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Immunohistochemical localization of metallothionein in human testicular embryonal carcinoma cells

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Summary. The presence of high levels of metallothionein (MT) in developing mammalian cells is well documented. It has been suggested that the developmental profile and gene expression of MT is similar to that of the so-called oncodevelopmental gene products such as a-fetoprotein. In this study tissue sections of nine human embryonal carcinomas of the testis were tested by means of the avidin-biotin peroxidase complex for the presence of MT. The antigen was localized in variable amounts in the cytoplasm and nucleus in tumour cells in all cases. There was evidence that immunoreactivity was related to the histological growth pattern of tumour cells. These findings suggest that MT may be considered an oncodevelopmental product which could be useful as a tumour marker. In addition, the histology of these tumours might predict MT expression; this may prove of value in testing the hypothesis of MT-related emergence of drug-resistant cell lines in the course of treatment of tumours with metal-containing chemotherapeutic agents.

Key words: Metallothionein – Immunocytochemistry – Embryonal carcinoma

Introduction

Metallothioneins (MT) are intracellular metalloproteins of low molecular weight (6000–7000 daltons), high content of cysteinyl residues (33%) and high affinity for heavy metal ions including zinc, copper, cadmium, mercury, silver, and platinum (Cherian and Goyer 1978; Kagi and Nordberg 1979; Templeton et al. 1984). Increased amounts of intracellular MT are present in mammalian liver during gestation and in early postnatal period with the levels declining to very low concentrations in adult life (Ryden and Deutsch 1978; Riordan and Richards 1980; Banerjee et al. 1982). This reflects the increased synthesis of MT in hepatocytes during fetal life due to their higher requirements for essential metals, especially zinc, necessary for various metabolic processes (Webb and Cain 1982). MT mRNA was observed in the developing liver and the endodermal yolk sacs of mice suggesting a very early expression of its gene in mammalian development; furthermore, MT mRNA was shown to be re-expressed in liver and germ cell carcinomas (Andrews et al. 1984). These findings suggest that MT could be considered an oncodevelopmental product similar to a-fetoprotein with possible analogous cellular and tissue distribution and potential as a tumour marker. Recently MT has been also localized in thyroid tumours suggesting a more diverse expression of this product in neoplasia (Nartey et al. 1987).

The production of MT by tumour cells has been proposed as a possible mechanism for the intracellular inactivation of metal-containing chemotherapeutic compounds, which appears to be a major factor contributing to the resistance of some tumours to platinum compounds (Eastman and Richon 1985).

These observations prompted us to study the localization of MT in human testicular embryonal carcinomas by an immunocytochemical method using a specific polyclonal rabbit antibody to rat liver MT which shows cross-reactivity with human MT (Tohyama and Shaik 1978).

Materials and methods

Routinely processed, formalin fixed, paraffin-embedded blocks of 9 embryonal carcinomas of testicular origin resected before

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the institution of any treatment to the patients (between the years 1983 and 1987) were retrieved from the files of the Pathology department of St. Joseph's Hospital, London, Ontario.

The patients ranged in age from 18 to 43 years, the mean age being 28 years. One patient presented to the hospital with para-aortic lymph node metastasis after initial orchidectomy at another hospital and before any adjuvant therapy. Four patients (including the one with lymph node metastasis) were diagnosed as pure embryonal carcinomas. Four patients had also foci of seminoma in various proportions (up to 75% in one case). Two patients had an endodermal sinus tumour component and 2 immature teratomatous elements. One case had syncytiotrophoblastic elements in addition. Blocks were selected from areas consisting predominantly of embryonal carcinoma elements. In two cases 2 and in one 3 blocks of tissue were tested.

The tissue sections were stained with the avidin-biotin peroxidase technique (Hsu et al. 1981). Four-micron sections were deparaffinized, hydrated in graded alcohols and washed with phosphate buffered saline, pH 7.3. The sections were incubated with 20% normal swine serum for 30 min to block nonspecific binding sites. Then the tissue sections were covered with rabbit anti-MT serum (1:100) or control normal rabbit serum or rabbit anti-MT serum preabsorbed with rat liver MT for 60 min at room temperature. After washing, the slides are incubated first with biotinylated swine anti-rabbit IgG (linking antibody), then avidin-biotin horseradish peroxidase complex, (ABC kit, Vector Lab., Inc., Burlington, CA) and developed with diaminobenzidine (DAB) in 0.33% hydrogen peroxide substrate. Antibody to rat liver MT was prepared in rabbits in Dr. Cherian's Laboratory and its cross-specificity was tested in a previous study (Banerjee et al. 1982). All tissue sections were lightly counter-stained with haematoxylin.

