[CANCER RESEARCH 61, 7375–7378, October 15, 2001]

Advances in Brief

A Polymorphism in Endostatin, an Angiogenesis Inhibitor, Predisposes for the Development of Prostatic Adenocarcinoma[1]

Paula Iughetti, Oscar Suzuki, Paulo H. C. Godoi, Venâncio Avancini Ferreira Alves, Andrea L. Sertié, Todd Zorick, Fernando Soares, Anamaria Camargo, Eloísa S. Moreira, Celso di Loreto, Carlos Alberto Moreira-Filho, Andrew Simpson, Glaucius Oliva, and Maria Rita Passos-Bueno[2]

Centro de Estudos do Genoma Humano, Departamento de Biologia, Instituto de Biociências, USP, São Paulo 05508-900 [P. I., O. S., A. L. S., T. Z., M. R. P-B.]; Instituto de Química de São Carlos, São Carlos 13560-970 [P. H. C. G.]; Departamento de Patologia, Faculdade de Medicina, USP 01246-903 [V. A. F. A., C. d. L.]; Instituto Ludwig, São Paulo 01509-010 [F. S., A. C., E. S. M.]; Departamento de Imunologia, Instituto de Ciências Biomédicas, USP [C. A. M-F.]; and Instituto de Física de São Carlos, USP, São Carlos 13560-970 [G. O.], Brazil

Abstract

We have performed association studies between a novel coding single nucleotide polymorphism (D104N) in endostatin, one of the most potent inhibitors of angiogenesis, and prostate cancer. We observed that heterozygous N104 individuals have a 2.5 times increased chance of developing prostate cancer as compared with homozygous D104 subjects (odds ratio, 2.4; 95% confidence interval, 1.4–4.16). Modeling of the endostatin mutant showed that the N104 protein is stable. These results together with the observation that residue 104 is evolutionary conserved lead us to propose that: (*a*) the DNA segment containing this residue might contain a novel interaction site to a yet unknown receptor; and (*b*) the presence of N104 impairs the function of endostatin.

Introduction

Prostate cancer is the most common male malignancy and the second leading cause of cancer-related deaths in American men (1). It has been estimated that although 42% of males have prostate carcinoma identified by postmortem examination, only 9.5% will have a clinical diagnosis in their lifetime, and only 2.9% actually will die of the disease (2). Hence, the development of prognostic tests is essential to identify those patients who would benefit from more vigilant surveillance. Although the majority of prostate cancer cases are sporadic, it has long been recognized that familial clustering exists, with an increased relative risk of affected families (3). Segregation analysis of prostate cancer suggests the presence of at least one major susceptibility locus that may account for up to 10% of all cases (3). Indeed, at least six putative prostate cancer susceptibility loci (five on autosomal chromosomes and one on the X chromosome) have already been mapped through linkage analysis (4, 5). In addition to these putative major susceptibility genes, it is believed that alterations of other genes could be associated with the risk and/or progression of this type of tumor, including both sporadic and familial cases (6).

Angiogenesis, or the formation of new blood vessels from preexisting endothelium, is a fundamental step in tumor progression and metastasis (7, 8). A wide range of both stimulatory and inhibitory molecules mediates the sequential steps involved in angiogenesis. Endostatin, a M_r 20,000 cleavage product of the COOH-terminal domain of collagen XVIII (NC1), has been added recently to this list. Endostatin induces inhibition of endothelial cell proliferation and migration as well as apoptosis through mechanisms still not completely understood (8–11). Higher serum levels of endostatin induced experimentally in mice and rats seem to cause regression of various types of solid tumors, including prostate cancer (12–14). In addition, Down's syndrome patients, who have higher serum levels of endostatin because of three copies of the *COL18A1* gene,[3] have a decreased incidence of solid tumors, including prostate cancer (15). Thus, lower levels or an impaired function of endostatin might be associated with a higher risk of developing malignant solid tumors or with a worsened prognosis of the disease. Recently, as a result of a systematic analysis of the *COL18A1* gene, we identified 20 polymorphic variants, but only one missense mutation (D104N) located in the COOH-terminal globular domain NC1 of collagen XVIII, the encoding region for endostatin (Table 1).[4] We hypothesized that the presence of an asparagine instead of aspartic acid at this position may lead to an impaired function of endostatin and could therefore be associated with the progression or aggressiveness of solid tumors. Our analysis of this cSNP[5] in 181 prostate cancer patients and 198 control individuals suggests that this change is predisposing to the development of malignant tumors of the prostate.

