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Serum and Tissue Copper Content in Two Mammary Adenocarcinomas with Different Biological Behaviour

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Abstract—*We have determined serum copper levels in BALB/c female mice subcutaneously inoculated with two related mammary adenocarcinoma tumors. Serum copper content increased progressively with tumor size in animals bearing either variant, and reached levels up to four times those of control mice. In contrast, copper levels detected by cytochemistry in tumor cells are higher in the slow growing tumor variant. It is suggested that the stronger angiogenic effect previously described for this variant could be related to its higher cellular copper content.*

INTRODUCTION

SERUM copper levels have been found to be elevated in patients with a variety of malignant lymphoproliferative disorders, such as leukemia, Hodgkin's disease and non-Hodgkin's lymphoma, and in a variety of tumors of digestive organs, osteosarcoma, cervix, uterus, breast and bronchus cancer [1–8]. In these studies, copper levels had been determined in patients already bearing different kinds of tumors at different clinical stages and with a variable course of the disease. Plasma ceruloplasmin (Cp), an α 2 glycoprotein which accounts for 85–95% of copper in blood, has been investigated in very few experimental models where the Cp levels were followed during the evolution of tumor growth. Ungar-Waron *et al.* [9] observed that Cp levels increased 4–8 fold during the progression of Vx-2 rabbit carcinoma, often before tumors could be detected by palpation. On the other hand, in rabbits challenged with different antigens, Cp levels did not vary significantly; while in pregnant rabbits, Cp levels increased up to 3 fold [9]. Linder *et al.* [10] found increased Cp levels in rats bearing transplantable mammary tumors, showing that the faster the growing rate of the tumor, the higher the Cp levels.

The present study was undertaken to evaluate the serum copper levels in mice bearing two mammary adenocarcinomas of the same origin but with different biological behaviour. Specifically, we analyzed whether serum copper levels correlated with tumor latency, tumor size and tumor growth rate. In addition, in order to shed some light on the metabolism of copper during tumor growth, we determined in the same animals by means of a histochemistry technique the copper present in the tumor cells, in the liver, in a resting and in a lactating mammary gland. The results show that mice bearing either tumor variant had increased levels of serum copper, although mice carrying the most aggressive tumor showed an earlier increase. In contrast, the slow growing tumor line exhibit significantly higher number of copper positive cells, as detected by histochemistry.

MATERIALS AND METHODS

1. Animals and tumors

The murine mammary adenocarcinoma M3 originated spontaneously in a female BALB/c mouse from a resting mammary tissue. The original M3 tumor has been maintained by subcutaneous transplants in syngeneic mice. MM3 was obtained by subcutaneous trocar implants of M3 lung metastases into syngeneic mice [11]. Once MM3 achieved growth and metastatic characteristics with stable behaviour, it was subsequently maintained by s.c. grafts of tumor tissue. M3 adenocarcinoma shows a latency of 6–8 days and tumor size reaches about 25 mm 25 days after s.c. transplantation. Tumor develops in 100% of the mice, which die after 35 days. Forty percent of the animals show lung metastases with an average of six nodules per lung. The MM3 tumor grows slower than the

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M3 variant and reaches 12 mm 25 days after transplantation. Ninety-five percent of the mice develop tumors and all die after 50–55 days with high incidence of lung metastases.

Tumor size was recorded at different times after implantation with graduated calipers, by measuring two diameters of the tumor through the skin and the size was calculated as $\sqrt{d_1 \times d_2}$. Tumor size was measured at different times for the M3 and the MM3 variant, because MM3 has a slower growth rate than M3. Forty-one BALB/c female mice were inoculated with the M3 tumor and 51 BALB/c female mice were inoculated with the MM3 tumor. After tumor development, animals were divided into three groups according to tumor size: (1) 0–10 mm; (2) 10.1–20 mm; (3) 20.1–30 mm.

Fifty-five normal BALB/c female mice of the same age were used as controls.

