Chapter 10

Thymidine phosphorylase in cancer cells stimulates human endothelial cell migration and invasion by increasing the secretion of angiogenic factors

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ABSTRACT

Thymidine phosphorylase (TP), also known as the platelet-derived-endothelial cell growth factor, is often overexpressed in tumors and plays a role in tumor aggressiveness and angiogenesis. Here, we determined whether TP increased tumor invasion and whether TP-expressing cancer cells stimulated angiogenesis. Angiogenesis was studied by exposing human umbilical vein endothelial cells (HUVECs) to conditioned medium (CM) derived from cancer cells with a high and no TP expression (Colo320TP1=CT-CM, RT112/TP=RT-CM and Colo320=C-CM, RT112=R-CM) after which the migration (wound healing assay) and invasion (transwell-assay) were determined. The involvement of several angiogenic factors were examined by RT-PCR, ELISA and blocking antibodies. Tumor invasion was not dependent on intrinsic TP expression. CT-CM and RT-CM stimulated HUVEC-migration and invasion by about 15% and 40%, respectively when compared to C-CM and R-CM. Inhibition of TP and dR by TPI and L-dR, respectively, blocked migration and reduced the invasion by about 50-70%. TP activity in HUVECs was increased by CT-CM, but not by RT-CM. RT-PCR revealed a higher mRNA expression of bFGF (Colo320TP1), IL-8 (RT112/TP) and TNF-α. VEGF expression levels were lower in Colo320TP1 than in Colo320 cells, but was not different between the RT112s. Blocking antibodies targeting these factors decreased both the migration and invasion that was induced by the CT-CM and RT-CM, except for IL-8 in CT-CM and bFGF in RT-CM. In our cell line panels, TP did not increase the tumor invasion, but stimulated the migration and invasion of HUVECs by 2 different mechanisms. Hence, TP targeting seems to provide a potential additional strategy in the field of anti-angiogenic therapy.

INTRODUCTION

The platelet derived endothelial cell growth factor (PD-ECGF) is also known as thymidine phosphorylase (TP). Numerous immunohistochemical and TP-activity studies have shown an increased TP expression and activity in a wide-range of solid tumors, compared to normal healthy tissues[1,2,3,4]. A high TP expression in these tumor sites has clearly been related to a high microvessel density, the induction of metastasis and a poor prognosis for the patient.[5,6,7] The location of TP expression varies between tumor type and grade and has been reported to be highly expressed in tumor cells[8], the invasive part or the tumor[5,9,10] or in the tumor stromal cells[11,12,13,14]. From these studies about TP, we conclude that TP is involved to two different but overlapping actions. First, it is related to a higher invasive

property and metastatic potential of the cancer cells[1,2,15]. Secondly, it is related to an increased angiogenesis. The mechanisms underlying TP aggressiveness in cancer cells are possibly by comparable cellular events as for that of angiogenesis. However, the exact molecular mechanisms behind these actions remain unclear.

TP catalyzes the reaction of thymidine (TdR) to deoxyribose-1-phosphate (dR-1-P) and thymine. From this reaction, deoxyribose (dR) can be formed[16]. It is believed that dR plays a role in the angiogenic effects of TP, since dR can be secreted from the cells. dR has shown angiogenic properties in various *in vitro* and *in vivo* studies[17,18,19]. Exposure of endothelial cells to dR and TP stimulated both the migration and invasion, and activated the focal adhesion kinase (FAK) and p70/S6k[17,20]. FAK plays an important role in the invasion and migration of cells and also in cell death regulation[21]. P70/S6k is the important downstream kinase of mTOR, regulating cell proliferation, metabolism and also angiogenesis[22]. In addition to angiogenesis, the mTOR-FAK signaling pathway also seems to be involved in the invasive potential of TP in cancer cells. Besides sugars that are produced by TP conversion of TdR, other mechanisms are possibly involved as well. A high TP expression has been related to an increased secretion of angiogenic factors, such as IL-8 and bFGF[23]. Moreover, TP is often co-expressed with the important angiogenic factor vascular endothelial growth factor (VEGF)[24]. However, whether TP can regulate VEGF is unclear.

In order to reduce tumor aggressiveness and angiogenesis, TP inhibitors have been synthesized. TPI is a very potent and specific inhibitor of TPI[25] and does not inhibit uridine phosphorylase (UP). Another possibility to inhibit one of the downstream biological actions of TP is by addition of L-deoxyribose, a stereoisomer of dR[26]. Although L-dR is often used to study TP functioning, dR is not the only product that is responsible for the pro-angiogenic activity of TP[4,23]. Therefore, the use of specific TP inhibitors are evaluated for their anti-angiogenic potential in cancer cells.

It is not exactly known how cancer cells with a high TP expression can stimulate endothelial cells to form new blood vessels. The aim of the present study was to evaluate the role of TP in cancer cells on the proliferation, migration and invasion of endothelial cells and to identify several potential angiogenic factors in their involvement in these process. Therefore, we used conditioned medium from TP expressing cells to determine whether tumor cells secrete molecules that stimulate angiogenesis. Since the TP expression in cancer cells also increase their aggressiveness, we also determined the level of invasion and intracellular signaling of colon cancer and bladder cancer cell lines, with and without TP expression.

