Spontaneous Rupture of Hepatocellular Carcinoma and Vascular Injury

L. X. Zhu, PhD; X. P. Geng, MB; S. T. Fan, MD

Hypothesis: Because spontaneous rupture of hepatocellular carcinoma (HCC) is one kind of bleeding complication related to the blood vessels, the possible mechanism of this rupture should occur on the blood vessel itself. Our hypothesis, which has not yet been investigated, is that the vascular integrity of HCC might be damaged during vascular injury.

Design: We examined semiquantitatively the expression of von Willebrand factor, elastin, neutrophil elastase, type IV collagen, and collagenase in 23 specimens of HCC with spontaneous rupture by immunohistochemistry, and compared them with 30 specimens of HCC without rupture.

Results: There was a significant decrease of von Willebrand factor, proliferation of degenerated elastin, abnormal distribution of neutrophil elastase, degradation of type IV collagen, and increase in collagenase production around the blood vessels in ruptured HCC. Since the decreased expression of von Willebrand factor is an indicator of vascular injury and elastase and collagenase are present in inflammatory processes, we postulate that the vascular injury probably exists before spontaneous rupture of HCC occurs. The blood vessel dysfunction resulting from the degeneration of elastin and the degradation of type IV collagen can render the blood vessels stiff and weak, causing them to split easily when the vascular load increases from hypertension or minor mechanical trauma.

Conclusion: Spontaneous rupture of HCC may be related to the vascular dysfunction.

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PATIENTS AND METHODS

PATIENTS

During 1989 to 1995, specimens from 23 patients with ruptured HCC who had undergone heptectomy in the Department of Surgery, Queen Mary Hospital, Hong Kong, China, were taken for the experimental studies. Another 30 patients with nonruptured HCC, who had undergone heptectomy in the same period, were randomly selected for the control group. The clinical characteristics of the 53 patients are shown in Table 1.

ENZYME-LINKED IMMUNOSORBENT ASSAY

The specimens of plasma from the patients were collected and stored at −80°C. With this method, the first antibody (rabbit anti-human vWF immunoglobulin; Dako, Copenhagen, Denmark) was diluted with a coating buffer (sodium carbonate, sodium bicarbonate, pH 9.6) and then added to the plastic plates for coating at 4°C overnight. After blocking the plasma by normal bovine serum, the dilution range of the plasma would be 1:200 to 1:6400 for each specimen. To reduce errors, the specimens were applied to 2 plates and the average data of the 2 plates were taken as the final result. The second antibody (mouse anti-human vWF immunoglobulin; Dako) and horseradish peroxidase–conjugated third antibody (horseradish peroxidase–conjugated goat antimouse immunoglobulin; Dako) were then added for 1 hour at 37°C. Then, o-phenylenediamine diluted with substrate buffer was added to the incubation for 15 minutes at 37°C. Finally, 2-mol/L sulfuric acid was added to stop the enzymatic reaction, and then the plates were read by a spectrophotometer.

IMMUNOHISTOCHEMISTRY

The specimens without necrosis and hemorrhage tissue were paraffin-embedded and cut into 4-μm thick sections. These sections included the junction of tumor and nontumorous liver.

Standard immunoperoxidase technique for ABC (avidin biotin complex) method was performed. The antibodies used included vWF (Dako, at 1:20 dilution), elastin at 1:1000 dilution (Sigma-Aldrich; St Louis, Mo), neutrophil elastase at 1:100 dilution (Dako), type IV collagen at 1:10 dilution (Dako) and collagenase at 1:100 dilution (Chemicon International Inc, Temecula, Calif). All antibodies were monoclonal. The slides were incubated at 37°C with primary antisera for 30 minutes. Secondary biotinylated immunoglobulin (rabbit anti-mouse biotinylated immunoglobulin at 1:100 dilution; Dako) and freshly prepared streptavidin-biotinylated horseradish peroxidase complex were applied with incubation for 30 minutes at 37°C. The staining process was completed after diamino benzidine–hydrogen peroxide solution stain and counterstain. Positive control slides were prepared with human HCC tumor tissue for vWF, elastin, elastase, type IV collagen, and collagenase that were identified to be positive previously. The negative control was buffer solution that replaced the primary antisera. Controls were repeated for every new batch of tests.

COUNTING

The spectrophotometer (A5022; Anthos Labtec Instruments, Sydney, Australia) at 492 nm and a computer (Externa 310, Texas Instruments, Taipei, Taiwan) were used to read and quantitate the parameters of enzyme-immunosorbent assay (ELISA).

