Jpn. J. Cancer Res. 82, 1428–1435, December 1991

Aberrant Elevation of Tyrosine-specific Phosphorylation in Human Gastric Cancer Cells

Eisuke Takeshima,[1] Michinari Hamaguchi,[2,3] Tadashi Watanabe,[1] Seiji Akiyama,[1] Masato Kataoka,[1] Yukano Ohnishi,[1] Hengyi Xiao,[2] Yoshiyuki Nagai[2] and Hiroshi Takagi[1]

[1]*Department of Surgery II and* [2]*Research Institute for Disease Mechanism and Control, Nagoya University School of Medicine, Tsurumai-cho 65, Showa-ku, Nagoya 466*

Phosphotyrosine-containing proteins in various human cancer cell lines were studied by immunoblotting with anti-phosphotyrosine antibody. Of 29 cell lines derived from oral epidermoid cancer, esophageal cancer, gastric cancer, colon cancer, pancreatic cancer, hepatocellular carcinoma and malignant melanoma, 3 of the 6 gastric cancer cells showed aberrant elevation of tyrosine-specific phosphorylation. On the other hand, both esophageal cancer cells and colon cancer cells, which were reported to have amplified epidermal growth factor receptor and activated $p60^{v\text{-}src}$ kinase, respectively, showed no apparent elevation of tyrosine-specific phosphorylation, and their profiles of phosphorylation were similar to that of normal human fibroblasts. Two gastric cancer cells, NUGC-4 and MKN-45, showed similar profiles of phosphorylation but their responses to growth factors differed from each other. Tyrosine phosphorylation in NUGC-4 was strongly activated by treatment with epidermal growth factor and quickly reduced by the acid treatment which is effective in removing growth factors from cellular surface receptors. On the contrary, phosphorylation in MKN-45 did not respond to either growth factor or acid treatment. These results suggest that NUGC-4 and MKN-45 have tyrosine kinases which are activated by different mechanisms but share similar substrates.

Key words: Phosphotyrosine — Anti-phosphotyrosine antibody — Tyrosine kinase — $p60^{c\text{-}src}$ — Human gastric cancer

Since the discovery of *src* gene, more than 50 distinct oncogenes of viral and cellular origins have been identified. These oncogenes are grouped into several classes on the basis of their structure and function,[1] and many of them belong to the groups that encode tyrosine-protein kinases. Extensive studies have been made to identify oncogenes that encode tyrosine-protein kinase in human cancer cells, and several lines of evidence suggested that oncogenes of tyrosine kinase-type are involved in the generation and immortalization of various types of human cancer. Overexpression or aberrant expression of epidermal growth factor (EGF)[4] receptors has been reported in cell lines derived from gastric cancer and squamous cancer of various origins such as skin, tongue, esophagus, gingiva and vulva.[2-4] *trk* and *met* oncogenes that were isolated from colon carcinoma[5] and from osteosarcoma,[6] respectively, were shown to be active in transfection assay. In chronic myelogenous leukemia cells, the c-*abl* gene product showed constitutive activation of kinase.[7] Activation of $p60^{c\text{-}src}$ protein kinase was widely observed in human colon carcinoma cells and tissues.[8] However, in these studies, the methods for the identification of oncogenes were limited to *in vitro* kinase assay or DNA-transfection assay, which occasionally caused activation of tyrosine kinase during the transfection step by rearrangement of the kinase gene.[9] Thus, it is uncertain whether *in vivo* tyrosine phosphorylation is elevated in these human cancer cells. Moreover, the overall picture of tyrosine-specific phosphorylation in various human cancers is still largely unclear.

Recently, a method to detect phosphotyrosine (PTYR)-containing proteins was developed by utilizing specific sera recognizing PTYR residues.[10-14] We have examined the specificity of these sera in analysis of substrated of $p60^{v\text{-}src}$ kinase, and found that these antisera could effectively recognize most, if not all, of the PTYR-containing proteins in RSV-transformed cells.[14] We used this technique to detect PTYR-containing proteins in human cancer cells. In this report, we show that a number of human gastric cancer cell lines have elevated level of tyrosine-specific phosphorylation, and that phosphorylation in one of these cell line, MKN-45 does not respond to growth factor stimulation.

MATERIALS AND METHODS

Cells Cell lines derived from human gastric cancer, NUGC-2, NUGC-3 and NUGC-4, were established in our laboratory as described previously.[15] Cell lines

[3] To whom correspondence should be addressed.

