Homeostasis of Zinc and Iron in Mouse B16 Melanoma

Kedar N. Prasad, Charles R. Ahrens, and Judith M. Barrett

Department of Radiology, University of Colorado Medical Center, Denver, Colorado 80220

SUMMARY

Zinc and iron homeostasis in mouse B16 melanoma was studied by measuring whole-body turnover, organ retention, and organ uptake of $^{65}$Zn and $^{59}$Fe at various times after tumor transplantation. Results suggested that zinc and iron homeostasis was impaired at the advanced stage of tumor development. At 18 days, the spleen $^{59}$Fe uptake in mice with melanomas was considerably higher than in the control, which was primarily due to extramedullary erythropoiesis. The major portion of $^{59}$Fe (92—95 percent) in the spleens of both mice with melanomas and the control animals was in the supernatant fraction, in which 92 percent of $^{59}$Fe was nondialyzable. In mice with melanomas, cycloheximide treatment prevented the increase in spleen $^{59}$Fe uptake completely, whereas in the control animals, the antibiotic had no effect on the spleen $^{59}$Fe uptake. The spleen $^{59}$Fe uptake in both mice with melanomas and the control mice was insensitive to actinomycin D treatment. A certain degree of anemia was associated with the mice with melanomas, as evidenced by a low hematocrit value, a low retention of $^{59}$Fe in the blood, and a relatively high number of reticulocytes in the peripheral blood.

INTRODUCTION

Zinc and iron are physiologically essential. The concentration of these trace metals in melanin granules is high (7). Whether the homeostasis of zinc and iron is impaired during the growth of melanoma is unknown. Tumor tissues of spontaneous mammalian adenocarcinoma and dibenzanthracene-induced spindle-cell sarcoma in mice take up abnormally large amounts of $^{65}$Zn (3). Another study reports (11) that organ $^{65}$Zn uptake in tumor-bearing mice is similar to that of controls, but $^{60}$Co and $^{64}$Cu metabolism is altered. This paper describes the homeostasis of zinc and iron in mouse B16 melanoma, using $^{65}$Zn and $^{59}$Fe. Changes in whole-body turnover, organ retention, or organ uptake of radioactive trace metals is considered to be a reflection of a defective homeostasis.

MATERIALS AND METHODS

Male black BAF6/J mice weighing from 16 to 20 gm were maintained on Purina chow and water ad libitum for 5—7 days before experimentation. The transplantation method involved a subcutaneous implantation of macerated tumor bits (melanoma B16) into the right axillary region by a Becton Dickinson BD13 needle. Tumor growth was divided into three periods, according to tumor size. Using $^{65}$Zn and $^{59}$Fe, the homeostasis of zinc and iron in mice with melanomas was studied at 5—6 days (undetectable tumor), 10 days (medium-size tumor), and 18—20 days (large-size tumor, no metastasis).

Radioisotopes. Carrier-free $^{65}$Zn (International Chemical and Nuclear Inc.) and ferrous citrate $^{59}$Fe (specific activity, 24.4 mc/mg, Abbott, Inc.) were diluted with 0.1 N HCl (10 mc/ml).

Whole-Body Turnover of $^{65}$Zn and $^{59}$Fe. Mice were injected i.p. with $^{65}$Zn (1 mc/mouse) or $^{59}$Fe (1.5 mc/mouse) 6 days after transplantation, and whole-body radioactivity was assayed by a well-type scintillation detector for a 13-day period. Each mouse was placed into a 50-ml plastic tube which was filled with sponge gauze to maintain a constant counting geometry. It was necessary to hold the gauze in place because the animal tended to displace the gauze. The initial whole-body radioactivity was counted at 1 hour after injection of $^{65}$Zn or $^{59}$Fe. The whole-body counts at various postinjection times were expressed as percent of initial counts.

Organ Retention of $^{65}$Zn and $^{59}$Fe. At the end of the whole-body turnover study, the animals were decapitated. Parts of the pancreas, liver, blood, tumor, and the entire spleen, kidney, testis, and femur were excised, rinsed in saline, blotted on sponge gauze, and transferred into a pretarred 10-ml plastic culture tube. The organ weight was recorded, and the radioactivity was assayed by a well-type scintillation detector. Cpm/gm of tissue were converted to dpm/gm and expressed as percent of initial whole-body dpm. The organ retention of $^{65}$Zn or $^{59}$Fe in mice with melanomas then was expressed as percent of control.

