ANTIOXIDANTS & REDOX SIGNALING
Volume 2, Number 3, 2000
Mary Ann Liebert, Inc.

Original Research Communication

Detection of Thioredoxin in Gastric Cancer: Association with Histological Type

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ABSTRACT

Thioredoxin (TRX) is a redox-active protein with multiple intra- and extracellular functions. This protein exists ubiquitously in all life forms, from primitive living cells, such as *Escherichia coli* and yeast, to higher mammals. Recently, augmentation of the expression and transcription level of TRX has been reported in tumors of various organs. In this study, we examined the expression of TRX in gastric cancer with respect to its histological type and depth of invasion. The association with cell proliferation was also studied. Results of histochemical analysis of surgical specimens as well as cytochemical analysis and Northern blot analysis of gastric cancer cell lines indicated that TRX is predominantly expressed in undifferentiated type gastric cancer rather than in the differentiated type. Neither the depth of tumor invasion nor cell proliferation significantly determined the staining intensity for TRX. Antiox. Redox Signal. 2, 519–528.

INTRODUCTION

THIOREDOXIN (TRX) is a ubiquitous protein with two half-cystine residues that are redox-active in various reactions. It is present in species ranging from prokaryotes to mammalian cells with its preserved amino acid sequence (-Cys-Gly-Pro-Cys-) (Holmgren, 1985). TRX was purified from *Escherichia coli* in 1964 as a hydrogen donor for ribonucleotide reductase, which plays a role in the synthesis of a deoxyribonucleotide precursor (Laurent *et al.*, 1964). Subsequently, TRX was purified from the rat and characterized (Luthman and Holmgren, 1982), and the distribution of this protein was immunohistochemically studied in mammals (Rozell *et al.*, 1985; Hansson *et al.*, 1988). The first study on human TRX involved cultured human fibroblasts from cases with 5-oxoprolinuria and cystinosis (Larsson *et al.*, 1978).

Later research identified two distinctive virus-related proteins that were revealed to be human TRX (Tagaya *et al.*, 1989). Those proteins are 3B6-IL-1 (Wakasugi *et al.*, 1987), an interleukin-1 (IL-1) like substance produced by Epstein-Barr virus (EBV)-transformed human B cell line (3B6), which is secreted from virus-transformed human lymphocytes, and adult T-cell leukemia-derived factor (ADF) (Teshigawara *et al.*, 1985), which induces IL-2 receptor/Tac from its producer cell, the human T-cell leukemia (ATL) virus-1 (HTLV-1) cell line. Several other proteins in mammalian cells were also designated differently at first by terms

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such as a component of early pregnancy factor (Tonissen and Wells, 1991), eosinophil cytotoxicity-enhancing factor (Balcewicz-Sablinska *et al.*, 1991), and glucocorticoid receptor activating factor (Grippo *et al.*, 1985). They have all since been revealed to be human homologues of TRX, which reflects the multifunctional nature of TRX.

One of the most important functions of TRX is its cytoprotective activity. TRX protects cells against a variety of stresses such as ultraviolet irradiation, hydrogen peroxide (H_2O_2) (Okamoto *et al.*, 1994), activated neutrophils (Nakamura *et al.*, 1994), tumor necrosis factor (TNF), and anti-Fas antibody (Matsuda *et al.*, 1991), all of which have reactive oxygen species (ROS)-mediated cytotoxicity. TRX also plays a role in controlling transcription factors such as AP-1 and NF-κB (Matthews *et al.*, 1992; Meyer *et al.*, 1993; Schenk *et al.*, 1994) through regulation of the redox state of the cell.