[4] Abbreviations: EGF, epidermal growth factor; PTYR, phosphotyrosine; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PDGF, platelet-derived growth factor

derived from esophageal cancer, TE-1, TE-2 and TE-3, were kindly supplied by Dr. T. Nishihira of Tohoku University.[16] Cell lines derived from lung cancer, QG-56, QG-90 and Darby, were kindly supplied by R. Ueda of Aichi Cancer Center. Cell lines derived from colon cancer, SW-480, SW-1083, SW-1116, SW-1222, SW-1417 HT-29, cell lines derived from lung cancer, Luci-7, Luci-10 and Luci-13, cell lines derived from malignant melanoma, SK-MEL-36, SK-MEL-37 and SK-MEL-40 and a cell line derived from pancreatic cancer, Panc-1, were donated by Memorial Sloan Kettering Cancer Center. Cell lines derived from gastric cancer, MKN-45 and Kato-III, cell lines derived from hepatoma, HLE and HLF, a cell line derived from pancreatic cancer, Paca-2, and a cell line derived from vulvar cancer, A431, were supplied by the Japanese Cancer Research Resources Bank. Human fibroblasts were supplied by Dr. S. Saga of Nagoya University. A rat cell line, 3Y1, and SR-RSV-transformed 3Y1 (SR3Y1) were described elsewhere.[17]

Preparation of anti-PTYR antibody An affinity-purified antibody that specifically recognized PTYR residues was prepared as described previously.[14] Briefly, v-*abl*-encoded protein expressed in bacteria, which can autophosphorylate on tyrosine residues,[18] was partially purified and used to immunize rabbits. Antibody specifically recognizing PTYR residue was purified from the sera by affinity chromatography, using PTYR-coupled Sepharose as previously described.[14]

Immunoblotting Subconfluent cell cultures were washed with ice-cold phosphate-buffered saline (PBS) containing 0.5 m*M* sodium orthovanadate, and quickly solubilized with a buffer for gel electrophoresis which contains 2% sodium dodecyl sulfate (SDS) and 5% mercaptoethanol. The protein concentration was determined with a protein assay kit (Bio-Rad Laboratories), and 100 μg of proteins was subjected to SDS-7.5% polyacrylamide gel electrophoresis (PAGE). After electrophoresis, proteins were transferred electrophoretically to polyvinylidene difluoride (PVDF) membranes (Millipore), and probed with anti-PTYR antibody or anti-EGF receptor antibody (ICN) followed by ^{125}I-protein A (ICN). Transfer, blocking, antibody incubation, and ^{125}I-protein A incubation were performed as described.[14] Autoradiography was performed on X-ray film with an intensifying screen at -80°C for 24 h, unless otherwise indicated.

Inhibition of anti-PTYR antibody staining by PTYR To examine the specificity of antibody, PVDF filters to which protein bands were transferred were probed with anti-PTYR antibody in the presence of 5 m*M* phosphotyrosine (Sigma) and reacted with ^{125}I-protein A. Phosphoserine and phosphothreonine were used as controls.

Treatment of cells with growth factors Cells were starved for 2 h with serum-depleted medium, and subsequently stimulated with 5 n*M* epidermal growth factor (Wakunaga) or 1 half-maximal unit/ml of platelet-derived growth factor (Collaborative Research Inc.).

Treatment of cells with acidic medium Subconfluent cells were washed with PBS and subsequently incubated at 37°C for the indicated times in minimum essential medium containing 0.25% bovine serum albumin; the pH was adjusted to 3.7 with HCl.[19-21] After the treatment, cells were washed with ice-cold PBS, lyzed, subjected, to PAGE, and analyzed by immunoblotting with anti-PTYR antibody. Viability of the cells after acid treatment was examined by means of the trypan blue exclusion test and we found that more than 90% of cancer cells used in this experiment survived.

RESULTS

Tyrosine-specific phosphorylation of cellular proteins in cells derived from human cancers Cell lines derived from various human cancers were analyzed for PTYR-containing proteins by immunoblotting with anti-PTYR antibody. The cell lines used and their relative levels of tyrosine phosphorylation with respect to SR3Y1 cells are shown in Table I. Human fibroblasts, rat 3Y1 cells and SR3Y1 (RSV transformant of 3Y1) were used as negative and positive controls, respectively. As shown in Fig. 1, we found that three of six gastric cancer cells (NUGC-4, MKN-45 and Kato-III) had dramatically elevated levels of tyrosine phosphorylation. Although their origins and morphology are different (Fig. 3), the profiles of phosphorylation of these gastric cancer cells were remarkably similar to each other. To estimate the relative content of PTYR in these gastric cancer cells, each lane of immunoblotted filters was excised and its radioactivity was measured with a liquid scintillation counter. As shown in Table I, these gastric cancer cells had approximately 45–65% of PTYR compared with that of RSV-transformed 3Y1, strongly suggesting that tyrosine kinase(s) is aberrantly activated in these cells. Compared with these gastric cancer cells, most of the cell lines derived from esophageal cancer, colon cancer, hepatoma and lung cancer showed a moderate elevation of tyrosine-specific phosphorylation. We found that several proteins were phosphorylated in these cells but the phosphorylation profiles were essentially the same as that of fibroblasts (Fig. 1), suggesting that these PTYR-containing proteins are components involved in the normal cellular pathway of growth regulation. Yamamoto *et al.*[2] reported that cell lines derived from esophageal cancer (TE-1, TE-2 and TE-3) show amplified expression of EGF receptor. However, we found this overexpression did not lead to the aberrant phosphorylation of cellular proteins, although phosphorylation of a protein band (185 kDa) corresponding to EGF receptor was observed (Figs. 1 and 4). Bolen *et al.* reported that colon cancer

