Copper Histochemistry of 5 Murine Tumors and Their Respective Metastases

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Abstract. Tissue copper content has been evaluated in 4 murine mammary adenocarcinomas, 1 murine lung adenocarcinoma and their respective metastases. Histochemical techniques have been used to analyze copper distribution in tumor tissues. It is observed that the degree of copper staining is inversely related to tumor differentiation. As the copper level reflects one of the metabolic changes in the host carrying the tumor, it is suggested that it could be used as a good marker for tumor differentiation.

Introduction

Abnormal biodistribution of trace metals in tumor-bearing animals has already been described by Apelgot et al. [1] in 1986. They have observed higher amounts and higher uptake of copper in malignant tissue of tumor-bearing rats. It has also been described in breast cancer [2–5], malignant neoplasms of the female reproductive system [6], hepatic and duct cancer, and stomach and large bowel cancer [7, 8]. A higher chemically quantified copper accumulation has been noted than in the corresponding nonmalignant tissue.

Only few results have been reported concerning the histochemical localization of copper in tumor tissue. Haratake et al. [7] and Vecchio et al. [9] have reported copper binding proteins in a fibrolamellar liver cell carcinoma. Studying the oral mucous and basal skin of squamous cell carcinoma, Bedrick et al. [10] determined by histochemical methods the presence of high amounts of copper in invasive cells.

In a previous work, studying two murine mammary adenocarcinomas with a different incidence of lung metastases we described

the intracellular copper distribution, as well as the elevation of serum copper [11]. In the same tumor system, higher levels of ceruloplasmin during tumor evolution were observed [12].

However, the relationship between the presence of copper among tumors presenting with different degrees of differentiation and their metastases has not yet been studied.

Materials and Methods

Tumors

Four murine mammary adenocarcinomas and 1 murine lung adenocarcinoma have been studied. All tumors were maintained by subcutaneous transplants into syngeneic BALB/c female mice.

M1 is a spontaneous papillar mammary adenocarcinoma with a homogeneous pattern. The cells are regular and homogeneous in size. At time of death, mice present a higher incidence (95%) of small lung metastatic nodules with peritumoral lymphocyte infiltration. Degenerative areas are present in the lung metastases.

M3 is a spontaneous pseudoglandular mammary adenocarcinoma; the cells are heterogeneous in size, with oval cells alternating with round cells. It has a moderate incidence of lung metastases (45%) and a low incidence of liver metastases with medium size at time of death (45 days).

MM3 is a pseudoglandular mammary adenocarcinoma, a cellular subline obtained by subcutaneous transplantation of lung metastases from the M3 tumor. The cells are homogeneous in size, mostly oval. The metastatic lung capacity of MM3 is higher than M3 (90%), the metastases are regular and large in size. Growth times from subcutaneous inoculation of the tumor to its palpability were longer than for M3; the tumor killed the animal in about 45–60 days [13].

S13 is a spontaneous mammary adenocarcinoma, obtained after implantation and excision of foreign body, which shows an anaplastic transformation without any glandular structure. The cells are homogeneous, round and small in size. It shows large lung metastases, high in incidence (95%). The timelag between tumor inoculation and the death of the animal was approximately 30 days.

P is a spontaneous lung adenocarcinoma with scanty pseudoglandular structure. The cells are large, the common pattern of cells is oval to polygonal. It has a moderate incidence of lung metastases (35%) which often appear with interstitial pneumonia characterized by interstitial inflammation. The average survival for these tumor-bearing mice is similar to the survival time of animals inoculated with MM3.

Lung metastatic nodules of M3, MM3, and S13 essentially revealed the same morphologic characteristics of the subcutaneous transplants of their respective primary tumors.

Our concept of the degree of differentiation derives from two parameters: the metastatic behavior associated with the morphologic features: M1 could be considered a well-differentiated adenocarcinoma, M3 a semidifferentiated adenocarcinoma, MM3 an undifferentiated adenocarcinoma, S13 an undifferentiated adenocarcinoma with 'sarcomatoid' tumor elements, and P a semidifferentiated adenocarcinoma.

To avoid individual variability, primary tumors and metastases were obtained from the same tumor-bearing mice.