Several recent studies on the relationship between TRX and malignancies have shown that the level of TRX protein or mRNA in the tissue (Fujii *et al.*, 1991a; Kusama *et al.*, 1991; Nakamura *et al.*, 1992; Wakita *et al.*, 1992; Gasdaska *et al.*, 1994; Berggren *et al.*, 1996) or in the serum (Wakasugi *et al.*, 1998) tends to be elevated in cases with solid tumor. However, the factors that contribute to this elevation have not yet been clarified. The present study focuses on the expression of TRX in gastric cancer, with respect to its association with several probable factors, including histological type of the tumor, depth of invasion, and cell proliferation.

MATERIALS AND METHODS

Patients

Forty-two patients who underwent surgery for gastric cancer at the National Cancer Center Hospital, Tokyo, were selected for this study. Gastric tumors of these patients were evaluated in terms of histology and depth of invasion. Histologically, the tumors were classified as differentiated or undifferentiated based on the level of histological differentiation according to the general rules for the gastric cancer study in surgery and pathology (Kajitani, 1981). The differentiated tumor type included well and moderately differentiated tubular adenocarcinoma, whereas the undifferentiated tumor type included poorly differentiated adenocarcinoma, signet-ring cell carcinoma, and mucinous carcinoma. On the basis of these classifications, 19 tumors were differentiated and 23 were undifferentiated. Twenty-one tumors were early cancer confined to the mucosal or submucosal layer whereas the other 21 were advanced cancer with invasion to the muscularis propria, subserosa, or serosa.

Other clinicopathological factors such as age, gender, tumor size, and state of nodal metastasis were compared between the two groups with different histology, as well as between the two groups with different levels of tumor invasion.

Cell culture

Five gastric cancer cell lines derived from gastric cancers of various levels of differentiation were used in this study. Two were derived from differentiated type adenocarcinoma of the stomach, MKN28 and MKN74, and three were established from undifferentiated type adenocarcinoma, OKAJIMA, KATO-III, and MKN45. All of the gastric cancer cell lines were maintained in RPMI-1640 supplemented with 10% fetal calf serum (FCS) under 5% CO_2 in air at 37°C. 3B6, an EBV-transformed B cell line, was cultured in RPMI-1640 supplemented with 8% FCS as a positive control.

Antibodies

Recombinant TRX was kindly provided by Basic Research Laboratory, Ajinomoto Co., Inc. (Kawasaki, Japan). We raised two kinds of anti-TRX polyclonal antibodies (poAbs), one against carboxy-terminal oligopeptides and the other against amino-terminal oligopeptides of TRX. Synthetic peptides of the carboxyl terminus (-Ser-Gly-Ala-Asn-Lys-Glu-Lys-Leu-Glu-Ala-Thr-Ile-Asn-Glu-Leu-Val-COOH) and amino terminus (NH_2-Val-Lys-Gln-Ile-Glu-Ser-Lys-Thr-Ala-Phe-Gln-Glu-Ala-Leu-Asp-) were injected subcutaneously into rabbits together with complete Freund adjuvant at the first immunization, and then incomplete Freund adjuvant after the second immunization every 14 days. After eight

injections, serum was obtained from the rabbits and decomplemented at 56°C for 30 min. The resultant poAbs were purified by a protein A column (Ampure PA kit: Amersham, Buckinghamshire, UK) according to the manufacturer's recommendations. The specificity of all of the antibodies to TRX was confirmed by immunoblotting analysis using rTRX (Wakasugi *et al.*, 1990). The antibody against carboxy-terminal oligopeptides of TRX, which showed superior immunoreactivity in our preliminary immunohistochemical study, was used in this study.

Immunochemical staining

Surgically removed stomach was fixed with 10% formaldehyde and embedded in paraffin. Sections were cut 4 μm thick and placed on a slide. One slide containing a tumor and nontumor boundary was selected as representative samples in each case. Staining was performed using a Vectastain elite kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions.