Table I. Classification of Human Cancer Cell Lines according to the Degree of Phosphorylation

Degree of phosphorylation		Origin	Cell line	Histology
Strong	65%	stomach	MKN-45	poorly differentiated adenocarcinoma
(45–65%)	45%		Kato-III	signet ring cell cancer
	52%		NUGC-4	poorly differentiated adenocarcinoma
Moderate		esophagus	TE-1	well differentiated squamous cell carcinoma
(10–25%)			TE-2	poorly differentiated squamous cell carcinoma
			TE-3	well differentiated squamous cell carcinoma
		stomach	NUGC-2	poorly differentiated adenocarcinoma
			NUGC-3	poorly differentiated adenocarcinoma
			MKN-1	adenosquamous cell carcinoma
		colon	SW-480	adenocarcinoma
			SW-1083	adenocarcinoma
			SW-1116	adenocarcinoma
			SW-1222	adenocarcinoma
			SW-1417	adenocarcinoma
			HT-29	adenocarcinoma
		liver	HLE	nondifferentiated hepatoma
			HLF	differentiated hepatoma
		lung	Luci-7	large cell carcinoma
			Luci-13	ND
			QG-56	squamous cell carcinoma
			QG-90	small cell carcinoma
			Darby	ND
		melanocyte	SK-MEL-37	malignant melanoma
			SK-MEL-40	malignant melanoma
Weak		mouth	KB	oral epidermoid carcinoma
(<9%)		pancreas	Panc-1	ND
			Paca-2	ND
		lung	Luci-10	adenosquamous cell carcinoma
		melanocyte	SK-MEL-36	malignant melanoma
Control	100%	fibroblast	SR3Y1	*src*-transformed 3Y1
	9%	fibroblast		human, embryonic

ND: no data.

cells had activated $p60^{c\text{-}src}$ kinase.[8] We used the same cell lines (SW-480, SW-1116, SW-1417, and HT-29), and confirmed activation of $p60^{c\text{-}src}$ (Xiao and Hamaguchi, unpublished results). However, we found that the levels and profiles of tyrosine phosphorylation in these cell lines were similar to those of normal human fibroblasts. Thus, the activation of $p60^{c\text{-}src}$ observed *in vitro* in these colon cancer cells is not accompanied with apparent elevation or qualitative change of *in vivo* phosphorylation.

Cell lines derived from pancreatic cancer (Panc-1 and Paca-2), oral epidermoid cancer (KB), lung cancer (Luci-10) and malignant melanoma (SK-MEL-36) showed weak, though definite, levels of phosphorylation (Fig. 1). It is likely that tyrosine kinases are not constitutively activated in these cancer cells. We found that two PTYR-containing proteins of 110 kDa and 130 kDa were most prominent and widely detected among different types of cancer as well as 3Y1 and normal fibroblasts. It is possible that these proteins are the major tyrosine-phosphorylated components in the cellular pathway regardless of growth factor stimulation.

To assess the specificity of anti-PTYR antibody, we examined the inhibitory effect of phosphoamino acids upon antibody-antigen recognition with two gastric

