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Angiogenic Factor Thymidine Phosphorylase Increases Cancer Cell Invasion Activity in Patients with Gastric Adenocarcinoma

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Abstract

We investigated the biological role of thymidine phosphorylase (TP), an angiogenic factor, in gastric cancer cell migration and invasion and explored a therapeutic approach for high TP-expressing tumors using TP enzymatic inhibitor (TPI) and rapamycin. We established TP cDNA overexpressing gastric cancer cell lines (MKN-45/TP and YCC-3/TP) and did invasion and adhesion assays with Matrigel-coated transwell membranes. The related signal pathway using recombinant human TP (rhTP), deoxy-D-ribose (D-dRib), and signal pathway inhibitors (wortmannin, LY294002, and rapamycin) was investigated. First, AGS and MKN-1 gastric cancer cell lines showed dose-dependent up-regulation of invasiveness through Matrigel following treatment with rhTP or D-dRib. TP-overexpressing cancer cell lines displayed increased migration and invasion activity, which doubled with rhTP and D-dRib treatment. This activity depended on the enzymatic activity of TP, and TP stimulated the adhesion of cancer cells onto Matrigel and induced actin filament remodeling. Finally, we showed that this activity is related to increased phosphatidylinositol 3-kinase activity in TP-overexpressing cells and that combination treatment with rapamycin and TP enzymatic inhibitor produces an additive effect to abrogate TP-induced invasion. Taken together, TP increases the migration and invasion of gastric cancer cells, especially in TP-expressing cells. Therapies targeting TP might diminish the propensity for invasion and metastasis in gastric cancer. (Mol Cancer Res 2008;6(10):1554–66)

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Introduction

Migration and invasion of cancer cells, important characteristics associated with malignancy, are involved in the genesis of metastatic disease. Cell migration and invasion depend on cellular interactions with the extracellular matrix and are further regulated by factors produced and secreted by tumor cells and the surrounding stromal cells. Thymidine phosphorylase (TP) is a nucleoside metabolism enzyme, identical to the angiogenic factor platelet-derived endothelial cell growth factor (1). TP catalyzes the breakdown of thymidine to thymine and the sugar 2-deoxy-D-ribose-1-phosphate and induces endothelial cell chemotaxis *in vitro* and angiogenesis *in vivo* (1). TP modulates both angiogenesis and apoptosis under hypoxic conditions, suggesting that TP plays an important role in cancer progression (2, 3). The activity and expression of TP in carcinoma of the esophagus, stomach, colorectum, pancreas, and lung are significantly higher than in the adjacent nonneoplastic tissue (4).

TP is expressed not only in tumor cells but also in tumor-associated stromal cells. Several investigations have suggested that such stromal expression is substantially associated with an unfavorable prognosis in various cancers (5). However, a correlation between the expression and activity of TP and cancer progression, especially invasion and metastasis, has been observed mostly from analyses using clinical samples. High TP expression in the primary tumor is a risk factor for both hepatic and lymph node metastasis in patients with gastric adenocarcinoma (6, 7). Significant correlations have been observed between TP expression and extrapancreatic neural plexus invasion or the presence of postoperative hepatic metastasis in patients with ductal adenocarcinoma of the pancreas (8). TP mRNA levels are 33-fold higher in invasive bladder cancer than in superficial tumors and 260-fold higher than in normal bladder tissue (9). A few preclinical studies have shown that transfection of TP cDNA into the RT112 bladder cancer cell line transforms its superficial phenotype into an invasive one (10). Moreover, the TP-overexpressing KB head and neck cancer cell line displays a greater metastatic tendency to the liver in an animal model (11). All these findings imply that TP contributes to the invasion and metastasis of cancer cells through mechanisms other than angiogenesis, although significantly less is known about the biological action of TP in this respect. Furthermore, recent studies have shown that TP enzymatic inhibitor (TPI) can suppress chemotaxis and basement membrane invasion of cancer cells (12). Previously,

we have shown that 2-deoxy-D-ribose (D-dRib), which is a dephosphorylation product of 2-deoxy-D-ribose-1-phosphate, is a downstream mediator of TP functions (2, 13).

Gastric adenocarcinoma is the second most common cause of cancer-related mortality worldwide (14). Even with the rapid advancement of treatment options, the prognosis for gastric cancer is still unsatisfactory due to early metastasis and recurrence and the highly invasive and metastatic nature of gastric cancers. The molecular basis behind the induction of invasive activity remains largely unknown. In this study, we investigated the biological role of TP in cancer cell invasion in gastric adenocarcinoma, and we explored a therapeutic approach for high TP-expressing tumors using TPI and rapamycin as antimetastatic therapy.

Results

Relationship between TP Expression and Gastric Cancer Cell Invasion Activity through Matrigel

First, we investigated the protein expression of TP in gastric cancer cell lines. TP expression was detected in 6 (YCC-2, YCC-6, YCC-9, YCC-16, AGS, and NCI-N87) of 12 cell lines. When the cell lines were divided into two groups according to TP expression, 321 $\pm$ 213 cells per well migrated through Matrigel after 24 hours in the TP-expressing group compared with 132 $\pm$ 73 cells per well in the TP-nonexpressing group ($P = 0.067$).

Next, we examined the influence of TP on the invasiveness of gastric cancer cells using a Matrigel-coated transwell assay. When the lower chamber was treated with recombinant human TP (rhTP) or D-dRib, the catalytic product of thymidine by TP, TP-expressing AGS cells showed dose-dependent up-regulation of invasion activity through Matrigel. This activity peaked at 2.6- and 2.4-fold above the basal level with 40 ng/mL rhTP or 40 μmol/L D-dRib, respectively (Fig. 1A). MKN-1 cells, which had no detectable expression of TP, also showed dose-dependent up-regulation of invasiveness, although the effect was not remarkable as with AGS cells. Invasive activity increased 1.6- and 1.7-fold above the basal level after treatment with 40 ng/mL rhTP or 40 μmol/L D-dRib, respectively (Fig. 1B). These findings suggest that both TP and D-dRib influence the invasion activity of cancer cells and that this activity might be intrinsic for TP.

TP Expression in Cancer Cells Promotes Invasion Activity through Matrigel

To further analyze the biological roles of cancer cell–intrinsic TP in migration and invasion, we established two TP-overexpressing gastric cancer cell lines (YCC-3/TP and MKN-45/TP) that expressed TP more highly than their respective mock-transfected counterparts (YCC-3/CV and MKN-45/CV; Fig. 2A). The concentration of TP was 5.8- and 4.3-fold higher in MKN-45/TP and YCC-3/TP cells, respectively, compared with each mock transfectant (data not shown). Next, we investigated whether the additional TP expression affected cell growth. The two TP-overexpressing transfectants had significantly more rapid cell growth than their parental or mock transfectant counterparts. The doubling times of YCC-3/TP and MKN-45/TP cells were 32 and 33 hours, respectively, whereas those of YCC-3/CV and MKN-45/CV cells were 37 and 38 hours, respectively ($P = 0.012$; Fig. 2B).

Migration ability was evaluated using wound-healing assays. Both TP-overexpressing cell lines displayed increased motility. A remarkable rate of wound closure was observed in TP-overexpressing cells compared with control cells (Fig. 3A). In addition, MKN-45/TP cells showed significant invasion through Matrigel (1.9-fold) compared with that of MKN-45/CV or parent cells ($P < 0.001$; Fig. 3B). This effect was even greater after treatment with rhTP or D-dRib. Although treatment in the lower chamber with 40 ng/mL rhTP or 40 μmol/L D-dRib induced migration in all of the MKN-45–based cell lines (parent MKN-45, MKN-45/CV, and MKN-45/TP cells), the higher rate was most pronounced in the MKN-45/TP cells, where it was 2.2- and 3.6-fold compared with untreated MKN-45/TP and parent cells, respectively (Fig. 3B). These findings suggest that TP and its catalytic product, D-dRib, can promote cancer cell invasion depending on the intracellular level of TP.