A Power Macintosh 7200/120 computer (Apple Computers; N/H Image 1.59 by Wayne Rasband, San Jose, Calif) and a microscope (Carl Zeiss Axioplan, Munich, Germany) were used to identify and quantitate the different parameters in relation to the cells or substance with positive immunohistochemical staining in peritumoral regions (within 1 mm of the edge of the tumor). On each of these regions, 9 different fields were selected randomly for quantitation. The parameters assessed included (1) vascular areas defined by endothelial cells that stained positive for vWF, (2) the thickness of elastin layers in vessel walls, (3) the thickness of type IV collagen in blood vessel walls, (4) the total number of cells that stained positive with neutrophil elastase, and (5) the results of collagenase staining on blood vessels recorded as positive or negative according to presence or absence in every specimen. The data were counted as the sum of the results of the 9 fields.

STATISTICS

The Wilcoxon rank sum test was used to compare the variables of the group with ruptured HCC and the group with nonruptured HCC. The χ² and Pearson tests were used to compare the positive rates and the correlations, respectively. Computations were performed with Statistical Product and Service Solutions program (SPSS Inc, Chicago, Ill).

platelets and the endothelial cells to release the growth factors, such as transforming growth factor-β, platelet-derived growth factor, and basic fibroblast growth factor.9,11 During vascular injury, proliferated elastin is synthesized in smooth muscle cells by the stimulation of transforming growth factor-β.14 As it becomes degraded by elastase, which is released at the same time, the structure of proliferated elastin becomes fragmented and loses its elasticity. Under normal physiologic conditions, the synthesis of collagenase is low or absent. However, when vascular injury occurs, fibroblast growth factor stimulates the endothelium to synthesize collagenase,15 and platelet-derived growth factor stimulates smooth muscle cells to secrete collagenase.16 When collagen is exposed to collagenase, the property of collagen is lost and fails to protect the vascular wall from rupturing.

The hypothesis of our research is that the vascular integrity of HCC might be damaged during vascular injury, which has not been investigated before. In this study, immunohistochemistry was used to find the alteration of the quantity and distribution of vWF, elastin, elastase, type IV collagen, and collagenase in specimens of ruptured HCC.
RESULTS

CLINICAL CHARACTERISTICS OF PATIENTS

There were no significant differences between patients with ruptured HCC and nonruptured HCC in terms of age, sex, cirrhosis, tumor stage, portal vein invasion, and presence of tumor encapsulation (Table 1). In patients with ruptured HCC, the median activated partial thromboplastin time (APTT) test was longer, and the median diameter of ruptured tumors was larger than that of nonruptured ones.

IMMUNOHISTOCHEMICAL STUDY FINDINGS

Von Willebrand Factor

In the ELISA test, vWF expression was found to be significantly lower in the plasma of patients with ruptured HCC than of patients with nonruptured HCC (Figure 1A).

In the immunohistochemistry test, the vWF was expressed by the endothelium of blood vessels. In the peritumoral regions, the number of areas in the blood vessels that stained positively for vWF were also significantly lower in patients with ruptured HCC than in those with nonruptured HCC (Table 2, Figure 1B).

Except for the APTT test, there was no significant relationship between clinical characteristics and vWF results. The expression of vWF in plasma was inversely related to the APTT (Rs = −0.25, P < .05).

Elastin and Elastase

In the immunohistochemistry test, the elastin in the vessel walls at the peritumoral regions was significantly thicker in patients with ruptured HCC than that in patients with nonruptured HCC (Table 2). Proliferation of the elastin around the blood vessel predominated in patients with ruptured HCC but this was rarely seen in patients with nonruptured HCC. However, in the specimens of ruptured HCC, the elastin was usually either degenerated (Figure 2A) or fragmented (Figure 2B). Intact and unfragmented elastin fibrils (Figure 2C) were found diffusely in nonruptured HCC specimens.

In the immunohistochemistry study, no significant differences were found in the number of positive cells for neutrophil elastase at the peritumoral region (Table 2). However, the distribution of neutrophil elastase was different between the 2 groups. In the patients with rup-