MATERIALS AND METHODS

Chemicals

Thymidine (TdR) and L-deoxyribose (L-dR) were obtained from Sigma-Aldrich Chemicals (Zwijndrecht, The Netherlands). The thymidine phosphorylase inhibitor, TPI, was provided by Taiho Pharmaceuticals (Tokushima, Japan). The IL-8 blocking antibody was purchased from Abcam (Cambridge, UK) and was used at a concentration of 50 ng/ml, bFGF blocking antibody was obtained from Sigma and was used at a concentration of 50 ng/ml, the TNF-α antibody was used at concentrations of 50 ng/ml and avastin was used at a concentration of 100 ng/ml. Anti-focal adhesion kinase, anti-Akt, anti-MAPK, anti-p70/S6k antibodies were purchased from Cell Signalling Technology Inc. (Danvers, MA, USA). Anti-β-actin antibody was obtained from Sigma.

Cell lines

Human colon cancer cell lines Colo320, Colo320TP1[27] (TP-transfected), RT112 and RT112/TP[23] (TP-transfected) were cultured as monolayers in DMEM, supplemented with 20 mM Hepes and 10% FCS in 25 cm^2 culture flasks (Greiner Bio-One, Frickenhausen, Germany). Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cords and were cultured in M199 medium, supplemented with 10% FCS (PAA laboratory GmbH, Cölbe Germany), 10% human serum (HS) and ECGF. All cells were maintained in a humidified 5% CO_2 atmosphere at 37℃. For all experiments in which HUVECs were used, at least 3 different isolations (e.g. donors) were examined for their (angiogenic) response.

TP enzymatic activity

TP enzymatic activity was performed by HPLC measurement of TdR conversion to thymine as described previously[28]. In brief, cells were exposed to TdR for 6 h, after which the medium was collected. 25 µl 80% trichloroacetic acid (TCA) was added to 200 µl medium to denaturate the proteins. Samples were left on ice for 20 min. Subsequently, samples were centrifuged at 14000 g at 4ºC for 10 min. The supernatant was transferred to a new vial, and the pH was neutralized. Samples were mixed and centrifuged for 1 min at 14000 g. The upper aqueous layer was used for analysis of TdR and thymine by HPLC analysis for nucleosides with UV detection as described previously[29].

Concentrating conditioned medium

Cancer cells were seeded (2 - 2.5 x10^6 cells/T75 culture flask (Greiner Bio-One, Alphen a/d Rijn, The Netherlands) in exactly 15 ml medium and grown under standard culture conditions. After 3 days, the medium was collected and filtered through a 0.22 µm filter to remove any floating cells. Subsequently, the medium was concentrated 20x using Amicon Ultra 15 Centrifugal filters (Milipore, Billerica, MA). This concentrated medium was stored at -20ºC in aliquots. For each experiment, the concentrated medium was thawed and diluted 1:20 in HUVEC-medium. From this diluted medium, the conditioned medium (CM) is referred as C-CM (Colo320), CT-CM (Colo320 TP1), R-CM (RT112) and RT-CM (RT112/TP).

Migration assay

HUVEC migration was determined using the wound healing assay as described previously[30]. HUVECs were seeded (100000 cells/well) in duplicates in 1% gelatin coated wells of a 24 wells plate (Corning, Schiphol, Nederland). Cells were grown till confluence and a scratch wound was applied in two perpendicular directions with a sterile pipet tip. Subsequently, cells were washed 2 times with HBSS and cells were exposed to the various conditioned media, diluted in HUVEC-medium with 5% FCS and 5% HS. When blocking antibodies were used, the medium was 30 min pre-incubated in the diluted conditioned medium. Wounds were captured at 2.5x magnification with a microscope (TCS 4D, Leica, Jena, Germany), and Q500MC software (Leica) at time points 0, 3 and 6 h. At all indicated time points, the wound width was measured in four areas and compared with the initial width at the 0 h time point and were set relative to the positive control (ECGF; set to 100%) and the negative control (set at 0%).

Invasion assay

The invasion assay was carried out as described previously[30], using transwell chambers with a fluorescence-blocking 8 µm pore polycarbonate filter insert (#35-1152; HTS Fluoroblock Insert, Falcon, Becton Dickinson Labware, Bedford, MA) in 24 wells plates (#35-3504; Falcon, Becton Dickinson). The insert was coated overnight at room temperature (RT) with 100 µl matrigel (50 ng/ml in PBS; Sigma). For HUVEC invasion, the bottom compartment was coated with 1% gelatin. Cells (50000/insert) were seeded in medium with 1% FCS and 1% HS without ECGF. In the bottom compartment, the CM diluted in HUVEC-medium with 1% FCS and 1% HS was added. HUVECs were allowed to invade for 8 h. Thirty min before analysis, 5 µM calcein-AM was added to the lower compartment and fluorescently labelled cells were counted. The level of invasion was set relative to the positive control and normalized to the negative control. Cancer cells (200000/insert) were

seeded in serum free medium. In the bottom compartment, medium with 10% serum was added as chemoattractant. Cells were allowed to invade for 18 h. Thirty minutes before analysis, 5 µM calcein-AM was added to the lower compartment and fluorescently labelled cells were counted in the lower compartment.

Endothelial cells proliferation

At 24 h after seeding HUVECs (100 000 cells/T25 flask (Greiner Bio-One), cells were exposed to CM diluted in HUVEC medium containing 5% FCS and 5% HS, a concentration at which the HUVECs did not proliferate. Cell numbers were counted after 3 and 6 days exposure using a counting chamber (Bürker-Türk, Paul Marienfeld GmbH & Co. KG, Germany).