2. *Determination of serum copper*

Animals were killed by exsanguination after ether anesthesia. Plastic tubes used for blood collection and for sera storage were carefully cleaned with 1% EDTA and rinsed with copper-free bidistilled water [12]. Sera were kept at −40° C until used for analysis. Total serum copper levels were determined on 200 μl serum samples by the colorimetric Bathocuproin method [13] using the Merckotest 3319 reagent kit. This method for copper quantification, is based on the use of an acid hydroquinone solution (90 nM) which dissociates the copper from the protein complexes and converts it to the reduced Cu^+ form. After deproteinization with trichloracetic acid (20%), copper level in solution is determined spectrophotometrically at 480 nm with a specific copper reagent (sulfonated bathocuproin). Standard curve shows lineal absorbance between 0.5 ppm and 2 ppm. As an internal standard, pooled normal human sera was used throughout the experiments.

3. *Cytochemical methods*

Cellular copper levels were detected by cytochemistry. All solutions were prepared with distilled–deionized water and the surgery instruments used for tissue manipulation were washed with 1% EDTA. Paraffin-embedded tissues fixed in neutral formaldehyde were sectioned 5–10 μm thick and stained either with: (1) rubeanic acid for 24 hr (Uzman's method) and counterstained with light hematoxilin 15 sec or May Grunwald 1 : 20; with this technique as little as 6 ng copper stains black–green intracellularly with fair specificity [14, 15]; or (2) diphenylcarbazone for 2–4 hr, where as little as 2 ng copper stains red–violet intracellularly [15]. As positive controls for copper staining, either 20-day-old mouse liver or chick embryo liver were used [15]. Five different M3 and five different MM3 tumors 0–10 mm in size were processed; 100 sections for each tumor were analyzed. Livers from three mice bearing M3 and MM3 tumors were also analyzed (60 sections for each liver). As controls, cytochemistry was also performed on resting and lactating mammary tissues.

4. *Statistical analysis*

Quantitative differences between serum copper levels in the different groups were determined by the Student *t*-test. Galton's linear regression analysis [16] and correlation coefficients for serum copper levels and tumor size were performed. Quantitative differences between positive copper cells in the two related adenocarcinomas were determined by the χ^2 test.

RESULTS

BALB/c female mice were implanted s.c. with two variants (M3 and MM3) of a mammary adenocarcinoma showing different biological behaviour. Serum copper levels were determined in these mice after the tumors reached different sizes. The results, summarized in Table 1, show that mice bearing tumors under 10 mm in size had serum copper levels not significantly different from those present in normal animals. Nevertheless, mice bearing the rapidly growing variant M3 showed a tendency for a faster increase in the copper levels. Earlier increase of serum copper levels in some of the mice bearing M3 tumors could be correlated with a worse prognosis, as these animals always presented a shorter survival time than animals bearing MM3. Once the tumors reached 10 mm in size, serum copper levels began to increase steadily and proportionally to the size of the tumor, reaching levels up to 4-fold higher than those present in control mice. No significant differences were found in copper levels in mice bearing tumors similar in size of either variant. Regression and correlation analysis (Galton's test) show significant correlation between tumor size and serum copper levels: $r = 0.40$ for M3 tumor and $r = 0.60$ for MM3 tumor.

Cellular copper levels were investigated by cytochemistry. Copper granules were present in M3 tumor mostly around cell nuclei and occasionally spread in the cytoplasm (Fig. 1). MM3 tumor cells also show the presence of copper, although the stain was mainly associated to the nuclei. In addition, more copper positive cells were localized near the vascular channel (Fig. 2). The number of copper positive cells present in MM3 tumors (46% ± 2%) was significantly higher than that present in M3 tumors (20% ± 2%). χ^2 analysis showed that the five different tumors studied within each

