Tumor Progression Is Associated with a Significant Decrease in the Expression of the Endostatin Precursor Collagen XVIII in Human Hepatocellular Carcinomas

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Abstract

Endostatin inhibits angiogenesis and tumor growth in mice. The role of its endogenous precursor collagen XVIII in human cancer is unknown. In normal tissues, two variants of collagen XVIII, namely, the short and long forms regulate tissue specificity, the long form being almost exclusively expressed by hepatocytes in the liver. We analyzed RNA arrays from 57 hepatocellular carcinomas (HCCs) with common and variant-specific probes and investigated the relationships between collagen XVIII expression and angiogenesis by measuring the CD34-positive microvessel density. Low collagen XVIII expression by tumor hepatocytes was associated with large tumor size (r = –0.63; P < 0.001) and replacement of trabeculae with pseudoglandular-solid architecture (χ² = 28; P < 0.001), which indicate tumor progression. Tumors expressing the highest collagen XVIII levels were smaller and had lower microvessel density (P = 0.01) than those expressing moderate levels; and HCCs with the lowest collagen XVIII levels approached a plateau of microvessel density, which indicated that a decrease in collagen XVIII expression is associated with angiogenesis in primary liver cancer. HCCs recurring within 2 years of resection showed 2.2-fold lower collagen XVIII mRNA than nonrecurring ones (P = 0.02). The findings relied on the hepatocyte-specific long form. Thus, the endogenous expression of the endostatin precursor decreases along with tumor progression in HCCs.

Introduction

Angiogenesis is essential for tumor growth and metastasis and is thought to depend on a balance between endogenous stimulators and inhibitors (1). One of such inhibitors, endostatin, suppresses tumor growth in mouse models (2). It is generated by proteolytic cleavage of the COOH-terminal domain of collagen XVIII (2–4); and, accordingly, endostatin-containing polypeptides were identified in mouse tissues (5) expressing high levels of collagen XVIII (6–8). Collagen XVIII is a component of most epithelial and vascular basement membranes (5, 7) and is expressed at high levels by hepatocytes (8). In normal tissues, two variants of collagen XVIII, the short and long forms, regulate tissue specificity. In humans, the short form is found in basement membranes and is mainly expressed by capillary vessels and myofibroblasts (7). The long form is almost exclusively found in the liver (6), is expressed at strikingly high levels by hepatocytes, deposited along liver sinusoids (7), and regulated by liver-enriched transcription factors, all of which indicate that it is a liver-specific gene product (9). The short and long forms of collagen XVIII originate from the use of two alternate promoters and have variant NH₂-terminal noncollagenous domains of 303 and 493 amino acids but share the downstream collagenous and noncollagenous domains, including the endostatin module (6; Fig. 1A).

The biological role of collagen XVIII in human cancer is unknown. In normal adult liver, hepatocytes are arranged in trabeculae, i.e., one-cell thick cords separated by sinusoid vessels lined by fenestrated endothelial cells. HCC, the malignant transformation of hepatocytes, gradually loses these specialized features along the spectrum of tumor progression (10). With increasing tumor size, thin trabeculae are replaced with several-cell-thick tumor hepatocyte cords with increasingly frequent areas of glandular-like (pseudoglandular) and solid patterns (10) and CD34-positive endothelial cells, the latter indicating tumor angiogenesis (11, 12). Using RNA arrays of liver biopsy samples from 57 HCCs, we show that collagen XVIII expression decreases with increasing tumor size, loss of the trabecular architecture, and development of a vascular network. The findings indicate that tumor progression and angiogenesis are associated with decreased expression of the liver-specific form of collagen XVIII in HCCs.

Materials and Methods

Antisera and Nucleic Acid Probes. Fig. 1A shows antibodies and cDNA probes that recognize collagen XVIII sequences. All-type XVIII antibody (7) and the corresponding cDNA probe (6) detect a sequence common to all of the variants in the NH₂-terminal noncollagenous domain. Short and long cDNA probes recognize the short and long variant forms of the NH₂-terminal noncollagenous domain, respectively (6, 7). The cDNA probes for albumin, procollagen α1(IV), and laminin γ1 and a 25-mer oligo probe for 18S rRNA were prepared as described previously (8). The monoclonal anti-CD34 antibody (clone QB-END10; Novocastra, Newcastle, United Kingdom) was used as a marker for angiogenic endothelial cells. Secondary antibodies were peroxidase-conjugates, goat-antirabbit, or antioimmus IgG (Bio-Rad, Hercules, CA).