Sections were deparaffinized and immersed in methanol containing 0.3% H_2O_2 for 30 min to block endogenous peroxidase activity. The sections were then heated in 10 m*M* citrate buffer, pH 6.0, using a microwave oven (90°C, 15 min) for antigen retrieval. After blocking endogenous avidin and biotin, the sections were incubated with normal goat serum to reduce nonspecific antibody binding. Two μg/ml of anti-TRX poAb against carboxy-terminal peptides or normal rabbit immunoglobulin as a negative control was applied to the sections, which were incubated for 60 min at room temperature, and then incubated with biotinylated second Ab and avidin–biotin peroxidase complex, developed with 3,3′-diaminobenzidine tetrahydrochloride (Dojindo Laboratory, Kumamoto, Japan) plus H_2O_2, and counterstained with Mayer's hematoxylin.

Staining was evaluated by two observers (N.N., A.O.), who individually judged the intensity of staining at least twice according to three grades; negative (−), weak (+), and strong (++). Differences in grading were resolved by consensus. Negative (−) staining indicated reactivity that was less than or equal to that in normal mucosa, and did not indicate a complete absence of immunoreactivity. Strong immunoreactivity (++) indicated intense staining as was seen in the ATL lymph node, and intermediate immunoreactivity was considered as weak staining (+). For slides that showed heterogeneous staining within a tumor, intensity was graded according to the highest intensity within the tumor.

Proliferating cell nuclear antigen (PCNA) is a nonhistone protein detected in the nucleus of proliferating cells in the late G_1 through S phase with declining levels in the G_2 and M phase. PCNA is considered as a marker of cell proliferation because of its close linkage to the cell cycle. Staining for PCNA was performed on serial sections of 42 cases to determine the relationship between the expression of TRX protein and cell proliferation. Anti-PCNA Ab (PC-10, Novocatsra Laboratories, Newcastle upon Tyne, UK) was used at a dilution of 1:100 on slides that had been pretreated by heating in a microwave oven (90°C, 10 min) in 1% zinc sulfate buffer. The PCNA labeling index (LI), which is the percentage of PCNA-positive tumor cells relative to the total number of tumor cells counted, was calculated by scoring a minimum of 1,000 cells in at least four high-power fields of diverse density of positive cells.

Gastric cancer cell lines were stained using the same avidin–biotin complex immunoperoxidase technique. For the immunocytochemical study, 3B6 served as a positive control. MKN28, MKN45, and MKN74, cultured in Lab-Tek Chamber Slides (Nunc, Inc. Naperville, IL), and three other cell lines, including 3B6, were washed with phosphate-buffered saline (PBS) and fixed in Bouin's solution (Muto Pure Chemicals, LTD., Tokyo, Japan) for 90 min on ice. The subsequent procedure, except for treatment with a microwave oven, was the same as described above.

Northern blot hybridization

Total RNA of the five gastric cancer cell lines was extracted with ISOGEN (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. Northern blot analysis was performed as described elsewhere (Akashi *et al.*, 1995). Briefly, 20 μg of total RNA was electrophoresed on agarose gel and transferred to a nylon membrane (Hybond-N^+, Amersham,

Buckinghamshire, UK). The membrane was then baked and hybridized with [α-^{32}P]dCTP-labeled human TRX cDNA probe at 42°C overnight. Total RNA from human peripheral blood lymphocytes (PBL) and 3B6 were run as negative and positive controls, respectively. For quantitation, the radioactivities of the bands of TRX and G3PDH, which was hybridized later on the same membrane as a control of the amount of RNA, were counted by a Bio-image-Analyzer (BAS2000, Fuji Photo Film, Tokyo, Japan), and the normalized radioactivity of TRX was calculated.

Statistical analysis

The significance of the relationship between intensity of staining for TRX and tumor characteristics (histological type and depth of invasion) was evaluated by Mann-Whitney's U test (Dixon and Massey, 1979). The Kruskal-Wallis rank sum test (Dixon and Massey, 1979) was used to evaluate the association between staining intensity of TRX and PCNA LI. In the analysis of clinicopathological factors, gender and nodal status were compared by Fisher's exact probability test, and age and tumor size were compared by Student's *t*-test. A p value of less than 0.05 was considered statistically significant.

