(1)

松下茂人 医研422

The Effect of a Thymidine Phosphorylase Inhibitor on Angiogenesis and Apoptosis in Tumors.

Shigeto Matsushita[1,2], Takao Nitanda[3], Tatsuhiko Furukawa[1], Tomoyuki Sumizawa[1], Ayako Tani[1], Kengo Nishimoto[1], Suminori Akiba[4], Kazutaka Miyadera[5], Masakazu Fukushima[5], Yuji Yamada[5], Hiroki Yoshida[3], Tamotsu Kanzaki[2], and Shin-ichi Akiyama[1,6]

[1]Department of Cancer Chemotherapy, Institute for Cancer Research, [2]Department of Dermatology, [3]1st Department of Pathology, [4]Department of Public Health, Kagoshima University, Sakuragaoka 8-35-1, Kagoshima 890-8520, Japan.

[5]Taiho Pharmaceutical Co. Ltd., Misugidai 1-27 Hanno 357, Japan.

[6]Address correspondence to: Shin-ichi Akiyama, Department of Cancer Chemotherapy, Institute for Cancer Research, Faculty of Medicine, Kagoshima University, Sakuragaoka 8-35-1, Kagoshima 890-8520, Japan. (FAX: 81-99-265-9687, TEL: 81-99-275-5490)

Running Title: Thymidine phosphorylase inhibitor suppresses thymidine phosphorylase function.

Key Words: thymidine phosphorylase, thymidine phosphorylase inhibitor, angiogenesis, apoptosis, platelet-derived endothelial cell growth factor.

Abbreviations: TP: thymidine phosphorylase; PD-ECGF: platelet-derived endothelial cell growth factor; TPI: thymidine phosphorylase inhibitor ; TdT : terminal deoxynucleotidyl transferase; PBS : phosphate-buffered saline; DAB : diaminobenzidine ; TUNEL : TdT-mediated dUTP-biotin nick end labeling

ABSTRACT

Thymidine phosphorylase (TP) is an enzyme involved in the reversible conversion of thymidine to thymine, and is identical to an angiogenic factor, platelet-derived endothelial cell growth factor (PD-ECGF). TP is expressed at higher levels in a wide variety of solid tumors than in the adjacent non-neoplastic tissues. Patients with TP-positive colon and esophageal tumors have a poorer prognosis than those with negative tumors. We have recently synthesized a new TP inhibitor (TPI), 5-chloro-6-[1-(2-iminopyrrolidinyl) methyl] uracil hydrochloride . We investigated the effect of TPI on angiogenesis by KB cells transfected with PD-ECGF cDNA, KB/TP, and a mock transfectant, KB/CV, using the mouse dorsal air sac assay model. We found that KB/TP had higher angiogenic ability than KB/CV, and TPI completely suppressed angiogenesis by KB/TP. Furthermore, TPI, at a dose of 50 mg/kg/day, considerably decreased the growth rate of KB/TP xenografted into nude mice. Microvessel density in KB/TP tumors was higher than in KB/CV tumors and TPI did not significantly change the density in both tumors. The apoptotic index in KB/TP tumors was significantly lower than in KB/CV tumors, and TPI significantly increased the apoptotic index in KB/TP but not in KB/CV tumors. These findings, taken together with previous reports, suggest that expression of TP plays an important role in tumor growth, and that the inhibitor of TP, TPI, suppresses tumor growth by increasing the proportion of apoptotic cells and probably inhibiting angiogenesis.

INTRODUCTION

Thymidine phosphorylase (TP; Enzyme Commission No. 2. 4. 2. 4.) catalyzes the reversible phosphorolysis of thymidine, deoxyuridine and their analogs to their respective bases and 2-deoxyribose-1-phosphate (1-3). TP also catalyzes the transfer of deoxyribose from one deoxynucleoside to another base to form a second deoxynucleoside (4-6). In mammals, TP consists of two identical subunits with a molecular weight of 55 kDa (7). We have previously shown that TP is identical to platelet-derived endothelial cell growth factor (PD-ECGF) (8, 9). TP stimulates chemotaxis and [^{3}H]thymidine incorporation by endothelial cells *in vitro* and has angiogenic activity *in vivo* (10-13). Recently, we demonstrated that the enzymatic activity of TP is indispensable for its angiogenic activity (10, 13). Among the degradation products of thymidine by TP, 2-deoxy-D-ribose, a dephosphorylated product derived from 2-deoxy-D-ribose-1-phosphate, has chemotactic activity *in vitro* and angiogenic activity *in vivo*. These findings suggest that the enzymatic products of TP may stimulate chemotaxis of endothelial cells and possibly other cells, causing angiogenesis (13).

Overexpression of TP in MCF-7 cells transfected with TP cDNA has no effect on their growth *in vitro*, but confers a growth advantage on them when they are xenografted into nude mice (14). Although TP has angiogenic activity, it has effects on prognosis of cancers independent of angiogenesis assessed by micro-vessel density in human colorectal carcinoma and renal cell carcinoma (15, 16). These observations suggest that TP has functions other than angiogenesis that affect tumor growth. It is very important to develop strong and selective inhibitors of TP, because they may inhibit angiogenesis and suppress progression of solid tumors. Recently, we generated a novel inhibitor of TP, 5-chloro-6-[1-(2-iminopyrrolidinyl) methyl] uracil hydrochloride (TPI) (Ki = 1 x 10^{-8} M) that had about 1,000-fold higher inhibitor activity than 6-amino-5-chlorouracil, one of the most potent TP inhibitors (17). In this study, we examined whether inhibition of TP activity by TPI in human epidermoid carcinoma KB cells affects tumor angiogenesis and growth.

MATERIALS AND METHODS

Growth factors and chemicals.

[^{14}C]Thymidine (56 mCi/mmol) was obtained from Moravek Biochemicals, Inc.(Brea, CA), rat anti-mouse CD31 from PharMingen (San Diego, CA), and terminal deoxynucleotidyl transferase (TdT) and biotin-16-2'-deoxyuridine-5'-triphosphate (Biotin-dUTP) from Boehringer Mannheim (Mannheim, Germany). TPI, a competitive inhibitor of thymidine phosphorylase (Fig. 1), was synthesized by Taiho Pharmaceutical Co. Ltd.

Animals and Cell Lines.

Male BALB/c mice and that of nude mice at 6-8 weeks old were used. KB/TP, human KB epidermoid carcinoma cells transfected with PD-ECGF cDNA, and KB/CV, a mock transfectant, were maintained in minimal essential medium containing 10% fetal calf serum. These cells were *mycoplasma*-free.

Transfection of PD-ECGF cDNA into KB cells.

PD-ECGF full length cDNA was kindly provided by Dr. K. Miyazono and Dr. C-H. Heldin (Ludwig Institute for Cancer Research, Uppsala, Sweden). A *Kpn*I-*Eco*RI fragment from pPL8 that encompassed the PD-ECGF coding region was cloned between the *Kpn*I and *Eco*RI restriction sites of pT7T318U (Pharmacia, Uppsala, Sweden). An *Xba*I-*Eco*RI fragment from pT7T318U was then cloned between the *Nhe*I and *Eco*RI restriction sites of the expression vector pBK-RSV (Stratagene, La Jolla, CA). The expression vector encoding PD-ECGF cDNA (RSV/TP) or the vecter alone (RSV) was transfected into KB cells by electroporation (18). After selection with Geneticin, expression of PD-ECGF/TP in each clone was determined by immunoblotting with anti-PD-ECGF/TP monoclonal antibody. One TP-positive clone transfected with RSV/TP (KB/TP cells) and one clone transfected with RSV (KB/CV cells) were further analyzed.

Preparation of cell lysate.

Cells were lyzed in 10 mM Tris-HCl (pH 7.5) by rapid freeze-thawing in liquid nitrogen. The lysates were centrifuged at 15,000 x g for 20 min at 4°C and the supernatants were

resolved by electrophoresis (19). Protein concentrations were determined by the method of Bradford (20).

Immunoblotting.

Each sample was resolved by 11% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS/PAGE) according to the method of Laemmli (21). Proteins in the gel were electrophoretically transferred to a sheet of polyvinylidene difluoride (PVDF) membrane, (Immobilon-P transfer membrane; Millipore, Bedford, MA) with Bio-Rad TRANSBLOT SD as described (22). The membrane was then incubated with monoclonal antibody against TP as previously described (23) and developed using the enhanced chemiluminescence Western blotting detection system (Amersham, Buckinghamshire, United Kingdom).