Normal Tissues

Salivary gland and adrenal cortex from BALB/c mice were used.

Histochemistry

Primary tumors, lung and liver metastases and normal tissues were analyzed. Lung metastases from a P tumor were not studied because tumor cells could not be clearly identified in the area of pneumonia.

Solutions were routinely prepared with distilled and deionized water. The instruments employed to obtain tissue samples were washed with 1% EDTA solution. Tissue samples were obtained and fixed with neutral formaldehyde for 24 h. Fifty 5- to 10-μm-thick sections of paraffin-embedded tissue for each sample were obtained and stained with: (1) Rubeanic acid (dithiooxamide) at 36 °C for 24 h. The sections were then rinsed with 70% ethanol to remove background, and counterstained with Harris hematoxylin for 15 s [14]; (2) diphenylcarbazone at 36 °C for 2–4 h; after rinsing with double distilled water, the tissue sections were counterstained with Harris hematoxylin for 15 s [15]. Technical controls were per-
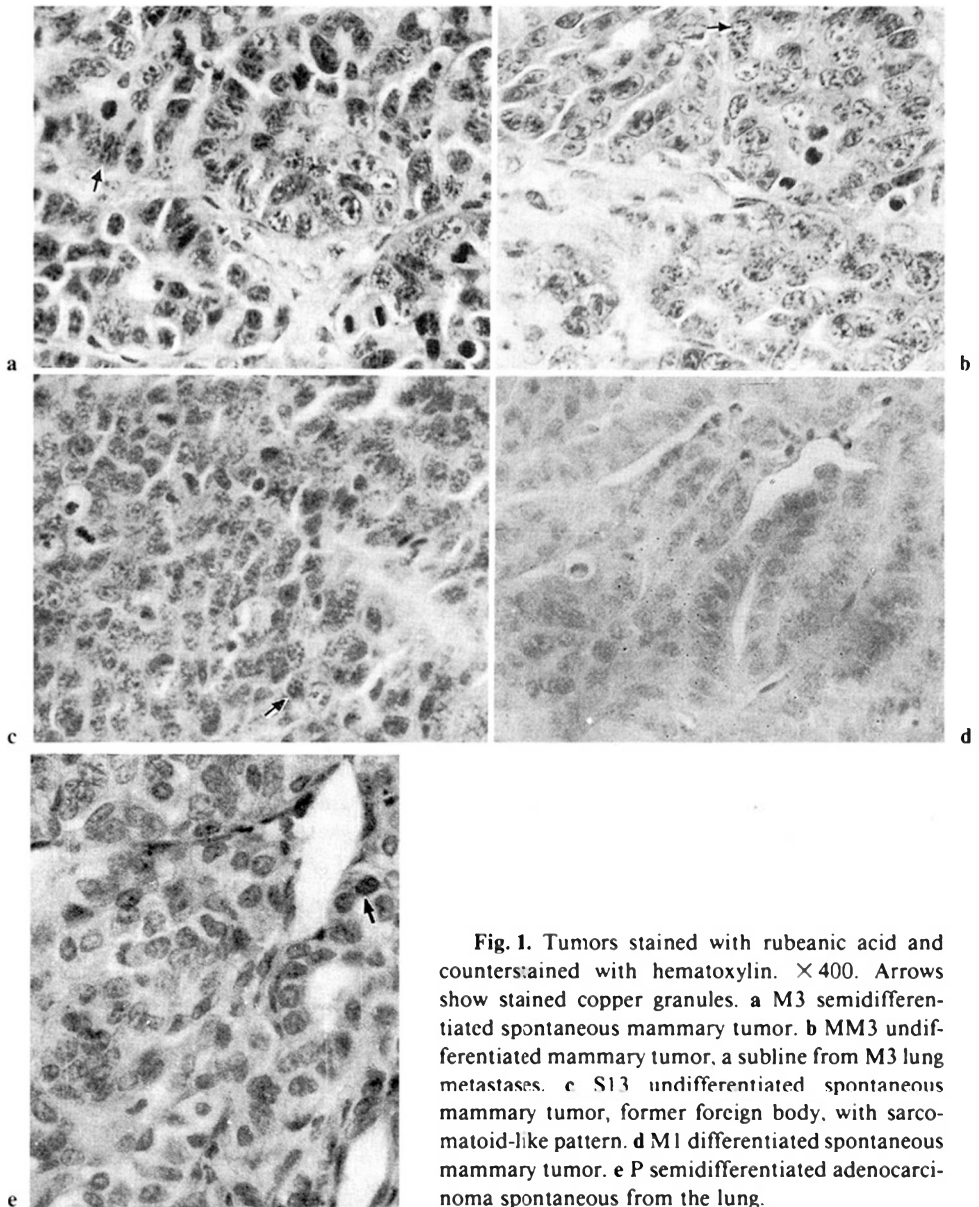
Fig. 1. Tumors stained with rubeanic acid and counterstained with hematoxylin. ×400. Arrows show stained copper granules. **a** M3 semidifferentiated spontaneous mammary tumor. **b** MM3 undifferentiated mammary tumor, a subline from M3 lung metastases. **c** S13 undifferentiated spontaneous mammary tumor, former foreign body, with sarcomatoid-like pattern. **d** M1 differentiated spontaneous mammary tumor. **e** P semidifferentiated adenocarcinoma spontaneous from the lung.