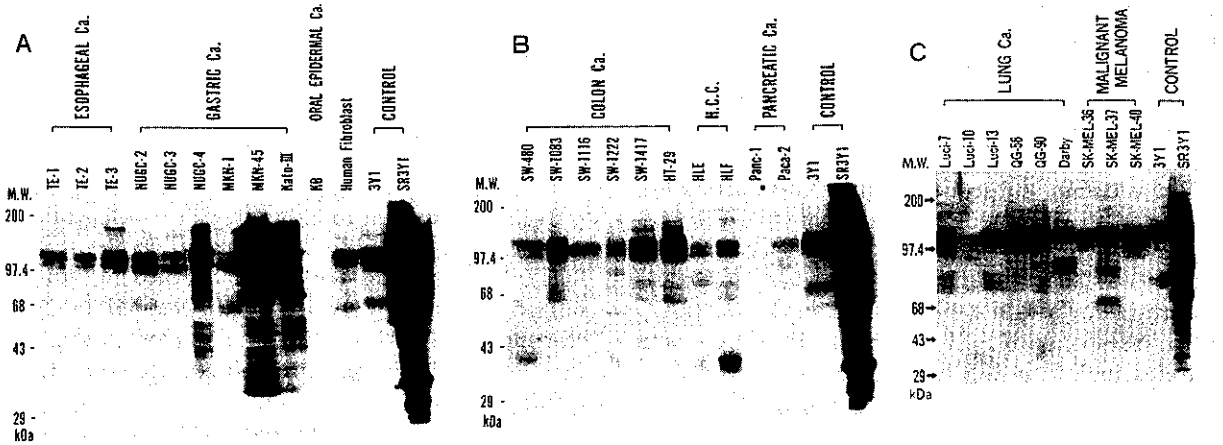

Fig. 1. Detection of PTYR-containing proteins in human cancer cell lines. Each cell lysate (100 μg) was analyzed for its PTYR-containing proteins by immunoblotting with anti-PTYR antibody. (A) Cell lines derived from esophageal cancer, gastric cancer, and oral epidermal cancer. (B) Cell lines derived from colon cancer, hepatocellular carcinoma, and pancreatic cancer. (C) Cell lines derived from lung cancer, and malignant melanoma. Human fibroblasts, rat 3Y1 cells and *src*-transformed 3Y1 (SR3Y1) were used as controls.

cancer cell lines (MKN-45 and NUGC-4) which showed prominent elevation of tyrosine-specific phosphorylation (Fig. 2). While addition of phosphoserine and phosphothreonine to anti-PTYR antibody solution had no effect on the results, phosphotyrosine completely blocked the reactivity of our antibody, indicating that the recognition is highly specific for phosphotyrosine residues of these cellular proteins.

Immunofluorescent staining of PTYR-containing proteins in human cancer cell lines To examine the subcellular localization of major PTYR-containing proteins of cells which showed elevated level of phosphorylation, cells were fixed and reacted with anti-PTYR antibody and an FITC-labeled secondary antibody. As shown in Fig. 3, all cell lines tested showed strong fluorescence mainly in the submembranous area, but not in the nuclear area. The submembranous fluorescence was strongly granular for the gastric cancer cells that showed dramatic elevations of phosphorylation. These results suggest that some of the PTYR-containing proteins in the human cancer cell lines may be components in signal transduction through the plasma membrane.

Expression of EGF receptor in cancer cell lines and effect of growth factor treatments on the tyrosine phosphorylation The expression level of EGF receptor in the cells which have elevated tyrosine phosphorylation was examined by immunoblotting with anti-EGF receptor antibody. As shown in Fig. 4, esophageal cancer cells (TE-1 and TE-2) had elevated expression of EGF receptor as previously reported.[2] We found two hepatoma cells examined (HLE and HLF) had extremely elevated

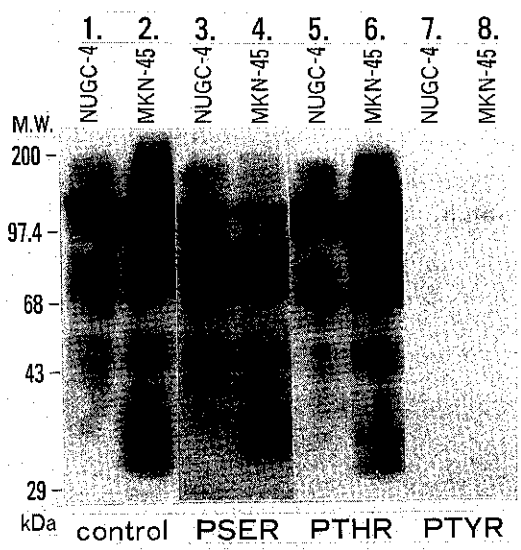

Fig. 2. Inhibition of detection of PTYR-containing proteins by phosphotyrosine. Cell lysates from NUGC-4 and MKN-45 were examined by immunoblotting with anti-PTYR antibody in the absence (control) or presence of phosphoserine (PSER), phosphothreonine (PTHR) and phosphotyrosine (PTYR).

expression of EGF receptor. The gastric cancer cell lines that had elevated level of PTYR (NUGC-4, MKN-45 and Kato-III) showed similar degrees of EGF receptor expression, slightly higher than those of colon cancer cells but lower than those of esophageal cancer cells.

We next examined the effect of EGF treatment on tyrosine-specific phosphorylation in tumor cells that showed high expression of EGF receptor. A cell line A431, derived from human epidermoid cancer, which has an abnormally elevated expression of EGF receptor[4] was used as a positive control. Cells were starved with serum-free medium, subsequently stimulated with EGF and subjected to immunoblotting with anti-PTYR antibody. As shown in Fig. 5A, tyrosine-specific phosphorylation of A431, HLF, TE3 as well as the fibroblasts was suppressed by serum depletion and was dramatically increased in response to EGF treatment. PTYR-containing proteins including 185 kDa protein possibly corresponding to the EGF receptor in TE3 and HLF (Fig. 1) were strongly phosphorylated by EGF treatment, suggesting that phosphorylation of these proteins is growth factor-dependent. On the other hand, two gastric cancer cells which had elevated phosphorylation showed different responses to EGF treatment, although their phosphorylation profiles were very similar to each other. In MKN45, serum depletion from the medium did not suppress the phosphorylation (data not shown). Moreover, neither the level nor the profile of phosphorylation was changed by the addition of EGF. Therefore it is likely that tyrosine kinase is activated in MKN-45, regardless of EGF stimulation. In contrast, depletion of serum resulted in a strong suppression of the phosphorylation in NUGC-4, although some PTYR-containing proteins remained phosphorylated even after the depletion, and EGF treatment dramatically increased the phosphorylation. Thus, most of the tyrosine-specific phosphorylation in NUGC-4 seemed to be growth factor-dependent.