Materials and Methods

Patients and Controls. A total of 181 prostate cancer cases (61 Caucasians, 11 blacks, and 109 unclassified) of mean age 65.8 years (SD, 8.1 years) comprises the case group, 50 from the Hospital do Câncer and 131 from the Hospital Oswaldo Cruz, both situated within the city of São Paulo, Brazil. All cases had fully documented histopathological diagnosis and were graded according to Gleason's system. A total of 198 non-cancer individuals (106 Caucasians, 83 blacks, and 9 unclassified) of mean age 52.7 years (SD, 14.1 years), who were relatives of patients with neuromuscular disorders and with negative history for prostate cancer, were considered as controls.

DNA was obtained from blood (in 66 cases and all control individuals) or from paraffin-embedded tissues (115 cases). Standard protocols for DNA extraction from each of these tissues were used.

Genotype Analysis. The missense change D104N (amino acid position in endostatin, which corresponds to amino acid position 1437 and nucleotide 4349G→A of the cDNA medium form of collagen XVIII) leads to the creation of a restriction site for *Mse*I. This change was analyzed through the amplification of a 169-bp fragment using the primers 5′-cacggtttctcttccaggac-3′ and 5′-ctctcagagctgctcacacg-3′, followed by restriction endonuclease digestion

Received 4/26/01; accepted 8/27/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by grants from the Fundação de Amparo a Pesquisa do Estado de São Paulo, Conselho Nacional de Pesquisa e Desenvolvimento, and the Programa de Núcleos de Excelência. M. R. P-B. and G. O. are supported in part by an International Research Scholars grant from the Howard Hughes Medical Institute.

[2] To whom requests for reprints should addressed, at Departamento de Biologia, Instituto de Biociências, USP, Rua do Matão 277, CEP: 05508-900, Sao Paulo, SP, Brazil. Phone: 55-11-38187563; Fax: 55-11-38187419; E-mail: passos@ib.usp.br.

[3] T. S. Zorick, Z. Mustacchi, S. Y. Bando, M. Zatz, C. A. Moreira-Filho, B. Olsen, and M. R. Passos-Bueno. High serum endostatin levels in patients with Down's syndrome: implications for improved treatment and prevention of solid tumors, Eur. J. Hum. Genet., in press.

[4] O. Suzuki, A. L. Sertié, J. Murray, F. Kok, M. Monteiro, M., and M. R. Passos-Bueno. Identification of novel pathogenic and polymorphic mutations in the *COL18A1* gene, manuscript in preparation.

[5] The abbreviations used are: cSNP, coding single nucleotide polymorphism; OR, odds ratio; CI, confidence interval.

Table 1 *COL18A1 variants and their frequency*[a]

Localization	Nucleotide change	Type of mutation	Frequency
Exon 03	c679A→G	Silent	30%[b]
Exon 07	c1408G→A	Silent	16%
Exon 07	c1417C→G	Silent	33%
Exon 07	c1420C→T	Silent	8%
Exon 07	c1426G→T	Silent	33%
Exon 10	c1804T→C	Silent	10%[b]
Exon 11	c1855C→T	Silent	50%
Exon 11	c1870C→T	Silent	50%
Exon 11	c1885C→A	Silent	50%
Exon 13	c2008C→T	Silent	8%
Exon 19	c2500T→C	Silent	15%[b]
Exon 21	c2561A→G	I841V	16%
Exon 35	c3376C→T	Silent	16%
Exon 42	c4349G→A	D1437N	12%[c]
Intron 06	g70362G→A	intronic	16%
Intron 06	g70377–70378 del AT	intronic	16%
Intron 25	g87517–87518 ins CCACTGCCCTCCCG	intronic	12%
Intron 31	g91374 del C	intronic	10%
Intron 38	g102233–102275 del 42bp	intronic	30%
Intron 41	g105865–105866 ins AC	intronic	62%