formed: (1) with 1% EDTA solution for 24 h and specific staining; (2) without EDTA solution or specific staining, but with hematoxylin for 15 s.

In order to count the positive copper-staining cells, slides were examined with a magnification of ×1,000. $10 \times 10^3 – 15 \times 10^3$ cells were counted in each primary tumor and $2 \times 10^3 – 5 \times 10^3$ cells were counted in each metastatic nodule. The results were expressed in average percentage of copper-positive cells. Significant differences between positive cells in the tumors and in the metastases were determined according to the χ^2 criterion.
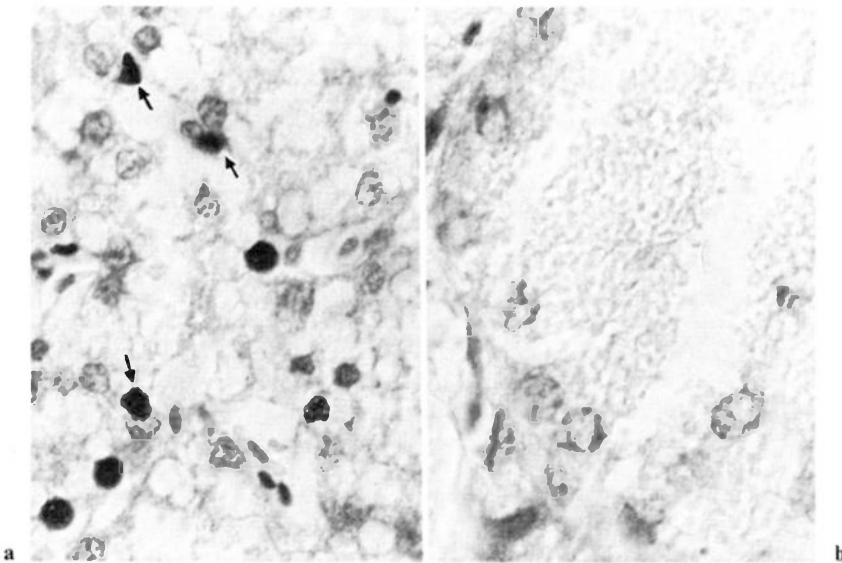
Fig. 2. Normal tissues from BALB/c mice. × 1,000. Granules are stained with rubeanic acid and counterstained with hematoxylin. **a** Adrenal cortex showing the fascicular structure of the tissue. The granules (arrows) are heavily stained and cytoplasmic. **b** Submaxillary salivary gland showing an acinar structure without copper granules. The lack of contrast is due to abundant presence of mucin.

Results

All the tumors examined, no matter their origin, showed cells histochemically staining for copper.

Furthermore, no differences in the localization of intracellular copper were observed between tumors with different degrees of differentiation. Copper granules were always observed in the nuclear and perinuclear areas (fig. 1). The cells showing the stronger staining for copper were located near the connective vascular axis and within the area of tumor invasion.

The normal tissues show cytoplasmatic-positive granules, with different degrees of staining. The adrenal cortex shows more cells staining for copper than salivary glands (fig. 2).