We next examined whether the enzymatic activity of TP is required for its invasion-promoting function. We inhibited TP enzymatic activity with TPI ($K_i = 2 \times 10^{-8}$ mol/L) and determined whether it could reverse invasion activity. TPI was not cytotoxic as previously reported (1, 3). We did not observe any adverse effects on cell viability with the tetrazole assay up to 125 μmol/L (data not shown). Pretreatment with 10, 50, and 100 μmol/L of TPI resulted in a dose-dependent abrogation of TP-induced invasiveness in MKN-45/TP cells, whereas the parent cells and MKN-45/CV cells were only mildly affected by TPI (Fig. 4A and B).

TP Promotes Actin Remodeling and Cell Adhesion on Matrigel

Cancer cell invasion is accompanied by cellular changes in adhesion to the extracellular matrix and is powered by actin filament polymerization, remodeling, and subsequent signal transduction. We investigated the effect of TP on cellular adhesion to the extracellular matrix. When AGS cells were treated with 40 ng/mL rhTP or 40 μmol/L D-dRib, they responded with a 2.3-fold increase in adhesion activity (Fig. 5A). This effect also depended on the expression level of TP. MKN-45/TP cells showed greater baseline adhesion activity onto Matrigel than the mock transfectant or parent cell lines. The effect of TP was more pronounced, up to 4-fold greater after rhTP or D-dRib treatment than in untreated parent cells (Fig. 5B).

Next, we examined actin filament remodeling after treatment with rhTP or D-dRib using immunofluorescence in AGS cells. As shown in Fig. 5C, untreated AGS cells were round and lacked a definitive filamentous actin (F-actin) structure. In contrast, cells treated with either rhTP or D-dRib contained actin filaments redistributed into rosette- and podia-like structures. These findings indicate that TP influences the adhesion activity of cancer cells to the surrounding matrix while at the same time inducing actin filament remodeling.

TP-Related Invasion Activity Is Dependent on the Phosphatidylinositol 3-Kinase Pathway

The activation of integrins and their respective signaling pathways is one of the primary mechanisms for inducing

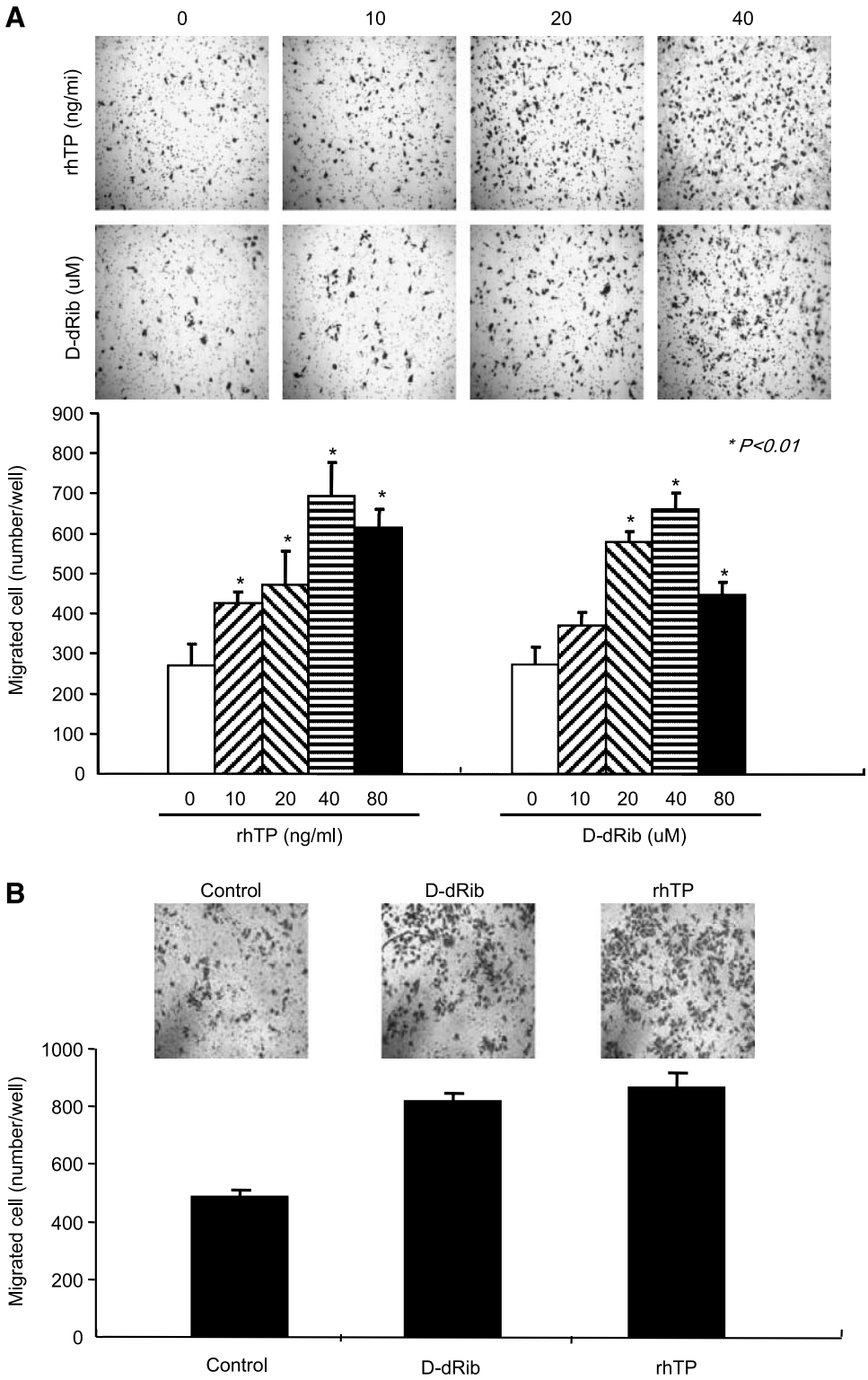


FIGURE 1. Treatment of rhTP and D-dRib stimulates gastric cancer cell migration through Matrigel. **A.** Cells (1×10^5) of AGS cell line. **B.** The same number of MKN-1 was added to the upper compartment of Matrigel-precoated transwell chamber and allowed to migrate for 36 h at 37°C. Cell migration was stimulated by the addition of each purified factors of rhTP or D-dRib to the lower chamber. Columns, mean; bars, SD. *, $P < 0.01$, compared with untreated control.

migration and invasion. We examined whether TP or D-dRib could enhance focal adhesion formation in cancer cells. We determined the activity of focal adhesion kinase (FAK) by assessing the phosphorylation status of $Tyr^{397/576}$ residues using immunoblotting. We noted, however, an already high baseline expression of phosphorylated FAK in untreated condition. The FAK level was not remarkably different according to time or dose when the cells were treated with rhTP or D-dRib (data not shown). Accordingly, we concluded that TP-induced migration activity is not dependent on FAK.