We next examined the effect of platelet-derived growth factor (PDGF) on the phosphorylation of these cells (Fig. 5B). We found that NUGC-4 and MKN-45 did not respond to PDGF treatment, and tyrosine phosphorylation in these cells remained unchanged after the treatment.

Effect of acid treatment on tyrosine-specific phosphorylation in MKN-45 cells Since various cancer cells are known to produce growth factors by themselves that stimulate autocrine growth of cells, it is possible that unknown growth factors other than EGF and PDGF might have activated the tyrosine phosphorylation of these gastric cancer cells. To test this possibility, we examined the effect on phosphorylation of a brief acid treatment, which has been shown to remove the growth factors such as EGF and PDGF bound to cellular receptors and thereby suppress growth factor-induced tyrosine phosphorylation, rapidly and reversibly.[19-21] It was reported that this treatment did not destroy the capability of EGF receptor to autophosphorylate in response to EGF after acid treatment,[20] nor did it affect the $p60^{v\text{-}src}$ kinase activity in v-*src*-transformed mouse fibroblasts.[21] It is, therefore, likely that the decrease of phospho-

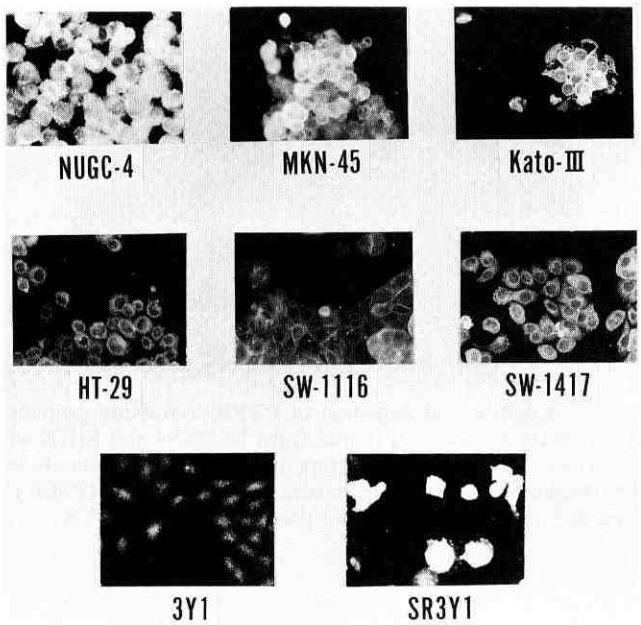

Fig. 3. Immunofluorescent staining of human cancer cells with anti-PTYR antibody. Three cell lines derived from gastric cancer (NUGC-4, MKN-45 and Kato-III) and three cell lines derived from colon cancer (HT-29, Sw-1116 and SW-1417) were fixed and stained with anti-PTYR antibody. 3Y1 and SR3Y1 were used as controls.

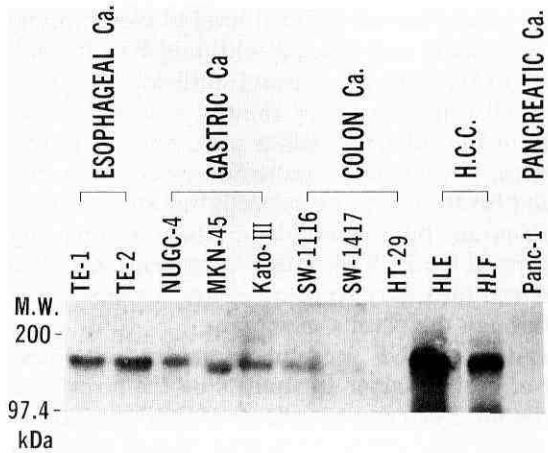

Fig. 4. Detection of EGF receptor in human cancer cells. The expresion levels of EGF receptor in human cancer cells derived from esophageal cancer, gastric cancer, colon cancer, hepatocellular carcinoma, pancreatic cancer, oral epidermal cancer and malignant melanoma were examined by immunoblotting with anti-EGF receptor antibody.