Tissue Samples. Liver tissue samples (n = 125) were obtained from patients hospitalized at Rennes or Bordeaux University Hospitals in France, between May 1991 and December 1997. Samples consisted of 57 HCCs, 47 matching nontumor areas, and 21 histologically normal liver controls. Thirty-three HCC patients underwent tumor resection and 24, liver transplantation. Histologically normal liver controls were from 8 liver donors inadequate for transplantation and 13 patients undergoing resection of liver metastases. Access to human tissues complied with French laws and with the guidelines of the Ethics Committee (Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale de Rennes) (13). Specimens were routinely processed for histology, i.e., H&E-saffron and Sirius red staining. On macroscopic analysis of the resected specimen, tumor size was defined as the largest diameter of the tumor (cm) or the diameter of the largest tumor in the case of

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3 The abbreviation used is: HCC, hepatocellular carcinoma.
multiple HCCs. Histological patterns and grades were classified using standard systems, as described previously (10).

**Tissue Processing, RNA Extraction, and Analysis.** The procedures were performed as described previously (13, 14). After macroscopic examination by a pathologist, representative samples were fixed in formalin, embedded in paraffin for histopathological routine diagnosis, and analyzed independently. A part of the fresh material was frozen in liquid nitrogen and stored at −80°C until use. Before RNA extraction, 5-μm frozen serial sections were stained with methylene blue and observed under light microscopy. Tissue blocks (0.1–1 g) that allowed a matching diagnosis with the pathology reports were homogenized for RNA extraction by the guanidinium thiocyanate/cesium chloride method. For dots blots, each RNA sample was blotted in triplicate at 1.25, 2.5, and 5 μg/μl onto nylon membranes using a filtration manifold. All of the samples were run in the same experiment and exposed simultaneously to the same film under optimized conditions, yielding a linear relationship between the densitometry signal and the amount of RNA loaded (range tested, 1–5 μg RNA; r, 0.97–0.99). Densitometry scanning was performed with the Denslab software package (Bioprobe Systems, Les Ulis, France). Signal normalization was done with a 25-mer oligonucleotide probe for 18S rRNA, and values were expressed as specific mRNA:18S ratios.

**Immunohistochemistry and Determination of Microvessel Density.** Standard immunoperoxidase techniques were used (8). As for RNA extraction (see above), areas representative of HCC allowing diagnosis and grading that matched the pathology reports were selected on H&E staining of frozen blocks. Microvessel density counting was performed and analyzed as described previously (11, 15) by immunostaining with anti-CD34 at 1:25 dilution. Neovascular hot spots were searched for on duplicate slide sets for each sample; these areas were frequently situated at or near the margin of the tumors. Microvessels in hypoplastic or sclerotic areas within the tumor were not taken into account. Sections were scanned at low power (×40); the five areas with the greatest density of distinct CD34-positive microvessels were selected and a ×200 field in each was counted by two independent observers (O.M. and B.C.), unaware of the clinical, pathological, mRNA expression, or other relevant data, using an Olympus BX60 microscope. There was no significant interobserver difference. The mean value of the counted five fields was considered as the microvessel density of each sample. Data were expressed as number of microvessels/740 μm².

**Statistical Analysis.** Comparisons between groups of independent samples were made using the Mann-Whitney U test or the Kruskal-Wallis test, as indicated in the “Results and Discussion.” Associations between categorical variables were assessed using the χ² test. Correlation between continuous variables was studied by Spearman’s rank-order coefficients. Probability values < 0.05 were considered significant. The Statistica 4.3B software package (StatSoft Inc., 1993) was used.