Next, we determined if the invasion activity promoted by TP is mediated by the phosphatidylinositol 3-kinase (PI3K) pathway. Recent reports have shown that PI3K induces actin filament remodeling through Akt and $p70^{S6K}$ (15, 16). When AGS cells were treated with 40 ng/mL rhTP or 40 μmol/L D-dRib, the level of phosphorylated Akt increased as early as 6 hours after treatment. The levels of phosphorylated $p70^{S6K}$ also increased (Fig. 6A). TP induced the maximum activity of $p70^{S6K}$ at 12 hours after treatment, whereas D-dRib induced earlier $p70^{S6K}$ activity, starting at 6 hours.

We then analyzed the role of the PI3K pathway in TP-induced invasion activity by determining the effect of PI3K inhibition on cell migration. AGS cells were pretreated with the PI3K inhibitors LY294002 and wortmannin for 1 hour before treatment with rhTP or D-dRib. As expected, the addition of PI3K inhibitors abrogated the phosphorylation of $p70^{S6K}$ completely and significantly attenuated the stimulation of migration by both rhTP and D-dRib (Fig. 6B and C). Treatment with PI3K inhibitors also reduced the invasion function in TP-overexpressing cells, such as MKN-45/TP, but not in the

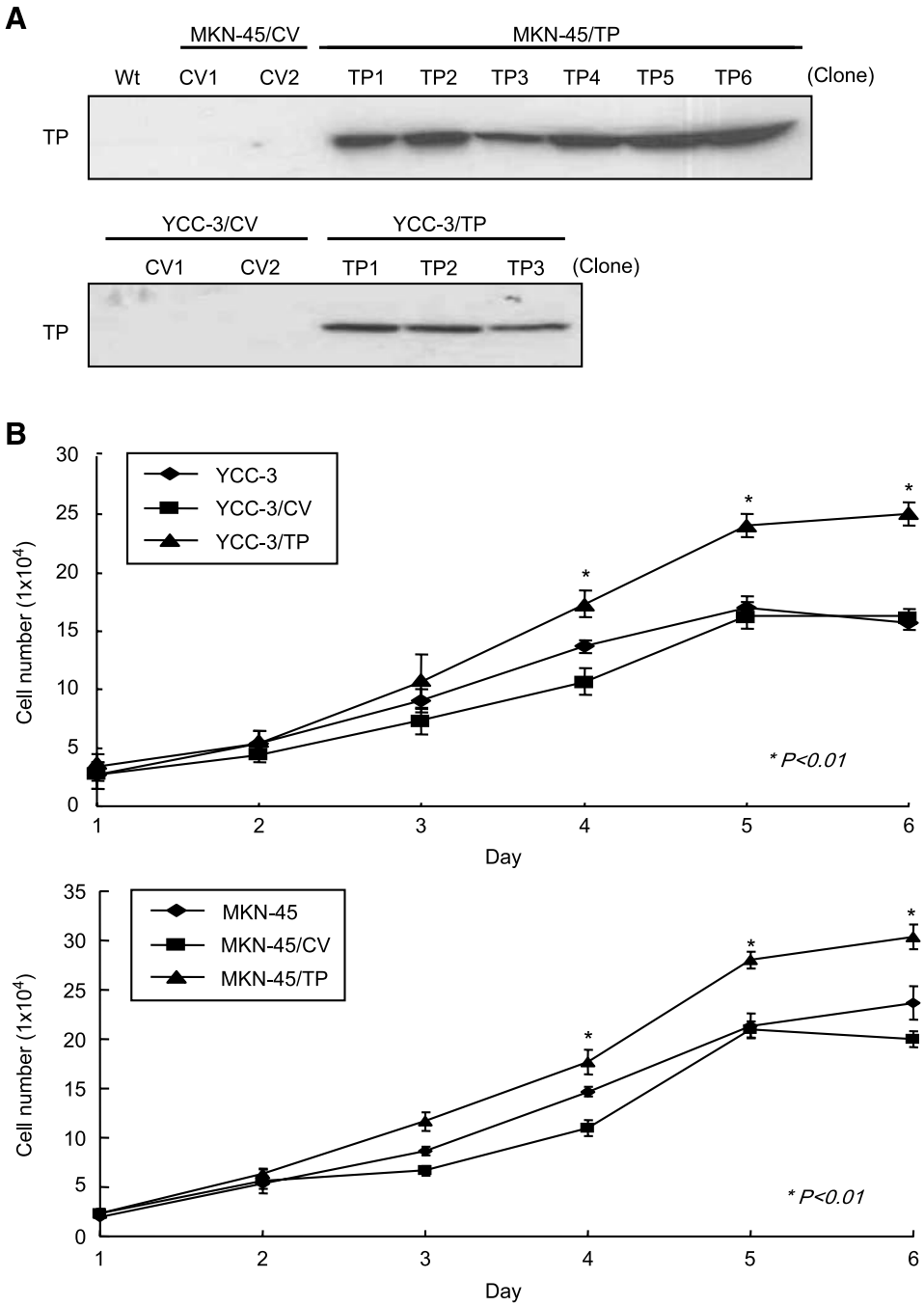


FIGURE 2. TP-overexpressing cells displayed enhanced migration ability. **A.** The two TP-overexpressing gastric cancer cell lines YCC-3/TP and MKN-45/TP had high TP expression, as evidenced by immunoblotting for TP. **B.** Doubling times of YCC-3/TP and MKN-45/TP cells.

accompanying parental or mock transfectant cell lines (Fig. 6D). These results indicate that PI3K activation might be required for TP-related invasion.

TP Invasion Activity Is Inhibited by a Combination of Mammalian Target of Rapamycin Inhibitor and TPI

We hypothesized that mammalian target of rapamycin (mTOR), an upstream regulator of $p70^{S6K}$, would be directly associated with the TP-related migratory effect. MKN-45/TP cells showed more mTOR activity than MKN-45/CV cells. Moreover, pretreatment of cells with 100 nmol/L rapamycin 24 hours before treatment with rhTP or D-dRib significantly attenuated the mTOR activation induced by rhTP and the migration effect of both TP and D-dRib (Fig. 7A-C).

Finally, we speculated that attenuation of TP-related invasion activity could be potentiated if both the innate enzymatic TP activity of cancer cells and the downstream signaling pathway for cellular movement were inhibited. Accordingly, we investigated the additive activity of TPI and rapamycin in TP-overexpressing cells. As expected, the simultaneous addition of 100 μmol/L TPI and 100 nmol/L rapamycin did not induce significant cytotoxicity with the tetrazole assay (data not shown). As shown in Fig. 7D, concomitant treatment of TPI and mTOR additively inhibited invasion activity, which was greater in MKN-45/TP cells compared with the mock transfectant cell line.