Western blotting

Cells were exposed to various conditions, as indicated. After exposure, cells were washed twice with ice-cold PBS and lysed in lysis buffer (Cell Signalling Technology Inc.). Cell lysates were scraped, transferred into a vial and centrifuged at 11 000g at 4ºC for 10 min. Supernatants were transferred to a new vial and protein amounts were determined by the Bio-Rad assay, according to the manufacturer's instruction (Bio-Rad Laboratories, Veenendaal, the Netherlands). From each condition 30 µg of protein was separated on a 8-12% SDS-PAGE and electroblotted onto polyvinylidenedifluoride (PVDF) membranes (Millipore Immobilon^{TM} –FL PVDF, 0.45 µm). Subsequently, the membranes were blocked for 1 h at room temperature (RT) in Odyssey blocking buffer (Odyssey blocking buffer #927-40003, Westburg, Leusden, the Netherlands) and incubated overnight at 4°C with the primary antibodies (dilution 1:1000-10000 in Odyssey blocking buffer 1:1 diluted with PBS-T (PBS with 0.05% Tween-20). The membrane was washed 5 times in PBS-T and incubated with the secondary antibody (1:10000 goat-α-mouse-IRDye (800CW;#926-32210 and 680;#926-32220) or goat-α-rabbit-IRDye (800CW;926-32211 and 680;#926-32221), Westburg) for 1 h at RT in the dark. After incubation, the membrane was washed in PBS-T and followed by 5 min washing in PBS without Tween-20 to decrease the background signal. Subsequently, the bands were scanned using an Odyssey Infrared Imager (Westburg), 84 µm resolution, 0 mm offset and with high quality[31].

RT-PCR

Colo320, Colo320 TP1, RT112 and RT112/TP cells were cultured for 3 days, after which cells were collected and RNA was isolated using the Qiagen RNeasy kit (Qiagen Benelux B.V., Venlo, The Netherlands), according to the supplier's protocol. Possible genomic DNA

contamination was removed by on-column DNaseI treatment for 20 min at RT. Concentration and quality of the RNA was analysed on the NanoDrop ND-1000 (Nanodrop Technologies Inc., Wilmington, USA). A total amount of 100 ng total RNA was used for cDNA synthesis with the iScript cDNA synthesis kit (Bio-Rad) according to the supplier's protocol. Quantitative PCR was performed with the iCycler (Bio-Rad) in a total volume of 25 µl on 30 ng cDNA with the iQ SYBR Green Supermix (Bio-Rad) and 400 nM forward and reverse primer. Primers were synthesised by Eurogentec and targeted against, β-actin, β2 microglubulin, cyclophilin and HPRT (for which the targets were normalized to) and the angiogenic factors VEGF-A, bFGF, PLGF, TNF-α, and IL-8[32,33].

VEGF, IL-8 and TNF-a detection

VEGF secretion was determined using a Quantikine human VEGF immunoassay ELISA (# DVE00; R&D systems, Inc, Minneapolis, MN), IL-8 by a human IL-8 ELISA kit (Becton Dickinson) and TNF-α Sanquin PeliKine human TNF-α ELISA kit (Ref. M1920). The ELISAs were performed according to manufacturer's instructions. In brief, concentrated medium was examined undiluted or diluted 20x and 200x. Subsequently, VEGF, IL-8 and TNF-α concentrations were determined and calculated in relation to the calibration curve.

Statistical analysis

For calculating significant differences between the parental and the transfected cells or between treated and untreated samples, the two-tailed paired Student's t-test was used. The values were considered significantly different when $p<0.05$.

RESULTS

The role of TP in cancer cell invasion

In order to determine whether TP expression in cancer cells result in an increased aggressiveness, Colo320, RT112 cells and their TP-transfected variants were examined on their invasion capacity. Colo320 and Colo320 TP1 cells hardly invaded, while RT112 and RT112/TP cells had a high invasion capacity (Figure 1A). Colo320 and RT112 had no TP activity, while their transfected variants had comparable TP activity (Figure 1B). There was no relation between the level of intrinsic TP expression and level of invasion. Furthermore, the invasive potential was not increased by stimulating TP activity with TdR or by the main secreted product of this reaction dR (Figure 1A). The TP inhibitor, TPI, did not affect the invasion of these cancer cell lines. Taken together, these data suggest that intrinsic TP

expression in RT112/TP and Colo320 TP1 cancer cells does not affect their invasive characteristic.

Expression levels of phospho-FAK and phospho-p70/S6k were lower in TP expressing cells

To determine whether differences in invasion between the two cell types was related to a different expression level of the FAK-Akt-p70/S6k pathway, the expression and phosphorylation levels were determined by Western blotting (Figure 1C). All cell lines constitutively expressed FAK and Akt and p70/S6k. Phosphorylation levels of FAK and Akt were higher in RT112 and RT112/TP, which might underlie to their higher invasion capacity. Interestingly, compared to the parental cell lines, phosphorylated-FAK and phosphorylated-p70/S6k were observed to be lower in the TP-transfected variants, although the invasion levels were not different between the cell lines.