RESULTS

Patient characteristics

The clinicopathological features of the patients are listed in Table 1. There was a significant difference in nodal status and tumor size between early and advanced cancer cases, and a significant age difference between the two groups with different histology. No patient had distant metastasis in the liver or in the peritoneum.

Expression of TRX protein in surgical specimens of gastric cancer

Because human TRX and ADF are identical, a tumor cell-infiltrated lymph node of ATL was employed as a positive control. We conducted a preliminary immunohistochemical study to confirm its immunoreactivity to our polyclonal antibody against the carboxyl terminus oligopeptide of TRX (Fig. 1).

Gastric mucosa was faintly stained by the anti-TRX antibody (Fig. 2A), and this staining served as a negative control. TRX staining in cancerous tissue was most prominently observed in the nucleus of signet-ring cell carcinoma or poorly differentiated adenocarcinoma (Fig. 2B,C). Most of the cases graded as having a strong immunoreactivity (++) had this type of staining pattern. The cytoplasm of the cancer cells tended to be stained with various intensity (Fig. 2B,C,D) in most of the cancer tissue samples. Histological localization of TRX either in the nucleus or cytoplasm in the cancerous tissue varied among the cases. There was also heterogeneity of staining intensity within individual samples of cancerous tissue. Only one differentiated early cancer sample, which had the same staining intensity as that in the adjacent noncancerous mucosa, was graded as negative (−).

Table 1. Clinicopathological Factors of Patients

	Histology[a]			*Depth of invasion*[b]		
	Differentiated	*Undifferentiated*	p	*Early*	*Advanced*	p
Age	63.6 ± 10.1	54.4 ± 12.9	0.015	57.6 ± 12.5	59.5 ± 12.6	0.617
Gender						
Male	16	15	0.29	14	17	0.48
Female	3	8		7	4	
Tumor size	48.8 ± 27.0	63.2 ± 37.9	0.12	40.0 ± 20.8	71.5 ± 37.8	0.0018
Lymph-node metastasis						
Yes	11	10	0.48	4	17	0.0001
No	8	13		17	4	

[a]Early, mucosal and submucosal cancer; advanced, cancer with muscularis propria or further invasion.
[b]Differentiated, well and moderately differentiated tubular adenocarcinoma; undifferentiated, poorly differentiated adenocarcinoma, signet-ring cell carcinoma, and mucinous carcinoma.

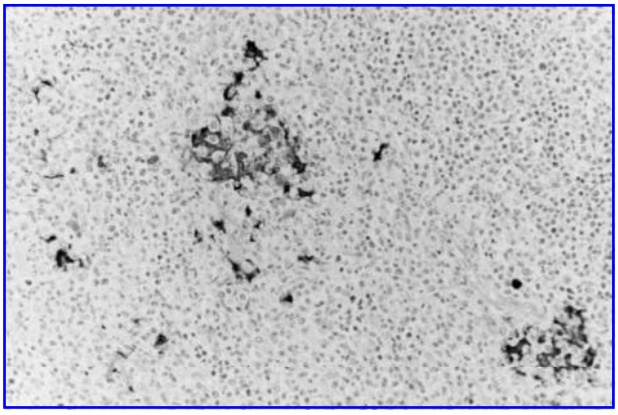

FIG. 1. Immunohistochemical staining of TRX in ATL lymph node. Some tumor cells infiltrating the lymph node are stained with polyclonal antibody against the carboxyl terminus of TRX.

Table 2 provides a summary of the staining intensity of gastric cancer in terms of histology (differentiated or undifferentiated) and the depth of tumor invasion (early or advanced). A statistically significant difference was observed in intensity between differentiated and undifferentiated tumors. On the other hand, the staining of early cancer did not differ significantly from that of advanced cancer. There was no significant correlation between PCNA LI and the staining intensity of TRX ($p = 0.92$).