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Table 1. Clinical Characteristics of 53 Patients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>RHCC Group (n = 23)</th>
<th>NHCC Group (n = 30)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, median (range), y</td>
<td>50 (38-76)</td>
<td>53 (5-82)</td>
<td>.62</td>
</tr>
<tr>
<td>Sex, M/F</td>
<td>21/2</td>
<td>26/4</td>
<td>.60</td>
</tr>
<tr>
<td>HBsAg positive, No. (%)</td>
<td>20/23 (87)</td>
<td>25/30 (83)</td>
<td>.08</td>
</tr>
<tr>
<td>APTT, median (range), s</td>
<td>28.5 (23.9-39.6)</td>
<td>21.1 (20.1-35.3)</td>
<td>.001</td>
</tr>
<tr>
<td>PT, median (range), s</td>
<td>11.9 (8.9-16.6)</td>
<td>12.1 (10.4-15.9)</td>
<td>.48</td>
</tr>
<tr>
<td>Cirrhosis, No. (%)</td>
<td>12/16 (75)</td>
<td>18/21 (66)</td>
<td>.41</td>
</tr>
<tr>
<td>Tumor stage (TNM), No.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>0</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>8</td>
<td>10</td>
<td>.40</td>
</tr>
<tr>
<td>III</td>
<td>11</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Portal vein invasion, No. (%)</td>
<td>1/15 (7)</td>
<td>6/24 (25)</td>
<td>.15</td>
</tr>
<tr>
<td>Tumor encapsulation, No. (%)</td>
<td>5/11 (45)</td>
<td>5/16 (31)</td>
<td>.59</td>
</tr>
<tr>
<td>Tumor size (largest diameter), median (range), cm</td>
<td>10.5 (2.5-15.0)</td>
<td>5.5 (1.0-18.0)</td>
<td>.007</td>
</tr>
</tbody>
</table>

* RHCC indicates ruptured hepatocellular carcinoma; NHCC, nonruptured hepatocellular carcinoma; HBsAg, hepatitis B surface antigen; APTT, activated partial thromboplastin time; and PT, prothrombin time.

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tured HCC, neutrophil elastase was absent around the blood vessels where there was elastin proliferation, but if elastase was present, elastin proliferation was absent. This means that the blood vessels in patients with ruptured HCC would be surrounded by proliferated elastin or infiltrated neutrophils only. In patients with ruptured HCC, elastin proliferation was predominant. However, normal elastin without neutrophil infiltration was found predominantly in patients with nonruptured HCC. Due to the rare incidence of neutrophil infiltration, the significant difference from the elastase test cannot be determined.

There was no relationship between the patients’ clinical characteristics and elastin or elastase results.

**Type IV Collagen and Collagenase**

In the immunohistochemistry study, the thickness of the blood vessels at the peritumoral region with positive staining for type IV collagen was significantly less in the group with ruptured HCC than in the group with nonruptured HCC (Figure 3, Table 2).

Collagenase was expressed by the endothelial cells or the smooth muscle cells at the peritumoral regions in 22 (96%) of 23 cases with ruptured HCC, but in only 16 (53%) of 30 cases with nonruptured HCC. The positive rates were significantly different between the 2 groups (Table 2). The type IV collagen around the blood vessels was either thinner or absent where the collagenase stained positive. The thickness of type IV collagen in the vessel walls was inversely related to collagenase expression in the blood vessels (Rs = –0.297, $P < .001$).

There was no relationship between the patients’ clinical characteristics and type IV collagen or collagenase results.

### Table 2. Comparison of Parameters in 2 Groups of Patients*

<table>
<thead>
<tr>
<th>Markers</th>
<th>RHCC Group (n = 23)</th>
<th>NHCC Group (n = 30)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>vWF, µm&lt;sup&gt;2&lt;/sup&gt;</td>
<td>152.0 (5.0-660.0)</td>
<td>363.5 (31.0-1187.0)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Elastin thickness, µm</td>
<td>19.0 (5.0-69.0)</td>
<td>9.5 (1.0-33.0)</td>
<td>.002</td>
</tr>
<tr>
<td>Neutrophil elastase, No./1000 cells</td>
<td>37 (11-27)</td>
<td>38 (11-139)</td>
<td>.80</td>
</tr>
<tr>
<td>Type IV collagen thickness, µm</td>
<td>5.0 (1.0-32.0)</td>
<td>13.0 (3.0-61.0)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Collagenase, % positive</td>
<td>96</td>
<td>53</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

*Data are given as median (range) unless otherwise specified. RHCC indicates ruptured hepatocellular carcinoma; NHCC, nonruptured hepatocellular carcinoma; and vWF, von Willebrand factor.

In this study, the pathological changes of the blood vessels were found mainly at the peritumoral region (within 1 mm of the edge of the tumor) but not at the center of the tumor. This is because the blood supply to the tumor is the richest at the peritumoral region<sup>26</sup> and thus many blood vessels could then be counted in this limited field.

Since vWF is synthesized and present in the endothelium, a lower expression of vWF would imply that endothelial function has been damaged in patients with ruptured HCC. When endothelial function is damaged, the growth factors, such as transforming growth factor-β, platelet-derived growth factor, and fibroblast growth factor, will be released to start the process of the pathological changes in the blood vessels.