Discussion

This study sought to examine the interaction between TP expression and invasion activity in cancer cells and to determine whether the $p70^{S6K}$ pathway plays a role in

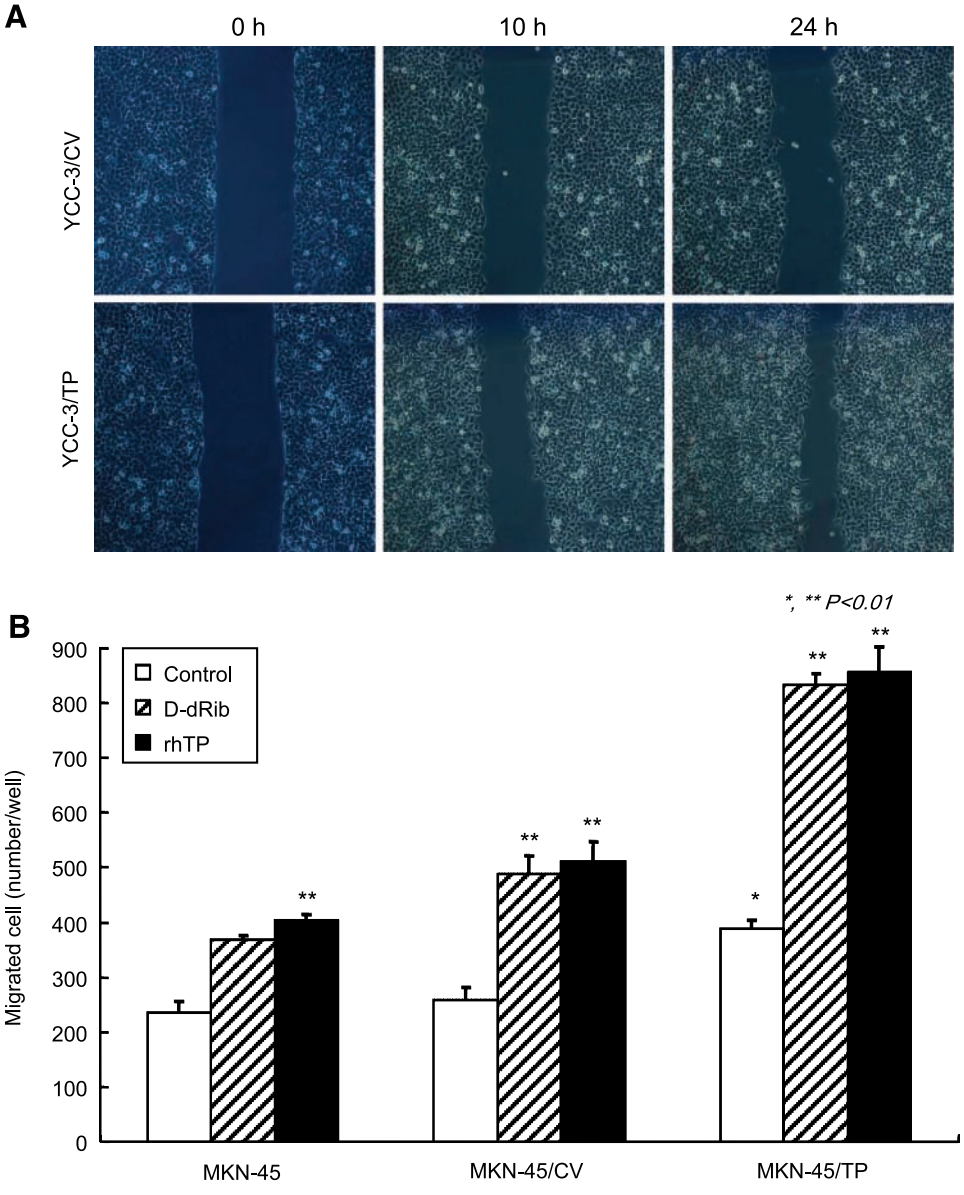


FIGURE 3. A. Confluent monolayers of cells were scratched with a sterile 200-μL pipette tip to form a wound area and then cultured in 0.5% serum medium for 10 and 24 h. Cell migration distances were calculated and compared with the initial distance from the wound area. **B.** MKN-45 cells (1×10^5) were added to the upper compartment of a Matrigel-precoated transwell chamber and allowed to migrate for 36 h at 37°C. Cell migration was stimulated by the addition of either purified 40 ng/mL rhTP or 40 μmol/L D-dRib to the lower chamber. Columns, mean; bars, SD. *, $P < 0.01$, compared with MKN-45 cells; **, $P < 0.01$, compared with untreated controls.

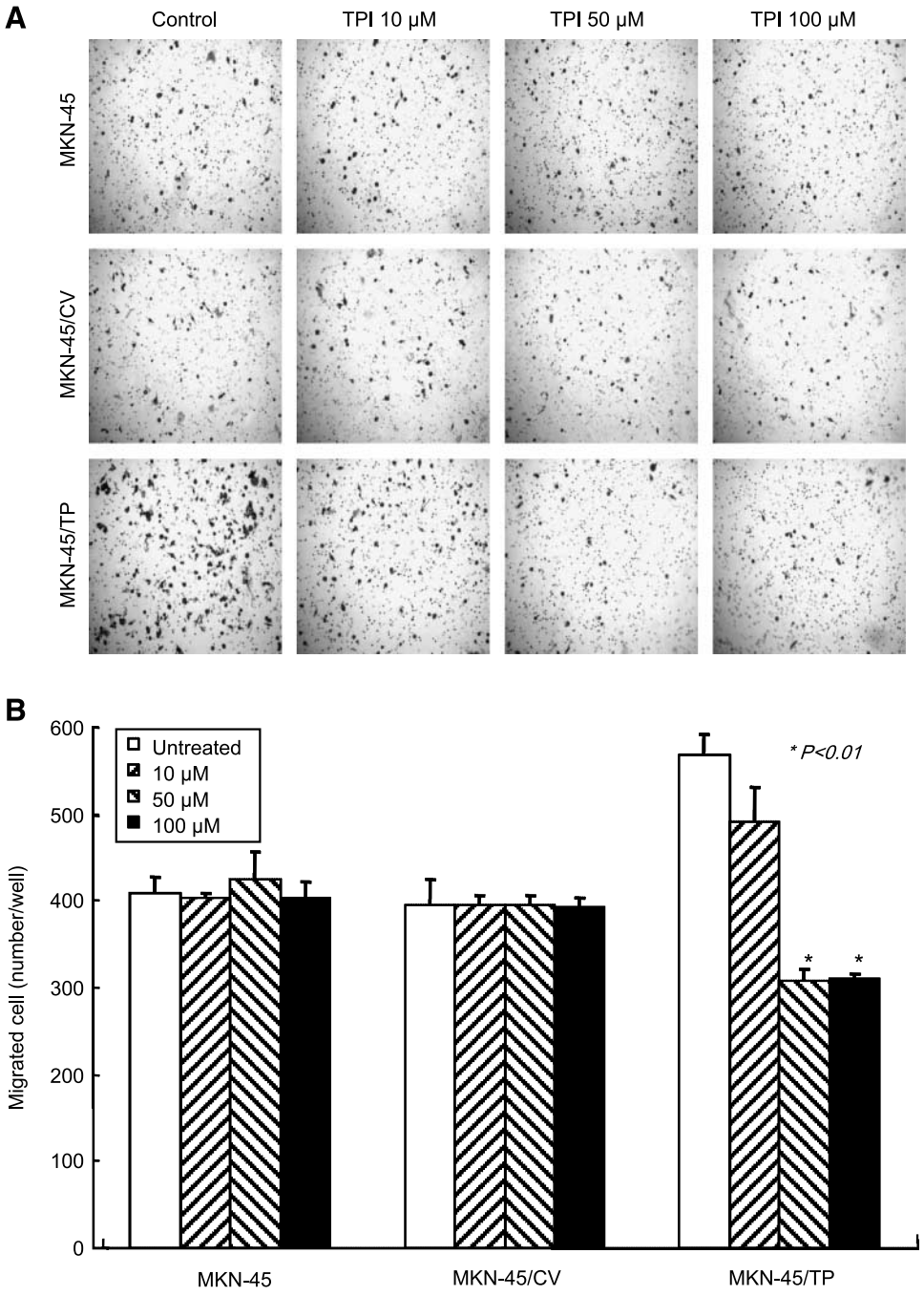


FIGURE 4. TP-induced migration ability was dependent on TP enzymatic activity. MKN-45 cells (1×10^5) were added to the upper compartment of a Matrigel-precoated transwell chamber. TPI was added at concentrations of 10, 50, and 100 μmol/L to the upper compartment and cells were allowed to migrate for 36 h at 37°C. **A.** Representative figures of Matrigel. **B.** Quantitative results are represented. Columns, mean; bars, SD. *, $P < 0.01$, compared with TPI-untreated MKN-45/TP cells.

regulating TP-induced invasion. TP is a known angiogenic factor, and despite evidence that it is overexpressed in various solid tumors, the mechanism by which it promotes angiogenesis has not been elucidated. TP lacks a signal sequence for secretion, and there have been no reports that TP exerts angiogenesis through specific receptors, as most angiogenic factors do (17). TP releases the monosaccharide 2-deoxy-D-ribose-1-phosphate, which, after dephosphorylation, can depart from the cell as the cell-permeable D-dRib. D-dRib also displays angiogenic activity. Addition of thymidine to platelets that have high levels of TP gives rise to thymine and D-dRib in the extracellular medium (18, 19). Consequently, D-dRib is considered an effector of TP-related angiogenesis in endothelial cells.

