POLYCYTHEMIA IN HEPATOCELLULAR CARCINOMA-BEARING B6C3F1 MICE

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Abstract: Secondary polycythemia was observed in hepatocellular carcinoma-bearing B6C3F1 mice which were used as control group animals in 2-year carcinogenicity studies. Statistically significant increases in erythrocyte count, hematocrit value, hemoglobin concentration, and absolute reticulocyte count were noted in the peripheral blood of hepatocellular carcinoma-bearing mice as compared with non-tumor-bearing mice. A decrease in mean corpuscular volume and mean corpuscular hemoglobin value was also noted in hepatocellular carcinoma-bearing mice. Mean corpuscular hemoglobin concentration and relative reticulocyte count in hepatocellular carcinoma-bearing mice were comparable to those in non-tumor-bearing mice. In addition, the plasma erythropoietin level in hepatocellular carcinoma-bearing mice was significantly higher than that in hepatocellular adenoma-bearing mice and that in non-tumor-bearing mice. Therefore, the hematological changes observed in hepatocellular carcinoma-bearing mice were diagnosed as secondary polycythemia. At necropsy, the spleen in hepatocellular carcinoma-bearing mice was dark-red and enlarged 2 to 4 times compared with that in non-tumor-bearing mice. Histopathologic examination revealed proliferation of the erythroid cells and megakaryocytes in the spleen and bone marrow in hepatocellular carcinoma-bearing mice, suggesting an increase in erythropoietic activity. These findings indicate that increased levels of plasma erythropoietin in hepatocellular carcinoma-bearing mice stimulate erythropoiesis in the hematopoietic organs and result in proliferation of microcytic and hypochromic erythrocytes. Possible mechanisms for the induction of secondary polycythemia in hepatocellular carcinoma-bearing mice are discussed. (J Toxicol Pathol 9: 233–240, 1996)

Key words: Polycythemia, Hepatocellular carcinoma, B6C3F1 mice, Erythropoiesis, Erythropoietin

Introduction

C57BL/6 × C3H F1 (B6C3F1) mice are usually used in carcinogenicity bioassays of medical drugs and chemical compounds and are known to have a relatively high incidence of spontaneous hepatocellular neoplasms, especially the aged males of this strain1–4. At necropsy, an increased volume of peripheral blood (hypervolemia) and an enlarged spleen are often seen in hepatocellular carcinoma-bearing B6C3F1 mice. However, there have been no detailed studies to determine if there is any correlation between spontaneous hepatocellular tumors and these phenomena as far as we known.

In the present study, hematological changes and plasma levels of erythropoietin were examined in hepatocellular carcinoma-bearing B6C3F1 mice.

Materials and Methods

Experiment 1

1. Animals

B6C3F1 mice were obtained from a commercial breeding colony (CLEA Japan Inc., Tokyo and Charles River Japan Inc., Kanagawa) and used as control group animals for carcinogenicity bioassays conducted at the Drug Safety Research Laboratories, Takeda Chemical Industries, Ltd. (Hikari, Yamaguchi). They were maintained in individual cages in environmentally-controlled animal rooms, allowed free access to tap water and a powdered labolatory animal diet (CE-2, CLEA Japan Inc., Tokyo) and necropsied at 111 or 112 weeks of age. From the necropsy results and histopathological examinations described later, 123 male animals were the subjects in this study: 67 mice had no tumors of any type (non-tumor–bearing mice; control group) and 56 mice had one or more tumors (tumor group).
2. Hematology

On the day of necropsy, blood samples were withdrawn from the abdominal vein of all animals with heparinized syringes under ether anesthesia. Part of the heparinized blood was used for hematological examination. The values of the following items were determined or calculated with an automated hematology analyzer (ELT-8/ds, Ortho Instruments, E-5000, or R-2000 Toa Medical): erythrocyte count, leukocyte count, platelet count, hematocrit value, hemoglobin concentration, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and reticulocyte count.

