Role of Metallothioneins in Irradiated Human Rectal Carcinoma

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BACKGROUND. Metallothioneins (MT) are low-molecular weight, metal-binding proteins that play a role in cellular proliferation and differentiation, as well as in cellular defense mechanisms. They act as scavengers of free radicals produced by irradiation. A number of in vitro and in vivo studies have linked overexpression of cellular MT with tumor cell resistance to radiation. This is the first study that investigates whether MT expression is involved in the radioresistance of rectal carcinoma.

METHODS. Using a mouse monoclonal antibody, MT expression was analyzed by immunohistochemistry on surgical samples ($n = 85$) from 85 patients with locally advanced rectal carcinoma who were treated preoperatively with a hyperfractionated and accelerated radiotherapy schedule and on tumor biopsies ($n = 13$) obtained before treatment. The potential correlations between MT expression and pathologic variables and survival were examined.

RESULTS. MT were expressed strongly in both the cytoplasm and nucleus of tumor cells in 7 biopsy and 42 surgical samples. A comparison of MT expression in biopsy and surgical specimens showed that MT expression did not change after irradiation in most cases. Against all expectations, MT were expressed more frequently in tumors from responders than in those from the nonresponders ($P = 0.02$). There was no correlation between MT expression and tumor stage, histology after radiotherapy, or survival.

CONCLUSION. These findings do not support the hypothesis that MT overexpression at the end of radiotherapy is a marker for radiation resistance. *Cancer* 2002;95:1003–8. © *2002 American Cancer Society.*

Failure to control local tumor growth and recurrence is due to the acquired or inherent radioresistance of the tumors. Over the last decade, there has been increasing interest in biologic markers to stratify cancers according to their response to chemotherapy or radiotherapy. Even if several factors are known to cause resistance to chemotherapeutic drugs and radiation, there have been only a few reports on the prediction of response to radiotherapy. Metallothioneins (MT) represent a group of low-molecular weight cysteine-rich intracellular proteins that bind and detoxify heavy metal ions.[1,2] Synthesis of MT is induced in a variety of normal and tumor tissues by these metal ions, as well as by endogenous factors such as glucocorticoids, cytokines, and vitamin D.[3,4]

MT are involved in a transient response to any form of stress or injury, providing a cytoprotective mechanism against the potentially damaging effects of alkylating agents and oxygen-derived free radicals, particularly those produced by irradiation.[1,5] Therefore, induc-

tion of MT synthesis may be one of the most important defense mechanisms against DNA-damaging agents.[6–13] Supporting this hypothesis, several groups have found that an increased level of MT in cultured cells or xenograft tumors is associated with resistance to ionizing radiation.[6,11,12,14] However, there is no study demonstrating such a correlation in human primary tumors treated with only neoadjuvant radiotherapy.

By using immunohistochemistry, MT are detectable at a basal level in a variety of normal and tumoral human tissues.[15–20] Increased expression is found frequently in patients with colorectal adenomas and adenocarcinomas,[21,22] hepatocellular carcinomas,[23] testicular embryonal carcinomas,[24] thyroid tumors,[25] transitional cell carcinomas of the bladder,[26] and breast carcinomas.[27]

Our goal was to determine whether MT expression has any prognostic value for tumor response and survival in patients with locally advanced rectal carcinomas treated with preoperative hyperfractionated and accelerated radiotherapy (HART).