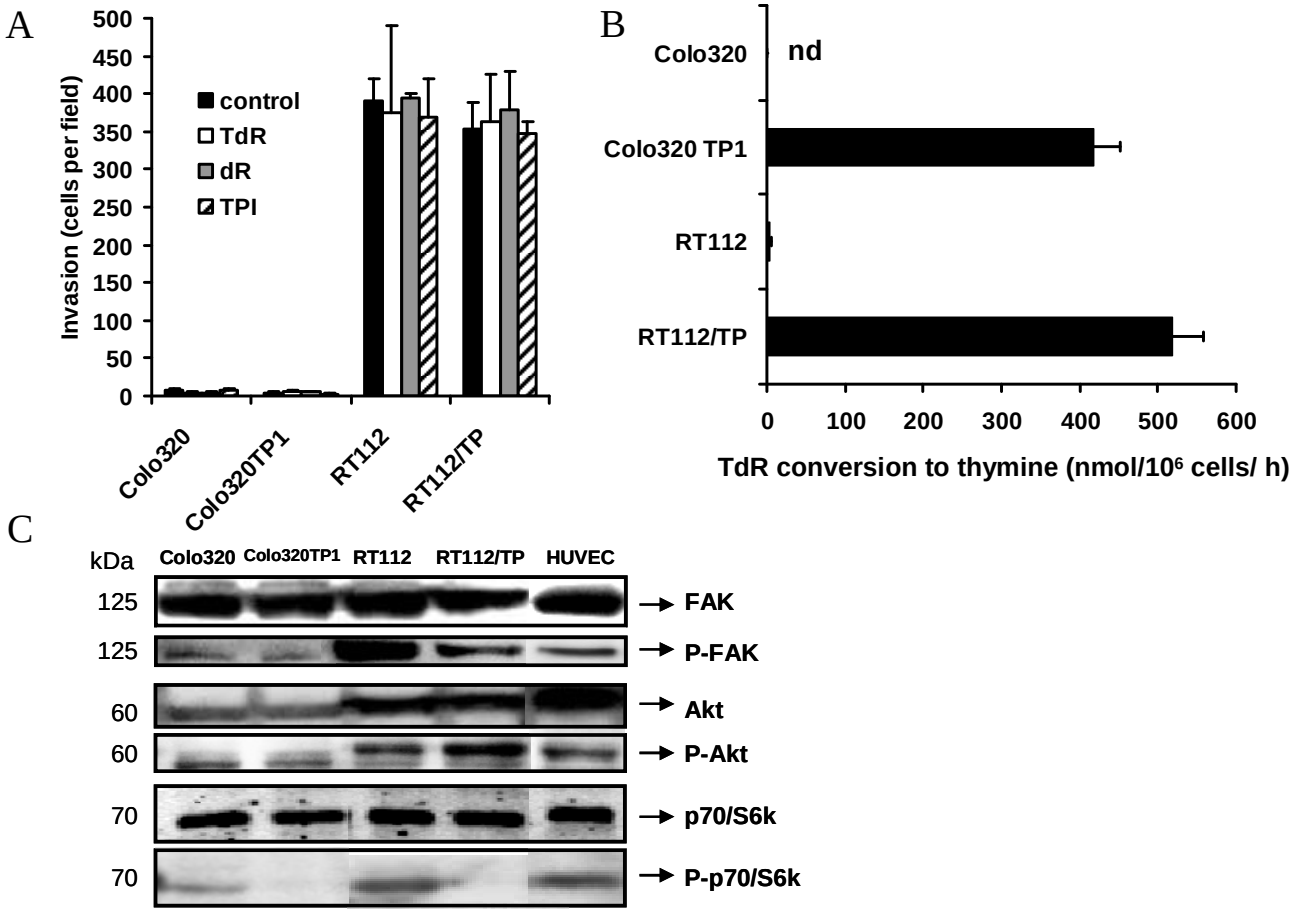

Figure 1 - The role of TP in cancer cells. A. Invasion capacity of the various cell lines after 24 h of invasion through an extracellular matrix (50 ng/ml matrigel) using the transwell invasion assay. Values are means of at least 3 independent experiments ± SEM. B. TP activity of the various cancer cells. Colo320 had no detectable TP activity. Values are means of at least 3 independent experiments ± SEM. C. Western blot of cancer cell lines expression of the kinases (phosphorylated) FAK, Akt and p70/S6k in Colo320, Colo320 TP1, RT112, RT112/TP and in HUVEC cells.

CM from TP expressing cancer cells does not stimulate endothelial cell proliferation

In order to determine whether cancer cells with a high TP expression can stimulate proliferation, HUVECs were exposed to the CM of the cancer cells and cells were counted after 3 and 6 days of growth. Therefore we used a condition at which cell did not proliferate. However, at these used conditions (5% of both FCS and HS), the CM did not stimulate the proliferation of the HUVECs at all (data not shown). This indicates that cancer cells did not secrete products that directly stimulated HUVEC proliferation.