Assay of TP activity.

Enzyme activity was measured with the radiometric assay (24). The reaction mixtures contained 5mM sodium phosphate (pH7.0), lysates (100μg protein), 0.4mM [^{14}C]thymidine (56mCi/mmol), and TPI concentration of 0μM and 10μM in a volume of 50 μl. After incubation at 37°C for 1h, the reactions were stopped by incubation at 95° C for 2 min. After centrifugation at 10,000 x g for 10 min, aliquots of the reaction mixture were spotted on PEI-cellulose chromatography plates (Merck, Darmstadt, Germany) which were developed by ascending chromatography in 0.5M LiCl at room temperature for 1h. The nucleoside and base were identified under ultraviolet light, scraped from the plate and analyzed for radioactivity. Enzyme specific activity was expressed as the amount of thymine (nmol) formed/mg protein/h.

Effects of TPI on in vivo tumor angiogenesis.

To examine the effect of TPI on tumor-associated angiogenesis, we used the mouse dorsal air sac assay model (25). KB/TP and KB/CV cells were washed twice with PBS and suspended in PBS at 6.7 x 10^5 cells/ml. A Millipore chamber (diameter, 10 mm; thickness, 2 mm; filter pore size, 0.22 μm; Millipore Co., Bedford, MA) was filled with 150 μl (1 x

10^5 cells) of either cell suspension or PBS and implanted s.c. into an air sac formed previously in the dorsum of 7-8 week-old male BALB/c mice by injection of an appropriate volume of air. TPI (50mg/kg/day) or vehicle alone was administered intraperitoneally every 12h for 4 days. On day 4, the implanted chambers were removed from the subcutaneous fascia of the treated animals. Angiogenic response was assessed under a dissecting microscope by determining the number of newly formed blood vessels longer than 3 mm with the characteristic zigzagging pattern of tumor cell-induced new vasculature in the subcutaneous side of the skin area which had been in contact with the chamber. The angiogenic response was graded as 0, 1, 2, 3, 4, and 5 according to the numbers of newly formed blood vessels, 0, 1, 2, 3, 4, and more than 5, respectively (26).

Effects of TPI on tumor growth.

KB/TP and KB/CVcells were suspended in PBS at 10^7cells/ml and 0.1ml was injected into the shoulders of 7-8 week-old male BALB/c nude mice. When the tumor size reached a volume of approximately 50-150mm^3, three different concentrations of TPI (50, 100, or 200 mg/kg/day) or vehicle alone was administered intraperitoneally every 12h for 22 days. Tumors were measured in two dimensions by calipers every other day and the volume was calculated as $width^2$ x length x 0.5. The effect of TPI on tumor weight was determined using multiple regression analysis adjusting for tumor type and observation period.

TP staining.

Tumors embedded in paraffin were cut into 3-μm thick sections. The sections were deparaffinized with xylene and dehydrated with 98% ethanol. Endogenous peroxidase was blocked by covering the sections with 0.3% (v/v) H_2O_2 in absolute methanol for 20 min at room temperature and the sections were then incubated with polyclonal antibody against TP (19). Antibody binding was detected by sequential incubation with biotinylated anti-rabbit IgG and streptavidin-peroxidase complex. Immune complexes were visualized by incubating the sections with 0.5 mg/ml diaminobenzidine (DAB) and 0.03% (v/v) H_2O_2 in PBS for 3 min.

Microvessel staining.

Tumors were embedded in OCT compound (Sakura Finetek, Torrance, CA), snap-frozen in liquid nitrogen and stored at -80° C. Cryostat sections were fixed in acetone for 10 min at 4° C and immunostained with anti-mouse CD31 (PharMingen, San Diego, CA). Antibody binding was detected by sequential incubation with biotinylated anti-rat IgG (Vecter Laboratories, Inc., Burlingame, CA) and streptavidin-peroxidase complex. Immune complexes were visualized by incubating the sections with 0.5 mg/ml DAB and 0.03 % (v/v) H_2O_2 in PBS.

Microscopic analysis was done at 400-fold magnification to obtain accurate microvessel counts which are expressed as vessels/mm^2.

TUNEL staining and evaluation of apoptosis.

TdT-mediated dUTP-biotin nick end labeling (TUNEL) was performed according to a modification of the method described by Gavrieli et al.(27). Tumors were fixed with 10% formaldehyde in PBS, embedded in paraffin , and cut into 3-μm thick sections. The sections were deparaffinized with xylene and dehydrated with 98% ethanol. Nuclei in the tissue sections were stripped of proteins by incubation with 20 μg/ml proteinase K (Life Technologies, Rockville, MD) for 20 min at room temperature and the slides were then washed in double distilled water (DDW) for 5 min. Endogenous peroxidase was blocked by covering the sections with 0.3% (v/v) H_2O_2 in absolute methanol for 20 min at room temperature. The sections were rinsed with DDW, and immersed in TdT buffer [30 mM Tris-HCl (pH 7.2), 140 mM sodium cacodylate, 1 mM cobalt chloride, 0.025% (w/v) bovine serum albumin]. Twenty-five units/ml TdT and 0.5 μM biotin-dUTP in TdT buffer were added to cover the sections which were then incubated at 37°C for 60 min. The reaction was terminated by transferring the slides to 2x SSC. After washing with DDW, the sections were incubated with avidin and biotinylated horseradish peroxidase complex in PBS for 30 min. After washing in PBS, the immune complexes were visualised by incubating the sections with 0.5 mg/ml DAB and 0.03% (v/v) H_2O_2 in PBS for 3 min. The sections were counterstained with haematoxylin and mounted.

The apoptotic index was estimated by determining the percentage of apoptotic cells seen under a light microscope at 400-fold magnification. A minimum of 3,000 cells were counted in the sections of the tumors. Positively stained tumor cells with the morphological characteristics of apoptosis were identified using standard criteria (28, 29).

Statistical analysis

Multiple regression analysis and Student's *t* test (30) were done using the statistical analysis program StatView J-4.5 (Abacus Concepts, Inc., Berkeley, CA).

RESULT

Expression level of TP.

We examined the levels of TP expression in KB cells transfected with TP cDNA (KB/TP) or RSV plasmid vector (KB/CV) by immunoblot analysis using anti-TP monoclonal antibody. KB/TP cells expressed high levels of TP and KB/CV cells expressed TP only slightly, as shown in Fig. 2. TP activity in KB/TP cells was 114-fold higher than that in KB/CV cells ($P<0.0001$), and the TP activity in both cell lines was inhibited by 10 μM TPI. TP activity in KB/TP cells treated with TPI was 1.6 % of that in untreated KB/TP cells (Table 1). TPI could not inhibit both human and mouse uridine phosphorylase activities (data not shown).

Effect of TPI on in vivo angiogenesis.

The inhibitory effect of TPI on angiogenesis 4 days after implantation of a chamber containing KB/TP or KB/CV cells was analysed using the mouse dorsal air sac assay (Fig.3, 4). The angiogenesis index (±SEM) in KB/TP (4.8±0.2) cells was significantly higher than that (2.2±0.86) in KB/CV cells ($P=0.0186$). Furthermore, the angiogenesis index in TPI-treated KB/TP (2.0±0.95) cells was significantly lower than in untreated KB/TP cells ($P=0.0203$). The control chamber containing PBS produced no angiogenic response (data not shown). Angiogenesis was not suppressed by 6-amino-5-chlorouracil at 30 mg/kg/day (almost the molar equivalent of 50mg TPI /kg/day) in KB/TP or KB/CV cells (data not shown).

Expression of TP in experimental tumors.

KB/TP and KB/CV cells were xenografted into nude mice to examine the effect of TPI on tumor growth. Expression of TP in KB/TP tumors was determined by immunohistochemical staining using a polyclonal antibody against TP (Fig. 6 A). The cytoplasm of almost all KB/TP tumor cells in the non-necrotic area was strongly stained, but necrotic tumor cells were not. TP activity in experimental tumors was measured using

a radiometric assay (Table 1) and it was significantly higher in KB/TP tumors than in KB/CV tumors ($P<0.0001$). TP activity in KB/TP tumors was significantly lower than in KB/TP cells ($P<0.0001$). In contrast, TP activity in KB/CV tumors was significantly higher than that in KB/CV cells ($P=0.0057$).

Effects of TPI on tumor growth.

