.nih.gov

International Short Course: Ventilation Design, September 3–7, 2001, Utrecht, The Netherlands. Contact: Mieke Lumens, PhD, Institute for Risk Assessment Sciences, Utrecht University, P.O. Box 80175, 3508 TD Utrecht, The Netherlands. Phone: 31.317482080; Fax: 31.317485278; Email: MLumens@vet.uu.nl; Website: http://www.slm.wau.nl/ehp/.

18th International Symposium on Polycyclic Aromatic Compounds, September 13, 2001, Kingsgate Conference Center, University of Cincinnati, OH. Contact: University Conferencing, University of Cincinnati, PO Box 210031, Cincinnati, OH 45221-0031. Phone: 513.558.1810; Fax: 513.558.0385; Email: ISPAC18@uc.edu; Website: http://ispac.tor.ec.gc.ca.

1st Symposium of the International Society for Chemosensitivity Testing in Oncology, September 14–15, 2001, Homburg/Saar, Germany. Contact: Dr. U. Reinhold, Department of Dermatology, The Saarland University Hospital, D-66421 Homburg/Star, Germany. Phone: 49.6841.163832; Fax: 49.6841.163845; Email: hadneu@med-rz.uni-sb.de.

International Meetings in Sardinia: New Trends in Molecular Carcinogenesis, September 23–26, 2001, Alghero, Italy. Contact: Dr. Francesco Fox or Dr. Rosa M. Pascale, Department of Biomedical Sciences, Div. of Experimental Pathology and Oncology, Via P. Marcella 4, 07100 Sassari, Italy. Phone: 079.228307/228105; Fax: 079.228485/228305; Email: ffox@ssmain.uniss.it or patsper@ssmain.uniss.it; Website: http://www.uniss.it/dipartimenti/dsb/meeting.

8th Hong Kong International Cancer Congress, September 26–28, 2001, Hong Kong Academy of Medicine Building, 99 Wong Chuk Hang Road, Hong Kong. Contact: 8th HKICC Congress Secretariat, Department of Surgery, University of Hong Kong Medical Center, Queen Mary Hospital, Hong Kong. Phone: 852.2818.0232/852.2855.4235; Fax: 852.2818.1186; E-mail: mededcon@hku.hk.

Children’s Cancer Institute Australia for Medical Research’s Symposium: Childhood Cancer From Mechanisms to Therapeutics, a satellite symposium to 33rd meeting of SIOP, Swiss-Grand Hotel, Bondi Beach, Sydney, Australia, October 7–9, 2001. Contact: Convention Associates. Phone: 613.9887.8003; Fax: 613.9887.8773; E-mail: convention@optushome.com.au; Website: www.ccia.org.au.

International Symposium on Leukemia, Lymphoma and AIDS: Pathogenesis and Treatment of the International Association for Comparative Research on Leukemia and Related Diseases, October 7–11, 2001, Padova–Venice, Italy. Contact: Dr. Luigi Chieco-Bianchi, IACRLRD, Dept. of Oncology and Surgical Sciences, Via Gattamelata 64, I-35128 Padova, Italy. Phone: 39.049.8215802/4; Fax: 39.049.8072854; E-mail: oncology@ux1.unipd.it; Website: http://www.sistemacongressi.com/leukemia2001/default.htm.
Sixth Annual International Society of the Study of Xenobiotics Meeting, October 7–11, 2001, Munich, Germany. Contact: Nancy Holahan. Phone: 301.983.2434; Fax: 301.983.5355; email: nholahan@issx.org.

33rd Meeting of the International Society of Pediatric Oncology (SIOP), October 10–13, 2001, Brisbane Convention & Exhibition Center, Queensland, Australia. Contact: Nicole Perrin. Phone: 61.7.3858.5580, Fax: 61.7.3858.5510; E-mail: siop2001@im.com.au.

Second Annual Scientific Workshop of the National Cancer Institute’s Early Detection Research Network: Genomics, Epidemiology, Proteomics, and Bioinformatics for Early Cancer Detection and Risk Assessment, Seattle, WA, October 14–16, 2001. Contact: Lora Kutkat, National Cancer Institute, 6130 Executive Plaza North, Room 313, Rockville, MD 20852. Phone: 301.594.7635; Fax 301.402.8990; E-mail: lora.kutkat@nih.gov.

13th Biennial Congress of Asian Surgical Association, November 1–4, 2001, Shangri-la Hotel, Singapore. Contact: Congress Secretariat, 13th Biennial Congress of Asian Surgical Association, c/o Academy of Medicine, Singapore, 142 Neil Road, Singapore 088871. Phone: 65.223.8968; Fax: 65.225.5155; Email: main_ac@academyofmedicine.edu.sg; Website: http://www.academyofmedicine.edu.sg.