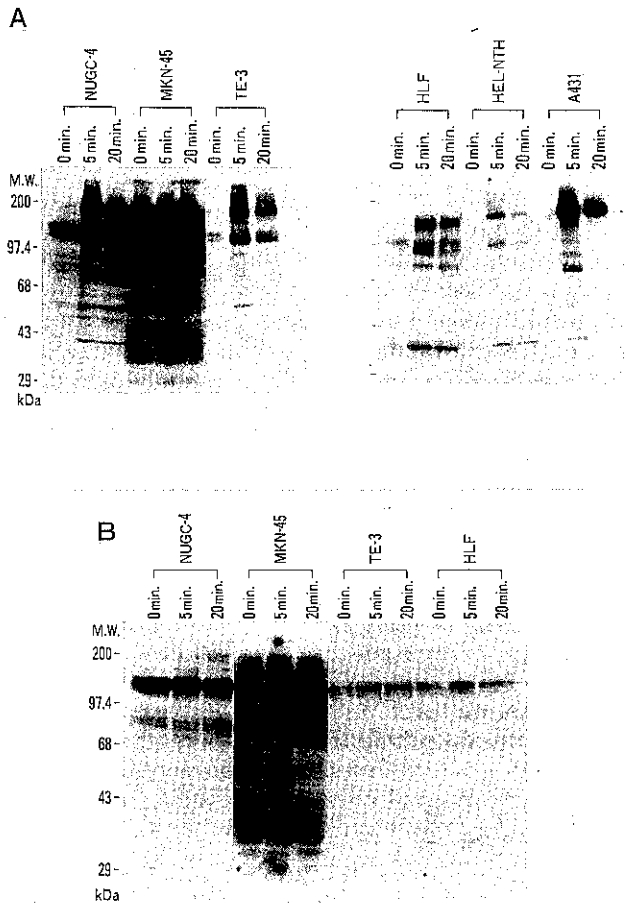

Fig. 5. PTYR-containing proteins in cells stimulated with EGF or PDGF. Subconfluent cell cultures were starved with serum-depleted medium and subsequently stimulated with EGF (A) or PDGF (B). Cells were harvested at the indicated times and PTYR-containing proteins in each lysate were examined by anti-PTYR antibody.

tyrosine by the treatment is due to dephosphorylation. We found that 3Y1 responded well to the acid treatment with a rapid reduction of tyrosine-specific phosphorylation, whereas its transformant, SR3Y1, was resistant and tyrosine phosphorylation remained unchanged (Fig. 6). When gastric cancer cells NUGC-4 and MKN-45 were treated with acid, tyrosine phosphorylation in the former was dramatically reduced within a short time of treatment. The tyrosine phosphorylation in the latter, however, was not changed by acid treatment. Thus, these results are consistent with that of EGF treatment and strongly suggest that most of the tyrosine phosphorylation in NUGC-4 is growth factor-dependent, whereas some tyrosine kinase(s) is constitutively activated in MKN-45 regardless of growth factor stimulation.

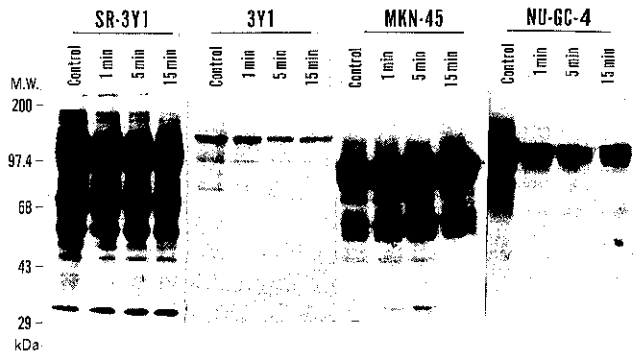

Fig. 6. PTYR-containing proteins in cells treated with acidic medium. Subconfluent cell cultures were treated with acidic medium (pH 3.7) for the indicated times. Then cells were harvested and PTYR-containing proteins in each lysate were examined by immunoblotting with anti-PTYR antibody.

DISCUSSION

The results presented in this report demonstrate that human tumor cell lines of different origins vary in the level of tyrosine-specific phosphorylation, some of them showing a dramatically elevated level. Complete blocking of the antibody reaction by phosphotyrosine confirmed the specificity of anti-PTYR antibody. Of the 29 cell lines examined, we found that many gastric cancer cell lines (3 of 6 cell lines examined) had aberrant elevation of tyrosine-specific phosphorylation. These three cell lines showed similar profiles of phosphorylation, suggesting homologous signaling pathways were activated in these cells. However, they differed in the growth factor dependency of phosphorylation. In MKN-45 cells that showed most prominent elevation of phosphorylation, depletion of serum from medium or stimulation of cells with growth factors changed neither the level nor the profile of tyrosine phosphorylation. Thus, it is likely that tyrosine kinase(s) is constitutively activated in MKN-45 cells, although we cannot rule out the posibility that tyrosine-phosphatases are systematically suppressed in these cells. On the contrary, tyrosine-specific phoshorylation in NUGC-4 was stimulated by EGF treatment and was sensitive to acid treatment. Therefore, tyrosine phosphorylation in NUGC-4 seems to be growth factor-dependent. However, we found that several cell lines derived from esophageal cancer (TE-1, TE-2 and TE-3) and hepatoma (HLE and HLF) had higher levels of EGF receptor expression than that of NUGC-4, yet the levels of their tyrosine phosphorylation were far lower than that of NUGC-4. Thus, tyrosine phosphorylation in NUGC-4 seems to be amplified by some unidentified mechanism such as systemic suppression of tyrosine

phosphatase, which may contribute to autocrine growth of this cell line.