We examined the growth of the cells xenografted into nude mice and the effect of TPI on tumor growth (Fig. 5). KB/TP tumors grew significantly faster than KB/CV tumors. The mean weight of TPI-treated tumors was significantly less than that of untreated tumors ($P<0.0001$). The growth of KB/TP tumors in mice treated with 50 mg/kg TPI was inhibited by 30.8 % compared to that in control mice treated with vehicle alone. Meanwhile, the growth of KB/CV tumors in mice treated with 50 mg/kg TPI was suppressed by 18.7 % compared to that in control mice treated with vehicle alone. The growth of KB/TP tumors was more effectively inhibited by TPI than of KB/CV tumors ($P=0.0199$). Body weight loss was less than 10 % in all of the mice (data not shown).

Number of microvessels in experimental tumors.

Sections of experimental tumors were stained with anti-mouse CD31 antibody (Fig. 6 B) and the numbers of vessels were counted. The average density of vessels/mm^2 is shown in Fig. 7. The average number of vessels (±SEM) in KB/TP (51.6±4.1) tumors was significantly higher than in KB/CV (29.8±1.8) tumors ($P=0.0006$). KB/TP tumors treated with TPI contained less vessels (45.0±1.9) than untreated KB/TP tumors, but the difference was not significant. Furthermore, the numbers of vessels were compared when the tumor weight (±SEM, n=6) of KB/TP (226.3±21.0mg) and KB/CV(226.2±15.7mg) were similar. The average number of microvessels (±SEM, n=6) in KB/TP (87.2±4.9) tumors was significantly higher than in KB/CV (41.6±4.9) tumors ($P<0.0001$).

Apoptotic index in experimental tumors.

Apoptosis was quantified *in situ* at a cellular level by labeling fragmented DNA with the TUNEL technique (Fig. 6 C). The proportion of apoptotic cells in the experimental tumors

is shown in Fig. 8. The apoptotic index (±SEM) in KB/CV (1.54±0.12%) tumors was significantly higher than in KB/TP (1.07±0.08%) tumors (P=0.0052). The apoptotic index in KB/TP (1.36±0.05 %) tumors treated with TPI was significantly higher than in untreated KB/TP tumors (P=0.01).

DISCUSSION

Angiogenesis is not only involved in tumor growth and distant metastasis but also is an important early step in carcinogenesis. It is a complex multistep process regulated by a number of angiogenic factors (31, 32). PD-ECGF was initially cloned as a novel angiogenic factor distinct from other endothelial cell growth factors (11). Previous studies demonstrated that PD-ECGF was identical to TP (8, 9), and that it stimulated chemotaxis and [^{3}H]thymidine incorporation by endothelial cells *in vitro* and had angiogenic activity *in vivo* (10-13). Expression of PD-ECGF in transformed fibroblasts in nude mice resulted in increased tumor vascularity (11). Overexpression of TP in MCF-7 cells transfected with TP cDNA had no effect on growth *in vitro*, but conferred a growth advantage when these cells were xenografted into nude mice (14). Recent studies showed that TP is expressed in a wide variety of solid tumors (15, 16, 23, 33-36), but the role of TP in tumor proliferation was unknown.

Our study showed that, *in vivo*, KB cells with high TP activity grew faster than those with low TP activity. The density of blood vessels in KB tumors with high TP activity was higher than in KB tumors with low TP activity suggesting that TP was involved in angiogenesis in KB tumors. Recently, we have newly synthesized a novel selective inhibitor of TP, TPI, which has 1,000-fold higher inhibitory activity than 6-amino-5-chlorouracil, and does not inhibit another enzyme involved in pyrimidine nucleotide metabolism, uridine phosphorylase. We demonstrated that TPI partially suppressed the growth of TP expressing tumors, but did not significantly suppress angiogenesis in the tumors. However, in the mouse dorsal air sac assay model, TPI completely suppressed the angiogenesis induced by KB/TP. Administration of TPI was started after the tumor reached a volume of 50-150 mm^3 in the nude mice model. While it was started immediately after implantation of the chamber in the mouse dorsal air sac assay model. Considerable experimental evidence indicates that vascular endothelial cell growth factor (VEGF) (37) is not necessary for growth after tumors reach a certain size, and suppression of VEGF has no effect on the expansion of the large tumors because of the up-regulation of other angiogenic factors like bFGF and TGF-α in large tumors (38). These findings, taken

together, suggest that TP might be important in the early stage of tumor angiogenesis through remodelling of the existing vasculature (36), and other angiogenic factors might be involved in angiogenesis after tumors reach a certain size.

In the nude mice model, TPI did not inhibit angiogenesis but still had an inhibitory effect on tumor growth, suggesting that TP has some role(s) in tumor growth other than angiogenesis and recently we proposed another role for TP in the progression of solid tumors besides angiogenesis. TP conferred resistance to apoptosis induced by hypoxia and the degradation products of thymidine were involved in the resistance (unpublished data). The proportion of apoptotic cells assessed by the TUNEL assay in KB/TP tumors was significantly lower than in KB/CV tumors, and TPI abolished the difference. This suggests that TP conferred the resistance to apoptosis. The finding that TP can confer resistance to apoptosis, in addition to its angiogenic activity, may explain why breast carcinoma cells that overexpressed TP had increased growth *in vivo* without increased vessel density (14). In accordance with this, patients with TP-positive tumors have a poorer prognosis than those with TP-negative tumors, whereas microvessel density is not a significant prognostic factor in colorectal and renal cell carcinomas (15, 16).

Our study showed that TP activity in KB/TP tumors was significantly lower than in KB/TP cells *in vitro* ($P<0.0001$). The considerable extent of necrosis in KB/TP tumors may be related to the low TP activity in the tumors. In contrast, TP activity in KB/CV tumors was significantly higher than in KB/CV cells *in vitro* ($P=0.0057$), although the necrotic lesions were also found in KB/CV tumors. It has been shown that tumor necrosis factor α, interleukin 1 and inteferon γ up-regulate TP expression in tumor cells (39, 40) and these cytokines may have induced TP expression in KB/CV cells in nude mice. Furthermore, the center of tumors with a certain size is supposed to be hypoxic. Griffiths and her colleagues reported that hypoxia regulated and influenced the levels of expression of PD-ECGF/TP *in vitro* and *in vivo* (41). Therefore, TP may have been induced by hypoxic conditions in KB/CV tumors.

Angiogenesis is also important for metastasized cells to grow in their target lesions (42). The density of microvessels in primary carcinoma lesions significantly correlates with metastasis (42). We had previously demonstrated that TPI alone or TPI in combination with 5-fluorothymidine had anti-metastatic activity in mice bearing an experimental liver metastasis of TMK-1 gastric cancer or a spontaneous liver metastasis of Co-3 colon cancer (17). Furthermore, we also showed in this study that TPI could completely inhibit early events of angiogenesis in the mouse dorsal air sac assay model. TPI may be valuable in the therapy of some patients with locoregional recurrent and metastatic TP expressing tumors. In summary, we have demonstrated that the newly synthesized inhibitor of TP, TPI, competitively inhibits TP activity, inhibits angiogenesis induced by TP, increases the proportion of apoptotic cancer cells in TP-positive tumors, and suppresses the growth of the tumors. While the precise mechanisms for these phenomena remain to be elucidated, these findings suggest the possibility that TPI alone or in combination with other anti-cancer agents may suppress locoregional recurrence and metastasis of TP expressing tumors.

Acknowledgments

Supported in part by Grants-in-Aid from the Ministry of Education, Science and Culture and the Ministry of Health and Welfare, Japan; and by a research grant from the Princess Takamatsu Cancer Research Fund. We thank Ms. Yukino for her exellent technical assistance.

REFERNCES

(1) Iltzsch, M. H., Kouni, M. H., and Cha, S. Kinetic studies of thymidine phosphorylase from mouse liver. Biochemistry, *24:* 6799-807, 1985.

(2) Friedkin, D., and Roberts, D. The enzymatic synthesis of nucleosides. J. Biol. Chem., *207:* 245-256, 1954.

(3) Krenitsky, T. A., Koszalka, G. W., and Tuttle, J. V. Purine nucleoside synthesis, an efficient method employing nucleoside phosphorylase. Biochemistry, *20:* 3615-3621, 1981.

(4) Zimmerman, M., and Seidenberg, J. Deoxyribosil transfer. Thymidine phosphorylase and nucleoside deoxyribosyltransferase in normal and malignant tissues. J. Biol. Chem., *230:* 2618-2621, 1964.