Organ Uptake of $^{65}$Zn and $^{59}$Fe. Mice were injected i.p. with $^{65}$Zn (1 mc/mouse) or $^{59}$Fe (1.5 mc/mouse) at 5, 10, and 18 days after tumor transplantation and were decapitated 1 hour later. Radioactivity in the pancreas, liver, spleen, kidney, testis, blood, tumor, and whole femur was assayed as described above. Cpm/gm of tissue were converted to dpm/gm and then organ radioactivity in mice with melanomas was expressed as percent of control.

Dialysis of Spleen $^{59}$Fe. To evaluate whether the increased spleen $^{59}$Fe uptake in mice with melanomas represents a dialyzable or nondialyzable fraction, the following experiment was performed. Mice were injected i.p. with $^{59}$Fe (0.4 mc/gm) 18 days after tumor transplantation and were decapitated 1 hour later. Three spleens from mice with melanomas and five
from the controls were pooled separately. A 2.5 percent spleen homogenate was prepared in Tris-buffer (pH 7.3); the supernatant was dialyzed against the same buffer at 4°C for a 17-hour period.

Effect of Cycloheximide on Spleen 59 Fe Uptake. To measure whether the increased spleen 59 Fe uptake in mice with melanomas depended upon the protein synthesis, cycloheximide, a protein inhibitor (1, 5, 13), was used. Cycloheximide (20 mg) was dissolved in 0.2 ml of absolute ethyl alcohol and then diluted with 3.8 ml of saline (5 mg/ml). Mice were injected i.p. with cycloheximide (1 mg/mouse); after 1 hour, they were injected i.p. with 59 Fe (1.5 μc/mouse) and were decapitated 1 hour later. The control animals received an equivalent volume of ethanol-saline mixture before the administration of 59 Fe. Cycloheximide (1 mg/mouse) inhibited protein synthesis in 94—96 percent of the controls (12). The spleen cpm was converted to dpm.

Effect of Actinomycin D on Spleen 59 Fe Uptake. Since data showed that a portion of spleen 59 Fe uptake in mice with melanomas depended upon protein synthesis, the question arose whether the synthesis of this protein requires DNA-dependent RNA synthesis. Therefore, spleen 59 Fe uptake after the administration of actinomycin D, a RNA inhibitor (10), was studied. Actinomycin D was dissolved in saline (100 μg/ml). Mice were injected i.p. with actinomycin D (1 μg/gm); after 4 hours, 59 Fe (1.5 μc/mouse) was administered i.p. and they were decapitated 1 hour later. The control animals received an equivalent volume of saline before the administration of 59 Fe. The antibiotic (1 μg/gm) inhibits RNA synthesis completely 4 hours after its injection (12).

Histology of Spleen, Liver, and Kidney. During this investigation, enlargement of the spleen was noted in mice with melanomas. Therefore, for a histologic study, the spleen was fixed in 10 percent neutral buffered-formalin or Bouin’s fluid 5, 10, and 18 days after transplantation, and sections were stained with eosin-hematoxylin or Giemsa stain. The histology of the liver was also studied to assess the extramedullary erythropoiesis. The kidney histology was investigated to locate the protein precipitate which might have resulted due to lysis of RBC.

To estimate the degree of anemia, the hematocrit value was determined, and the peripheral blood smear in Wright stain was studied.

RESULTS

Zinc and iron homeostasis in mice with melanomas was investigated by measuring the whole-body turnover, organ retention, and organ uptake of 65 Zn and 59 Fe.

Whole-Body Turnover of 65 Zn and 59 Fe. Whole-body turnover of 65 Zn was slower in mice with melanomas than in the controls; however, 59 Fe turnover was similar in both groups (Chart 1). The latter was expected.

Organ Retention of 65 Zn and 59 Fe. Chart 2 shows that the pancreas, kidney, liver, testis, and spleen of mice with melanomas retained more 65 Zn than those of the controls. The kidney, liver, and testis retained more 59 Fe and the blood less 59 Fe than those of the controls, whereas the spleen and femur showed no significant change compared to the controls.
Tumor Retention of $^{65}$Zn and $^{59}$Fe. Tumor retention of $^{65}$Zn was less than that of $^{59}$Fe. Retention of these trace metals by the tumor was considerably greater than the uptake (Tables 1, 2).

Organ Uptake of $^{65}$Zn and $^{59}$Fe. In mice with melanomas, $^{65}$Zn uptake by the pancreas, liver, spleen, kidney, testis, and blood, during the entire observation period, and $^{59}$Fe uptake by the liver, kidney, testis, and blood, 5 and 10 days following transplantation, were similar to those of the controls. However, at 18 days spleen $^{59}$Fe uptake in mice with melanomas was considerably higher than that of the controls. Chart 3 demonstrates the change in spleen weight and spleen $^{59}$Fe and spleen $^{65}$Zn uptake as a function of the posttransplantation time. Femur $^{59}$Fe uptake in mice with melanomas was about 25 percent higher than the controls at 5, 10, and 18 days after transplantation.