3. Necropsy and histopathology

After terminal body weight was determined, each animal was necropsied. All the tissues/organs designated in the protocol including the liver, spleen, and bone marrow (femur) were fixed in neutral phosphate-buffered 10% formalin. Tissues were routinely processed, embedded in paraffin, cut at a thickness of 4 μm, stained with hematoxylin and eosin, and examined using a light microscope. The diagnostic criteria for hepatocellular tumors was based on the criteria proposed by the National Toxicology Program.

Experiment 2

1. Animals

B6C3F1 mice were obtained from commercial breeding colonies (Charles River Japan Inc., Kanagawa) and used as control group animals under the same conditions as described for Experiment 1. From the necropsy result and histopathological examinations described later, 31 male animals were the subjects in this study: 15 mice had no tumors of any type (control group), 6 mice had hepatocellular adenoma (adenoma group), and 10 mice had hepatocellular carcinoma (carcinoma group).

2. Hematology

Hematological examination was conducted as described for Experiment 1.

3. Plasma levels of erythropoietin

Blood samples taken from the abdominal vein were centrifuged at 7,500 x g for 10 minutes to obtain plasma for the erythropoietin assay. Plasma erythropoietin concentrations were determined by an RIA method (Mitubishi Chemicals B.C.L.). The plasma samples were stored in a freezer until the assay was performed.

4. Necropsy, organ weight, and histopathology

Necropsy and histopathological examinations were conducted as described for Experiment 1. The liver and spleen weights were determined.

5. Statistics

The data on hematology, plasma erythropoietin concentration and organ weights were tested by Bartlett’s test for homogeneity of variance. If the variances were homogeneous, a one way analysis of variance was applied. When the results indicated a significant difference among the groups, the Dunnet test was performed to compare each of the tumor group means simultaneously with the control mean. If the variances were heterogeneous, the Kruskal-Wallis test was applied. When the results indicated a significant difference among the groups, the mean ranks of the tumor groups were compared with that of the control group, utilizing a Dunnet type test. All statistical tests were conducted at the 5% and 1% two-tailed probability levels. Dunnet’s test was performed using the SAS function PROBMC. The correlation between plasma erythropoietin concentration and erythrocyte count in the peripheral blood was examined by Pearson’s method.

Results

1. Hematology (Tables 1 & 2)

Experiment 1

All parameters in tumor-bearing mice were comparable to those in non-tumor-bearing mice. Upon analysis by tumor type, statistically significant increases in erythrocyte count, hemoglobin concentration, hematocrit value, and leukocyte count were noted in hepatocellular carcinoma-bearing mice when compared to non-tumor-bearing mice. Statis-
### Table 1. Hematological Data in Tumor-bearing B6C3F1 Mice