(5) Gallo, R. C., Perry, S., and Breitman, T. R. The enzymatic mechanisms for deoxythymidine synthesis in human leukocytes. J. Biol. Chem., *242:* 5059-5068, 1967.

(6) Krenitsky, T. A. Pentosyl transfer mechanisms of the mammalian nucleoside phosphorylases. J. Biol. Chem., *243:* 2871-2875, 1968.

(7) Desgranges, C., Razaka, G., Rabaud, H., and Bricaud, H. Catabolism of thymidine in human blood platelets: purification and properties of thymidine phosphorylase. Biochim. Biophys. Acta, *654:* 211-218, 1981.

(8) Furukawa, T., Yoshimura, A., Sumizawa, T., Haraguchi, M., Akiyama, S., Fukui, K., Ishizawa, M., and Yamada, Y. Angiogenic factor. Nature, *356:* 668,1992.

(9) Sumizawa, T., Furukawa, T., Haraguchi, M., Fukui, K., Ishizawa, M., and Akiyama, S. Thymidine phosphorylase activity associated with platelet-derived endothelial cell growth factor. J. Biochem. (Tokyo), *114:* 9-14, 1993.

(10) Haraguchi, M., Miyadera, K., Uemura, K., Sumizawa, T., Furukawa, T., Yamada, K., Akiyama, S., and Yamada, Y. Angiogenic activity of enzymes. Nature, *368:* 198, 1994.

(11) Ishikawa, F., Miyazono, K., Hellman, U., Drexler, M., Wernstedt, C., Hagiwara, K., Usuki, K., Takaku, F., Risau, W., and Heldin, C. H. Identification of angiogenic

activity and the cloning and expression of platelet-derived endothelial cell growth factor. Nature, *338:* 556-562, 1989.

(12) Miyazono, K., Okabe, T., Urabe, A., Takaku, F., Heldin, C. H., Purification and properties of an endothelial cell growth factor from human platelets. J. Biol. Chem., *262:* 4098-4103, 1987.

(13) Miyadera, K., Sumizawa, T., Haraguchi, M., Yoshida, H., Konstanty, W., Yamada, Y., and Akiyama, S. Role of thymidine phosphorylase activity in the angiogenic effect of platelet-derived endothelial cell growth factor/thymidine phosphorylase. Cancer Res., *55:*1687-1690, 1995

(14) Moghaddam , A., Zhang, H. T., Fan, T. P. D., Hu, D. E., Lees, V. C., Turley, H., Fox, S. B., Gatter, K. C., Harris, A. L., and Bicknell, R. Thymidine phosphorylase is angiogenic and promotes tumor growth. Proc. Natl. Acad. Sci. USA, *92:* 998-1002, 1995.

(15) Takebayashi, Y., Akiyama, S., Akiba, S., Yamada, K., Miyadera K., Sumizawa, T., Yamada, Y., Murata, F., and Aikou T. Clinicopathologic and prognostic significance of an angiogenic factor, thymidine phosphorylase, in human colorectal carcinoma. J. Natl. Cancer Inst., *88:* 1110-1117, 1996.

(16) Imazono, Y., Takebayashi, Y., Nishiyama, K., Akiba, S., Miyadera, K., Yamada, Y., Akiyama, S., and Ohi, Y. Correlation between thymidine phosphorylase expression and prognosis in human renal cell carcinoma. J. Clin. Oncol., *15:* 2570-2578, 1997.

(17) Miyadera, K., Emura, T., Suzuki, N., Akiyama, S., Fukushima, M., and Yamada, Y., Novel functional antitumor nucleoside TAS-102, combined form of F3dThd and its modulator (2): Inhibitory effect of TPI on tumor-derived angiogenesis and metastasis. Proc. Am. Assoc. Cancer Res. Annu. Meet., *39:* 609, 1998.

(18) Potter, H., Weir, L., and Leder, P. Enhancer-dependent expression of human kappa immunoglobulin genes introduced into mouse pre-B lymphocytes by electroporation. Proc. Natl. Acad. Sci. USA, *81:* 7161-7165, 1984.

(19) Yoshimura, A., Kuwazuru, Y., Furukawa, T., Yoshida, H., Yamada, K., and Akiyama, S. Purification and tissue distribution of human thymidine phosphorylase; high

expression in lymphocytes, reticulocytes and tumors. Biochim. Biophys. Acta, *1034:* 107-113, 1990.

(20) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., *72:* 248-254, 1976.

(21) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond.), *227:* 680-685, 1970.

(22) Andersen, J. K. Electroblotting of multiple gels: a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. J. Biochem. Biophys. Methods, *10:* 203-209, 1984.

(23) Takebayashi, Y., Yamada, K., Miyadera, K., Sumizawa, T., Furukawa, T., Kinoshita, F., Aoki, D., Okumura, H., Yamada, Y., Akiyama, S., and Aikou, T. The activity and expression of thymidine phosphorylase in human solid tumors. Eur. J. Cancer, *32:* 1227-1232, 1996.

(24) Gan, T. E., Hallam, L., Pilkington, G. R., and van der Weyden, M. B. A rapid and simple radiometric assay for thymidine phosphorylase of human peripheral blood cells. Clin. Chim. Acta, *116:* 231-236, 1981.

(25) Sakamoto, N., Tanaka, N. G., Tohgo, A., and Ogawa, H. Heparin plus cortisone acetate inhibits tumor growth by blocking endothelial cell proliferation. Cancer J., *1:* 55-57, 1986.

(26) Oikawa, T., Sasaki, M., Inose, M., Shimamura, M., Kuboki, H., Hirano, S., Kumagai, H., Ishizuka, M., and Takeuchi, T. Effects of cytogenin, a novel microbial product, on embryonic and tumor cell-induced angiogenic responces *in vivo*. Anticancer Res., *17:* 1881-1886, 1997.

(27) Gavrieli, Y., Sherman, Y., and Ben-Sasson, S.A. Identification of programmed cell death in situ via specific labelling of nuclear DNA fragmentation. J. Cell Biol., *119:* 493-501, 1992.

(28) Thompson, C. B. Apoptosis in the pathogenesis and treatment of the disease. Science (Washington DC), *267:* 1456-1462, 1995.

(29) Ansari, B., Coates, P. J., Greenstein, B.D., and Hall, P. A. *In situ* end-labeling detects DNA strand breaks in apoptosis and other physiological and pathological states. J. Pathol., *170:* 1-8, 1993.

(30) Zar J.H. Biostatistical analysis. 3rd edition. New Jersey : Prentice Hall, 1996.

(31) Folkman J. What is the evidence that tumors are angiogenesis dependent ? J. Natl. Cancer Inst., 82: 4-6, 1990.

(32) Folkman J., Shing Y. Angiogenesis. J. Biol. Chem., 267: 10931-10934, 1992.

(33) Takebayashi, Y., Miyadera, K., Akiyama, S., Hokita, S., Yamada, Y., Akiba S., Yamada, Y., Sumizawa, T., and Aikou T. Expression of thymidine phosphorylase in human gastric carcinoma. Jpn. J. Cancer Res., *87:* 288-295, 1996.

(34) Maeda, K., Chung, Y. S., Ogawa, Y., Takatsuka, S., Kang, S. M., Ogawa, M., Sawada, T., Onda, N., Kato, Y., and Sowa, M. Thymidine phosphorylase/platelet-derived endothelial cell growth factor expression associated with hepatic metastasis in gastric carcinoma. Br. J. Cancer, *73:* 884-888, 1996.

(35) Koukourakis, M. I., Giatromanolaki, A., O'Bryne, K. J., Comley, M., Whitehouse, R. M., Talbot, D. C., Gatter, K. C., and Harris, A. L. Platelet-derived endothelial cell growth factor expression correlates with tumor angiogenesis and prognosis in non-small cell lung cancer. Br. J. Cancer, *75:* 477-481, 1997.

(36) Fox, S.B., Westwood, M., Moghaddam, A., Comley, M., Turley, H., Whitehouse, R.M., Bicknell, R., Gatter, K.C., and Harris, A.L. The angiogenic factor pletelet-derived endothelial cell growth factor / thymidine phosphorylase is up-regulated in breast cancer epithelium and endothelium. Br. J. Cancer, *73:* 275-280, 1996.

(37) Shweiki, D., Itin, A., Soffer, D., and Keshet, E. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature (Lond.), *359:* 843-845, 1992.