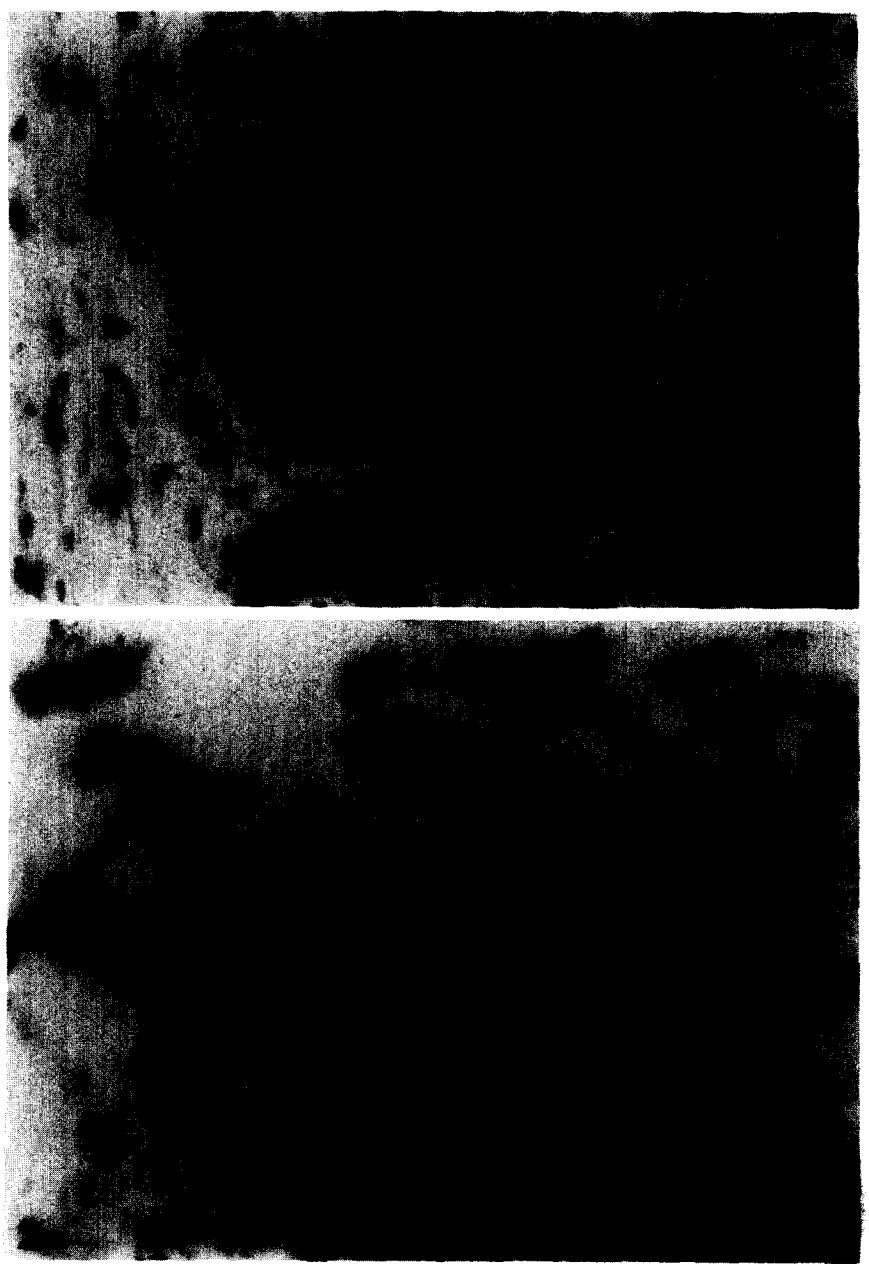

Fig. 1. *Paraffin section of mammary adenocarcinoma of mouse stained for copper by diphenilcarbazone technique. The metal is present in some cells* × 400.