MATERIALS AND METHODS

Patients

This study included 105 consecutive patients with locally advanced rectal carcinoma (T3–T4, N0, or N1 or any T but N1) who were eligible for Trial 93-01. These patients were treated with HART at the Institute of Pathology, Lausanne, between 1992 and 1998.[28,29] This protocol was reviewed by the local ethics committee. Informed consent was obtained from all patients. Satisfactory immunostaining with MT antibody was achieved in 85 of these 105 patients. Subsequent analysis was limited to these patients. They ranged in age from 28 to 85 years (median age, 63 years) and the male-to-female ratio was 1:2. Before the start of treatment, all patients underwent a complete clinical examination, blood count, assessment of renal and liver function, and a carcinoembryonic antigen assay. Histologic confirmation of malignancy was available for all patients but pretherapeutic biopsies were retrievable for only 34 patients. Distant metastatic disease was excluded by chest X-ray, abdominal ultrasound, and thoracoabdominal computed tomography (CT) scan. The assessment of the local extension of the tumor was based on digital rectal examination, completed by rectal ultrasound and CT scan. In total, 54 patients were staged T3, 14 were N1, and 30 were T4, 6 of whom were N1 and 1 of whom was T2N1. All patients received the same radiotherapy schedule, which was delivered with a linear accelerator with a minimal energy of 6 MV (1.6 Gy twice a day, with 6 hours as the interfraction interval). The total dose was 41.6 Gy in 26 fractions administered over 2.5 weeks. The interval between the end of radiotherapy and surgical resection was kept as short as possible. Surgery was usually performed within 6 days (the median was 5 days).

Macroscopic Examination of Surgical Specimens

The surgical specimens were opened through the anterior wall and fixed in 10% buffered neutral formalin for 24 hours. The whole tumor and attached mesorectum were sliced serially in 3–4-mm slices and the whole tumor was included for histologic examination. For assessment of perirectal lymph nodes, adipose tissue was removed after tumor sampling and cleared in a Carnoy solution for 24 hours.

Samples

In 21 of 34 pretherapy biopsies, the material was fixed in sublimated formol. The remaining 13 biopsies and surgical specimens were fixed in 10% neutral formalin for 12–24 hours at room temperature. Tumor samples were embedded in paraffin. From the tissue blocks, 4-μm thick sections were obtained and stained with hematoxylin and eosin according to standard procedure. For each case, one paraffin block with normal mucosa adjacent to the carcinoma was selected for the detection of MT protein expression by immunohistochemistry.

Histologic Assessment

All 85 irradiated rectal tumors were reviewed by the same pathologist (H.B). The tumors were classified according to the World Health Organization criteria of intestinal carcinoma[30] and staged according to the TNM classification.[31] Tumor regression was graded according to the presence of residual tumor cells and the extent of fibrosis. Grade 1 was defined by the absence of residual cancer and fibrosis extending through the layers of the rectal wall, Grade 2 was characterized by the presence of residual cancer cells and fibrosis, and Grade 3 was characterized by the absence of any tumor regression.

Immunohistochemistry

Four-μm thick tissue sections were mounted on aminopropylmethoxysilane-coated glass slides, deparaffinized in xylol, taken through to absolute alcohol, blocked for endogenous peroxidase with 1% H_2O_2 in methanol (45 mn), and rehydrated through graded alcohols. They were subjected to microwave oven heating for 15 minutes in 10 mM citrate buffer, pH 6.0, and rinsed in Tris-buffered saline (TBS; Tris 0.05 M, NaCl 0.9%, pH7.6). To reduce nonspecific binding, they were incubated for 10 minutes in normal goat

FIGURE 1. Nontumoral rectal mucosa showing low-level metallothionein (MT) immunostaining. Some absorptive epithelial cells bordering the luminal surface and crypts show MT positivity.

FIGURE 2. Islets of carcinomatous cells from a rectal carcinoma specimen treated preoperatively with radiotherapy showing cytoplasm and/or nuclear metallothionein immunoreactivity.

serum (Pel-Freez Biologicals, Rogers, AK) 1:30 in TBS. After a 30-minute incubation with the primary monoclonal antibody (MoAb; mouse monoclonal anticlone E9 Metallothionein, Dako, Glostrup, Denmark) diluted 1:500 in TBS containing 5% nonfat dry milk (TBS-nfdm), the sections were incubated for 30 minutes with biotinylated horse antimouse immunoglobulins (Vector, Burlingame, CA) diluted 1:400 in TBS-nfdm. They were also incubated for 30 minutes in ABC-peroxidase complex solution (Vector) prepared according to the manufacturer's instructions. Peroxidase activity was revealed with 5-5, diaminobenzidine as chromogen and the sections were counterstained in Mayer's acid-free hematoxylin. As a negative control, the primary MoAb was replaced by a mouse hybridoma supernatant of similar isotype (IgG1).