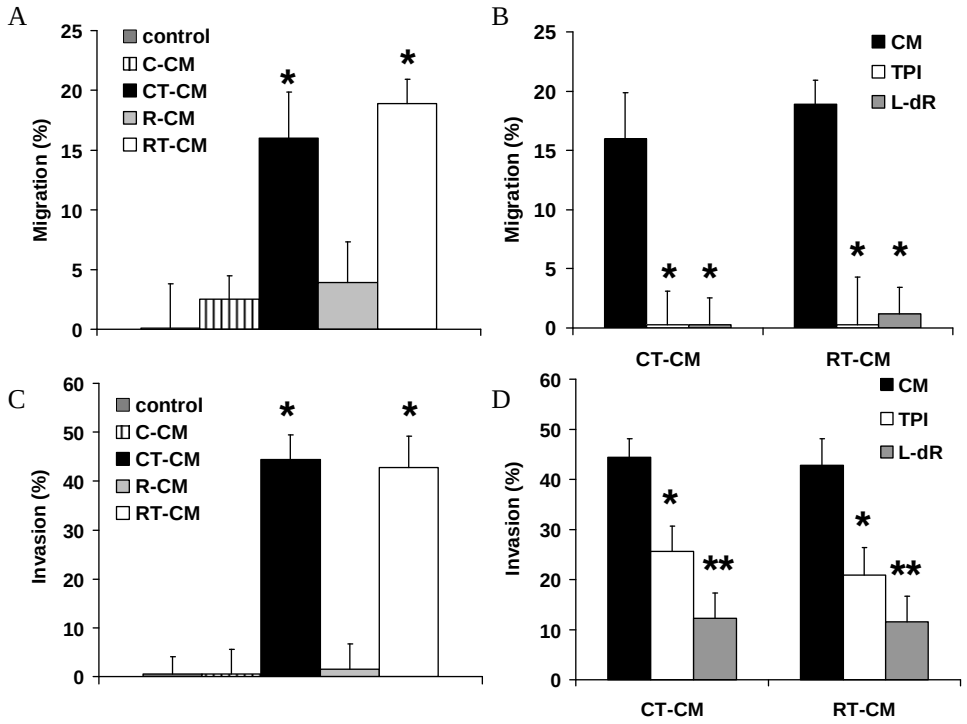

Figure 2 - Migration and invasion of HUVECs. **A.** Migration after 6 h stimulation with R-CM, R/TP-CM, C-CM or C/TP-CM. EC (endothelial cell) medium is the medium in which the concentrated medium was diluted 20x. Values represent means of at least 5 independent experiments ± SEM. Significant differences between the CM and EC medium are indicated in the graph *p<0.01. **B.** Migration after 6 h stimulation of HUVECs with the CM with and without TPI and L-dR. All values represent means of at least 3-5 independent experiments ± SEM. Significant differences between CM alone and CM plus inhibitor (*p<0.01) are indicated in the graph. **C.** Invasion stimulation after 8 h stimulation with R-CM, R/TP-CM, C-CM or C/TP-CM. EC medium is the medium in which the concentrated medium was diluted 20x. Values represent means of at least 5 independent experiments ± SEM. Significant differences between the CM and EC medium are indicated in the graph *p<0.005. **D.** Invasion after exposure of HUVECs with the CM with and without TPI and L-dR. All values represent means of at least 3-5 independent experiments ± SEM. Significant differences between CM alone and CM plus inhibitor (* p<0.05, ** p<0.005) are indicated in the graph.

CM from cancer cells with a high TP expression can stimulate endothelial cell migration

In order to determine whether the CM derived from cancer cells with a high TP expression could stimulate the migration of the HUVECs, the wound healing assay was performed. We used experimental conditions comparable to the proliferation assay, since under these conditions the cells were viable but do not proliferate. The CM derived from TP expressing cells (RT-CM and CT-CM) stimulated the migration by about 15-20%, respectively. The CM derived from non-TP expressing cells (C-CM and R-CM) did not significantly increase the migration of the HUVECs (Figure 2A). Extracellular addition of TPI and L-dR to RT-CM and CT-CM reduced HUVEC migration back to control levels (Figure 2B), while these inhibitors did not affect the migration of the controls or the C-CM and R-CM (data not shown). This indicates that TP is involved in the induced migration.

CM from cancer cells with a high TP expression stimulate endothelial cell invasion

In order to determine whether the CM derived from cells with a high TP expression can increase the invasion capacity of the HUVECs, the transwell invasion assay was performed. In agreement with the wound healing assay, the CM derived from cells with a high TP expression attracted endothelial cells by about 40 to 45% (Figure 2C). The CM from cells with no TP expression did not significantly increase the invasion of HUVECs (Figure 2C). TPI reduced the invasion that was induced by RT-CM and CT-CM by almost 50% (Figure 2D). L-dR inhibited the RT-CM and CT-CM induced invasion by almost 90%. TPI and L-dR did not inhibit or stimulate the invasion of the controls (data not shown).

Activation of p70/S6k expression levels of endothelial cells

The differences in migration and invasion between HUVECs stimulated by the CM from TP expressing cells and non-TP expressing cells may be related to the activation of different intracellular signaling pathways. In order to study this, a Western blot was performed determining expression levels of (phosphorylated) FAK, Akt and p70/S6k. These kinases were previously reported to be involved in TP mediated cell migration and invasion[17,20] (Figure 3A). Upon stimulation of endothelial cells with the CM for 6 h, the p70/S6k signaling pathway was highly activated, compared to the negative control. However, p-p70/S6k was less phosphorylated after exposing cells to the RT-CM. Phosphorylated levels of mTOR and FAK were not changed at all. This indicates that these signaling molecules were probably not involved in the migration or invasion induced by the CM derived from TP expressing cells.