The specificity of the antibody for MT was tested with three different control experiments; the prior absorption of MT antibody with purified rat liver MT, the substitution of the antiserum by normal rabbit serum (1:100) and the omission of primary antiserum (anti MT) from the procedure.

Two variables were evaluated; intensity and extent of staining.

The staining intensity was graded on a scale of 0 to 3, (0) signifying no staining at all, (1) weak, (2) moderate, and (3) strong reactivity. The extent of staining was also quantified as a percentage of the whole tumour area of the tissue section.

The histological pattern of the tumours was examined and classified as glandular-papillary, solid or trabecular (Mostofi and Prize 1973; Mostofi 1980). The tumour area with a particular pattern was estimated and expressed as a percentage of the whole tumour area.

Results

The results of the MT staining and localization are summarized in Table 1. MT was localized in all 9 tumours and in all 14 sections tested. The intensity of the staining reaction was variable. Strong positivity (3) was observed in 6 tumours and in 9 total areas studied and moderate or weak (1–2) in 4 tumours and 5 areas. Background staining was graded from absent to weak and did not affect the interpretation of results. The background staining is attributed to diffusion of the MT antigen prior to fixation of the tissues.

None of the negative control procedures produced any staining, demonstrating the specificity of the antibody to MT.

Table 1. Growth patterns correlated with immunocytochemical expression of MT in nine human embryonal carcinomas

No.	Pattern			Intensity	Extent
	G-P	Solid	Trabecular		
1*. (a)	90%	10%	–	3	90%
(b)	10%	90%	–	1	5%
2.	30%	70%	–	3	50%
3**.	–	–	100%	1	90%
4.	10%	90%	–	3	50%
5.	10%	90%	–	1	5%
6.	50%	50%	–	3	50%
7.	100%	–	–	3	90%
8.	30%	70%	–	2	5%
9.	70%	30%	–	3	90%

* Tumor 1 showed two areas: (a), (2 sections) with glandular-papillary (G-P) pattern and (b), (one slide) with solid pattern
** Intense (grade 3) staining was observed in 10% of the tumour, with peripheral localization

Three tumours had a predominantly glandular-papillary pattern, four solid, one trabecular and one mixed. The extent of staining on the sections was estimated to range from 5% of the total tumour area in cases 1, 8 and 5 to 70 and 90% in cases 1, 7 and 9. In the three cases with extensive staining it was noted that the tumours had a prominent glandular-papillary pattern (Fig. 1 A). In addition, the intensity of staining also appeared to correlate with the same histological pattern; thus, four cases with the papillary-glandular growth pattern exhibited intense, grade 3, staining. However, two cases, 5 and 8 and one section of case 1, showing mostly a solid pattern of growth, displayed both less intense and less extensive staining which tended to be more prominent at the periphery. Similar peripheral staining was seen in the solid areas of other tumours (Fig. 1 B). Case 3 was exceptional with a highly organized trabecular pattern showing weak but extensive positivity and weak background staining; only 10% of the tumour area showed strong positivity and this was localized at the periphery.

At the cytological level, MT was present in both the cytoplasm and the nucleus of cells. The localization was variable among individual cells and groups of cells. Stained and unstained single cells or cell groups were present side by side (Fig. 2 A and B). No difference among cases were noted. The cytoplasm was more extensively stained; frequently, there was increased perinuclear positivity associated with negative nuclear immunoreactivity (Fig. 3 a). The nuclear staining was equally intense but less extensive; occasional cells showed nuclear staining alone (Fig. 3 B).