Dialysis of Spleen $^{59}$Fe. The spleen homogenates of mice with melanomas had twice as much $^{59}$Fe as the controls. The supernatant fractions of both the melanoma and the control spleen homogenates had 92–95 percent of homogenate $^{59}$Fe. After dialysis, the supernatant fractions of the melanoma and the control retained 92 percent of their initial radioactivity.

Effect of Cycloheximide and Actinomycin D on Spleen $^{59}$Fe Uptake. In the mice with melanomas, cycloheximide treatment prevented the increase in spleen $^{59}$Fe uptake completely, whereas in the control animals the antibiotic has no effect on the spleen $^{59}$Fe uptake (Chart 4). Actinomycin D had no effect on the spleen $^{59}$Fe uptake of either the melanomas or the controls 4 hours after its administration.

Tumor Uptake of $^{65}$Zn and $^{59}$Fe. No tumors were detected at 5 days after transplantation. At 18 days, the tumor uptake of $^{65}$Zn and $^{59}$Fe was less than at 10 days (Table 2).

### Table 1

<table>
<thead>
<tr>
<th>Radioisotopes</th>
<th>Tumor (dpm/gm × 10⁶)</th>
<th>Liver (dpm/gm × 10⁶)</th>
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<tbody>
<tr>
<td>$^{65}$Zn</td>
<td>6.2 ± 0.3</td>
<td>9.4 ± 0.9</td>
</tr>
<tr>
<td>$^{59}$Fe</td>
<td>16.4 ± 1.9</td>
<td>38.0 ± 7.0</td>
</tr>
</tbody>
</table>

Retention of $^{65}$Zn and $^{59}$Fe (tumor vs liver). Animals were injected i.p. with $^{65}$Zn (1 μc/mouse) or $^{59}$Fe (1.5 μc/mouse) 6 days after tumor transplantation and were killed 13 days later. The tumor radioactivity was compared with the liver of the same animal.

$^a$Standard error of the mean.

$^b$Number of animals.

### Table 2

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Posttransplantation time (dpm/gm × 10⁶)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 days</td>
</tr>
<tr>
<td>Tumor, $^{65}$Zn</td>
<td>Tumor undetectable</td>
</tr>
<tr>
<td>Liver, $^{65}$Zn</td>
<td>44.0 ± 8.0 (3)</td>
</tr>
<tr>
<td>Tumor, $^{59}$Fe</td>
<td>Tumor undetectable</td>
</tr>
<tr>
<td>Liver, $^{59}$Fe</td>
<td>32.0 ± 3.0 (9)</td>
</tr>
</tbody>
</table>

Uptake of $^{65}$Zn and $^{59}$Fe (tumor vs liver). Mice were injected i.p. with $^{65}$Zn (1 μc/mouse) or $^{59}$Fe (1.5 μc/mouse) and killed 1 hour later. The tumor radioactivity was compared with the liver of the same animal.

$^a$Standard error of the mean.

$^b$Number of animals.
Histology of the Spleen, Liver, and Kidney. The spleens of mice with melamomas showed hyperplasia of the lymphocytes at 10 days after tumor transplantation (Fig. 1b); however, at 18 days in addition to lymphocytic hyperplasia, extramedullary erythropoiesis was also present (Fig. 1c). The degree of extramedullary erythropoiesis in the spleens of mice with melamomas was compatible with the animals concurrent pathology. Fig. 1a represents the spleen of a control mouse. The livers of the mice with melamomas showed no extramedullary erythropoiesis and the kidneys did not contain any visible protein precipitate.

Peripheral Blood. At 18 days after transplantation, the hematocrit values in the control mice varied from 39 to 46 percent, whereas in the melanoma it ranged from 33 to 37 percent. At this time, the peripheral blood of the mice with melamomas showed high reticulocyte counts (6—10 percent) compared to about 3 percent in the controls. The erythrocytes appeared to be normal and there was no sign of hemolytic anemia. No erythrocyte parasites of the erythrocytes were found in the smear of peripheral blood of the melanoma. Platelet and leucocyte counts were similar to those of the controls, but a slight increase in neutrophils and a substantial decrease in lymphocytes were noticed.