To better understand the biological roles of cancer cell–derived TP and its interaction with stromal TP, we first investigated TP expression in several gastric cancer cell lines. We found that TP-expressing cells showed increased invasion through Matrigel. Notably, YCC-16 cells with high TP expression showed exceptionally higher levels of invasion. YCC-16 was first isolated from the blood-circulating cancer cells of a multiple metastatic cancer patient, implying that this

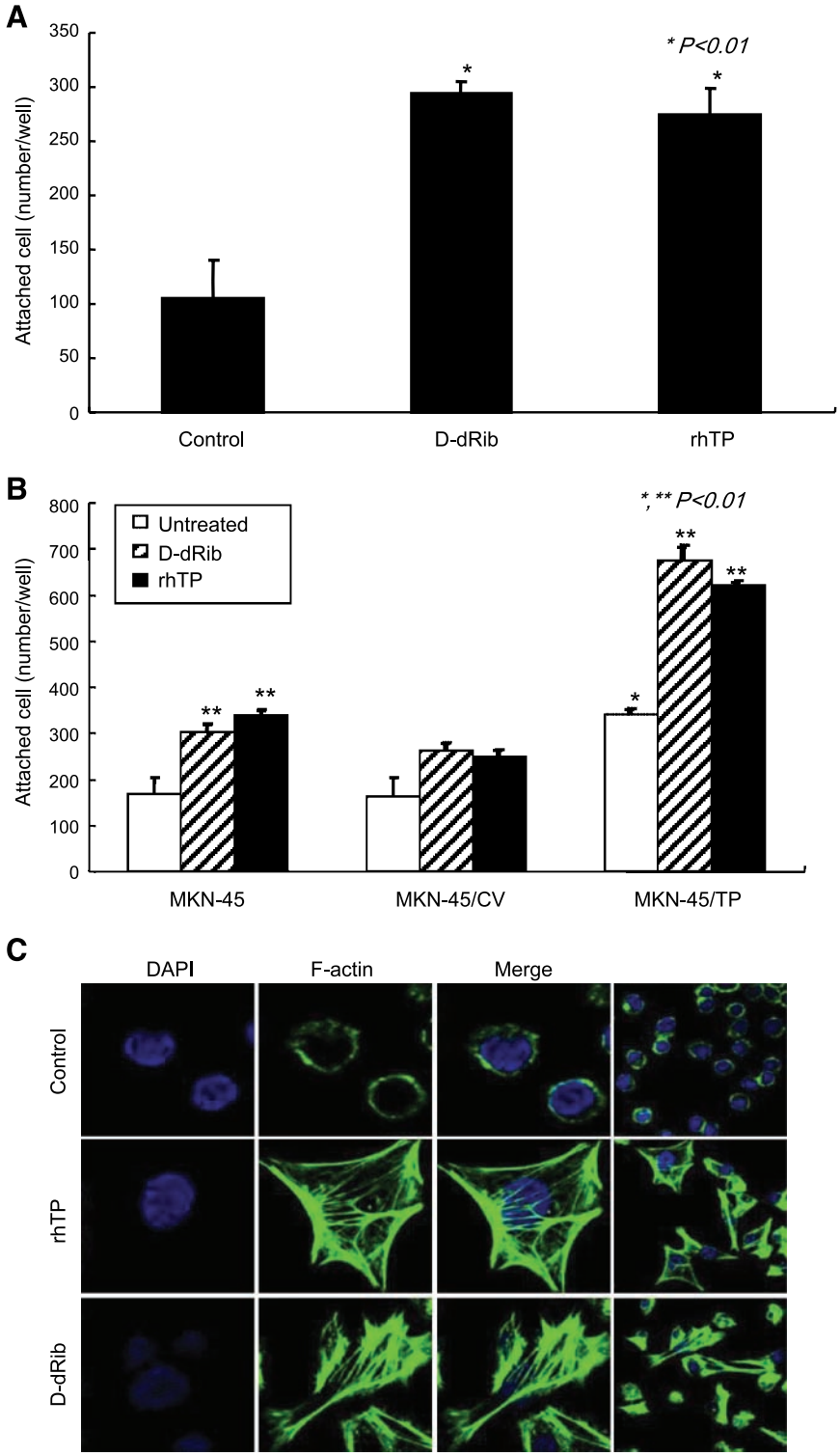


FIGURE 5. TP-induced migration ability was accompanied by increased adhesion activity related to TP. Cells (1×10^5) were seeded into a Matrigel-precoated 96-well plate, and the plate was incubated for 2 h at 36°C. Cell adhesion was stimulated with the addition of 40 ng/mL rhTP or 40 μmol/L D-dRib to the medium and attached cells were counted. **A.** AGS cells treated with rhTP and D-dRib. *, $P < 0.01$, compared with untreated controls. **B.** MKN-45 cells treated with rhTP and D-dRib. *, $P < 0.01$, compared with MKN-45; **, $P < 0.01$, compared with each cell line untreated. Columns, mean; bars, SD. **C.** AGS cells were treated with 40 ng/mL rhTP or 40 μmol/L D-dRib and incubated overnight with primary antibody against F-actin conjugated with Alexa Fluor 488 phalloidin at 4°C. Immunofluorescent images were detected with a confocal fluorescent microscope.

cell line reflects the active metastatic process. This finding provides indirect evidence that TP expression could be related to cancer cell movement and metastasis.

Cancer patients have elevated plasma TP levels (20). Although TP is not a secreted protein, high cellular turnover and substantial cell necrosis are thought to occur inside solid tumors, permitting intracellular enzymes to spill into the plasma, thereby raising plasma TP. For these reasons, we assumed that intracellular TP activity was proportional to the amount of D-dRib produced, although we did not directly measure the amount of D-dRib produced in TP-overexpressing cells (21). We observed that the addition of TP or D-dRib promoted invasion of gastric cancer cells and that this activity was more pronounced in TP-overexpressing cells. Our experimental treatment with TP or D-dRib in culture medium was designed to mimic a TP-rich microenvironment. In tumors, extracellular TP and metabolites of thymidine, including D-dRib and oxygen radicals, seem to either interact with cancer cells by various adhesion and chemotactic molecules or activate some signal pathway for invasion. Accordingly, we speculated that TP-expressing cells could potentiate an extracellular, TP-rich microenvironment. At present, we have very limited information about the roles of intracellular and extracellular TP, which may be different. Moreover, the proximal action site of TP/D-dRib within the cell remains unknown. Intracellular TP is a marker of high cellular turnover that may regulate another, as yet unidentified, signal pathway to promote cancer progression and metastasis. TP-dependent production of D-dRib may provide an energy source for cancer cells and chemotactic information for invasion. High-throughput techniques for universal gene or protein expression could help identify the holistic factors involved.