Bolen *et al.*[8] reported activation of $p60^{c\text{-}src}$ kinase *in vitro* in colon cancer cells. We obtained similar results with the same cell lines (Xiao and Hamaguchi, unpublished data). However, we found that this activation observed *in vitro* did not apparently cause *in vivo* elevation of tyrosine phosphorylation. On the contrary, so far as we have examined, elevation of tyrosine phosphorylation closely correlated with the transforming activity of various c-*src* mutants.[14, 22] It is, therefore, likely that activation of $p60^{c\text{-}src}$ kinase does not directly correlate with transformation of colon cells.

Immunofluorescence studies of human cancer cells with anti-PTYR antibody showed that the degree of fluorescence correlates well with the level of tyrosine-specific phosphorylation, and that fluorescence was specifically blocked with phosphotyrosine. These results suggest that anti-PTYR antibody is applicable to immunohistochemical characterization of human cancer tissues.

In summary, we found that cell lines derived from human gastric cancer have high incidence of activation of tyrosine-specific phosphorylation. No such aberrant elevation of tyrosine phosphorylation has been seen so far for other cancer cell lines derived from esophageal cancer, colon cancer, lung cancer, hepatoma and oral epidermoid cancer. Different responses to EGF treatment and acid treatment between NUGC-4 and MKN-45 suggest that tyrosine kinases activated in these cells are different in species. However, the similarity of phosphorylation profile suggests that homologous signaling pathways leading to cell growth and transformation may be activated in these cell lines. It is of importance to know what species of tyrosine kinase is activated in these gastric cancer cells. We observed activation of $p60^{c\text{-}src}$ kinase in these cells, especially in MKN-45 (Xiao *et al.*, unpublished results), although the mechanism of activation and its correlation to cell transformation are yet to be studied. Recently, anti-PTYR antibody was shown to be useful to clone tyrosine kinases by immunoscreening of a cDNA expression library. With this technique, several new tyrosine kinases were successfully cloned.[23–25] We are currently screening the cDNA library of gastric cancer cell lines with anti-PTYR antibody to identify the activated kinases.

ACKNOWLEDGMENTS

We are grateful to Dr. J. Wang for her gift of bacteria expressing v-*abl* protein and to Drs. T. Nishihara, R. Ueda, and S. Saga for their gifts of cancer cell lines. We are also grateful to Dr. H. Hanafusa for his continuous encouragement and support. We thank M. Sugata and F. Yamauchi for their excellent technical assistance. This work was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, Japan and a grant from the Foundation for Promotion of Cancer Research.

(Received July 4, 1991/Accepted September 13, 1991)

REFERENCES

1) Hunter, T. and Cooper, J. A. Protein-tyrosine kinases. *Ann. Rev. Biochem.*, **53**, 897–930 (1985).
2) Yamamoto, T., Kamata, N., Kawano, H., Shimizu, S., Kuroki, T., Toyoshima, K., Rikimaru, K., Nomura, N., Ishizaki, R., Pastan, I., Gamou, S. and Shimizu, N. High incidence of amplification of the epidermal growth factor receptor gene in human squamous carcinoma cell lines. *Cancer Res.*, **46**, 414–416 (1986).
3) Yasui, W., Sumiyoshi, H., Hata, J., Kameda, T., Ochiai, A., Ito, H. and Tahara, E. Expression of epidermal growth factor receptor in human gastric and colonic cacinomas. *Cancer Res.*, **48**, 137–141 (1988).
4) Ullrich, A., Coussens, L., Hayflick, J. S., Dull, T. J., Grey, A., Tam, A. W., Lee, J., Yarden, Y., Libermann, T. A., Shlessinger, J., Downward, J., Mayes, E. L. V., Whittle, N., Waterfield, M. D. and Seeburg, P. H. Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. *Nature*, **309**, 418–425 (1984).
5) Martin-Zanca, Hughes, S. H. and Barbacid, M. A human oncogene formed by the fusion of the truncated tropomyosin and protein tyrosine kinase sequences. *Nature*, **319**, 743–747 (1986).
6) Cooper, C. S., Park, M., Blair, D. G., Tainski, M. A., Huebner, K., Crose, C. M. and Vande Woude, G. F. Molecular cloning of a new transforming gene from chemically transformed human cell line. *Nature*, **311**, 29–33 (1984).
7) Konopka, J. B., Watanabe, S. M., Singer, J. W., Collins, S. J. and White, O. N. Cell lines and clinical isolates derived from Ph′-positive chronic myelogenous leukemia patients express c-*abl* proteins with a common structural alteration. *Proc. Natl. Acad. Sci. USA*, **82**, 1810–1814 (1985).
8) Bolen, J. B., Veillette, A., Schwartz, A. M., DeSeau, V. and Rosen, N. Activation of $p60^{c\text{-}src}$ protein kinase activity in human colon carcinoma. *Proc. Natl. Acad. Sci. USA*, **84**, 2251–2255 (1987).
9) Takahashi, M., Ritz, J. and Cooper, G. M. Activation of a novel human transforming gene, *ret*, by DNA arrange-