Expression of TRX in gastric cancer cell lines

All the cell lines showed heterogeneous staining in the cytoplasm. However, the nuclei of the cells were not stained with anti-TRX Ab. This lack of immunoreactivity in the nucleus might be due to the lack of a permeabilization procedure in the fixation method. 3B6 had the highest intensity for TRX, followed by the undifferentiated type cell lines MKN45, OKAJIMA, and KATO-III. The differentiated type cancer cell lines MKN28 and MKN74 were stained less intensely (Fig. 3).

Northern blot analysis revealed that all of the gastric cancer cell lines showed an elevated level of TRX mRNA compared to that of PBL. KATO-III and OKAJIMA showed higher levels of transcripts, similar to the level of the positive control 3B6, than did MKN28, MKN45 and MKN74 (Fig. 4).

DISCUSSION

The present results show that TRX level is elevated in gastric cancer tissue, with greater expression seen in the undifferentiated type than the differentiated type. A similar trend was shown by cytochemical analysis of the gastric cancer cell lines, as well as Northern blot analysis, although the latter technique demonstrated low levels of mRNA in the poorly differentiated gastric cancer cell line MKN45.

In normal rat tissues (Rozell *et al.*, 1985) and human fetal tissue (Fujii *et al.*, 1991b), immunoreactivity to anti-TRX Ab varies widely among cells with different characteristics. These authors reported that the characteristics associated with an increased level of TRX are protein synthesis, intracellular transport, and different forms of secretion. However, the contribution of other factors may be necessary to explain fully the actual staining reactivity. Gastric mucosa, for example, is composed of several kinds of exocrine cells with different reactivities to anti-TRX Ab (Hansson *et al.*, 1988); most of these constituent epithelial cells show weak reactivity, except for parietal cells, in which reactivity is remarkably high. In human cervix and endometrium, the mRNA level of TRX may be influenced by the hormonal state of the reproductive system (Maruyama *et al.*, 1997; Sahlin *et al.*, 1997).

Intracellular localization of TRX also demonstrates, at least in part, a dynamic change. TRX is known to regulate binding of transcriptional proteins, such as NF-κB (Matthews *et al.*, 1992) and AP-1 (Schenk *et al.*, 1994), to DNA through redox control of the cell. Translocation of TRX from the cytoplasm to the nucleus is induced by the ultraviolet irradiation, and its localization is reportedly associated with regulation of transcriptional factors (Hirota *et al.*, 1999).

Elevated TRX protein and mRNA levels in malignant tumors compared to their normal counterparts have been observed in several organs, including cervical squamous epithelium (Fujii *et al.*, 1991a), the liver (Nakamura *et al.*, 1992), lung (Gasdaska *et al.*, 1994), and colon (Berggren *et al.*, 1996). The importance of this phenomenon has not yet been clarified.

TRX reportedly promotes growth of a variety of cells (Biguet *et al.*, 1994; Iwata *et al.*, 1994;