Based on these findings, we examined whether the observed invasion activity was related to the enzymatic activity of TP. We previously showed that TP-overexpressing cell lines have high enzymatic activity (22). Treatment with TPI attenuated the migration-promoting effect of TP, especially in TP-overexpressing MKN-45 cells. AGS cells with weak TP expression also displayed attenuated migration with TPI, although not as much as in MKN-45/TP. MKN-45/CV or parent cells without detectable TP expression did not respond to TPI, suggesting that TP-related invasion activity is primarily dependent on innate TP activity. Intercellular variability of TP expression and its correlation with motility were also associated with enzymatic activity. Cancer cell migration is a process involving cell attachment and the degradation of tissue barriers caused by various proteolytic enzymes. Recent studies suggest that TP promotes the secretion of matrix metalloproteinase-1 through oxidative stress and that there is a correlation between TP and matrix metalloproteinase-2 mRNA levels (23, 24). TP might promote the expression of matrix metalloproteinases, leading to cancer cell invasion and metastatic potential.

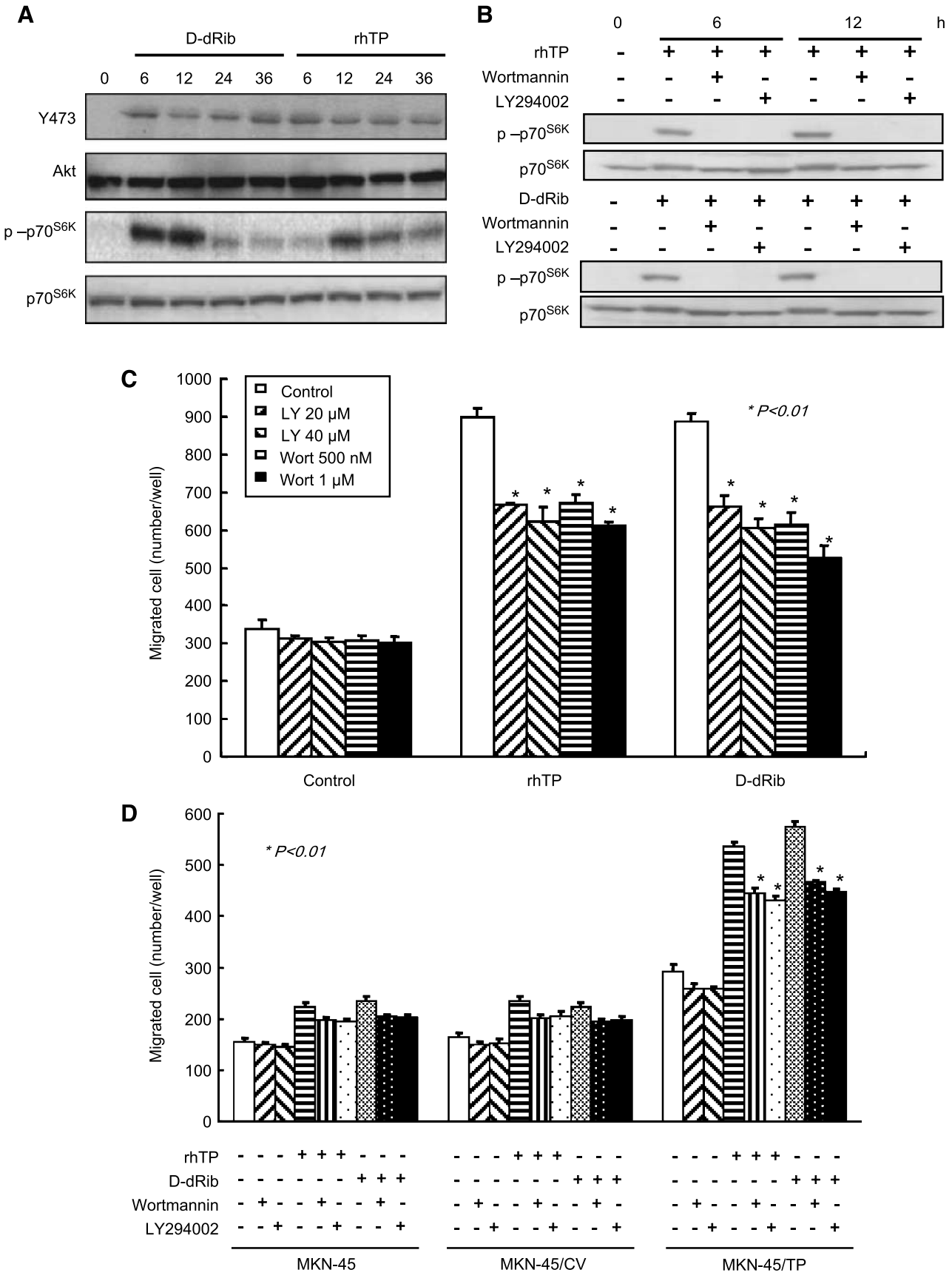
We speculated that the alteration of adhesion and actin remodeling act as functional indicators of migrating cancer cells. We showed that adhesion ability to Matrigel was also increased by treatment with TP or D-dRib, implying that the activation of signal pathways by contact with the extracellular matrix could play a major role in TP-related ability. These pathways could be divided into those that are focal adhesion related or growth factor related. Hotchkiss et al. (25) reported that TP and D-dRib stimulate the formation of focal adhesions and activate specific integrins for endothelial cell migration. We did not observe FAK activation in our experiments. FAK is activated by a phosphorylated tyrosine, and its own kinase activity is higher in cancer cells. The cells used in this experiment had a considerable amount of baseline FAK activation (26, 27).

Because TP and D-dRib are not present in normal serum, there is good reason to suspect that D-dRib attracts cells by acting as an energy source. Pig erythrocytes take up D-dRib more rapidly than D-ribose, suggesting that pentose sugars may enter the cell without a transporter because D-dRib is the less polar molecule (28, 29). Accordingly, we hypothesized that TP or D-dRib was able to modify the phosphorylation status of molecules involved in cancer angiogenesis or progression.

The finding that rapamycin inhibits TP-stimulated migration implicates the activation of $p70^{S6K}$ in this cellular response, which is consistent with other works showing that rapamycin inhibits the migration of vascular smooth muscle cells or endothelial cells (30-32). In those studies, the effects of rapamycin were primarily mediated by $p27^{kip}$, a cell cycle regulator that may be a downstream target of mTOR. We found that TP-overexpressing cells had significantly more rapid cell growth than parental or mock transfectant cells. The relationship between TP expression and cell proliferation remains debatable. Early reports asserted that TP enhances tumor growth *in vivo* but has no effect on growth *in vitro* (33, 34). More recent reports, however, suggest that high TP expression may modulate the cell cycle in head and neck cancer cells in association with the down-regulation of $p27^{kip}$ (35). The mechanism behind the rapid cell growth in our TP-expressing cells was outside the scope of our study, although this is the first report that TP may regulate cell proliferation in gastric cancer.