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of animals</th>
<th>Erythrocyte count (×10^6/μl)</th>
<th>Hemoglobin concentration (g%)</th>
<th>Hematocrit value (%)</th>
<th>MCV (μl)</th>
<th>MCH (pg)</th>
<th>MCHC (%)</th>
<th>Leukocyte count (×10^9/μl)</th>
<th>Platelet count (×10^9/μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No tumors</td>
<td>67</td>
<td>820 (1)</td>
<td>12.2</td>
<td>33.8</td>
<td>41</td>
<td>14.8</td>
<td>36.0</td>
<td>43</td>
<td>127.7</td>
</tr>
<tr>
<td>Tumors</td>
<td>56</td>
<td>871</td>
<td>12.6</td>
<td>35.0</td>
<td>41</td>
<td>14.6</td>
<td>36.0</td>
<td>51</td>
<td>134.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>203</td>
<td>6.8</td>
<td>1.0</td>
<td>2.0</td>
<td>24</td>
</tr>
<tr>
<td>Hepatocellular</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>carcinoma</td>
<td>9</td>
<td>1,255**</td>
<td>17.2**</td>
<td>46.1**</td>
<td>37*</td>
<td>13.6**</td>
<td>37.3</td>
<td>78**</td>
<td>123.8</td>
</tr>
<tr>
<td>Hepatocellular adenoma</td>
<td>14</td>
<td>821</td>
<td>11.6</td>
<td>32.3</td>
<td>40</td>
<td>14.2**</td>
<td>36.0</td>
<td>44</td>
<td>131.3</td>
</tr>
<tr>
<td>Alveolar/bronchiolar</td>
<td>11</td>
<td>817</td>
<td>12.0</td>
<td>33.8</td>
<td>41</td>
<td>14.7</td>
<td>35.8</td>
<td>51</td>
<td>135.4</td>
</tr>
<tr>
<td>adenoma</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>68</td>
<td>1.1</td>
<td>4.3</td>
<td>2.0</td>
<td>28</td>
</tr>
<tr>
<td>Harderian gland adenoma</td>
<td>7</td>
<td>816</td>
<td>12.3</td>
<td>34.2</td>
<td>42</td>
<td>15.1</td>
<td>35.9</td>
<td>47</td>
<td>142.6</td>
</tr>
<tr>
<td>Hemangioma, liver</td>
<td>3</td>
<td>716</td>
<td>10.6</td>
<td>30.3</td>
<td>43</td>
<td>15.2</td>
<td>35.4</td>
<td>54</td>
<td>161.4</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>3</td>
<td>817</td>
<td>11.9</td>
<td>34.8</td>
<td>43</td>
<td>14.6</td>
<td>34.4</td>
<td>39</td>
<td>104.7</td>
</tr>
<tr>
<td>Others</td>
<td>9</td>
<td>744</td>
<td>11.3</td>
<td>32.0</td>
<td>44</td>
<td>15.4</td>
<td>35.5</td>
<td>43</td>
<td>145.6</td>
</tr>
</tbody>
</table>

1): Mean, 2): S.D.
* : Significantly different from non-tumor-bearing mice, P<0.05
** : Significantly different from non-tumor-bearing mice, P<0.01

### Table 2. Hematological Data in Hepatocellular Tumor-bearing Mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of animals</th>
<th>Non-tumor-bearing mice</th>
<th>Hepatocellular tumor-bearing mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Hepatocellular adenoma</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Erythrocyte count (×10^6/μl)</td>
<td>Hemoglobin concentration (g%)</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>859 ± 44</td>
<td>12.5 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>982 ± 118</td>
<td>13.3 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1,244 ± 225**##</td>
<td>15.6 ± 2.7**##</td>
</tr>
</tbody>
</table>

a): Mean ± S.D.
MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin,
MCHC: mean corpuscular hemoglobin concentration
* : Significantly different from non-tumor-bearing mice, P<0.05
** : Significantly different from non-tumor-bearing mice, P<0.01
#: Significantly different from hepatocellular adenoma-bearing mice, P<0.05
##: Significantly different from hepatocellular adenoma-bearing mice, P<0.01
tically significant decreases in MCV and MCH were also noted in hepatocellular carcinoma-bearing mice. The MCH value was decreased in hepatocellular adenoma-bearing mice. The erythrocyte count, hemoglobin concentration and hematocrit and MCV values in hepatocellular adenoma-bearing mice were comparable to those in non-tumor-bearing mice. There were no statistically significant changes in mice that had one or more tumors excluding hepatocellular tumors.

Experiment 2

Statistically significant increases in erythrocyte count, hemoglobin concentration, hematocrit value, and absolute reticulocyte count were noted in hepatocellular carcinoma-bearing mice when compared to hepatocellular adenoma-bearing mice and non-tumor-bearing mice. Statistically significant decreases in MCV and MCH were also noted in hepatocellular carcinoma-bearing mice. Platelet and leukocyte counts and MCHC in hepatocellular car-

| Table 3. Plasma Erythropoietin Levels in Hepatocellular Tumor–bearing Mice |
|------------------|------------------|------------------|------------------|
|                   | Non-tumor-bearing mice | Hepatocellular tumor–bearing mice | Hepatocellular adenoma | Hepatocellular carcinoma |
| Number of animals | 15                | 6                 | 10                |
| Erythropoietin (mU/ml) | 25.5±4.5* | 25.7±4.1 | 38.8±9.6**## |

a): Mean±S.D.  
**: Significantly different from non-tumor-bearing mice, P<0.01  
###: Significantly different from hepatocellular adenoma–bearing mice, P<0.01  