Immunohistochemical expression of MT was not assessable on tissue biopsies fixed in sublimated formol. In the remaining biopsies ($n = 13$) and surgical specimens ($n = 85$), immunoreactivity for MT was evaluated semiquantitatively by two observers using a double-headed microscope (H.B, S.G) without knowledge of the tumor stage or the clinical data. Staining for MT in the normal rectal mucosa was used as a positive control (Fig. 1). Absorptive epithelial cells bordering the luminal surface and especially the basal part of the crypts showed MT positivity, generally localized in the paranuclear region and cell membranes and less frequently in the nuclei. If no crypt staining was obtained after two attempts, the sample was eliminated from the study for technical reasons. In tumor cells, MT immunoreactivity was observed in the cytoplasm and/or in the nuclei. Positive tumor nuclei and cytoplasm were scored separately as follows: 0 = less than 5% of immunostained cells; 1 = 5–30% of positive cells; 2 = 30–60% of positive cells; and 3 = greater
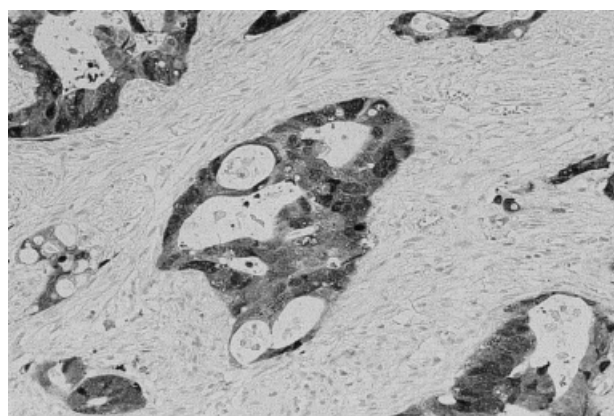
than 60% of positive cells (Fig. 2). The spindle-shaped stromal cells, intermingled with neoplastic glands, sometimes showed staining for MT.

Follow-Up and Statistical Analysis

Follow-up was available for all patients at the date set for collecting data. Overall survival (OS), disease-free survival (DFS), and local control were the endpoints for evaluating the prognostic significance of MT staining. All statistical analyses were conducted using JUMP 3.0 software (SAS Institute, Cary, NC). A *P* value less than 0.05 was statistically significant. The correlation between MT and pathologic factors such as tumor stage, histology, and tumor regression was determined by a Pearson chi-square test. Survival curves were estimated according to the Kaplan–Meier method for both cytoplasmic and nuclear MT positivity. The significance between groups for survival curves was estimated by the log rank test.

RESULTS

Pathologic Findings

Correlations between MT expression and histologic parameters in 85 surgical specimens of rectal carcinoma treated with preoperative radiotherapy are summarized in Table 1. Of the 85 rectal carcinomas, one was pT1 (1%), 16 were pT2 (19%), 56 were pT3 (66%), and 12 were pT4 (14%). Regional lymph node metastases were found in 49 patients (58%). Tumors were classified into well (27%), moderately (42%), and poorly differentiated adenocarcinomas (12%), and mucinous carcinomas (19%). None of the 85 tumors showed complete tumor regression (Grade 1). Partial tumor regression (Grade 2) was observed in 68 pa-

TABLE 1
Correlations between MT Expression and Histologic Parameters in 85 Surgical Specimens of Rectal Carcinoma Treated with Preoperative Radiotherapy