(38) Yoshiji, H., Harris, S.R., Thorgeirsson U.P. Vascular endothelial cell growth factor is essential for initial but not continued in vivo growth of human breast carcinoma cells. Cancer Res., *57:* 3924-3928, 1997.

(39) Ho, C.K., Ou, R.B., Hsu, M.L., Su, S.N., Yung, C.H., and Wang, S.Y. Induction of thymidine kinase activity and clonal growth of certain leukemic cell lines by a granulocyte - derived factor. Blood, *75:* 2438-2444, 1990.

(40) Eda, H., Fujimoto, K., Watanabe, S., Ura, M., Hino, A., Tanaka, Y., Wada, K., and Ishituka, H. Cytokines induce thymidine phosphorylase expression in tumor cells and make them more susceptible to 5'-deoxy-5-fluorouridine. Cancer Chemother. Pharmacol., *32:* 333-338, 1993.

(41) Griffiths, L., Dachs, G. U., Bicknell, R., Harris, A. L., and Stanford, I. J. The influence of oxygen tension and pH on the expression of platelet-derived endothelial cell growth factor/thymidine phosphorylase in human breast tumor cells grown in vitro and in vivo. Cancer Res., *57:* 570-572, 1997.

(42) Weidner, N., Semple, J.P., Welch, W.R., and Folkman, J. Tumor angiogenesis and metastasis-correlation in invasive breast carcinoma. N. Engl. J. Med., *324*: 1-8 1991.

Cell/Tumor	TPI	
	0	10 μM
KB/CV Cells	0.44 ± 0.04^{a}	0.22 ± 0.01^{e}
KB/CV Tumors	0.90 ± 0.08^{b}	0.56 ± 0.06^{f}
KB/TP Cells	50.31 ± 1.70^{c}	0.79 ± 0.07^{g}
KB/TP Tumors	13.65 ± 0.83^{d}	0.68 ± 0.04^{h}

Table 1. TP activity in KB cells and tumors. TP activity (nmol thymine / mg protein / h) was measured with a radiometric assay as described in Materials and Methods. Each value represents the mean ± SEM of three independent experiments. KB/TP is a subline of KB transfected with PD-ECGF cDNA. Statistical significance was determined by Student's *t* test. a vs b, P=0.0057, c vs d, P<0.0001, a vs c, P<0.0001, b vs d, P<0.0001, a vs e, P=0.0045, b vs f, P=0.0162, c vs g, P<0.0001, d vs h, P<0.0001

FIGURE LEGENDS

Fig. 1 Chemical structure of TPI.

Fig. 2 Immunoblot analysis of TP in KB/CV and KB/TP cells. KB/CV and KB/TP cytosols (100 and 10 μg protein, respectively) were separated by SDS/PAGE and transferred to a PVDF membrane. TP levels in the cytosols were determined using a monoclonal antibody against TP.

Fig. 3 The effect of TPI on TP-induced angiogenesis. Mice implanted with a chamber containing KB/TP (A, B) and KB/CV (C, D) cells were treated with vehicle (A, C) or 50mg/kg/day TPI (B, D). TPI strongly inhibited the KB/TP-induced formation of new blood vessels (b). Magnification (20 x).

Fig. 4 Inhibitory effect of TPI on the angiogenic response. Four days after implantation of a chamber containing KB/TP or KB/CV cells, the effect of TPI on angiogenesis was assessed. Statistical significance was determined by Student's *t* test. (mean ± SEM, n=5) *P=0.0186 **P=0.0203

Fig. 5 The effects of TPI on tumor weight. After tumor size reached about 50-150 mm^3, TPI at the indicated amounts was administered intraperitoneally for 22 days. Statistical significance was determined using multiple regression analysis adjusting for tumor type and observation period. (mean ± SEM, n=6)

Fig. 6 TP staining in KB/TP tumor, 200 x (A). Immunohistochemical analysis of vascularization and apoptosis. (B) Microvessel staining with murine anti-CD 31 monoclonal antibody (200 x). (C) Apoptotic cells were detected *in situ* by labelling fragmented DNA with TUNEL (400 x).

Fig. 7 The numbers of microvessels in the tumors. TPI-untreated KB/TP tumors contained significantly more vessels than TPI-untreated KB/CV tumors. The numbers of microvessels in the TPI-untreated KB/TP tumors were not significantly different from those in TPI-treated KB/TP tumors. Statistical significance was determined by Student's *t* test. (mean ± SEM, n=6) *P=0.0006

Fig. 8 Apoptotic indexes in the tumors. The proportion of apoptotic cells in TPI-untreated KB/CV tumors was significantly higher than in KB/TP tumors. The proportion of

apoptotic cells in TPI-treated KB/TP tumors was significantly higher than in TPI-untreated KB/TP tumors. Statistical significance was determined by Student's *t* test. (mean ± SEM, n=6) *P=0.0052 **P =0.01

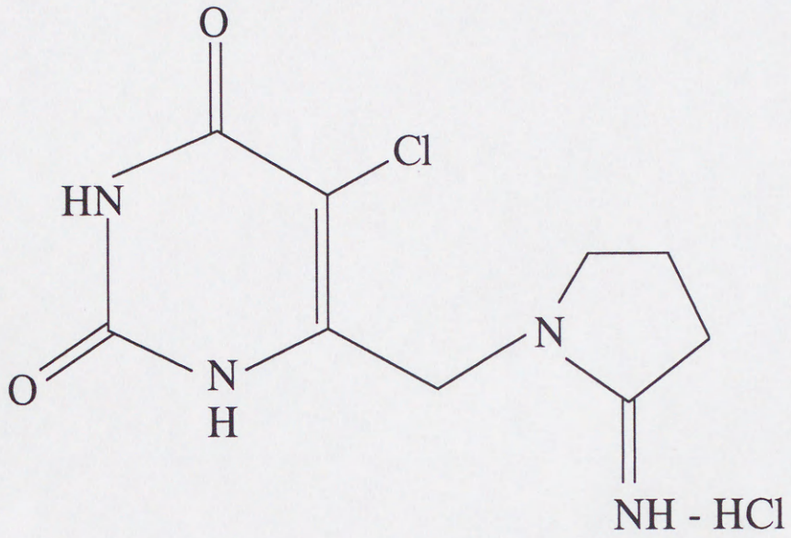
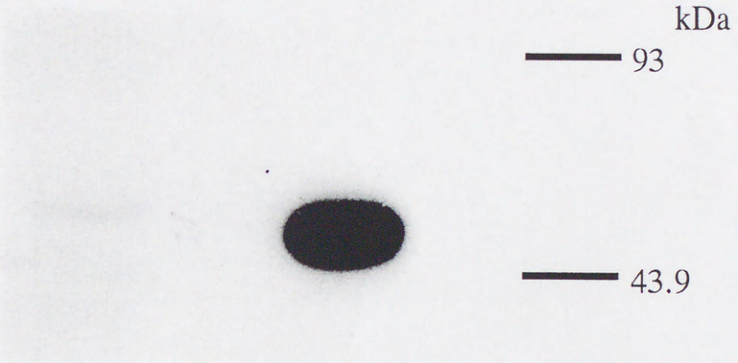


Fig. 1, Matsushita et al.



Cells	KB/CV	KB/TP
protein	100 μg	10 μg

Fig. 2, Matsushita et al.