ment. *Cell,* **42,** 581–588 (1985).
10) Rose, A. H., Baltimore, D. and Eisen, H. N. Phosphotyrosine-containing proteins isolated by affinity chromatography with antibodies to a synthetic hapten. *Nature,* **294,** 654–656 (1981).
11) Frackelton, A. R., Ross, A. H. and Eisen, H. N. Characterization and use of monoclonal antibodies for isolation of phosphotyrosyl proteins from retrovirus-transformed cells and growth factor-stimulated cells. *Mol. Cell. Biol.,* **3,** 1343–1352 (1983).
12) Pang, D. T., Sharma, B. R. and Shafer, J. A. Purification of the catalytically active phosphorylated form of insulin receptor kinase by affinity chromatography with o-phosphotyrosyl-binding antibodies. *Arch. Biochem. Biophys.,* **242,** 176–186 (1985).
13) Wang, J. Y. J. Isolation of antibodies for phosphotyrosine by immunization with a v-*abl* oncogene-encoded protein. *Mol. Cell. Biol.,* **5,** 3640–3643 (1985).
14) Hamaguchi, M., Grandori, C. and Hanafusa, H. Phosphorylation of cellular proteins in Rous sarcoma virus-infected cells: analysis by use of anti-phosphotyrosine antibodies. *Mol. Cell. Biol.,* **8,** 3035–3042 (1988).
15) Akiyama, S., Amo, H., Watanabe, T., Matsuyama, M., Sakamoto, J., Imaizumi, H., Kondo, T. and Takagi, H. Characteristics of three human gastric cancer cell lines, NUGC-2, NUGC-3 and NUGC-4. *Jpn. J. Surg.,* **18,** 438–446 (1988).
16) Nishihira, T., Kasai, M., Mori, S., Watanabe, T., Kuriya, Y., Suda, M., Kitamura, M., Hirayama, K., Akaishi, T. and Sasaki, T. Characteristics of two cell lines (TE-1 and TE-2) derived from human squamous cell carcinoma of the esophagus. *Gann,* **70,** 575–584 (1979).
17) Kawai, S. Transformation of rat cells by fusion-infection with Rous sarcoma virus. *J. Virol.,* **34,** 772–776 (1980).
18) Wang, J. Y. J. and Baltimore, D. Localization of tyrosine kinase-coding region in v-*abl* oncogene-encoded proteins in bacteria. *J. Biol. Chem.,* **260,** 64–71 (1985).
19) Garrett, J. S., Coughlin, S. R., Niman, H. L., Tremble, P. M., Giels, G. M. and Williams, L. T. Blockade of autocrine stimulation in simian sarcoma virus-transformed cells reverses down-regulation of platelet-derived growth factor receptors. *Proc. Natl. Acad. Sci. USA,* **81,** 7466–7470 (1984).
20) Sturani, E., Zippel, R., Toschi, L., Comoligo, P. M. and Alberghina, L. Kinetics and regulation of the tyrosine phosphorylation of epidermal growth factor receptor in intact A431 cells. *Mol. Cell. Biol.,* **8,** 1341–1351 (1988).
21) Giordano, S., Di Renzo, M. F., Ferracini, R., Chiado-Piat, L. and Comoglio, P. M. p145, a protein with associated tyrosine kinase activity in a human gastric carcinoma cell line. *Mol. Cell. Biol.,* **8,** 3510–3517 (1988).
22) Jove, R., Hanafusa, T., Hamaguchi, M. and Hanafusa, H. *In vivo* phosphorylation states and kinase activities of transforming $p60^{c\text{-}src}$ mutants. *Oncogene Res.,* **5,** 49–60 (1989).
23) Kornbluth, S., Paulson, K. E. and Hanafusa, H. Novel tyrosine kinase identified by phosphotyrosine antibody screening of cDNA libraries. *Mol. Cell. Biol.,* **8,** 5541–5544 (1988).
24) Pasquale, E. B. and Singer, S. J. Identification of a developmentally regulated protein-tyrosine kinase by using anti-phosphotyrosine antibodies to screen a cDNA expression library. *Proc. Natl. Acad. Sci. USA,* **86,** 5449–5453 (1989).
25) Ben-David, Y., Letwin, K., Tannock, L., Bernstein, A. and Pawson, T. A mammalian protein kinase with potential for serine/threonine and tyrosine phosphorylation is related to cell cycle regulators. *EMBO J.,* **10,** 317–325 (1991).