Tumor staging	MT negative No. (%)	MT positive No. (%)
pT1	0 (0)	1 (1)
pT2	7 (8)	9 (11)
pT3	30 (35)	26 (31)
pT4	8 (9)	4 (5)
Nodal staging		
pN0	18 (21)	21 (25)
pN1	27 (32)	19 (22)
Tumor differentiation		
Well differentiated carcinoma	13 (15)	10 (12)
Moderately differentiated carcinoma	17 (20)	19 (22)
Poorly differentiated carcinoma	6 (7)	4 (5)
Mucinous carcinoma	9 (11)	7 (8)
Tumor regression		
Complete (Grade 1)	0 (0)	0 (0)
Partial (Grade 2)	32 (38)	36 (42)
Absent (Grade 2)	12 (14)	5 (6)

MT: metallothionein.

TABLE 2
Cytoplasmic and Nuclear MT Immunoreactivity in a Series of 85 Surgical Specimens with Rectal Tumors Treated with Preoperative Radiotherapy

Nuclear MT	Cytoplasmic MT				Total (%)
	0	1	2	3	
0	43	1	2	0	46 (54)
1	2	15	4	2	23 (27)
2	0	3	6	1	10 (12)
3	0	0	0	6	6 (7)
Total (%)	45 (53)	19 (22)	12 (14)	9 (11)	85 (100)

MT: metallothionein.

tients (80%) and tumor regression (Grade 3) was absent in 17 cases (20%).

Immunohistochemistry confirmed MT expression in 7 and no MT expression in 6 of the 13 tumor biopsies performed before treatment. In the seven specimens expressing tumors, the immunostaining score varied from 1 to 3. In surgical specimens, MT expression was present in 42 carcinomas and absent in 43. The cytoplasmic and nuclear distribution of MT expression and their corresponding scores are summarized in Table 2. In 38 of the 42 positive cases, the MT protein was expressed both in the cytoplasm and in the nucleus and the expression score was most frequently 1 (tumor cells with MT expression < 30%). In two cases, MT were expressed only in the cytoplasm

and in two cases, only in the nucleus. When comparing MT expression in biopsies and surgical specimens, the MT expression score was identical in five patients and lower in five patients. In three cases, MT expression was present in the biopsies, but not in the surgical specimens.

MT were expressed in 37 of 68 tumors with partial tumor regression (Grade 2), but only in 5 of 17 tumors with no tumor regression (Grade 3; $P = 0.02$). There was no statistical correlation between MT expression and tumor stage, tumor grade, and differentiation (Table 1).

Survival Analysis
The median actuarial OS and DFS period was 52 months. The actuarial local recurrence rate at 2 and 5 years was 6.4% and 7.6%, respectively. During the follow-up (median = 40 months), 29 patients developed distant metastasis and 8 patients developed local recurrence. Forty patients died, 32 of whom died of their carcinoma.

There was no correlation between MT expression and local control, DFS, and OS by univariate analysis. To increase the number of patients per group, the individual scores of MT expression were also combined. Using this approach, MT expression also did not correlate with the above-mentioned endpoints.

DISCUSSION
The lack of reliable criteria to predict the outcome after preoperative radiotherapy for individual patients with advanced rectal carcinoma is a major problem. The precise mechanism of radioresistance in experimental and human studies is still unknown, and probably multifactorial. Much of the damage induced by ionizing radiation is caused by the oxygen-derived free radicals produced by the radiolysis of water in cells. In recent years, MT have emerged as an important factor in tumor development and progression because of the high Cu/Zn-containing proteins present in patients with malignancies.[1,5,8,32–36] In addition, MT have been implicated in the transient response to any form of stress or injury such as ionizing radiation and alkylating agents, providing a cytoprotective mechanism against the potential damaging effects of oxygen-derived free radicals.[1,2,37–40] Many studies support the hypothesis that MT, acting as hydroxyl radical scavengers, lead to radioresistance in normal and tumor cells.[6,7,9,11–13,39] However, if experimental studies have linked MT overexpression and radioresistance, there is no clinicopathologic study reporting a relationship in patients with rectal carcinoma treated with neoadjuvant therapy. The aim of this study was to evaluate by immunohistochemistry the possible correlation be-

tween MT expression and pathologic factors and survival in a series of patients with cancer treated with preoperative radiotherapy.