It seems unlikely that the effects of mTOR on migration in such cells are mediated exclusively by $p27^{kip}$, and we cannot exclude the possibility that a target of mTOR other than $p70^{S6K}$ mediates the effects of rapamycin on cancer cell motility. We also observed that treatment with rapamycin attenuated, but did not completely abrogate, TP-stimulated migration activity. We have been unable to identify the mechanism by which rapamycin influences TP-induced migration ability, but recent studies suggest that the phospholipase D pathway may play a critical role. Phospholipase D activates $p70^{S6K}$, an observation that may be explained by the phosphatidic acid–mediated activation of mTOR (36, 37). Some evidence suggests that phospholipase D activates the PI3K signaling pathway (38). Our experiments revealed Akt activation by rhTP and D-dRib, and TP-overexpressing cells displayed increased Akt activity, consistent with the findings of previous reports (39). It is possible, therefore, that rapamycin treatment may be required for the phospholipase D pathway in actin cytoskeletal reorganization.

The ability of both TPI and rapamycin to abrogate TP-induced migration has implications for clinical application. A



combined TPI and trifluorothymidine (TAS-102) agent is currently undergoing phase I studies (40). Our study provides the rationale for antimetastatic therapy combining a mTOR inhibitor (signal blocker) with TPI (enzymatic inhibitor) to increase potency, especially in high TP-expressing tumors. To conclude, this study showed that TP and D-dRib increased the invasion activity of gastric cancer cells, especially in TP-overexpressing cells. TP could be a therapeutic target for diminishing the propensity for invasion and metastasis in gastric cancer.

Materials and Methods

Reagents and Antibodies

rhTP was purchased from R&D Systems. D-dRib came from Sigma. Matrigel was purchased from BD Biosciences. TPI, a previously described specific inhibitor of TP activity, was synthesized by Taiho Pharmaceutical Co. Ltd. (11). The PI3K inhibitors wortmannin and LY294002 were supplied by Sigma and Calbiochem, respectively. Rapamycin was supplied by Sigma. Monoclonal antibody for immunoblotting and ELISA against TP was purchased from R&D Systems. Antibodies against Akt, phosphorylated Akt (Thr^{473}, Thr^{308}), p70 ^{S6K}, phosphorylated $p70^{S6K}$, and mTOR were purchased from Cell Signaling. Anti-phosphorylated mTOR antibody came from Biosource.

Cell Culture

The human gastric cancer cell lines AGS and NCI-N87 were supplied by the American Type Culture Collection. The gastric cancer cell lines MKN-1 and MKN-45 were provided by the Korean Cell Line Bank. YCC-1, YCC-2, YCC-3, YCC-6, YCC-7, YCC-9, YCC-11, and YCC-16 were gastric cancer cell lines established by Yonsei Cancer Center (Seoul, Korea) from advanced gastric cancer patients through isolation from ascites (YCC-1, YCC-2, YCC-3, YCC-6, YCC-7, YCC-9, and YCC-11) or peripheral blood (YCC-16). All cell lines were maintained in RPMI 1640 (Nissui) supplemented with 10% fetal bovine serum, 100 units/mL ampicillin, 100 μg/mL streptomycin, and 2 mmol/L glutamine (Life Technologies) in a 5% CO_2 humidified atmosphere at 37°C.

Establishment of TP-Overexpressing Cell Lines

Full-length TP cDNA expression vector (RSV/TP) and the empty vector [RSV; previously described (39)] were provided by Prof. Akiyama (Kagoshima University, Kagoshima, Japan). The plasmid was transfected into MKN-45 and YCC-3 cells by electroporation. After selection with geneticin, the expression of TP in each clone was determined by immunoblotting. A TP-positive clone (MKN-45/TP or YCC-3/TP) and a control vector–transfected clone (MKN-45/CV or YCC-3/CV) were selected for further analysis.

ELISA for TP

TP protein expression was analyzed by a direct (sandwich) ELISA with modifications, according to the manufacturer's instructions. Samples and the standard were diluted to the desired concentration with coating buffer and dispensed into each microplate well. After overnight incubation at 4°C, the plates were blocked for 1.5 h at 37°C. Anti-human TP primary antibody was added to each well, and the plates were incubated for 2 h at room temperature. After the plates were washed three times, goat anti-mouse IgG serum conjugated with peroxidase was added, and the plates were incubated for an additional hour at room temperature. A substrate reaction was done with a solution of the 3,3′,5,5′-tetramethylbenzidine substrate system (R&D Systems). The peroxide reaction was terminated with 1 mol/L phosphoric acid solution, absorbance was measured at 450 nm, and the TP level was determined with reference to the standard curve.

Cell Proliferation Assay

To measure cell growth, 1×10^4 cells were seeded on 24-well plates and cultured for 24 h. The cells were then cultured for 5 d in 10% fetal bovine serum. The cell growth rate was evaluated by trypan blue assay (Life Technologies) in triplicate. Doubling time was calculated by the following equation: $TD = T \times \log 2/\log(N/N_0)$, where TD = doubling time, T = time interval, N = end point cell number, and N_0 = initial cell number.

Wound-Healing Assay

Wound-healing assays were done according to the suggested methods. After cells were cultured to a 100% confluent monolayer in six-well tissue culture plates for 24 h, a sterile 200-μL pipette tip was used to scratch the cell monolayer to form a wound area. The wells were then cultured in medium with 0.5% serum for 8 and 24 h and then fixed with formalin. Migration and cell movement throughout the wound area was examined. The migration distances were calculated and compared with the initial size of the wound area, and the results are presented as a percentage of the closed wound.

Invasion Assay Using Transwell Filters

We conducted a modified version of the standard transfilter assay for invasion. Transwell filters (diameter, 6.5 mm; pore size, 8 μm; BD Biosciences) were coated on the lower side with 8 μg/μL Matrigel and placed on a 24-well plate containing medium supplemented with 0.1% bovine serum albumin.

FIGURE 6. TP induces cancer cell migration via the PI3K pathway. **A.** AGS cells were treated with either 40 ng/mL rhTP or 40 μmol/L D-dRib, and the level of phosphorylated Akt (Y473) and $p70^{S6K}$ was detected by immunoblotting. **B.** AGS cells were pretreated with the PI3K inhibitors LY294002 and wortmannin for 1 h before treatment with rhTP or D-dRib. Cell lysates were obtained at 6 and 12 h after PI3K inhibitor treatment, and changes in the phosphorylation of $p70^{S6K}$ were detected by immunoblotting. **C.** AGS cells were preincubated with LY294002 (20 and 40 μmol/L) or wortmannin (500 nmol/L and 1 μmol/L) and treated with either 40 ng/mL rhTP or 40 μmol/L D-dRib in the lower chamber. Cells were allowed to migrate for 36 h at 37°C. *, $P < 0.01$, compared with untreated control. **D.** MKN-45 cells were preincubated with LY294002 (40 μmol/L) or wortmannin (500 nmol/L) and treated with either 40 ng/mL rhTP or 40 μmol/L D-dRib in the lower chamber. Cells were allowed to migrate for 36 h at 37°C. Columns, mean; bars, SD. *, $P < 0.01$, compared with each cell line untreated.