[a,b] Variants identified through the sequencing of the 43 exons (and its flanking intronic regions) of the *COL18A1* in 8 patients with Knobloch syndrome (16–19). Allele frequency estimates are based on the initial screening or [b] the analysis of 50 control chromosomes.

[c] Polymorphism described in the present report.

Table 2 *Endostatin genotype distribution among control and patient groups*

Genotypes[a]	Subjects with racial classification		Total sample *n* (%)
	Caucasians (*n*/%)	Blacks (*n*/%)	
Controls			
DD	89 (84%)	76 (92.6%)	174 (87.9%)
DN	17 (16%)	7 (8.4%)	24 (12.1%)
Total	106 (100%)	83 (100%)	198 (100%)
Patients			
DD	43 (70.5%)	5 (45%)	135 (74.5%)
DN	18 (29.5%)	6 (55%)	45 (24.9%)
NN	0	0	1 (0.6%)
Total	61 (100%)	11 (100%)	181 (100%)

[a] $D^{\text{aspartic acid}}/N^{\text{asparagine}}$.

with *Mse*I. The PCR reaction consisted of 32 cycles of amplification and used the primers described above at concentrations of 4 μM, 100 ng of genomic DNA, PfxTaq DNA polymerase (Life Technologies, Inc.; 0.2 unit), and 250 μM of each deoxynucleotide triphosphate, at 94°C for 40 s, 57°C for 40 s, and 72°C for 1 min. The restricted digested PCR products were separated on 8% polyacrylamide gels stained with silver nitrate by standard procedures. In the presence of the mutation, two fragments of 101 and 68 bp were generated. All samples were done in duplicate to ensure genotyping accuracy. Analysis for the presence of the D104N mutation was also performed in DNA samples extracted from two different regions (a normal and a tumor one) of the paraffin block of 20 patients chosen randomly.

ELISA. ELISA analysis was performed in serum from 26 control individuals (13 heterozygous for the polymorphism D104N and 13 homozygous for the most common allele). Blood samples were collected in EDTA-containing tubes, and serum was separated and stored at −70°C for future use. ELISA for serum endostatin was performed using a commercially available assay (Accucyte; Cytimmune Sciences, Inc., College Park, MD), according to the manufacturer's instructions for usage. All measurements were performed in duplicate to ensure the accuracy of the data collected. The kit used has a sensitivity of 2 ng/ml, and typical interassay and intraassay variances were 10% or less.

Molecular Modeling and Mutant Structure Analysis. The human endostatin structure was obtained from the Protein Data Bank accession code 1BNL and analyzed using the programs GRASP (16), Swiss PDB Viewer (17), O (18), and Insight-II/Discover (19). The observed mutation was created by Insight-II and optimized by molecular energy minimization fixing all atoms except the side chains of D104N and its neighbors S102, K106, and T113.

The structures of mouse endostatin derived from collagen XVIII (PDB accession code 1KOE) and mouse endostatin derived from collagen XV (PDB accession code 1DY2) were also used for comparison.

Statistical Analysis. Gene and genotype frequencies in affected and normal individuals were compared by contingency table analysis using both χ^2 statistics and Fisher's exact test. The level of significance considered was 5% and 1 degree of freedom for χ^2 analysis. Hardy-Weinberg equilibrium was tested with the χ^2 statistic for the goodness-of-fit (1 degree of freedom). The OR and its CI were estimated by standard methods (20).