The lower level of vWF in the plasma of patients with ruptured HCC also suggests that the function of coagulation is poor. von Willebrand factor is the carrier protein of factor VIII, which is an essential factor in coagulation. Without the interaction of vWF, factor VIII cannot be moved to the bleeding site. Activated partial thromboplastin time is one of the most widely used screening tests for blood coagulation disorders.<sup>16</sup> The test reflects changes in clotting factors, including factor VIII. In the present study, the content of vWF was inversely related to APTT and the decreased expression of vWF prolonged the APTT (Table 1). However, the prothrombin time test reflects the changes of factors II, VII, and X only<sup>18</sup>; therefore, we found no significant difference from the test (Table 1). Poor coagulation can also aggravate the bleeding and cause a rupture in the vascular wall.

When vascular injury occurs, transforming growth factor-β will act on the smooth muscle cell to synthesize the excessive elastin. When the elastin is proliferated, the elastin will become degenerated or fragmented. Why this occurs is still unclear, but it may be due to the inflammatory reaction after the vascular injury.<sup>19</sup> The injured blood vessel becomes stiff and nonelastic as a result of the proliferation of the degenerated elastin. The capability of the vessel wall to resist stretching, which is in turn governed by the elastic modules of elastin fibers,<sup>20</sup> is greatly destroyed.

In patients with ruptured HCC in our study, the proliferation of degenerated elastin was found where elastase was absent around the blood vessel, and was absent where expression of elastase was positive. In the former case, the proliferation was activated by vascular injury; whereas, in the latter case, it was because of the infiltration of neutrophils from the bloodstream to the surrounding tissues.<sup>21</sup> Although the latter occurrence is not common, both suggest that the vascular injury existed in patients with ruptured HCC. However, patients who have a balanced synthesis and normal degradation of elastin will have no risk of vascular injury.

In this study, type IV collagen around the blood vessel was found to be thinner in patients with ruptured HCC because the type IV collagen is degraded by the collagenase, which is secreted during vascular injury. The damaged collagen causes the blood vessels to become weak,
because the vascular resistance to split mainly depends on the content of the collagen around the blood vessels. The breaking point of the collagen is several hundred kilograms per square centimeter and their extension at this point can still be minute. Its virtual inextensibility and high tensile strength (about 15-30 kg/mm²) is comparable weight for weight to that of steel. It has been reported that the degradation of collagen predisposes to rupture of blood vessels.

Under normal physiologic conditions, the synthesis of the collagenase is low or absent. However, when vascular injury occurs, fibroblast growth factor and platelet-derived growth factor can stimulate the endothelium and smooth muscle cells to synthesize collagenase, respectively. So in patients with ruptured HCC, the increased production of collagenase in the blood vessels indicates the presence of vascular injury. During vascular injury, collagen is degraded by collagenase and the capability of the vascular wall to resist a splitting force is decreased.

Based on the pathological changes mentioned above, we postulate that the vessel walls in patients with ruptured HCC have become stiff and weak. These blood vessels will then be subjected to rupture easily when there is an increase in vascular load related to portal hypertension or minor mechanical injury. Intratumoral pressure increases with the formation of hematoma, and the tumor ruptures if it is on the surface of the liver. If the tumor is deep inside the liver, the sudden increase in tumor size secondary to hematoma formation will split the overlying liver parenchyma, leading to hemoperitoneum (Figure 4).

It has been reported that ruptured HCC occurs in larger tumors frequently but small ones also can rupture. Similar results are seen in our study (Table 1). Due to the vascular injury, we thus postulate that a larger tumor can rupture more easily, because the larger one can be attacked more easily, but spontaneous rupture can also occur in smaller tumors due to the presence of abnormal vascular lesions.

The vascular injury mentioned herein was considered to be the cause rather than the result of the tumor rupture. First, the specimens that we selected were with-
out necrosis and hemorrhage tissue. Second, the pathological changes that occurred after hemorrhage were characterized as necrosis and proliferated collagen. In patients with ruptured HCC, collagen was present with degradation but without proliferation.

From this study, we postulate that a preexisting vascular injury including elastin proliferation and degeneration, abnormal distribution of neutrophil elastase, degradation of type IV collagen, and increased expression of collagenase characterized as necrosis and proliferated collagen. In patients with ruptured HCC, collagen was present with degradation but without proliferation.

From this study, we postulate that a preexisting vascular injury including elastin proliferation and degeneration, abnormal distribution of neutrophil elastase, degradation of type IV collagen, and increased expression of collagenase may all bring about a spontaneous rupture of HCC when the blood vessel encounters hypertension or minor mechanical injury. We conclude that spontaneous rupture of HCC may be related to vascular dysfunction.

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**Figure 4. Relationship between vascular injury and ruptured hepatocellular carcinoma (HCC).** TGF-β indicates transforming growth factor-β; PDGF, platelet-derived growth factor.