**Results and Discussion**

**Different Expression Profiles of Collagen XVIII in HCCs.** Immunohistochemistry (Fig. 1, B and C) and in situ hybridization (not shown) showed that tumor hepatocytes were a major source of all-type XVIII collagen in HCCs. The expression of this collagen type varied according to the tissue architecture of the tumors. Tumor cells were more intensely labeled in HCCs with a predominantly trabecular pattern (Fig. 1B) than in HCCs with mixed pseudoglandular and solid patterns (Fig. 1C). All-type XVIII mRNA was assessed in 125-sample dot blot arrays containing 57 HCCs, 47 matching nontumor areas, and 21 normal liver controls. All-type XVIII collagen was slightly (1.5-fold) increased in HCCs with respect to controls [HCCs, mean ± SD, 0.9 ± 0.5 (n = 57); controls, 0.6 ± 0.2 (n = 21); P = 0.02]. HCCs showed three distinct profiles of all-type XVIII expression, namely, higher than, similar to, and lower than the matching nontumor samples (Fig. 2A). In HCCs, the frequency distribution of densitometry data indicated a great dispersion of values, with a >20-fold difference between the cases expressing all-type XVIII at the highest and at the lowest levels (range, 0.13–2.78; median, 0.83; 25–75th percentiles, 0.44–1.2, respectively). Seventeen HCCs were above the 75th percentile (Fig. 2B), 27 HCCs were within the 25th and 75th percentile

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**Fig. 1.** A, schematic structure of the full-length human α1(XVIII) collagen variant chains and the probes used. The variant NH₂-terminal noncollagenous domains (white boxes) are respectively called SHORT and LONG. All of the variants share a part of the NH₂-terminal noncollagenous domain (hatched box), a highly interrupted collagenous stretch (thin interrupted line) with noncollagenous interruptions (hollow vertical boxes), and the COOH-terminal noncollagenous domain (black box), containing the angiogenesis inhibitor endostatin. B and C, immunoperoxidase staining of all-type XVIII in trabecular and pseudoglandular-solid HCCs. B, trabecular HCC. Tumor cells growing in cords show intense immunosignal for all-type XVIII. The endothelial lining of tumor sinusoids is intense immunosignal for all-type XVIII. The endothelial lining of tumor sinusoids is fainter than that in tumor vessels with thick, basement membrane-like bundles. C, pseudoglandular-solid HCC. The signal in tumor cells (t) is fainter than that in tumor vessels with thick, basement membrane-like bundles (arrows). Hepatocytes in the adjacent nontumor liver (nt) show more intense signal than the tumor cells. Sections were counterstained with hematoxylin. ×400.
The expression of all-type XVIII was compared with those of type IV collagen and laminin γ1, two major basement membrane components. As expected, the expression of type IV collagen mRNA was strongly correlated with that of laminin γ1 mRNA (Spearman’s r, 0.88; P < 0.001; n = 123). However, they were not associated with all-type XVIII (laminin γ1: r, 0.14; P = 0.1 (n = 123); collagen IV: r, 0.038; P = 0.67 (n = 123)), which demonstrated that these findings were specific.

The Decrease in Collagen XVIII Expression Is Associated with Higher Vascular Support, Increasing Tumor Size, and Changes in the Histological Architecture of HCCs. We hypothesized that the variability in the expression of the endostatin precursor collagen XVIII could reflect different angiogenic phenotypes of the tumors. Microvessel density was assessed by immunohistochemistry with anti-CD34 antibody in representative samples from the groups with high (n = 14), moderate (n = 20), and low (n = 9) all-type XVIII expression. The mean microvessel density of the high group was significantly lower than that of the moderate group (Fig. 2C). By contrast, the mean microvessel density of the low group was not statistically different from that of the other groups (Fig. 2C). Interestingly, the three groups differed in tumor size (Fig. 2D). Indeed, collagen XVIII expression decreased with increasing tumor size (Fig. 2, B and D). In addition, the scatter-plot of all-type XVIII mRNA and tumor size values showed a strong negative correlation between both variables (Fig. 2E). Thus, the moderate group showed a 1.8-fold increase in microvessel density and a 2-fold increase in tumor size with respect to the high group, whereas the further increase in size in the low group was not associated with significant changes in microvessel density (Fig. 2, C and D). These data agree with a recent report of a 1.4-fold increase in microvessel density between small (<2 cm) and medium-sized (2–5 cm) HCCs and with a minor, albeit not statistically significant, decrease in microvessel density in larger tumors (>5 cm; Ref. 12).