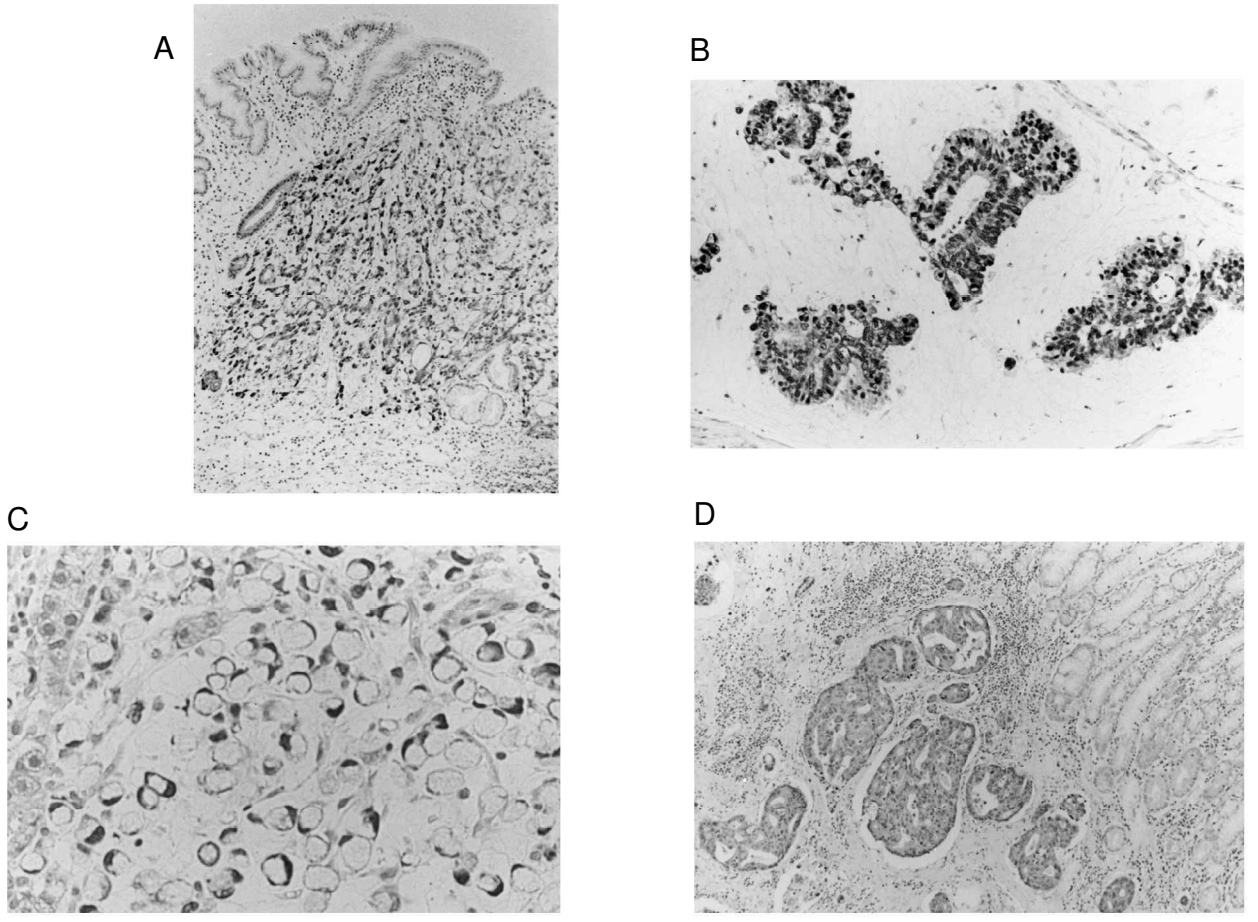

FIG. 2. Immunohistochemistry of gastric cancer. (A) Tumor and nontumor boundary of poorly differentiated adenocarcinoma confined to the mucosa. **(B)** Cancer cells in a mucous lake strongly stained for TRX, especially in the nucleus. **(C)** Signet-ring cell carcinoma with strong staining. **(D)** A border area of gastric cancer with positive staining and normal mucosa. These four cases were graded (++).

Powis *et al.*, 1994; Gasdaska *et al.*, 1995). Gallegos *et al.* (1996) demonstrated accelerated growth of a cancer cell line transfected with TRX cDNA *in vitro.* Even more rapid tumor growth was demonstrated *in vivo* by inoculating TRX transfectant to combined immunodeficient mice compared to inoculation with wild-type control cells (Baker *et al.*, 1997). This acceleration of tumor growth appears to be directly related to inhibition of apoptosis. Thus, the growth advantage brought by TRX seems to be attributable to at least two factors: growth stimulation of the cells by TRX itself, and inhibition of spontaneous apoptosis of the tumor cells. We did not observe a significant correlation between PCNA LI and TRX protein expression in the tumors in our study. Furthermore, depth of tumor invasion had no impact on the difference in staining intensity for TRX. Further investigation related to the overexpression of TRX and apoptosis in tumor should be conducted to clarify the role of TRX in tumorigenesis.