DISCUSSION

Zinc and iron homeostasis in mice with melamomas has been studied, measuring the whole-body turnover, organ retention, and organ uptake of $^{65}$Zn and $^{59}$Fe. The whole-body $^{65}$Zn turnover in melamoma is slower than in the controls. This difference is also reflected in a higher retention of organ $^{65}$Zn at the advanced stage of tumor development. Although whole-body $^{59}$Fe turnover in melanoma is similar to that of controls, there is a marked difference in organ retention (kidney, liver, and testis retained more, and blood retained less $^{59}$Fe than the controls). It seems that zinc and iron homeostasis is impaired at the advanced stage of tumor development, but the mechanism is unknown. The greater retention of $^{65}$Zn and $^{59}$Fe by the tumor indicates a very slow turnover of these trace metals in this tissue. Another possibility may be that a high percentage of iron retention may arise from hemorrhage into the tumor, with the subsequent concentration of iron in macrophages.

At 10 days after transplantation, the tumor is more vascularized and less necrotic than at 18 days, which may explain, in part, the reason for the higher tumor $^{65}$Zn and $^{59}$Fe uptake at 10 days; however, a difference in stable zinc and iron concentration could also account for this.

In mice with melamomas, organ $^{65}$Zn uptake, during the entire period of observation, and $^{59}$Fe uptake, at 5 and 10 days after transplantation are similar to that of the controls. The femurs of mice with melanomas pick up 25 percent more $^{59}$Fe than the controls, indicating an increased hematopoietic activity. At 18 days, the spleen $^{59}$Fe uptake in mice with melamomas is twice that of the controls. This appears to be related to the extramedullary erythropoiesis in the spleen. The dialysis experiment shows that a major portion of $^{59}$Fe (92—95 percent) in the spleen of both the melanoma and the control is in the supernatant fraction, in which 92 percent of $^{59}$Fe is nondialyzable. Inhibition of the portion of spleen $^{59}$Fe uptake in mice with melamomas by cycloheximide, an inhibitor of protein synthesis, suggests that $^{59}$Fe uptake by the spleen erythroid element is sensitive to protein inhibition. The exact mechanism of this phenomenon is unknown. However, erythroid elements of a spleen with melanoma are dividing at a relatively rapid rate, and therefore show greater sensitivity to cycloheximide than those of control. Cycloheximide has no effect on the spleen $^{59}$Fe uptake in the control animals, indicating that protein synthesis is not required for $^{59}$Fe uptake. Actinomycin D has no effect on the spleen $^{59}$Fe uptake of either mice with melanomas or control mice within 4 hours after its administration, which may imply that the template for the synthesis of that spleen protein, which is needed for a portion of spleen $^{59}$Fe uptake, is relatively stable, or that the synthesis of that protein does not require DNA-dependent RNA synthesis.

The relatively high retention of $^{59}$Fe by the livers of mice with melamomas is not due to extramedullary erythropoiesis, but the exact mechanism is unknown. A very high retention of $^{59}$Fe in the kidneys of mice with melanomas is an interesting phenomenon. The question arises whether the tumor has caused an exacerbation of erythrocytosis parasites of the erythrocytes with resultant lysis of cells and loss of hemoglobin into the kidney. The peripheral blood of the mice with melanomas showed no visible parasites of erythrocytes or any other sign of hemolytic anemia. The kidney also does not show any detectable amount of protein precipitate. The above findings indicate that the high retention of $^{59}$Fe by the kidney is not related to hemolysis; however, the exact mechanism of this phenomenon remains uncertain.

The mice with melanomas showed a certain degree of anemia as evidenced by a low hematocrit value, a low retention of $^{59}$Fe in the blood, and a high number of reticulocytes in the peripheral blood compared to the control. The problem of anemia in cancer has been discussed extensively by Price and Greenfield (9). Hyman (4) has shown that in the patient with malignant melanoma the life span of erythrocytes is reduced considerably and this may account for the anemia. In this study, a certain degree of anemia appears to be associated with melanoma; its etiology is unknown.

Enlargement of the spleen following tumor transplantation deserves a separate comment. Aplastic bone marrow induces splenomegaly associated with hyperplasia of lymphocytes (8). Certain strains of Friend leukemia virus also cause increases in spleen weight and $^{59}$Fe uptake (2, 6). In this study, splenomegaly associated with lymphocytic hyperplasia may be due to an immunologic response to tumor transplantation or to the melanoma virus.

REFERENCES

3. Heath, J. C., and Liquier-Milward, J. The Distribution and Func-
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Fig. 1. Section (a, x 30) of a control spleen. Section of a spleen with melanoma at 10 days (b, x 30) and 18 days (c, x 30) after transplantation. Note the lymphocytic hyperplasia in b and c.

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