As mentioned above, the 3 primary tumors (M1, M3 and S13) showed positive staining for copper; however, different degrees of apparent copper content were observed. Highly differentiated M1 showed a few cells staining with rubeanic acid; the semidifferentiated M3 tumor showed a significantly higher percentage of staining than M1, and the undifferentiated S13 tumor showed the same degree of staining as the MM3 tumor. The semidifferentiated pulmonary tumor P showed a low percentage of staining cells although it was not significantly lower than the M3 tumor (table 1).

The percentage of cells with copper reactivity was the same for the primary tumor and for the corresponding metastases (table 1). Copper granules kept the same intracellular localization and tissue distribution

Table 1. Percentage (means ± SD) of copper-stained cells

Tumors	Primary	Lung metastases	Liver metastases
S13	43.9 ± 7.0 (50)	39.6 ± 6.90 (49)	NP
MM3	44.0 ± 7.0 (50)	58.0 ± 7.1 (46)	NP
M3	27.5 ± 6.3 (50)	30.0 ± 9.0 (24)	27.0 ± 7.0 (39)
M1	9.8 ± 4.0 (50)	6.0 ± 4.3 (31)	NP
P	18.0 ± 6.0 (42)	NP	NP

Values in parentheses = Number of sections examined on the same biological sample; NP = not present. Test of significance (χ^2 test): S13 vs. M3 $p < 0.05$; M3 vs. M1 $p < 0.01$; M3 vs. MM3 $p < 0.05$. There are no significant differences between the subcutaneous tumors and their corresponding metastases.

in the metastases (fig. 3a). Moreover, lung metastases showed lighter stained granules (fig. 3b), while the cells close to the biliary ducts, in the liver, showed heavily stained granules (fig. 3c). In spite of their hepatic localization, the percentage of positive copper-staining cells in the liver metastases was the same as for the primary tumors (table 1).

Discussion

Metal accumulation in tumor tissue has been measured previously by several authors. Schwartz et al. [5] reported that the level of potassium, phosphorus and copper are higher in malignant than in normal tissues. Mulay et al. [2] found a significant increase in copper, magnesium, manganese and zinc levels in cancerous breast tissues compared with their normal counterparts. De Jorge et al. [16], using a colorimetric method, observed an increase in copper in cancerous tissue and suggested that one of the first disorders in the breast carcinoma tissue involves a preferential accumulation of copper.

In a previous work we analyzed 2 mammary tumors (M3 and MM3) with different biological and metastatic behavior for copper presence. In spite of these differences, both tumors showed positively staining copper cells with mainly nuclear and perinuclear localization of granules. However, the total number of positive cells was higher in MM3 than in M3 [11].

We are unable to compare tumor and normal tissues because the cellular localization of copper-positive cell granules is different; for example, in controls its localization is mainly cytoplasmatic. Besides, the different degree of copper staining in normal tissue could depend on the capacity to synthesize metallothioneins; i.e., adrenal cortex versus salivary glands or their metabolic state, as in active mammary gland [17].