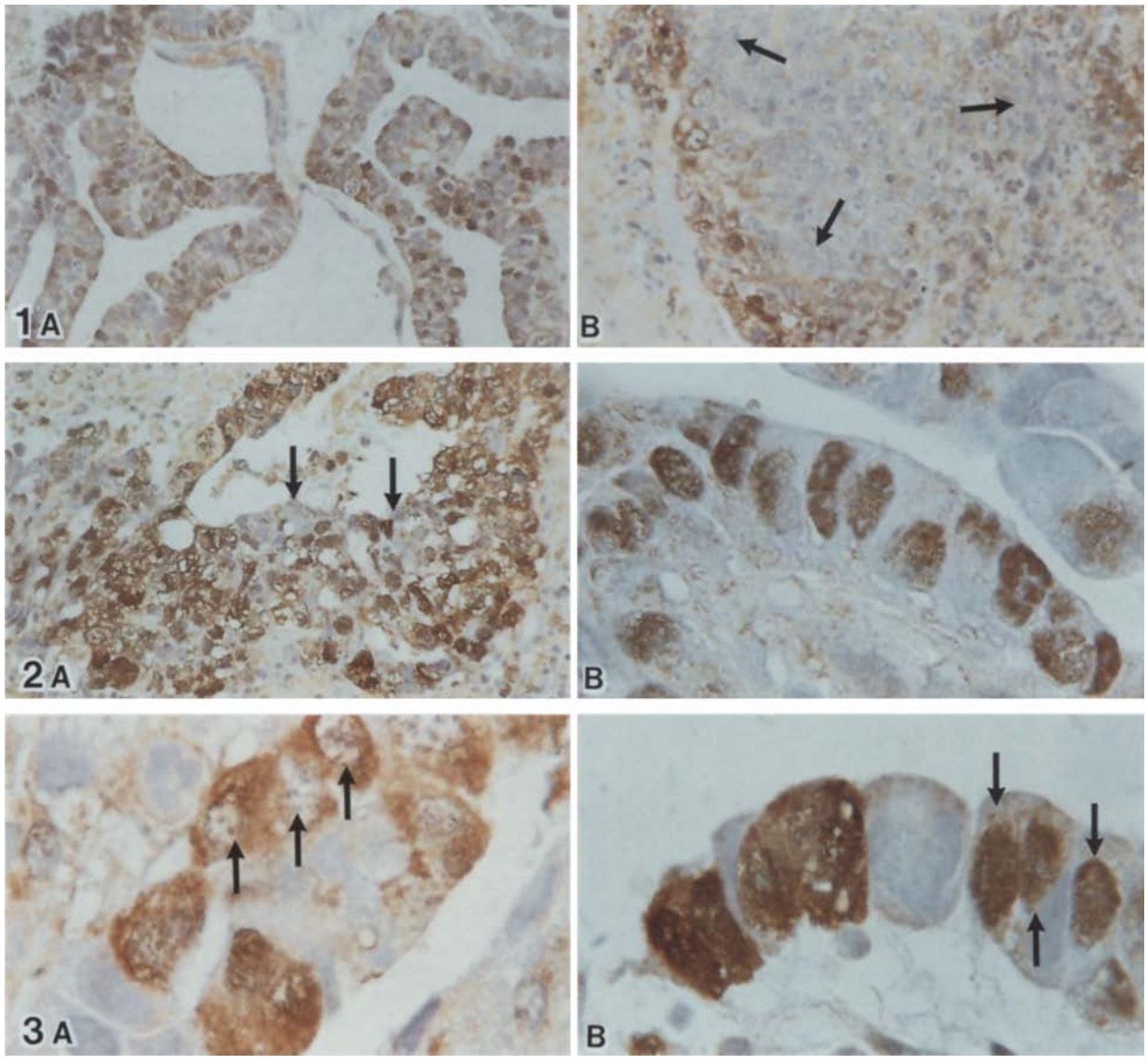

Fig. 1. A Extensive metallothionein (MT) immunoreactivity in neoplastic cells of tumours with glandular-papillary growth pattern (×125). **B** Peripheral MT positive staining of tumour areas with solid growth pattern. Arrows indicate periphery of tumour nodule (×125)

Fig. 2. A Single cells and groups of cells remain negative (*arrows*), while surrounded by strongly positive individual cells and cell groups (×125). **B** Alternating positive and negative cells in a row (×200)

Fig. 3. A Cytoplasmic and perinuclear staining; nuclei (*arrows*) remain negative (×500). **B** A row of tumour cells with concurrent cytoplasmic and nuclear positivity adjacent to cells with nuclear staining alone (*arrows*) (×500)

The seminomatous areas were negative or focally weakly positive. The foci of endodermal sinus tumours showed scattered moderately positive cells. Strong positivity of epithelial elements was seen in the teratomas.