Results and Discussion

Association between an Endostatin Polymorphism (D104N) and Prostate Cancer. The distribution of the genotypes for the polymorphism D104N is reported for controls and prostate cancer patients in Table 2. We did not observe any significant difference in the frequency of this polymorphism between Caucasians and blacks in controls ($P = 0.13$) or in patients ($P = 0.16$), and this variable was not considered in the majority of statistical analysis. In addition, we found very similar genotypic frequencies for this polymorphic system in the patients ascertained in the two hospitals (33% in patients from Hospital do Câncer and 22% in patients from Hospital Oswaldo Cruz). All patients were therefore considered as a unique group for analysis.

Both the patients' and controls' samples are in Hardy-Weinberg equilibrium ($\chi^2 = 1.82$, $P = 0.41$; $\chi^2 = 0.82$; $P = 0.66$, respectively), but the frequency of heterozygotes for the D104N polymorphism was significantly higher in patients as compared with the control group ($\chi^2 = 10.23$; $P = 0.0014$; Table 2).

These findings support for the first time a potential association between a polymorphism in endostatin, one of the most potent angiogenesis inhibitors, and prostate cancer. Our results predict that individuals heterozygous for N104 have a 2.5 times greater chance of developing prostate cancer (OR, 2.4; 95% CI, 1.4–4.2). Association studies have been biased by potential stratification. In our sample, we

Table 3 *Endostatin genotype distribution according to Gleason score and age in prostate cancer*

Genotypes[a]	Gleason score <7 (%)	Gleason score ≥7 (%)	Age <65 yr (%)	Age ≥65 yr (%)
DD	51 (75%)	68 (82%)	47 (70.1%)	79 (73.1%)
DN	17 (25%)	15 (18%)	20 (29.9%)	28 (26%)
NN	0	0	0	1 (0.9%)
Total	68 (100%)	83 (100%)	67 (100%)	108 (100%)

[a] $D^{\text{aspartic acid}}/N^{\text{asparagine}}$.

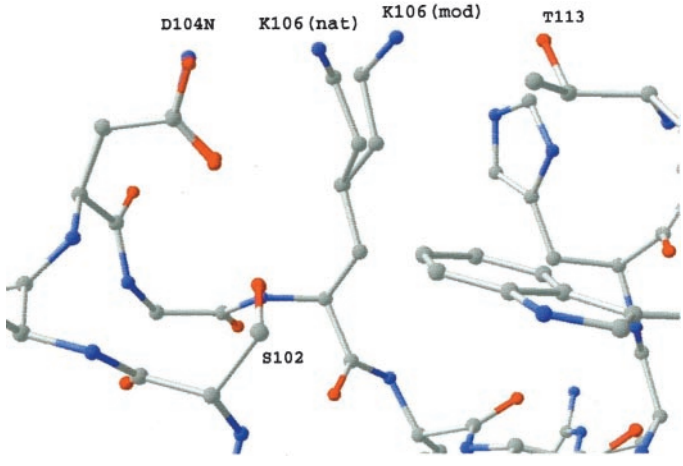

Fig. 1. Sobreposition between the modeled structures of human endostatin XVIII and the mutant D104N. The residues labeled in this figure were the only not fixed during the energy minimization protocol. The residue *K106(mod)* corresponds to the final conformation in the structure modeled (differs from the deposited structure), whereas *K106(mut)* corresponds to the final conformation calculated for the mutated D104N structure.