2. Plasma levels of erythropoietin (Table 3 & Fig. 1)

Mean plasma levels of erythropoietin for non-tumor–bearing mice and hepatocellular adenoma–bearing mice were 25.5 and 25.7 mU/ml, respectively, and the difference was not statistically significant. The mean value for hepatocellular carcinoma–bearing mice was 38.8 mU/ml, which was significantly higher than the values for non-tumor–bearing mice and hepatocellular adenoma–bearing mice. The correlation between plasma erythropoietin concentration and erythrocyte count in the peripheral blood was examined using the correlation coefficient, and the value was 0.70 (Fig. 1). Therefore, it was considered that the erythrocyte count correlated with the plasma erythropoietin level.

3. Necropsy and organ weights (Table 4)

At necropsy, the spleen in hepatocellular car-

Fig. 1. Erythrocyte count and plasma erythropoietin level in hepatocellular tumor–bearing mice.
Table 4. Body, Liver, and Spleen Weights in Hepatocellular Tumor-bearing Mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Non-tumor-bearing mice</th>
<th>Hepatocellular tumor-bearing mice</th>
<th>Hepatocellular adenoma</th>
<th>Hepatocellular carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of animals</td>
<td>15</td>
<td>6</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>37.1 ± 3.6*</td>
<td>39.1 ± 1.9</td>
<td>34.7 ± 2.2##</td>
<td></td>
</tr>
<tr>
<td>Organ weight (g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>1.71 ± 0.20</td>
<td>2.82 ± 0.38**</td>
<td>3.08 ± 0.84**</td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>0.89 ± 0.19</td>
<td>1.00 ± 0.19</td>
<td>2.01 ± 1.16**</td>
<td></td>
</tr>
<tr>
<td>Relative organ weight (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>4.63 ± 0.42</td>
<td>7.25 ± 1.26**</td>
<td>8.90 ± 2.38**</td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>0.24 ± 0.05</td>
<td>0.25 ± 0.05</td>
<td>0.58 ± 0.34**</td>
<td></td>
</tr>
</tbody>
</table>

a): Mean±S.D.
b): The values are expressed as a percentage of body weight
**: Significantly different from non-tumor-bearing mice, P<0.01
##: Significantly different from hepatocellular adenoma-bearing mice, P<0.01

Hepatocellular carcinoma-bearing mice was found to be dark-red and enlarged 2 to 4 times compared with that in non-tumor-bearing mice. No changes were observed in the spleen in hepatocellular adenoma-bearing mice.

The spleen weight in adenoma-bearing mice was comparable to that in non-tumor-bearing mice.

Fig. 2. Non-tumor-bearing mouse, spleen, HE, ×92
No abnormalities were observed.

Fig. 3. Hepatocellular carcinoma-bearing mouse, spleen, HE, ×92
Proliferation of erythroid cells and megakaryocytes was observed.
4. Histopathology (Figs. 2–4)

In the liver, hepatocellular adenomas were a well circumscribed lesion without a normal hepatic structure. Hepatocellular carcinomas were often composed of one or more confluent nodular areas separated by compressed hepatocytes and variable amounts of connective tissue. Local invasion of the adjacent hepatic parenchyma was observed, but metastases to other organs including the lung were not observed.

In the spleen, splenic cords were dilated and filled with numerous megakaryocytes and erythroid cells in various stages of maturation in hepatocellular carcinoma-bearing mice. Erythroid cells in each stage were observed in the extramedullary erythropoietic region; early stage erythroid cells with large nuclei and distinctive basophilic cytoplasm and later stage small erythroblasts with small nuclei and scanty cytoplasm were common, but mitotic cells were rare. No remarkable changes were observed in the spleen in adenoma-bearing mice.