Fig. 2. *MM_3 mammary adenocarcinoma stained for copper with rubeanic acid. The metal is mainly localized in granules at periphery of nucleus* 1000 × *positive cells are present near the vascular channel.*

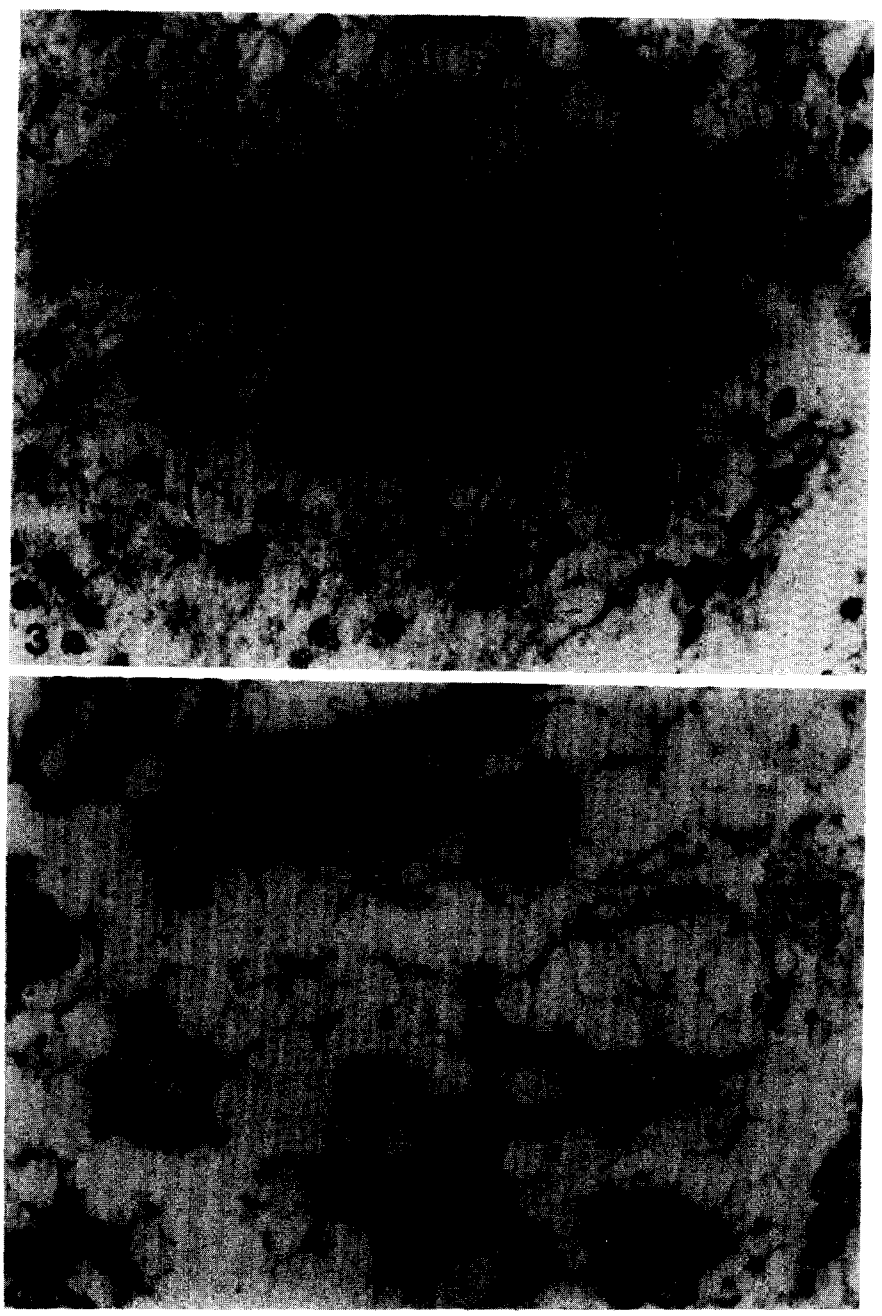

Fig. 3. Paraffin section of liver from chick embryo liver stained for copper by diphenylcarbazone technique. The metal is localized granules × 1000.

Fig. 4. Lactating mammary gland. Copper cytoplasmic granules in some cells diphenylcarbazone stain × 400.