Tumor growth is angiogenesis dependent (1). Thus, the vascular support of a tumor, that is, the amount of tumor supported by a unit amount of microvessels is low in growing mouse tumors, then increases asymptotically approaching the final tumor size as tumors attain a growth plateau (16). We thus calculated the vascular support for each HCC as tumor size: microvessel density ratios and plotted all-type XVIII expression (Fig. 2E) and vascular support (Fig. 2F) with respect to tumor size. Small and medium-sized HCCs clustered around the steep descending slope of the curve of all-type XVIII levels (Fig. 2E) and around the steep ascending slope of the curve of vascular support ratios (Fig. 2F), which indicated that a significant decrease in all-type XVIII expression was associated with increasing tumor size and the development of a vascular network in small and medium-sized HCCs.

All-type XVIII level was associated with the histological pattern mRNA values of HCCs (Table 1). Trabecular HCCs displayed the highest all-type XVIII levels (trabecular, (T) mean ± SD, 1.3 ± 0.61; group H and 2.8-fold lower than in group M and than in NT. C. microvessel density in HCCs, assessed by counting the number of immunohistochemically labeled CD34 (+) microvessels per 740 µm² (×200). The group H (n = 14) shows 1.9-fold lower microvessels density than group M (n = 20). Group L (n = 9) shows intermediate microvessel density counts not significantly different from the others. D. tumor size in the groups H, M, and L all-type XVIII (**, P = 0.01; *; P < 0.01; in B, C, and D). E. the scatter-plot shows a strong negative correlation between all-type XVIII and tumor size in 57 HCCs. F. vascular support in each HCC, assessed as tumor size: microvessel density ratios, represented with a logarithmic scale. Small and medium-sized tumors cluster around the ascending slope of the curve. Ratios increase steeply with increasing tumor size and tend to stabilize in large tumors, in which further increase in size is associated with lesser changes in microvessel density.
The mean all-type XVIII collagen levels in HCCs that recurred was 2.2-fold lower than in those HCCs that did not recur. The mean all-type XVIII collagen levels in HCCs that recurred was 5.7 ± 0.02 SD, 1.10 ± 0.69 (n = 16); recurring, 0.49 ± 0.40 (n = 8); P = 0.02, Mann-Whitney U test]. The mean tumor size in HCCs that recurred (120, 6.4 cm in tumors that recurred (P = 0.03; TG versus PS, P = 0.002). Trabeular HCCs were smaller than mixed pseudoglandular-solids (24); pseudoglandular-solids, 8.2 ± 4.9 cm (n = 12); P = 0.001). HCCs with trabecular and pseudoglandular patterns coexisting in similar amounts (trabeuculoglandular) were intermediate in size (6.2 ± 4.9 cm).

![Image](Fig. 3. The long variant accounts for the bulk of collagen XVIII forms in HCCs. Northern blots of collagen XVIII forms with the all-type XVIII probe, common to all variants (A), and short forms (Fig. 3B) showed identical 6- and 5-kb bands, whereas short form revealed a 4.5-kb band (Fig. 3C). All-type XVIII was strongly correlated with the long form (Fig. 3E), and slightly with the short form (Fig. 3F), which indicated that the long form accounts for the bulk of collagen XVIII forms in HCCs. By Northern blots, all-type XVIII (Fig. 3A) and long forms (Fig. 3B) showed identical 6- and 5-kb bands, whereas short form revealed a 4.5-kb band (Fig. 3C). All-type XVIII was strongly correlated with the long form (Fig. 3E), and slightly with the short form (Fig. 3F), which indicated that the long form accounts for the bulk of collagen XVIII forms in HCCs.)

**Table 1 Type XVIII mRNA expression and clinico-pathological features of HCCs**

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*All-type XVIII collagen mRNA levels in HCCs above the 75th percentile (high), within the 75th and 25th percentiles (moderate), and below the 25th percentile (low). Range, 0.13–2.78; median, 0.83; 25th–75th percentiles, 0.44–1.2, respectively. Dot blot analyses with 32P-labeled probes.*

*Mean ± SD.*

*NA, not applicable; HCV, hepatitis C virus; HBsAg, hepatitis B surface antigen; NS, not significant; NT, nontumor liver.

* Indicates tumor differentiation: I, most differentiated; IV, least differentiated.