In the bone marrow, hypercellularity constituted by proliferation of erythroid cells and megakaryocytes was observed in hepatocellular carcinoma-bearing mice. Erythroid cells in each stage were observed as in the spleen in hepatocellular carcinoma-bearing mice.

No remarkable changes were observed in the bone marrow in adenoma-bearing mice.

In other organs such as the kidneys, lung, and heart, age-related changes in hepatocellular carcinoma-bearing mice were comparable to those in hepatocellular adenoma-bearing and non-tumor-bearing mice.

Discussion

An increase in erythrocyte count, hemoglobin concentration, and hematocrit value and a decrease in MCV and MCH were observed in hepatocellular carcinoma-bearing male B6C3F1 mice. These changes indicate that the number of microcytic and hypochromic erythrocytes were increased in the peripheral blood of hepatocellular carcinoma-bearing male B6C3F1 mice. In addition, an increased
level of plasma erythropoietin was noted in hepatocellular carcinoma-bearing mice. None of these changes were observed in hepatocellular adenoma-bearing mice. Therefore, the hematological changes observed in hepatocellular carcinoma-bearing mice were diagnosed as secondary polycythemia characterized by proliferation of microcytic and hypochromic erythrocytes with an increased level of plasma erythropoietin. Although similar secondary polycythemia has been reported in human patients with hepatomas in which red blood mass and plasma volume have been increased, there have been no reports of proliferation of microcytic or hypochromic erythrocytes9-11.

Secondary polycythemia is a result of increased erythropoietin levels due to excessive production12-13. Erythropoietin levels are increased either as a compensatory physiologic response by the kidney to tissue hypoxia or as a result of autonomous production independent of tissue oxygen supply. One type of secondary polycythemia is seen in animals with teratology of Fallot. Other form of secondary polycythemia occurs in humans more commonly in association with certain types of kidney diseases (hydronephrosis, cysts, carcinoma and adenoma) and less commonly in association with neoplasms of the liver, cerebellar hemangioblastoma, pheochromocytoma, adrenal adenoma, and uterine fibroids. Polycythemia associated with space-occupying lesions in the kidneys and neoplasia of various organs appears to be the result of stimulation of erythropoiesis by lesion-related erythropoietin production14. Arterial oxygen saturation is normal in these forms of polycythemia. Tumor-induced secondary polycythemia has been reported in dogs with renal carcinoma15,16.

It is unlikely that increased levels of plasma erythropoietin are due to the disturbance or delay of its clearance from peripheral blood. Age-related renal changes found in hepatocellular carcinoma-bearing mice were comparable to those in nontumor-bearing mice. The half-life of erythropoietin is short; about 2-5 hours in rats17 and 7-10 hours in dogs18, although there is no information for mice.

The kidney is the principle site of erythropoietin production in the adult of many species. Studies on laboratory animals, sheep, and humans19 have indicated that extrarenal production of erythropoietin during the fetal and neonatal periods occurs primarily in the Kupffer cells in the liver. Erythropoietin production has been detected in tissue cultures of normal adult kidney and fetal liver cell lines20. In addition, hepatic erythropoietin production has been reported in experimentally induced severe anemia and hypoxia in rats and mice. Thus, hepatocytes and/or Kupffer cells have a potential of erythropoietin production.

It is conjectured that the malignant lesions (tumor hepatocytes) induce increased erythropoietin production, possibly through induction of local hypoxia because of tissue compression by a space-occupying lesion. The difference in tumor growth speed between hepatocellular carcinomas and adenomas may be the key to weather or not secondary polycythemia occurs in hepatocellular tumor-bearing mice.

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References
1. Haseman, JK, Huff, JE, Roo, GN, Arnold, JE, Boorman, GA, and McConnell, EE: Neoplasm observed in untreated and corn oil gavage control groups of F344/N rats and (C57Bl/6N × C3H/HeN)F1 (B6C3F1) mice. JNCI 75 : 975-984, 1985.