Pac Rim IV: An International Conference on Advanced Ceramics and Glasses, November 4–8, 2001, Outrigger Wailea Resort, Wailea, Maui, HI. Contact: The American Ceramic Society Customer Service Department. Phone: 614.794.5890; Fax: 614.899.6109; E-mail: customersrv@acers.org; Website: www.ceramics.org.

16th Annual Scientific Meeting of the Society for Biological Therapy, November 9–11, 2001, NIH Campus, Bethesda, MD. Contact: Meeting Manager, 611 Executive Plaza North, Room 313, Rockville, MD 20852. Phone: 301.954.6591; Fax: 301.402.8990; E-mail: lora.kutkat@nih.gov.

59th Electric Furnace Conference, November 11–14, 2001, Phoenix Civic Plaza Convention Center, Phoenix, AZ. Contact: The American Ceramic Society Customer Service Department. Phone: 614.794.5890; Fax: 614.899.6109; E-mail: customersrv@acers.org; Website: www.ceramics.org.

9th Asian Pacific Congress of Clinical Biochemistry, November 11–16, 2001, Ashok Hotel, New Delhi, India. Fax: 91.11.6011543; E-mail: cms@de13.vsnl.net.in.

3rd International Conference on Cancer-induced Bone Diseases, November 16–18, 2001, Awaji Yumebutai (Dream Stage), Awaji Island, Hyogo, Japan. Contact: Toshio Matsumoto, M.D., Local Organizing Committee, First Department of Internal Medicine, University of Tokushima School of Medicine, 3-18-15 Kuramoto-cho, Tokushima 770-8503, Japan. Phone: 81.88.633.7120; Fax: 81.88.633.7121; E-mail: toshimat@clin.med.tokushima-u.ac.jp; Website: http://square.umin.ac.jp/cibd/.

Third Annual Meeting on Nutrition and Cancer, November 28–30, 2001, Montevideo, Uruguay. Contact: Dr. William C. Cole, University of Colorado Health Sciences Center, 4200 East 9th Avenue, Denver, CO 80262-0278. Phone: 303.315.6190; Fax: 303.315.8993; Email: bill.cole@uchsc.edu.

18th World Congress of Digestive Surgery, December 8–11, 2001, Hong Kong Convention & Exhibition Centre, 1 Expo Drive, Wanchai, Hong Kong. Contact: Congress Secretariat, 18th World Congress of Digestive Surgery, c/o Department of Surgery, University of Hong Kong Medical Centre, Queen Mary Hospital, Hong Kong. Phone: 852.2818.0232/852.2855.4235; Fax: 852.2818.1186; Email: isdshk@hkucc.hku.hk.

Fourth International Symposium on Anti-Angiogenic Agents: Recent Advances and Future Directions in Cell Biology and Clinical Research, January 10–13, 2002, Adam’s Mark Hotel, Dallas, TX. Contact: Kim Pearson, The CBCE, 8445 Freeport Parkway, Suite 680, Irving, TX 75063. Phone: 972.929.1900 (x104); Fax: 972.929.1901; E-mail: symposia@theCBCE.com.

13th Annual Colorectal Disease Symposium: An International Exchange of Medical and Surgical Concepts, February 14–16, 2002, Marriott’s Harbor Beach Resort and Spa, Fort Lauderdale, FL. 25.5 Category I CME credit hours. Contact: Cleveland Clinic Florida, Department of Continuing Education, 2950 Cleveland Clinic Boulevard, Weston, FL 33331. Phone: 954.659.5490; Fax: 954.659.5491; E-mail: cme@ccf.org; Website: www.cmeccf.com.

**Corrections**

We, the undersigned, submit the following correction to our paper by X. Huang et al., entitled “Soluble Recombinant Endostatin Purified from Escherichia coli: Antiangiogenic Activity and Antitumor Effect,” which appeared in the January 15, 2001 issue of Cancer Research (pp. 478–481).


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In the article by S.-H. Song et al., entitled, “Transcriptional Silencing of Cyclooxygenase-2 by Hyper-methylation of the 5’ CpG Island in Human Gastric Carcinoma Cells,” which appeared in the June, 1, 2001 issue of Cancer Research (pp. 4628–4635), the first author’s name appeared incorrectly as Sang-Hyun Song. The author’s correct name is Sang-Hyun Song.
Soluble Recombinant Endostatin Purified from *Escherichia coli*: Antiangiogenic Activity and Antitumor Effect

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