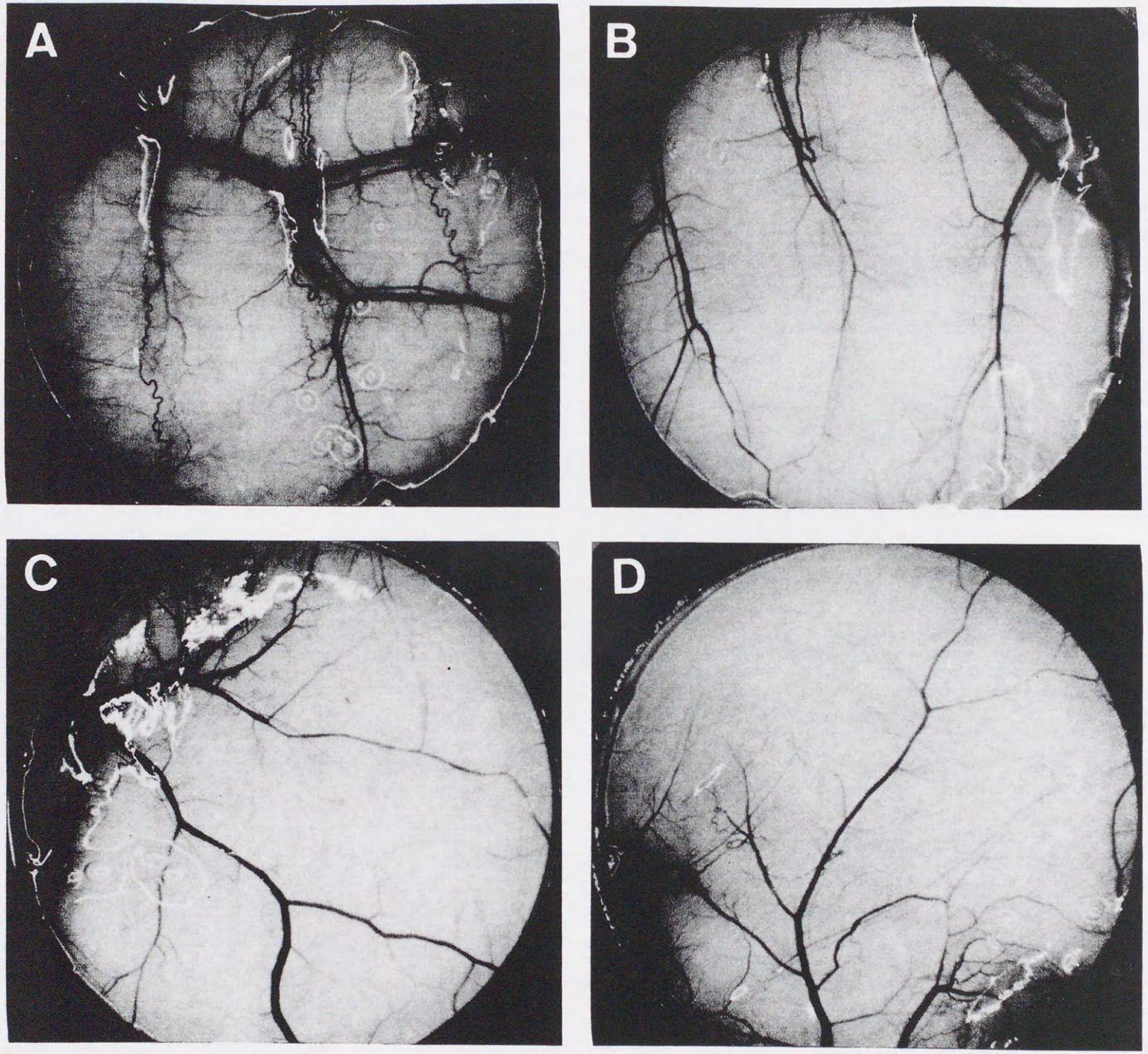


Fig. 3, Matsushita et al.

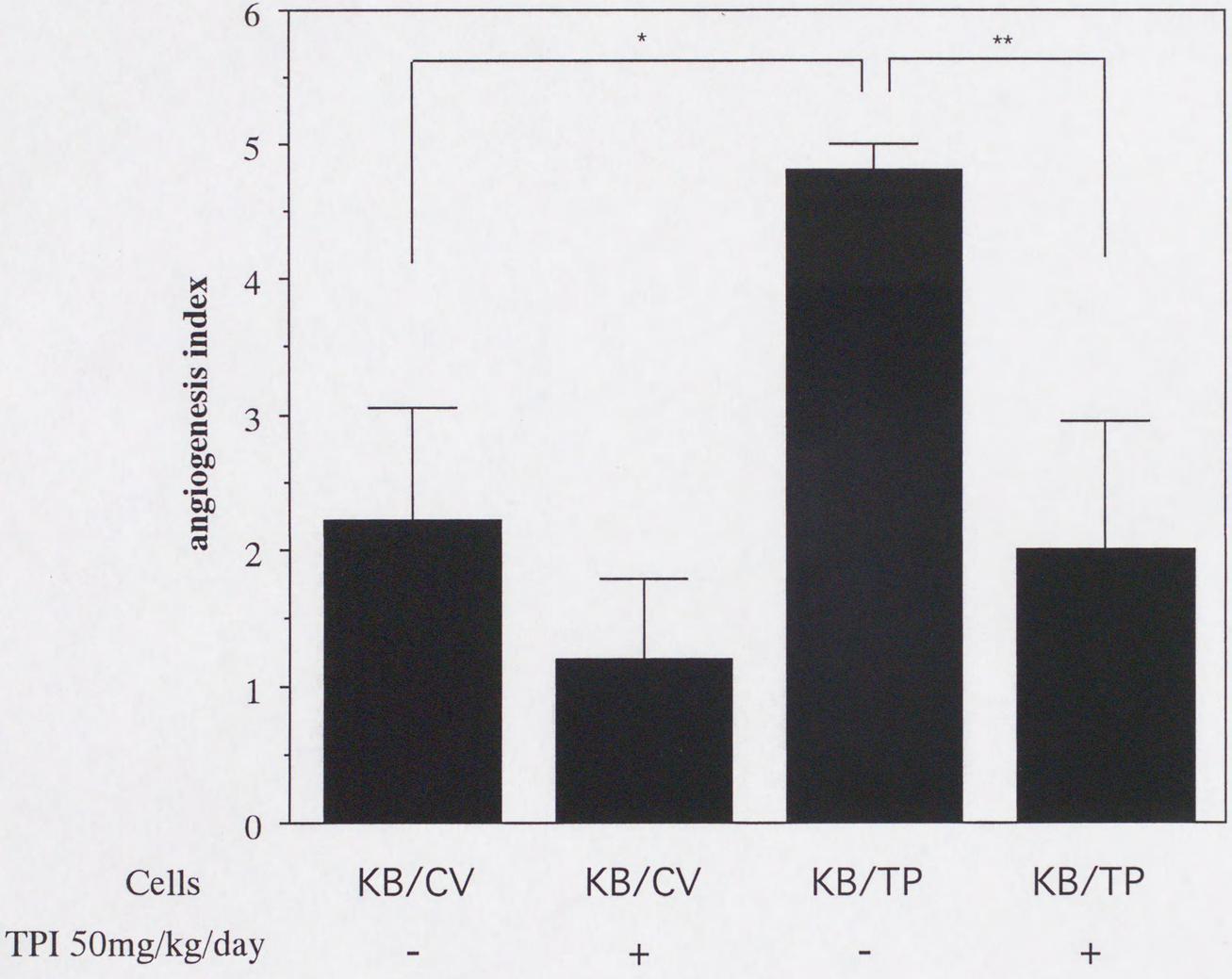


Fig. 4, Matsushita et al.