Table 1. *Serum copper levels of mice bearing M3 and MM3 tumors at different stages*

Tumor size (mm)	Serum copper levels (μg/100 ml) Controls Mean ± S.D.	M3 Mean ± S.D.	MM3 Mean ± S.D.
—	49.18 ± 18.86(56)	—	—
0–10	—	75.0 ± 34.28(8)	48.6 ± 25.80(14)
10.1–20	—	116.0 ± 61.20(22)	103.2 ± 69.63(25)
20.1–30	—	209.1 ± 105.28(11)	195.0 ± 64.81(12)

Serum copper was determined in normal mice and in mice bearing M3 and MM3 tumors at different growth stages, as described in Materials and Methods. Copper levels in mice with either tumor under 10 mm in size are not statistically different from control mice. Mice bearing either tumor over 10 mm in size have copper levels significantly higher than control mice ($P < 0.001$, Student's *t*-test). Figures in parenthesis represent the number of animals in each group.

group were 90–95% homogeneous. On the other hand, comparison of M3 with MM3, shows that both tumors are heterogeneous with a $P < 0.001$. As positive controls for copper staining, cytochemistry was also performed on chick embryo liver (Fig. 3) and on 20-day-old mouse liver, showing uniform cytoplasmic staining. Active lactating mammary tissues from normal mice were also studied, showing large cytoplasmic copper positive granules in several acinar cells (Fig. 4), while resting mammary glands (15 days after lactation) had few cytoplasmic granules in some acinar cells.

DISCUSSION

In the present work we have analyzed the serum copper levels of BALB/c mice subcutaneously inoculated with two related mammary adenocarcinoma tumors with different growth rate, different karyotype, different tumorigenicity, different metastatic potential (17) and angiogenic activity. In the initial phase of tumor growth, copper levels of mice bearing either tumor did not differ from levels present in control mice, although animals bearing the variant with faster growing abilities (M3) showed a tendency for higher levels. Once the tumors grew over 10 mm in size, copper levels increased progressively and proportionally to the size of the tumor, regardless of the different growth rate and prognosis of M3 and MM3. These results agree with those previously described for other experimental tumor models as well as in human cancers. Ungar-Waron *et al.* [9] and Linder *et al.* [10] showed that rats bearing fast growing tumors exhibited higher ceruloplasmin levels. Pizzolo *et al.* [18] observed increased serum copper levels in individuals with breast cancer, both at the onset of the disease and during active phase and suggested that copper level could be a useful parameter in evaluating the activity and spreading of the disease.

We have also analyzed cellular copper levels in the same mice bearing either M3 or MM3 tumors. Both tumor variants showed the presence of copper positive cells although MM3 tumors contained twice as many positive cells (specially with nuclear localization) than M3 tumors. This is probably related to the higher metastatic ability and angiogenic activity of the MM3 variant. On the other hand, normal lactating mammary glands showed high and homogeneous distribution of copper staining, while resting glands expressed lower stains. This would suggest an active role for Cu^{2+} during active metabolic activity. The different pattern of staining observed in normal glands and tumor mammary cells, suggests an alteration of cellular components and functions during malignant transformation. It is worth mentioning that Apelgot *et al.* [19] also found copper concentrated in the nuclei of Krebs ascitic cells in mice 20 hr after the injection of radioactive copper.

The fact that tissue copper localizes preferentially close to blood vessels in MM3 bearing mice, would suggest a flow of the metal from the blood to the tumor. In addition the higher copper levels in MM3 tumor cells could explain its stronger angiogenic activity assessed both on chorioallanthoic membrane and on endothelial cells *in vitro* (unpublished observations). McAuslan and Reilly [20] showed that copper ions exert chemotactic attraction of endothelial cells *in vitro* and Raju *et al.* [21] suggested that copper ions are involved in the sequence of events that lead to angiogenesis.

Increased serum copper levels observed in our system and increase of ceruloplasmin and copper in other models and in human malignancies, could be associated either with decrease catabolism or with enhanced synthesis of ceruloplasmin [22]. This enzyme is a copper amino-oxidase that can oxidize polyamines, which in turn are associated with tumor progression [23]. On the other hand,

high levels of plasma ceruloplasmin in neoplasia could be the host's response against high levels of toxic superoxide anions [24] due to reduced levels of superoxide dismutase. It was suggested that ceruloplasmin neutralizes superoxide ions [25] in place of the low activity of superoxide dismutase present in tumors [26–28].

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