There are four isoforms of MT (MT-I, MT-II, MT-III, and MT-IV) expressed in mammalian tissues. The MT-I and MT-II isoforms are the most predominant isoforms and are expressed ubiquitously in most mammalian organs and are regulated coordinately, whereas the MT-III and MT-IV isoforms are expressed specifically in the brain and in squamous epithelia. In the current study, we analyzed by immunohistochemistry the expression of MT in a series of rectal carcinomas treated by radiotherapy before surgical resection. Using an antibody that recognizes both MT-I and MT-II isoforms, MT immunoexpression was detected in normal rectal mucosa. Cytoplasmic and/or nuclear MT expression was also present in 42 of the 85 irradiated tumors. Comparing tumor biopsies taken before radiotherapy and tumor samples obtained from surgical specimens, we did not observe a significant increase in MT expression after irradiation. In three cases, MT expression in the tumor was higher before radiotherapy than after irradiation. There was no correlation between MT expression and tumor stage, tumor grade, or histologic types. In addition, MT expression was observed more frequently in the tumors of responders than in those of nonresponders. This finding was not associated with a poor prognosis. Therefore, our observation does not support the hypothesis that MT protect human rectal carcinomas against ionizing radiation.

These results contradict the data reported in some experimental studies. Therefore, we reviewed critically some of the most relevant in vitro and in vivo studies that examined the role of MT in the radioresistance of tumors or tumor cell lines. Renan and Dowman[12] observed that various tumor cell lines display increased levels after irradiation but that only some acquire radioresistance. This result indicates that if MT induction is one of the mechanisms involved in protection against radiation, this mechanism is cell line specific. However, in Renan and Dowman's study and in other in vitro experiments, the induction of MT expression was improved by various treatments with heavy metals, bismuth compounds, or salt before irradiation, conditions that do not occur in vivo.[11,12,41]

Different experimental approaches have demonstrated the dose–radiation dependence of MT expression.[42] Koropatnick et al.[39] observed that the MT content in rodent or human cells did not increase after a single exposure to X-rays. In mouse fibrosarcoma cells, multiple or fractionated moderate γ radiation doses are more effective in inducing MT synthesis than a single large dose.[9] MT synthesis is also induced by X irradiation in transplanted murine tumors (fibrosarcomas) in a dose-dependent manner.[43] However, the doses of ionizing radiation used to induce MT in these experimental conditions are much higher than the doses used in a clinical setting. Therefore, it is difficult to extrapolate these results to clinically relevant dose levels and fractionation irradiation.

Two in vivo studies on transgenic mouse models have also led to the conclusion that MT do not provide any protective role against ionizing irradiation. Lohrer and Robson[44] reported that transfection of tumor cell lines with a human MT gene does not provide a defense mechanism against irradiation. In vivo studies using transgenic mouse models that overexpress MT also indicate that MT do not protect the animal from irradiation damage.[45] Conrad et al.[46] found no difference in radiosensitivity between wild-type and MT$^{-/-}$ mice.

Conclusion

Most of the published reports on the induction of MT after exposure to ionizing radiation are on rodent xenograft tumor models or on cells in culture and at radiation dose levels and fractionation that are not clinically relevant. Therefore, it is difficult to extrapolate these experimental results to humans. Our results do not provide any evidence of a potential role for MT as a radioprotector in rectal tumors in humans undergoing preoperative irradiation.

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