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 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)pLysS strain from Promega [Madison, WI (7, 8)].

For small-scale expression screening, pTB01#4 was introduced into competent BL21(DE3)pLysS 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 inoculates were cultured in a 37° C shaker overnight at 280 rpm, and 50 ml of bacteria from each overnight culture were then transferred to a new tube containing 1 ml of the same broth, followed by continuous shaking culture at 37° C until A600 nm 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

The abbreviations used are: SRE, soluble recombinant endostatin; IPTG, isopropyl-1-thio-β-D-galactopyranoside; IRE, insoluble recombinant endostatin; FGF, fibroblast growth factor; SLS, sodium dodecyl sulfate.

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for 5 min at 4°C. The bacterial pellets were resuspended with 10 μl of 1× sampling buffer [50 m[HCl-Tris (pH 6.8), 100 mM DTT, 2% SDS, 0.1% bromphenol blue, and 10% glycerol] and analyzed by SDS-PAGE.

The clone with the highest expression efficiency was identified from the 1-ml expression screening experiment by SDS-PAGE and subsequently applied to a 1-liter scale expression. The culture and induction condition for 1-liter scale expression were the same as that used for the 1-ml culture modified by simply scaling up each component by 1000 times.

**Purification of Recombinant Endostatin from E. coli in an Insoluble Form.** To evaluate the biological activity of the soluble endostatin produced in this study, we used IRE as a reference for comparative studies. A purification procedure for recombinant endostatin from E. coli has been described previously (2). Briefly, bacteria pellet was collected with low-speed centrifugation, followed by lysis with 8 M urea. The lysate was then applied to a Ni2+-NTA column (Qiagen). After washing with 8 M urea containing 10 mM imidazole, endostatin was eluted with 8 M urea containing 250 mM imidazole.

The purification product were used as SDS-PAGE samples, respectively. Briefly, the SRE sample was serially diluted in distilled water to a final volume concentration in purified SRE was determined using a turbidimetric assay (10). 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 1X 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. 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 1X 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 volume 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 A280 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 A280 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 vitro 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 previously (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 Vectastain ABC according to the manufacturer’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 test 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 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. All other protocols were identical to the described procedure.

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, 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 series of animal experiments performed in our laboratory, this clone exhibited higher sensitivity to the growth-inhibitory effect of endostatin than the original 3LL tumor. Tumor cells were cultured in RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, and antibiotics. 3LL-C75 cells (1 × 106) were inoculated 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 treated 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 width2 × 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 possessing 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 dialyse in the soluble protocol did not contain any visible precipitates or fine particles and remained solubilized either at 4°C or after melting from −20°C storage. The levels of endostatin in purified SRE and IRE were lower than 8 endostatin unit/ml. When 10 μg of protein were analyzed by reducing SDS-PAGE, a single band of Mr 20,000 corresponding to endostatin was seen (Fig. 1). The identity of this homogeneous preparation was further confirmed by an endostatin-specific ELISA test (Cyntimmune Science, College Park, MD). Almost

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1 Unpublished data.
ANTITUMOR EFFECT OF SOLUBLE ENDOSTATIN FROM E. coli

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 inclusion bodies are partly but highly purified by repeated rinsing with a mild detergent SLS and reducing agent DTT were used to dissolve the inclusion bodies that vary from protein to protein. In our approach, detergent SLS and reducing agent DTT were used to dissolve the inclusion bodies that are not correctly folded, resulting in the production 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 inclusion body 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 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

Announcements

(Requests for announcements must be received at least three months before publication.)

FUTURE ANNUAL MEETINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH

2002 April 6–10, San Francisco, CA
2003 April 3–5, 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).

MOLECULAR MECHANISMS OF GASTROINTESTINAL CANCER DEVELOPMENT AND ITS CLINICAL IMPLICATIONS
(Joint Conference with the Korean Cancer Association)
September 10–14, 2001
Hotel Lotte, Seoul, Korea

Chairpersons
Jae-Gabh Park, Seoul, Korea
Sanford Markowitz, Cleveland, OH

CANCER AND CHROMOSOMAL ORGANIZATION
(Simultaneous conferences with separate and joint sessions)
October 17–21, 2001
Marriott Desert Springs Resort, Palm Desert, CA

Chairperson
Geoffrey M. Wahl, La Jolla, CA

EPIGENETICS OF CANCER
Chairperson
Peter A. Jones, Los Angeles, CA

MOLECULAR TARGETS AND CANCER THERAPEUTICS: DISCOVERY, BIOLOGY, AND CLINICAL APPLICATIONS
(Joint International Conference with NCI and EORTC)
October 29–November 2, 2001
Fontainebleau Hotel, Miami Beach, FL

Chairpersons
Carlos L. Arteaga, Nashville, TN
Edward A. Sausville, Rockville, MD
Jaap Verweij, Rotterdam, The Netherlands

NEW DISCOVERIES IN PROSTATE CANCER BIOLOGY AND TREATMENT
December 5–9, 2001
The Registry Resort, Naples, FL

Chairpersons
Kenneth J. Pienta, Ann Arbor, MI
Robert H. Getzenberg, Pittsburgh, PA
Donald S. Coffey, Baltimore, MD

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.

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/ehb/.

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. Marzella 4, 07100 Sassari, Italy, Phone: 070.228307/228105; Fax: 070.228485228305; Email: feo@msmain.uniss.it or patsper@msmain.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: 301.983.5357; 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, Shangrila 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, 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.

16th Annual Scientific Meeting of the Society for Biological Therapy, November 9–11, 2001, NIH Campus, Bethesda, MD. Contact: Meeting Manager, 611 East Wells Street, Milwaukee, WI 53202. Phone: 414.271.2456; Fax: 414.276.2146; Website: www.soobiother.com.

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.

ANNOUNCEMENTS

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).


Xiaojun Huang
Michael K. K. Wong
Qun Zhao
Zhenyu Zhu
Kent Z. Q. Wang
Nancy Huang
Caisheng Ye
Elieser Gorelik
Mengfeng Li

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.

Sang-Hyun Song

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 Yumenbutsu (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; Email: symposia@theCBCE.com.

13th Annual Colorectal Disease Symposium: An International Exchange of Medical and Surgical Concepts, February 14–16, 2002, Marriot’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 33333. Phone: 954.659.5490; Fax: 954.659.5491; E-mail: cme@ccf.org; Website: www.cmeccf.com.
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