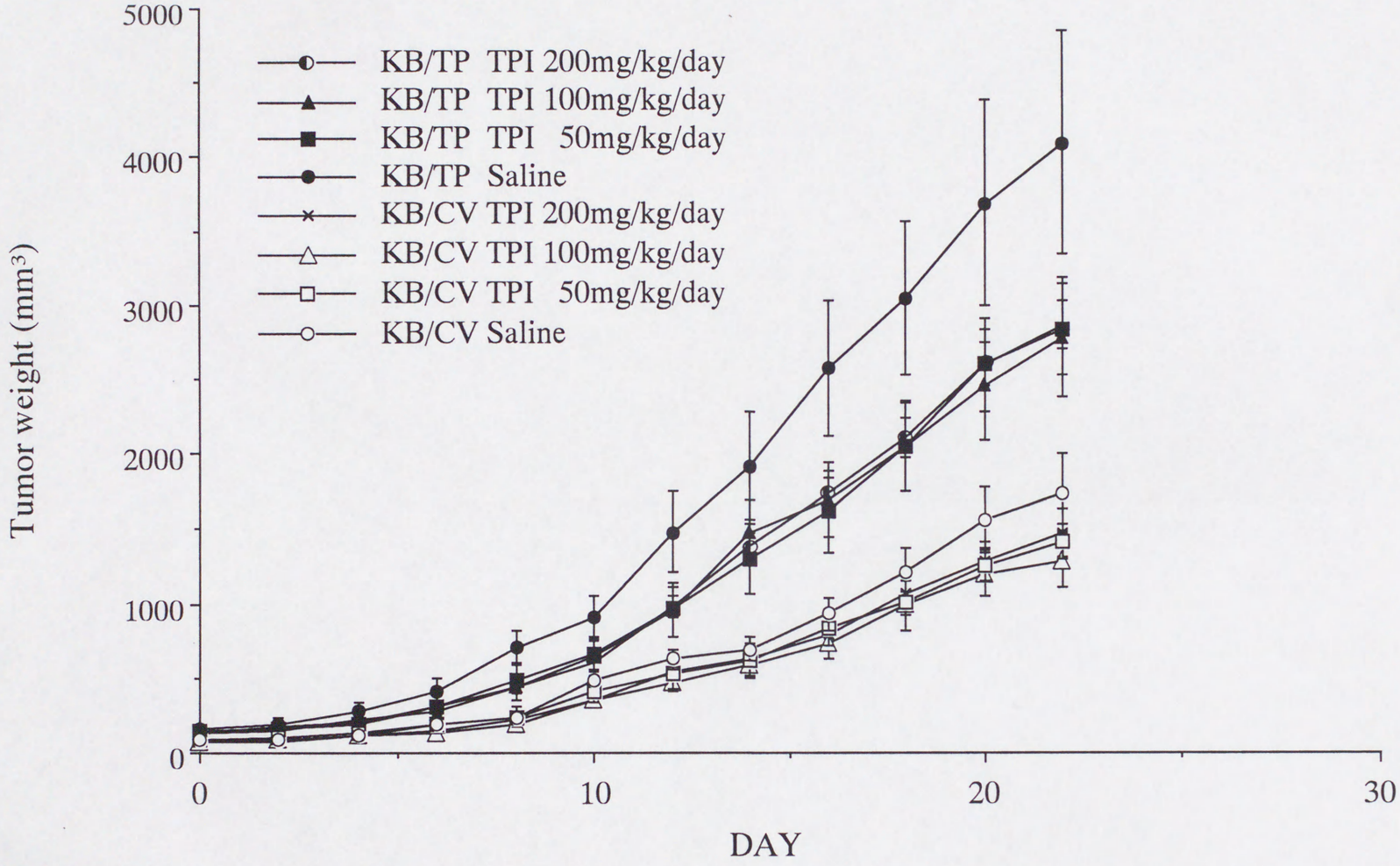


Fig. 5, Matsushita et al.

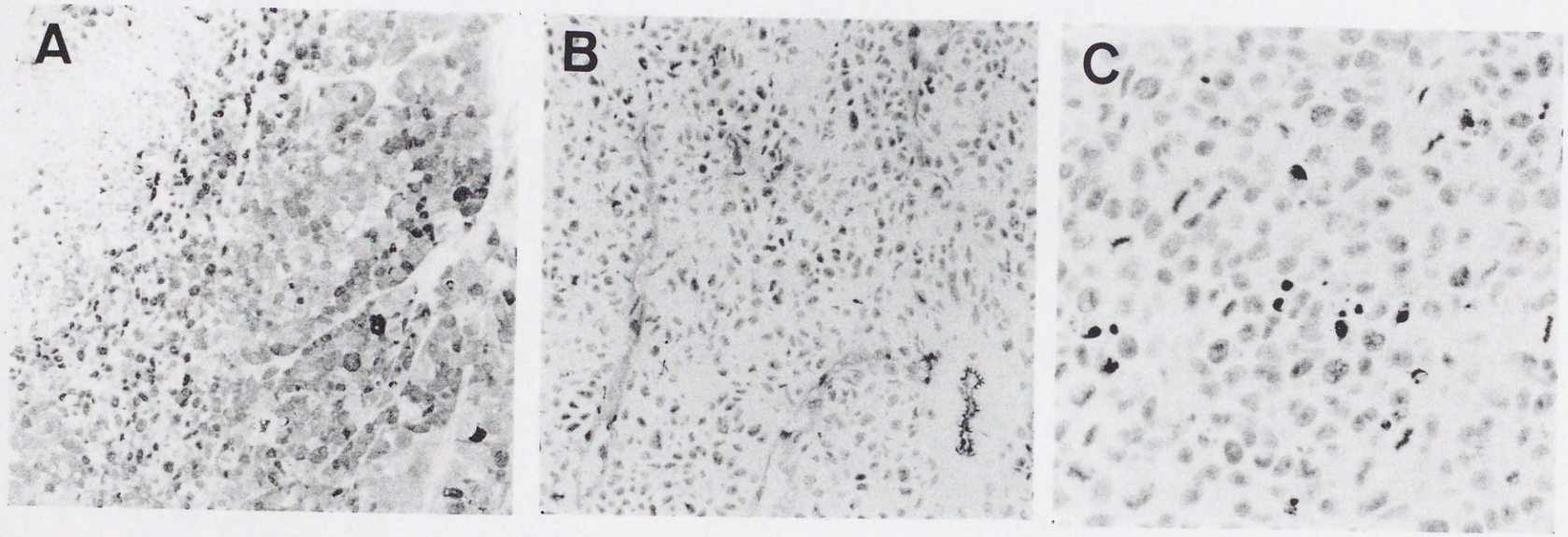


Fig. 6, Matsushita et al.

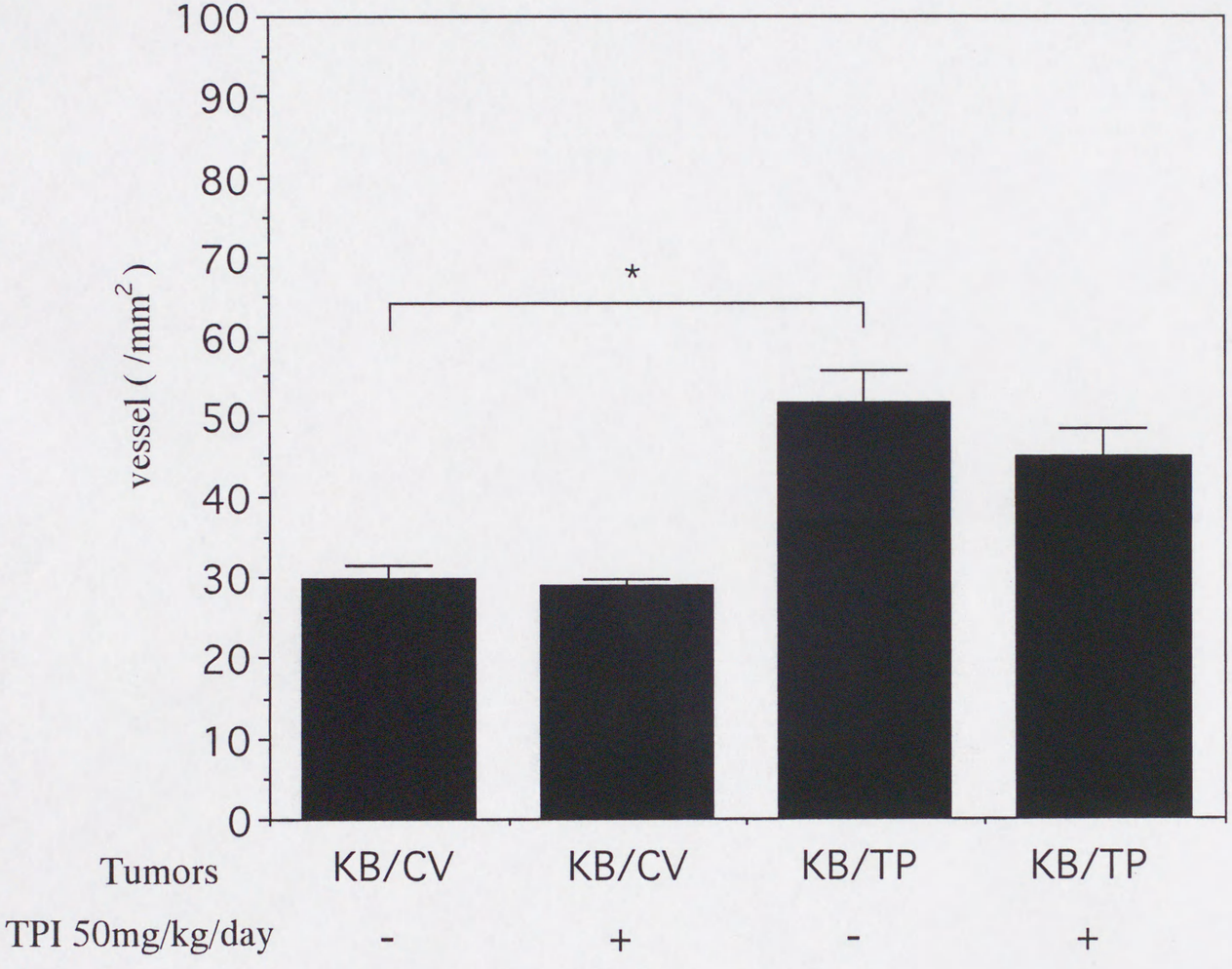


Fig. 7, Matsushita et al.