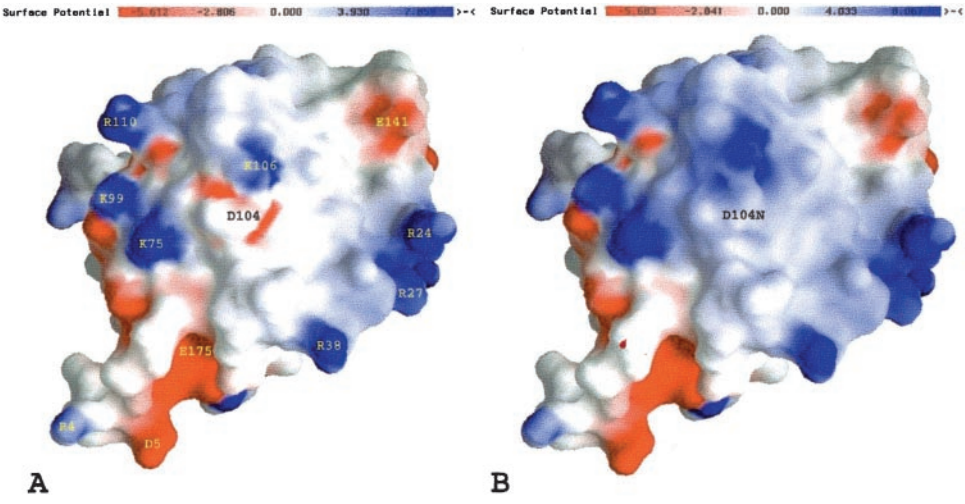

Fig. 2. Electrostatic surface potential of human endostatin XVIII (*A*) and the mutant D104N (*B*). *Red* and *blue* coloring represent negative and positive potentials, respectively. The orientation is about the same for both. Figure made with GRASP.

were able to obtain ethnic origin of just a small number of patients. However, we observed that the Caucasian cases have a significantly higher carrier frequency than the Caucasian controls ($P = 0.0488$; OR, 2.19; 95% CI, 1.0–4.6). The same was observed for the black population ($P = 0.0007$; OR, 13.03; 95% CI, 3.3–50.8). Therefore, even if the OR derived from the combined analysis has been slightly biased by potential stratification, the result is significant in both identifiable subsets themselves, confirming the results obtained with combined data. We did not observe any association between the presence of this mutation and Gleason score or age at diagnosis (Table 3), suggesting that this alteration is more related to the genesis of the tumor than to its aggressiveness. Recently, Musso *et al.* (21) reported that endogenous collagen XVIII levels in recurrent hepatocellular carcinomas were 2.2-fold lower than in those hepatocellular carcinomas that did not recur, a finding which is in agreement with our hypothesis that individuals with higher levels of endostatin might be less prone to develop solid tumors.

D104N Polymorphism Possible Leads to an Impaired Endostatin Function. We have aligned the sequences of both human and mouse endostatin XVIII as well as the endostatin-like molecule that is produced from the NC1 domain of collagen XV, which presents 61% sequence identity with that of collagen XVIII (22). This analysis showed that the aspartic acid at position 104 is conserved in all cases. In all of the three crystal structures, this residue is located at the surface of the molecule, partially buried through a hydrogen bond between OD1 (oxygen delta 1) and an internal serine residue (S102 in the human endostatin XVIII). The atom OD2 (oxygen delta 2) is surrounded by positively charged residues, and in the high-resolution mouse structures, it is interacting through a strong salt bridge to an arginine residue. In the human endostatin, however, this arginine corresponds to K106, the NZ (nitrogen zeta) of which is in close proximity to the OD2 of D104.

The modeling of the D104N mutant could encompass two possible arrangements for the asparagine side chain. In one configuration, the $-NH_2$ group would occupy the buried OD1 position, necessarily interacting with S102, which is unlikely to happen because the residue S102 is acting as a proton donor to D104 and a proton acceptor for the main chain -NH group of K106. Therefore, the second configuration is favored, with the $-NH_2$ group of the mutant N104 occupying the OD2 position of D104. Starting from a modeled mutant structure based on the second configuration, the molecular energy minimization shows little rearrangement of the surrounding residues to accommodate the new $-NH_2$ group of N104. On the other hand, the same procedure applied to the human structure resulted in a different conformation for K106, in terms of the distance to a salt-bridge to D104 (Fig. 1). Fig. 2 represents the electrostatic potential at the surface of both wild-type and the mutated structures, showing the altered charge distribution surrounding the D104N mutation.