Several studies were conducted concerning

TABLE 2. INTENSITY OF STAINING FOR TRX AND CHARACTERISTICS OF THE TUMOR; DEPTH OF INVASION AND HISTOLOGICAL TYPE

	Intensity of staining				
	n	−	+	++	p
Early[a]	21	1	11	9	0.513
Advanced	21	0	10	11	
Differentiated[b]	19	1	15	3	0.001
Undifferentiated	23	0	6	17	

[a]Early, mucosal and submucosal cancer; advanced, cancer with muscularis propria or further invasion.

[b]Differentiated, well and moderately differentiated tubular adenocarcinoma; undifferentiated, poorly differentiated adenocarcinoma, signet-ring cell carcinoma, and mucinous carcinoma.

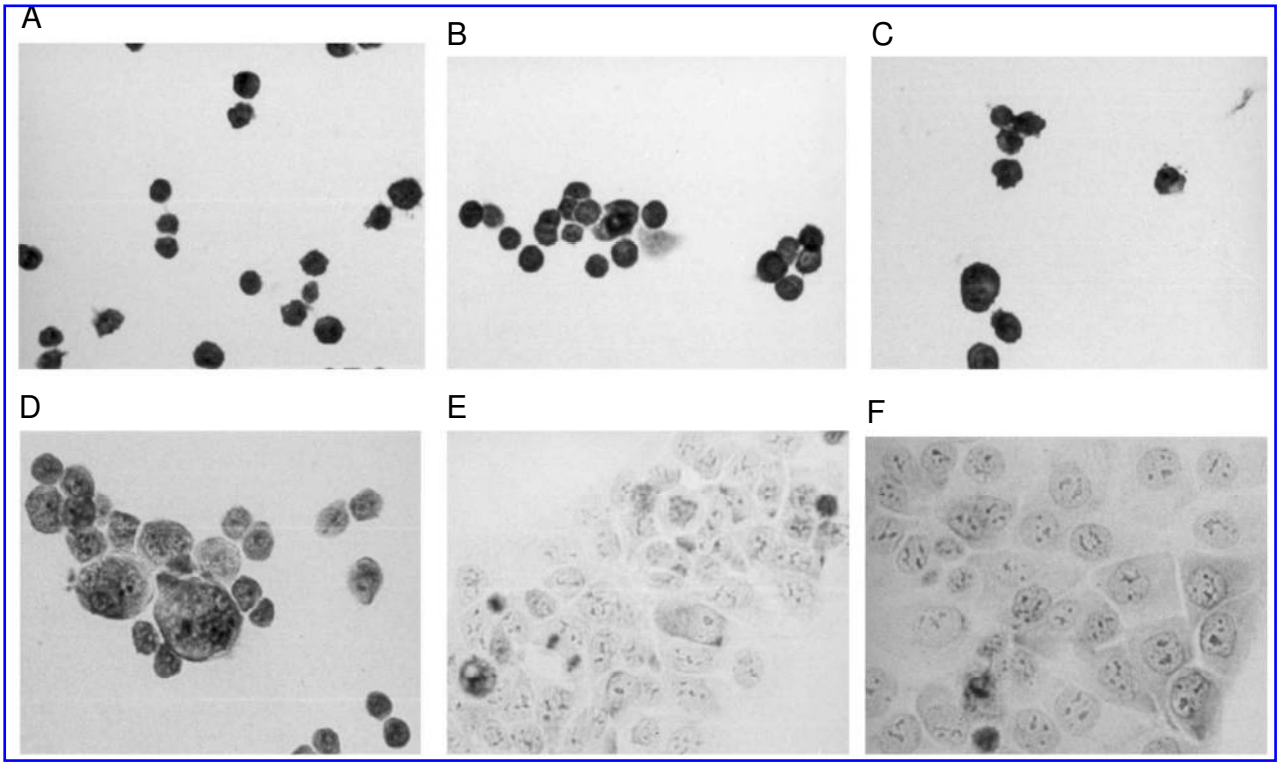

FIG. 3. Immunohistochemistry of cell lines of gastric cancer. (A) 3B6. **(B)** MKN45. **(C)** OKAJIMA. **(D)** KATO-III. **(E)** MKN74. **(F)** MKN28.