The primary mammary tumors used in the present work show a different degree of differentiation: the M1 is the most differentiated, the M3 is semidifferentiated, S13 is undifferentiated. The M1 has less copper-positive cells than M3, while S13 showed the highest percentage of copper-positive cells.
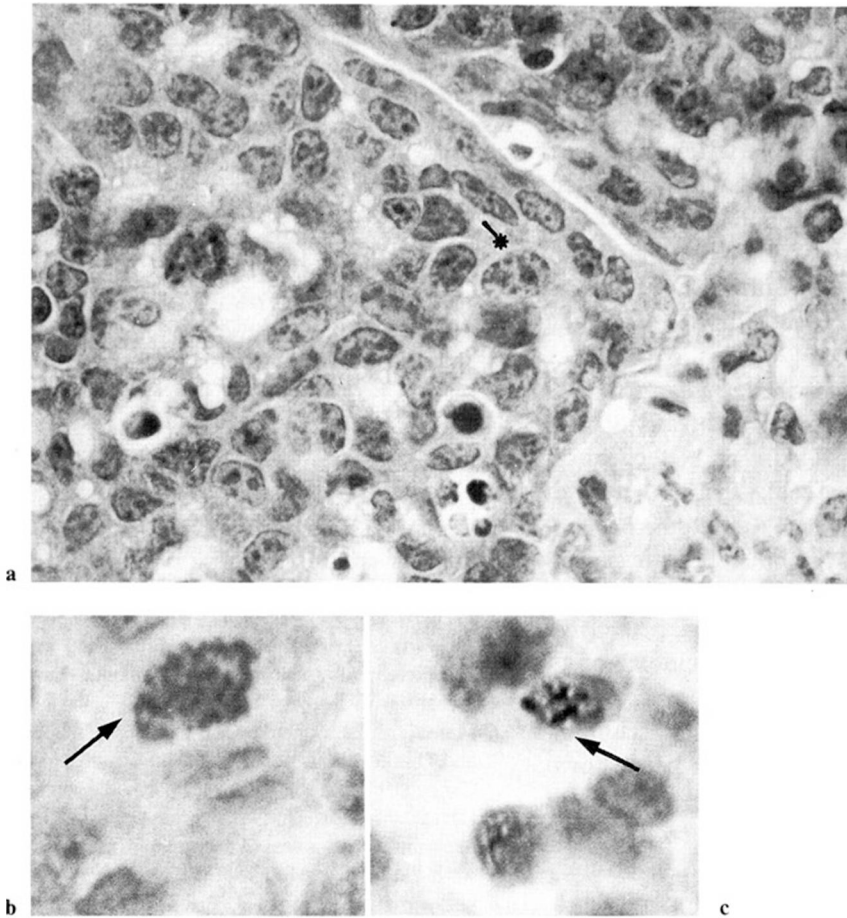
Fig. 3. All the samples are stained with rubeanic acid and counterstained with hematoxylin. **a** Lung metastasis from S13. Shows copper granules in the nucleus or near nuclear membrane. $\times 1,000$. **b** Detail of **a** (amplified twice) showing a cell from MM3 lung metastasis. Copper granules are located in the nucleus and near the nuclear membrane. **c** Detail (amplified twice) showing a cell from M3 liver metastasis. Copper granules are located in the nucleus. The copper stain is stronger than in lung metastases.

The comparison between all mammary subcutaneous tumors (M1, M3, MM3, S13) and their metastases showed that the percentage of copper-positive cells is preserved. Metastases of MM3, a parental subline of M3, showed a higher percentage of copper stained cells. MM3 showed the morphologic pattern of M3, but it has the same metastatic behavior as S13. The pattern of copper-positive cells seems to be related to tumor dissemination.

Copper in the cells is preferentially bound to metallothioneins, which allow the interchange of metals with the extracellular medium [18]. Histochemical methods only stain the copper-protein complex [14]. The

degree of staining in the tissue given by these histochemical methods could be related to amounts of copper [14].

The lung is a poor copper-containing tissue [19] and the liver is very rich in copper. The histochemical methods showed differences in staining between lung and liver metastases, and it is possible that the tumor establishes an equilibrium with respect to copper accumulation according to the organ where it is implanted [20].

As previously described, copper localization is peri- and intranuclear with scanty cytoplasmatic granules [11]. By histochemical techniques, Haratake et al. [7], Lefkovitz et al. [8] and Vecchio et al. [9] found positive copper staining in cells of human hepatic cancer. By the same method, Bedrick et al. [10] described copper staining in human invasive cells of basal and squamous carcinoma originating from skin and from oral mucosa.

A relationship between copper accumulation and tumor growth has also been shown in mammary tumors [17]. The percentage of copper-containing cells was directly correlated to the tumor growth rate in primary tumors originating in the mammary gland, M1, M3, and S13, while P, a semidifferentiated adenocarcinoma primarily located in the lung, and MM3, a subline originating from a subcutaneous transplant of M3 lung metastases, did not show the same relationship, their subcutaneous growth being slower than in M3 and S13.

The topographic localization of the cells with stronger staining, close to the blood vessels, would suggest that copper facilitates tumor invasion and growth. The copper content in primary tumors could be a good marker for evaluating metastatic behavior and tumor growth. These possibilities are presently under investigation.

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