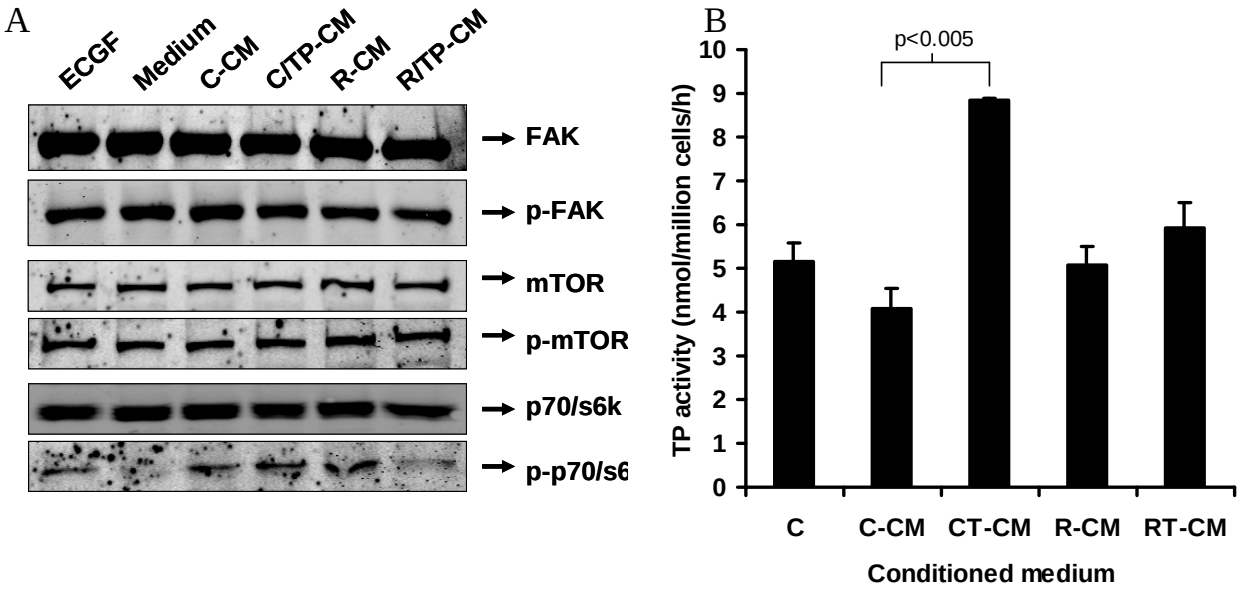

Figure 3 - A. Western blot of (phosphorylated) kinases after 6 h stimulation of HUVECs with the CM. As a positive control, complete medium with ECGF was used (ECGF). As negative control (medium), medium in which the concentrated medium was diluted was used (1% FCS and 1% HS). B. HUVEC TP enzymatic activity after stimulation with the various indicated CM. All values represent means of at least 3 independent experiments ± SEM. Significant differences between CM and control (C) medium are indicated in the graph.

TP enzymatic activity in HUVECs was increased by the CM from Colo320 TP1 cells

In order to determine whether the effect of the CM was related to an induction of TP expression of the HUVECs, TP enzymatic activity was determined. The C-CM and R-CM did not increase TP activity. CT-CM increased the TP activity of the HUVECs, compared to the C-CM (Figure 3B). However, RT-CM only slightly increased the TP activity of the HUVECs. The increase in invasion and migration induced by the CT-CM is possibly related to the increased TP expression of the HUVECs, and be an explanation why TPI (partially) inhibited the migration and invasion capacity of these cells.

Increased expression of IL-8, TNF-α and bFGF in TP expressing cells

The influence of the TP expressing cells on the increased migration and invasion of the HUVECs can be related to an increased expression and secretion of angiogenic factors. Therefore, we examined the mRNA expression levels of various important angiogenic factors in Colo320, Colo320 TP1, RT112 and RT112/TP cells (Figure 4A)[32]. The most

prominent difference between RT112 and RT112/TP cells was the expression of IL-8, which was much higher in RT112/TP cells (Figure 4B). However, in both Colo320 and Colo320 TP1 cells, IL-8 was not expressed. Other factors that were increased include TNF-α, and bFGF. The differences in mRNA expression levels between Colo320 and Colo320 TP1 cells were less compared to the differences in RT112 and RT112/TP cells. VEGF was not differentially expressed between the parental and transfected cell lines. TGF-α, which was expressed to a lower extent in Colo320 TP1 compared to Colo320 cells, and was higher expressed in RT112/TP than in RT112 cells. Taken together, various angiogenic factors were differentially expressed in TP expressing cells, compared to non-TP expressing cells, indicating a role for TP in modulating the expression of these angiogenic factors. The expression of these angiogenic factors were cell type dependent, indicating that TP is not the only factor that is involved.

In order to examine whether angiogenic factors were secreted, we selected VEGF, IL-8 and TNFα for further investigation (Figure 4C), since they are often implicated in stimulation of angiogenesis. VEGF was secreted at higher levels in C-CM than in CT-CM. R-CM had higher levels of VEGF than the C-CM, but the levels were not different in the transfected variant. In RT-CM the IL-8 levels were much higher, compared to the R-CM, which is in agreement with the mRNA expression levels (Figure 4C). In both C-CM and CT-CM, IL-8 was not detectable. TNF-α was hardly detectable in the conditioned medium nor in the medium with ECGF (data not shown).