The structure modeling analysis thus suggests that the human mutated D104N protein is stable, a result further supported by the finding that endostatin XVIII serum levels were similar both in carriers and non-carriers of this mutation (17.38 ± 5.55 ng/ml; 17.17 ± 5.99 ng/ml, respectively).

Recent studies have shown that inhibition of endothelial cell proliferation and migration of cultured endothelial cells might occur through the binding of an endostatin epitope of two clusters of arginine to heparin/heparan sulfate when angiogenesis is induced by FGF-2 (23). This heparin binding epitope does not include D104; however, it is possible that this amino acid and its surrounding residues are presented for the interaction with a yet undetermined target receptor. The existence of another interaction site in endostatin is also supported by the observation that inhibition of vascular endothelial growth factor-induced migration of endothelial cells is not dependent on its heparin-binding epitope. Thus, we hypothesize that the D104N mutation might decrease the ability of endostatin to bind other molecules, thereby impairing its ability to inhibit angiogenesis. The amino acid D104 is conserved in both humans and mice, and it is also conserved in endostatins from both collagen XV and XVIII, implying that this residue is in a functionally important region of endostatin, with a common role for both angiogenesis inhibitors. It will be important to perform functional analyses to test the above hypothesis. The confirmation of the association of N104 with prostate cancer in other populations will also be important to support the possible screening for this mutation as a predictive test for prostate cancer.

Acknowledgments

We thank Dr. Paulo A. Otto for valuable suggestions in statistical analysis, Dr. Zatz for helpful discussions, C. Urbani for secretarial assistance, and Elisângela Quedas, Silvia Bando, and Patrícia Mendonça for technical assistance.

References

1. Stanford, J. L., Just, J. J., Gibbs, M., Wicklund, K. G., Neal, C. L., Blumenstein, B. A., and Ostrander, E. A. Polymorphic repeats in the androgen receptor gene: molecular markers of prostate cancer risk. Cancer Res., *5:* 1194–1198, 1997.
2. Siegal, J. A., Yu, E., and Brawer, M. K. Topography of neovascularity in human prostate carcinoma. Cancer (Phila.), *7:* 2545–2551, 1995.