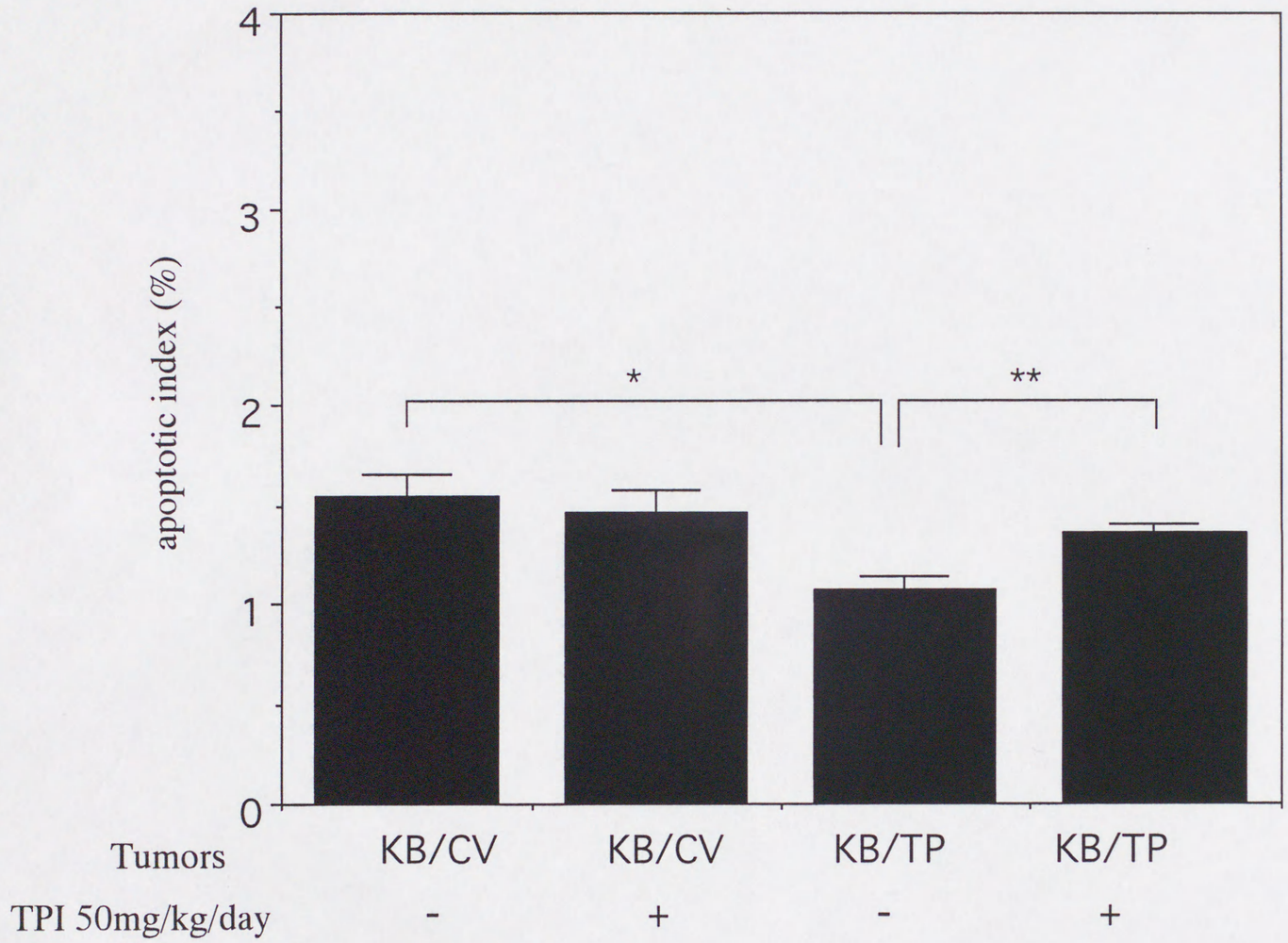
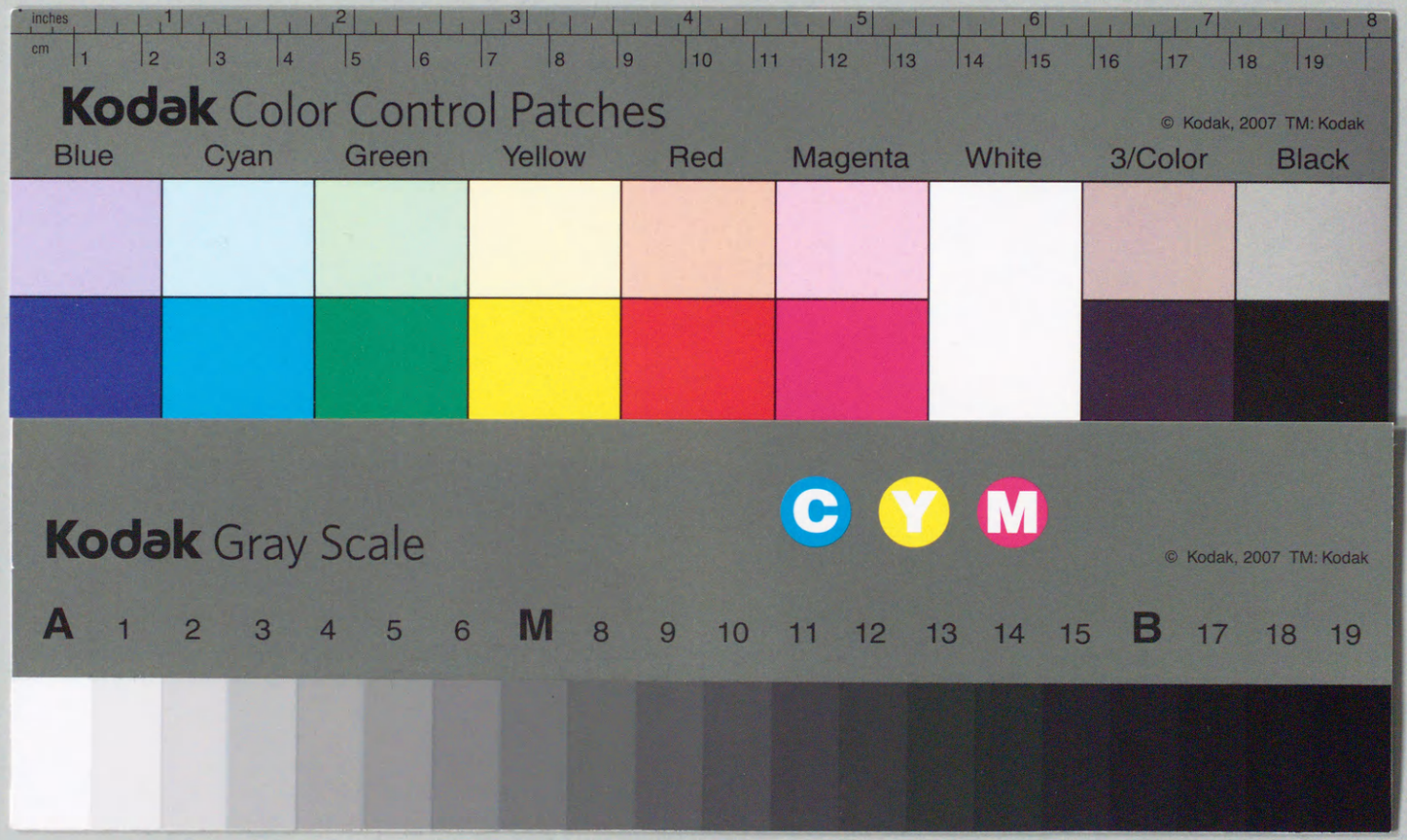


Fig. 8, Matsushita et al.


inches
cm
Kodak Color Control Patches
© Kodak, 2007 TM: Kodak
Blue
Cyan
Green
Yellow
Red
Magenta
White
3/Color
Black
Kodak Gray Scale
C
Y
M
© Kodak, 2007 TM: Kodak
A 1 2 3 4 5 6 M 8 9 10 11 12 13 14 15 B 17 18 19