ROS production in cancer (O'Donnell *et al.*, 1985; Okamoto *et al.*, 1994; Shimoda *et al.*, 1994; Szatrowski and Nathan, 1991; Toyokuni *et al.*, 1995). Because the cellular level of TRX increases in response to oxidative stress, ROS production may be one possible reason for the elevated TRX in cancer. Berggren *et al.* (1996) reported an elevation in TRX gene expression in a cancer cell line under hypoxia. Thus, elevated TRX levels in undifferentiated carcinoma may reflect greater oxidative stress in this type of cancer.

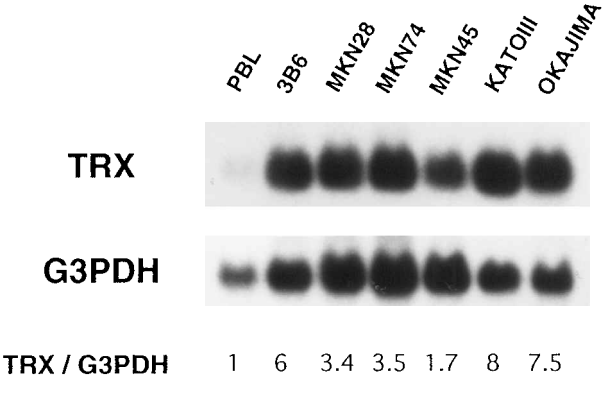

FIG. 4. Northern blot analysis of gastric cancer cell lines. Normalized radioactivity of TRX in each cell line is shown at the bottom as a relative value, which is the average of the results in two separate experiments.

Another possible cause of elevated TRX level is mucin production and/or secretion from cancer cells. Not only were mucinous carcinoma cells in mucous lake and signet-ring cells intensely stained, but intestinal metaplasia also frequently showed very intense immunoreactivity for TRX in our study, in stark contrast to adjacent nonmetaplastic epithelium (data not shown). It is noteworthy that the TRX level is elevated in intestinal metaplasia, which is regarded as part of the background in carcinogenesis.

In summary, the present results demonstrate that the cellular TRX level is elevated in gastric cancer and that it is strongly associated with the histological type of the tumor. In addition to the overall factors associated with overexpression of TRX in cancer, there may be certain chemical or biological characteristics of undifferentiated gastric cancers that are related to this elevation in cellular TRX.

ACKNOWLEDGMENTS

The authors thank Dr. Junji Yodoi for providing cDNA of TRX and Ms. Shinmura for her technical advice. This study was supported in part by a 2nd-Term Grant-in-Aid for the Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health and Welfare of Japan; by a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan; and by the Bristol-Myers Squibb Foundation.

ABBREVIATIONS

Ab, Antibody; ADF, ATL-derived factor; ATL, adult T-cell leukemia; EBER, Epstein-Barr virus-encoded small RNA; EBV, Epstein-Barr virus; FCS, fetal calf serum; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; H_2O_2, hydrogen peroxide; HTLV-1, human T-lymphotropic virus-1; IL-1, interleukin-1; LI, labeling index; moAb, monoclonal antibody; PBL, peripheral blood lymphocytes; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; poAb, polyclonal antibody; ROS, reactive oxygen species; rTRX, recombinant TRX; TNF, tumor necrosis factor; TRX, thioredoxin.

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Received for publication February 7, 2000; accepted April 10, 2000.

This article has been cited by:

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