Inhibition of IL-8, bFGF, TNF-α and VEGF reduced the migration and invasion

In order to determine whether TNF-α, VEGF, IL-8 and bFGF were related to the increased migration and invasion of the HUVECs, blocking antibodies were used that specifically inhibit their angiogenic modulation (Figure 4D). The induced migration by RT-CM was completely inhibited by blocking TNF-α, VEGF, IL-8 and bFGF. The induced migration and invasion by CT-CM were completely blocked by antibodies against TNF-α, VEGF and bFGF, but not by that against IL-8. This is in agreement with the lack of IL-8 mRNA expression and absence of protein secretion. Taken together, several angiogenic factors were involved in the TP-mediated modulation of the migration and invasion of HUVECs. The blocking antibodies against VEGF, IL-8 and bFGF inhibited the migration and invasion of R-CM to some extent, although to a lesser extent than that of RT-CM (data not shown). C-CM induced migration and invasion was not inhibited by any of the tested blocking antibodies. This indicates that IL-8, bFGF and possibly TNF-α and VEGF were involved in TP induced migration and invasion.

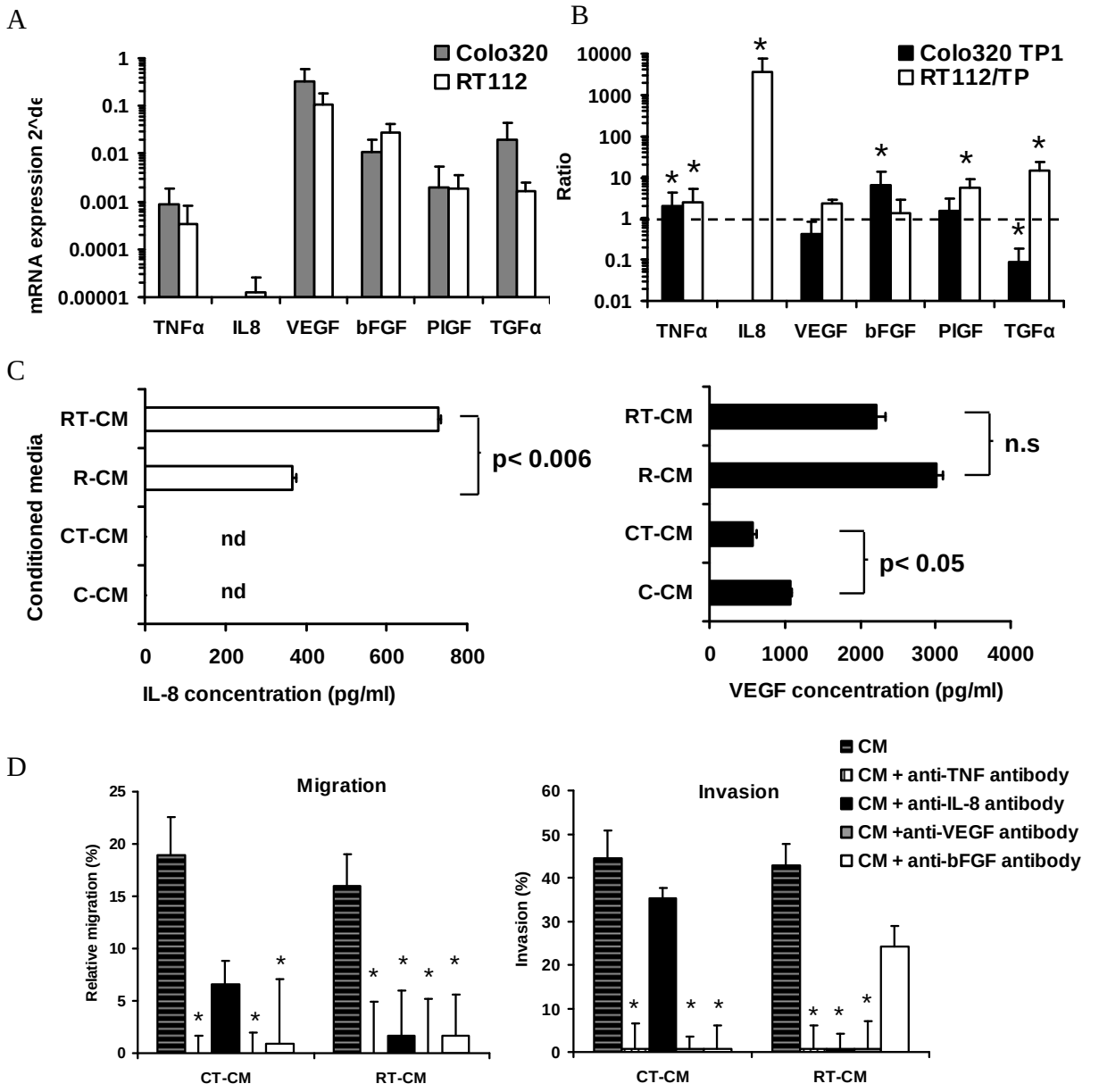

Figure 4 - Effect of TP expression of angiogenic factors and their effect on migration and invasion. A. mRNA expression levels of angiogenic factors in cancer cells by RT-PCR. Values represent means ± SEM. B. mRNA expression ratios between parental and TP transfected cell lines. Values represent means ± SEM. Significant differences between parental and TP transfected cells are indicated in the graph *p<0.05. C. IL-8 and VEGF concentration in the conditioned medium as determined by ELISA. Values represent means of at least 3 independent experiments ± SEM. Significant differences between the CM derived from TP transfected cells and the parental cells are indicated in the graph. D. Inhibition of migration and invasion by blocking antibodies of the differentially expressed angiogenic factors. As a control, IgG antibodies were used, which did not inhibit the migration or invasion at all. Values represent means of at least 5 independent experiments ± SEM. Significant differences are indicated in the graph *p<0.05.