Discussion

This study demonstrates the presence of MT in human testicular embryonal carcinomas by an immunohistochemical method. MT was strongly localized in sections of 6 tumours and in moderate or scanty amounts in 4 tumours. It became evident that different tumours and different areas in the same tumour express quite variable quantities of MT. MT staining was present in both the nucleus and the cytoplasm, either separately or concurently, of the embryonal carcinoma cells. The biological significance of the presence of MT in the cell cytoplasm and nucleus is not yet clearly under-

stood; it may reflect dedifferentiation of certain tumour components with the re-expression of a previously suppressed developmental protein (Riordan and Richards 1980; Banerjee et al. 1982).

A report on low molecular weight zinc binding proteins in rat testes suggests that these proteins are not metallothioneins, despite certain common properties (Waalkes and Peramtoni 1986). These testicular metalloproteins have low cystein content and high proportion of glutamate and lysine. Consistent with these results, the expression of MT gene in mouse is different from other tissues (Durnam and Palmiter 1981). A lack of induction of MT in the testes has been reported in mouse (Durnam and Palmiter 1981) and rat (Onosaka and Cherian 1981, 1982) upon exposure to metals such as cadmium and zinc. However, the presence of MT in embryonal carcinoma cells is not unexpected since MT mRNA has been found in abundance in normal yolk sacs and in "differentiated" teratocarcinoma cells in mice (Andrews et al. 1984).

The different histological growth patterns of embryonal carcinoma have been previously recognized and categorized as solid ("simulating seminoma"), acinar, tubular or papillary (Mostofi 1973, 1980).

Recently attempts have been made to correlate morphological and immunohistological variables and relate them to histogenetic models reflecting different stages of cell development and maturation (Jacobsen 1986).

In this study the variable expression of MT by embryonal carcinoma cells appeared to correlate with well-defined histomorphological patterns. Thus, tumours with a typical glandular-papillary pattern, corresponding to the epiblastic or ectodermal pattern of Jacobsen (1986) are strongly positive, while tumours growing in solid masses, designated embryoblastic by the same author, are negative or weakly to moderately positive mainly at the periphery. The peripheral positivity of tumour nodules with the solid pattern may be due to cellular heterogeneity as demonstrated by the multicell spheroid model in which the most actively proliferating cells are located in the outer cell layers (Sutherland 1988). These results suggest that the variability of MT expression may reflect the morphological heterogeneity of the tumour; it may also correlate with the degree of differentiation and maturation of the tumour cells. Thus, the solid pattern, which resembles the better differentiated seminomas (Mostofi 1980), may have lower Zn and MT metabolic requirements, as it may have less cellular metabolic activity and less rapid growth potential especially as far as the central parts are concerned. The seminomatous areas present in four of our cases were negative or weakly positive suggesting a similar interpretation. Finally, the trabecular pattern may represent an intermediate stage characterized by more rapid tumour growth, increased metabolic activity, and higher requirements for Zn and MT.

The significance of the nuclear MT expression may be related to the interactions of metal ions with various constituents of the nucleus; they may bind to histones, nucleolar RNA, nuclear proteins and modify gene expression and phosphorylation of regulatory proteins causing breakages, excisions, cross-links and transitions on DNA (Sunderman 1984); they regulate the activity of the MT gene the product of which, MT, can chelate and detoxify these metals. In addition, they may mediate the increased expression of viral oncogenes induced by hormones, through their interaction with transcriptional enhancers (Walters et al. 1981; Pfahl et al. 1983).

MT overexpression has been recently implicated as an important factor in the development of resistance to drugs by tumour cells; tumour cell lines with acquired resistance to cis-diaminechloroplatinum exhibited a high content of MT and were variably cross-resistant to other antineoplastic drugs such as chlorambucil and melphalan; a direct role of MT involvement in the acquisition of drug resistance was established by introducing a eukaryotic expression vector encoding MT into cells, a process that conferred resistance to these drugs over a wide range of concentrations, while significantly increasing the MT content of cells (Kelley et al. 1988).

The results of the present study show variable expression of MT in embryonal carcinomas cells in the same tumour and among different tumours; this may be related to the particular histological pattern of the tumour as a reflection of the stage of maturation and differentiation; in addition, the presence of tumor cell subpopulations with considerable heterogeneity in their expression of MT suggests that these cells spontaneously synthesize MT which may contribute to incomplete remissions and the emergence of drug resistance. Additional studies are clearly needed to elucidate the significance of MT as a possible tumour marker, as well as its clinical importance in treatment.

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