3. Carter, B. S., Beaty, T. H., Steinberg, G. D., Childs, B., and Walsh, P. C. Mendelian inheritance of familial prostate cancer. Proc. Natl. Acad. Sci. USA, *8:* 3367–3371, 1992.
4. Visakorpi, T. Molecular genetics of prostate cancer. Ann. Cirurg. Gynaecol., *88:* 11–16, 1999.
5. Tavitigian, S. V., Simard, J., Teng, D. H. F., Abtin, V., Baungard, M., Beck, A., Campi, N. J., Carillo, A. R., Chen, Y., Dayananth, P., Desrochers, M., Dumont, M., Farnham, J. M., Frank, D., Frye, C., Ghaffari, S., Gupte, J. S., Hu, R., Iliev, D., Janecki, T., Kort, E. N., Laity, K. E., Leavitt, A., Leblanc, G., McArthur-Morrison, J., Pederson, A., Reid, J. E., Richards, S., Schroeder, M., Smith, R., Snyder, S. C., Swedlund, B., Swensen, J., Thomas, A., Tranchant, M., Woodland, A-M., Labrie, F., Skolnick, M. H., Neuhausen, S., Rommens, J., and Cannon-Albright, L. A. A candidate prostate cancer susceptibility gene at chromosome 17p. Nat. Genet., *27:* 172–180, 2001.
6. Folkman, J. Tumor angiogenesis: therapeutic implications. N. Engl. J. Med., *285:* 1182–1186, 1971.
7. Hanahan, D., and Folkman, J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell, *8:* 353–364, 1996.
8. Sassaki, T., Fukai, N., Mann, K., Göhring, W., Olsen, B. R., and Timpl, R. Structure, function and tissue forms of the C-terminal globular domain of collagen XVIII containing the angiogenesis inhibitor endostatin. EMBO J., *17:* 4249–4256, 1998.
9. O' Reilly M. S., Boehm, T., Shing, Y., Fukai, N., Vasios, G., Lane, W. S., Flynn, E., Birkhead, J. R., Olsen, B. R., and Folkman, J. Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. Cell, *8:* 277–285, 1997.
10. Dhanabal, M., Ramachandran R., Waterman, M. J. F., Lu, H., Knebelmann, B., Segal, M., and Sukhatme, V. P. Endostatin induces endothelial cell apoptosis. J. Biol. Chem., *274:* 11721–11726, 1999.
11. Dixelius, J., Larsson, H., Sasaki, T., Holmqvist, K., Lu, L., Engstrom, A., Timpl, R., Welsh, M., and Claesson-Welsh, L. Endostatin-induced tyrosine kinase signaling through the Shb adaptor protein regulates endothelial cell apoptosis. Blood, *95:* 3403–3411, 2000.
12. Yoon S. S., Eto, H., Lin, C-m., Nakamura, H., Pawlik, T. M., Song, S. U., and Tanabe, K. K. Mouse endostatin inhibits the formation of lung and liver metastases. Cancer Res., *59:* 6251–6256, 1999.
13. Perletti, G., Concari, P., Giardini, R., Marras, E., Piccinini, F., Folkman, J., and Chen, L. Antitumor activity of endostatin against carcinogen-induced rat primary mammary tumors. Cancer Res., *60:* 1793–1796, 2000.
14. Yokoyama, Y., Green, J. E., Sukhatme, V. P., and Ramakrishnan, S. Effect of endostatin on spontaneous tumorigenesis of mammary adenocarcinomas in a transgenic mouse model. Cancer Res., *60:* 4362–4365, 2000.
15. Hasle, H., Clemmensen, I. H., and Mikkelsen, M. Risk of leukemia and solid tumors in individuals with Down's syndrome. Lancet, *355:* 165–169, 2000.
16. Nicholls, A., Sharp, K. A., and Honing, B. J. Proteins. GRASP program. *11:* 218–296, 1991.
17. Guex, N., and Peitsch, M. C. Swiss PDB Viewer. Electrophoresis, *18:* 2714–2723, 1997.
18. Jones, T. A., Zou, J-Y., Cowan, S. W., and Kjeldgaard, M. The "O" program. Acta Crystallogr., *A47:* 110–119, 1991.
19. Insight II/Discover program. Insight II Modeling Environment, release 98.0. San Diego: Molecular Simulations, Inc., 1998.
20. Fleiss, J. L. Statistical Methods for Rates and Proportions. New York: John Wiley & Sons, Inc., 1973.
21. Musso, O., Rehn, M., Theret, N., Turlin, B., Bioulac-Sage, P., Lotrian, D., Campion, J. P., Pihlajaniemi, T., and Clement, B. Tumor progression is associated with a significant decrease in the expression of the endostatin precursor collagen XVIII in human hepatocellular carcinomas. Cancer Res., *61:* 45–49, 2001.
22. Sasaki, T., Larsson, H., Tisi, D., Claesson-Welsh, L., Hohenester, E., and Timpl, R. Endostatins derived from collagens XV and XVIII differ in structural and binding properties, tissue distribution and anti-angiogenic activity. J. Mol. Biol., *301:* 1179–1190, 2000.
23. Hohenester, E., Sasaki, T., Olsen, B. R., and Timpl, R. Crystal structure of the angiogenesis inhibitor endostatin at 1.5 Å resolution. EMBO J., *17:* 1656–1664, 1998.