DISCUSSION

The present data show that conditioned medium derived from cells with a high TP expression, stimulate the migratory and invasive capacity of endothelial cells, by activating two different mechanisms. The increased TP expression in cancer cells stimulate the secretion of several angiogenic factors and also Colo320 TP1 medium increased the TP activity in HUVECs. The secretion of angiogenic factors was different between the two cell types. Since TPI inhibited both endothelial cell migration and invasion, the observed effects were related to the increased TP activity. On the other hand, in cancer cells, a high TP expression (cancer cells transfected with the TP gene) was not related to an increased invasion of the cells themselves, indicating that the invasive potential in our cell lines was not related to TP expression.

Limited studies related TP to angiogenesis[1,2]. From these studies, it was concluded that TP expression and activity was clearly related to the presence of angiogenesis. However, the mechanism behind angiogenesis that is induced by TP expressing cells is not completely understood. In order to study the angiogenic role of TP, exogenous TP or dR have been used[17,20,23]. However, other factors may also be involved[23]. Therefore, we used conditioned medium from TP expressing cells to determine whether tumor cells secrete pro-angiogenic molecules. TP expressing cells indeed secreted angiogenic factors (IL-8, bFGF) that stimulated endothelial cell migration and invasion, but not proliferation. The secretion of the angiogenic factors VEGF and IL-8 has been demonstrated before in RT112/TP cells by Brown *et al.*[23]. Our data are in agreement with their data, indicating that IL-8 plays a role in TP mediated migration and invasion in this cell line. However, we did not find an increased VEGF secretion by RT112/TP cells, possibly due to the low glucose levels that Brown *et al.* used in their study. The limited or even absent effect of the secreted VEGF in stimulating migration and invasion in our study is in agreement with observations from Hotchkiss, who reported that TP and VEGF are independent expressors[17]. In addition to IL-8, we demonstrate a possible role of bFGF and TNF-α in TP mediated stimulation of migration and invasion. bFGF and TNF-α are well documented angiogenic growth factors that stimulate endothelial cell migration[34,35,36,37]. Many angiogenic factors enhance each others effect to stimulate angiogenesis[37,38]. Inhibition of one of these factors can result in a complete inhibition of the angiogenic effects. Possibly a combination of these angiogenic factors could be responsible for the angiogenic effect of TP, since inhibition of one of these factors resulted in a complete inhibition of migration and invasion.

To study the role of TP in angiogenesis, L-dR is often used[39], including other inhibitors of TP[40,41]. Enzymatic inhibition of TP inhibited the induction of angiogenesis, indicating that

the enzymatic activity of TP in indispensable for these effects. L-dR inhibited the migration and invasion of endothelial cells[42], which indicates that dR produced by the cells can be one of the key factors in angiogenesis. Moreover, L-dR inhibited the formation of focal contacts between endothelial cells[17]. In the present study, we demonstrate that Colo320TP1 cancer cells increased the TP expression of HUVECs, which might increase their angiogenic potential. The inhibitors TPI and L-dR reversed these effects, indicating that TP and dR play a role. The development of TP inhibitors such as TPI provides potential for inhibiting migration and invasion of endothelial cells, and can be combined with various anti-cancer agents.

The molecular pathway that is activated upon stimulation of TP can be different between endothelial and cancer cells. In endothelial cells, exogenously added TP and dR activate integrins, subsequent downstream signaling pathways involving FAK and p70/S6k and the migration and invasion of endothelial cells[17,20]. FAK is a non-receptor tyrosine kinase that plays a role in cell migration and cell death[21]. However, conditioned medium only activated p70S6k, but not FAK, while the effects were not different between TP expressing cells and the non-TP expressing cells. Therefore, this molecular pathway may not be involved in TP dependent migration and invasion. Apparently, exogenous TP or dR inflicts other cellular responses than cells with intrinsically high TP expression or stimulating endothelial cells with conditioned medium from TP expressing cells.

TP expression in cancer cells might also influence their aggressiveness[26,43]. Our data show that intrinsic TP expression of Colo320, Colo320TP1, RT112 and RT112/TP cancer cells did not increase the invasiveness of caner cells themselves. Yu *et al.*[15] reported that gastric cancer cells had a higher invasive potential and activated FAK and p70/S6k phosphorylation levels. The difference between their data and our data is possibly related to the different tumor types. Other cell types may respond different to their TP expression, possibly due to different genetic profile[1,2]. Possibly, TP is not a key factor in the increased invasive potential of colorectal and bladder cancer cells, but other factors need to be differentially expressed as well. Future studies should investigate these co-factors important for the invasive potential of cancer cells with a high TP expression.

In conclusion, TP can stimulate endothelial cell migration and invasion, possibly by inducing the secretion of a combination of the angiogenic factors IL-8, bFGF and TNF-α and increasing endothelial cell TP activity. This underlines the important potential for developing TP inhibitors as anti-angiogenesis therapy. In addition, many anti-cancer agents can increase the TP expression of cancer cells. Therefore, combining TP inhibitors with these anticancer agents can have potential for targeting cancer dually.

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