**Low Collagen XVIII mRNA Levels Are Associated with Tumor Recurrence.** Two-year disease-free follow-up data were available for 24 HCC patients treated by tumor resection who had not received other preoperative or postoperative treatment before the onset of tumor recurrence and in whom the surgical resection margins were free of tumor. All of the patients were Child’s class A (17), indicating a compensated liver function. Eight subjects experienced tumor recurrence, and 16 remained disease-free within 24 months of resection. The mean all-type XVIII collagen levels in HCCs that recurred was 2.2-fold lower than in those HCCs that did not recur [not recurring, mean ± SD, 1.10 ± 0.69 (n = 16); recurring, 0.49 ± 0.40 (n = 8); P = 0.02, Mann-Whitney U test]. The mean tumor size in HCCs that did not recur was 5.7 ± 4.1 cm versus 11 ± 6.4 cm in tumors that recurred (P = 0.02). Microvessel density was higher in HCCs that recurred (196 ± 120, n = 8) than in nonrecurring ones (107 ± 35, n = 13; P = 0.04), consistently with previous findings in HCC (11) and other tumor types (15, 18). Patients in the nonrecurring and recurring groups did not differ in age, gender, prevalence of hepatitis C or B viral infection, alcohol abuse, incidence of microscopic intrahepatic metastases, liver fibrosis, cirrhosis, histological activity of the underlying liver disease, or the degree of tumor differentiation, as assessed using the Edmondson’s score (Ref. 10; not shown).

**The Long Form is the Major Type XVIII Collagen Variant in HCCs.** We asked whether the above findings were specific to one of the variant forms of collagen XVIII. The expression of the all-type XVIII long and short mRNAs were assessed by screening 123-sample dot blot arrays from 21 controls, 55 HCCs, and 47 matching nontumor liver samples. By Northern blots, all-type XVIII (Fig. 3A) and long forms (Fig. 3B) showed identical 6- and 5-kb bands, whereas short form revealed a 4.5-kb band (Fig. 3C). All-type XVIII was strongly correlated with the long form (Fig. 3E), and slightly with the short form (Fig. 3F), which indicated that the long form accounts for the bulk of collagen XVIII forms in HCCs.

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collagen XVIII expression in HCCs. In addition, the long form displayed the same clinicopathological associations in HCCs as all-type XVIII, but not as the short form (not shown).

Recent evidence indicates that the long form is a liver-specific gene product (6, 7, 9). We thus searched for the expression profile of the liver-specific gene albumin in 55 HCCs and 47 matching nontumor samples. Interestingly, HCCs in the low all-type XVIII expression group showed the lowest albumin mRNA levels, whereas those tumors in the high and moderate groups displayed similar albumin expression (Albumin:18S RNA ratios: nontumor samples, 2.1 ± 0.8; low all-type XVIII, 0.5 ± 0.1; high + moderate all-type XVIII, 1 ± 0.47; nontumor versus low, P < 0.001; high + moderate versus low, P < 0.001). Thus, HCCs in the low group were the largest and displayed the lowest collagen XVIII (Fig. 2, B and D) and albumin levels and approached the vascular support plateaux (Fig. 2, C and F). In addition, none of these tumors preserved a predominantly trabecular architecture (Table 1). Taken together, these data suggest that HCCs in the low all-type XVIII expression group were in a late stage of tumor progression and could be losing some of the phenotypic features of normal hepatocytes.

A higher expression of collagen XVIII in HCCs with respect to nonneoplastic liver was suggested in a preliminary in situ hybridization study that included three HCCs (19). In the present study, the analysis of 57 HCCs indicated that collagen XVIII expression decreases with increasing size and microvessel density of small and medium-sized tumors. Larger HCCs showed further decrease in collagen XVIII and albumin expression and replacement of trabecular with pseudoglandular and solid patterns. These changes relied on the long form, whose expression is regulated by liver-enriched transcription factors (9). Increasing tumor size heralds tumor progression and enhanced aggressiveness in HCCs and is associated with lower overall survival and higher recurrence rates after resection (20). Large HCCs display chromosomal damage (21), higher endothelial growth factor expression in hepatocellular carcinoma: its clinical and prognostic significance. Hepatology, 26: 1187–1209, 1997.


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