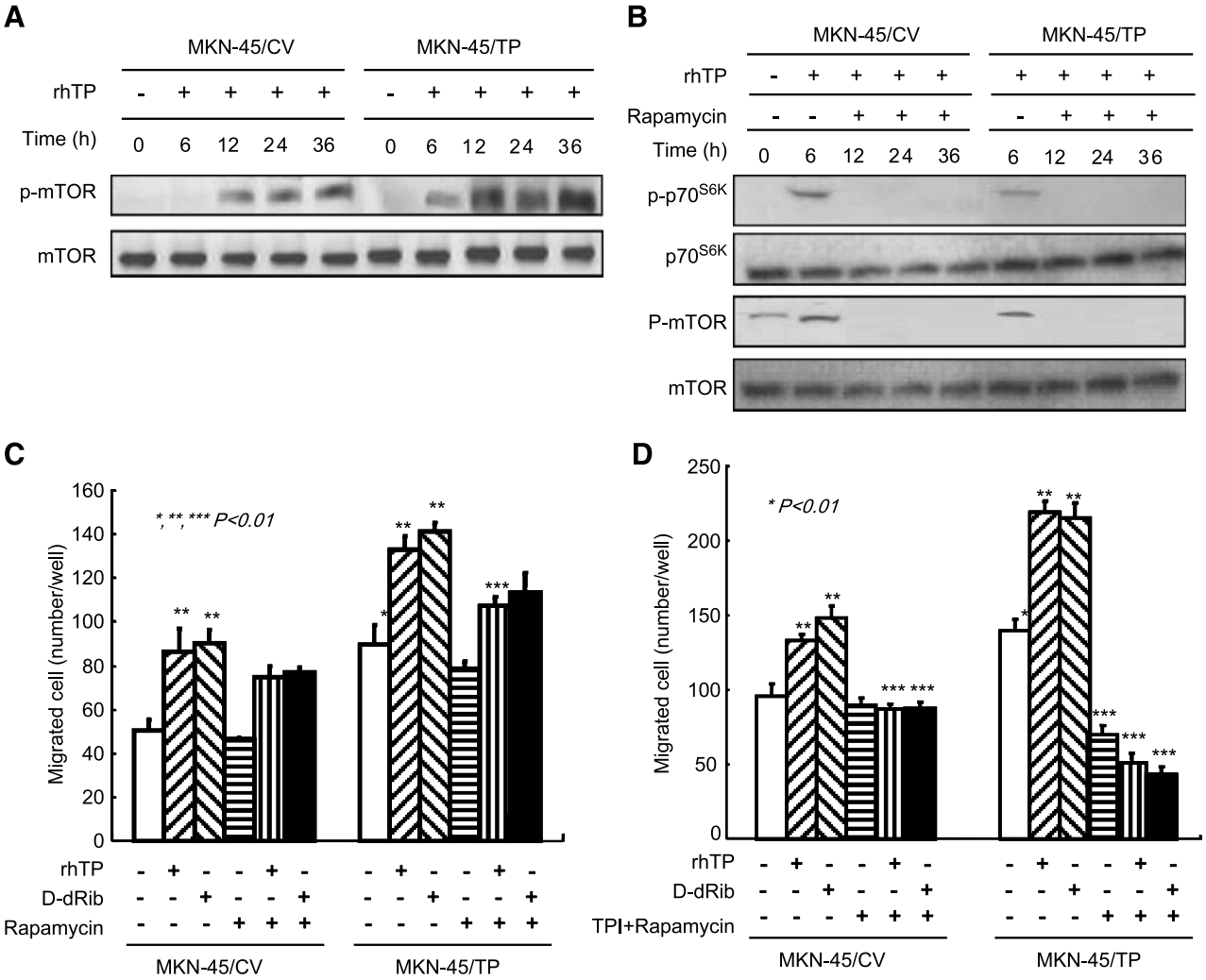


FIGURE 7. The migration ability induced by TP was inhibited by combination treatment with a mTOR inhibitor and TPI. **A.** MKN-45/CV and MKN-45/TP cells were treated with 40 ng/mL rhTP, and 16 time-dependent levels of phosphorylated mTOR were detected by immunoblotting. **B.** MKN-45/CV and MKN-45/TP cells were pretreated with 100 nmol/L of the mTOR inhibitor rapamycin for 1 h before treatment with 40 ng/mL rhTP. Cell lysates were obtained at 6, 12, and 24 h after treatment with the PI3K inhibitors, and changes in the phosphorylation of mTOR and $p70^{S6K}$ were detected by immunoblotting. **C.** MKN-45 cells were preincubated with 100 nmol/L rapamycin and treated with rhTP or D-dRib in the lower chamber. Cells were allowed to migrate for 36 h at 37°C. Columns, mean; bars, SD. *, $P < 0.01$, compared with MKN-45/CV cells; **, $P < 0.01$, compared with each cell line untreated; ***, $P < 0.01$, compared with each cell line treated with rhTP or D-dRib. **D.** MKN-45 cells were preincubated concurrently with 100 nmol/L rapamycin and 100 μmol/L TPI along with either 40 ng/mL rhTP or 40 μmol/L D-dRib in the lower chamber. Cells were allowed to migrate for 36 h at 37°C. Columns, mean; bars, SD. *, $P < 0.01$, compared with untreated controls; **, $P < 0.01$, compared with each cell line untreated; ***, $P < 0.01$, compared with each cell line treated with rhTP or D-dRib.

Cells were harvested with a cell dissociation solution (Sigma) and suspended in medium with 1% bovine serum albumin. Cells (1×10^5) were added to the upper compartment of a transwell chamber and allowed to migrate for 36 h at 37°C. Cell migration was stimulated by the addition of purified factors (rhTP or D-dRib) to the lower chamber. For protocols involving drug inhibitor treatment, the inhibitors were added to the upper compartment. After 36 h, nonmigrated cells on the upper side of membrane were removed with a cotton swab, and migrated cells on the bottom surface of the membrane were fixed in 3.7% paraformaldehyde in PBS and stained with crystal violet for 10 min at room temperature. Cell migration was quantified by counting the number of cells in three inserts. The data are expressed as the average number of cells per insert.

Cell-Matrigel Adhesion Assay

Harvested cells were suspended in medium with 0.1% bovine serum albumin, and 1×10^5 cells were seeded into 8 μg/μL Matrigel-precoated 96-well plates that were then incubated for 2 h at 36°C. Cell adhesion was stimulated by the addition of rhTP or D-dRib to the medium. After 2 h, the medium was removed and the attached cells were stained with crystal violet for 10 min at room temperature. Attached cells were counted.

Immunofluorescent Staining for F-Actin

Cells were seeded on Matrigel and stimulated with rhTP (40 ng/mL), D-dRib (40 μmol/L), or medium only (negative control) for 12 h. Cells were fixed with 3.7% paraformaldehyde in PBS for 10 min and permeabilized by incubation with

0.1% Triton X-100 in PBS (PBS-B) for 10 min. Cells were washed thrice with PBS and blocked with 10% fetal bovine serum in PBS-B for 60 min at room temperature. To stain F-actin, cells were incubated with primary antibody against F-actin conjugated with Alexa Fluor 488 phalloidin (Invitrogen) overnight at 4°C. Images were detected with a Zeiss LSM 510 Meta confocal fluorescent microscope.

Immunoblotting

Exponentially growing cells were seeded at 1×10^6 per 60-cm^2 dish and treated with rhTP (40 ng/mL) or D-dRib (40 μmol/L) for 36 h. Cells were harvested and resuspended in lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% NP40, 1 mmol/L sodium orthovanadate, 1 mmol/L NaF, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 mmol/L phenylmethylsulfonyl fluoride]. Whole-cell lysates were subjected to SDS-PAGE and blotted onto polyvinylidene difluoride membranes. Each membrane was then incubated with appropriate primary antibody overnight at 4°C followed by a peroxidase-linked secondary antibody for 1 h at room temperature. Membranes were developed by chemiluminescence according to the manufacturer's protocol (Amersham).

Statistical Analysis

Quantitative data are represented as the mean ± SD of at least three independent experiments. Statistical comparisons were done using Student's *t* test. Differences were regarded as significant when the *P* value was <0.05.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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