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Thymidine phosphorylase induces angiogenesis *in vivo* and *in vitro*: an evaluation of possible mechanisms

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1 Thymidine phosphorylase (TP) is elevated in the plasma of cancer patients, and has been implicated in pathophysiological angiogenesis. However, the downstream signals underlying this implication remain obscure. The purpose of the present study was to examine the effects of TP on the neovascularisation response *in vitro* and *in vivo*.

2 Both TP and its catalytic product, 2-deoxy-D-ribose-1-phosphate, and downstream 2-deoxy-D-ribose (2-DDR) promoted endothelial tubulogenesis *in vitro*, and the regeneration of a wounded monolayer of endothelial cells without exerting any mitogenic effect. *In vivo*, both TP and 2-DDR promoted the development of functional vasculature into an avascular sponge. A TP inhibitor, 6-amino-5-chlorouracil, was able to partially reverse the effects of TP, but had no effect on the 2-DDR-induced angiogenesis.

3 Enhanced monolayer regeneration was observed with TP-cDNA-transfected bladder carcinoma cells. The transfection of TP-cDNA, however, did not confer any proliferative advantage. The regeneration of TP overexpressing cells was associated with a time-dependent expression of the enzyme haeme-oxygenase (HO-1).

4 The present study demonstrates that both TP and its ribose-sugar metabolites induce angiogenesis by mediating a cohesive interplay between carcinoma and endothelial cells. The induction of HO-1 in TP-transfected cells suggests that it could be a possible downstream signal for the angiogenic effects of TP. Furthermore, reducing sugars have been shown to induce oxidative stress, and ribose could be a possible cause for the upregulation of HO-1, which has been implicated in the release of angiogenic factors. Therefore, we postulate that 2-DDR could be mediating the angiogenic effects of TP possibly through an oxidative stress mechanism and additionally getting integrated in the endothelial metabolic machinery.

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Abbreviations: TP, thymidine phosphorylase; 2-DDR, 2-deoxy-D-ribose; 2-DLR, 2-deoxy-L-ribose; HO1, haemeoxygenase-1; TP inhibitor, 6-amino-5-chlorouracil; VEGF, vascular endothelial growth factor; CO, carbon monoxide; EBM, endothelial basal medium; EGM, endothelial growth medium

Introduction

Angiogenesis or neovascularisation is finely choreographed through the interplay of the endothelial cells, angiogenic mediators, cytokines, growth factors and adhesion molecules, and can be said to involve three key events, namely migration, proliferation and maturation of the endothelial cells (Griffioen & Molema, 2000). Angiogenesis is of fundamental importance in several physiological processes including embryogenesis, wound healing and the menstrual cycle. It is however also implicated in certain pathological conditions, such as atherosclerosis, diabetic retinopathy, psoriasis and tumour growth (Griffioen & Molema, 2000).

Thymidine phosphorylase (TP), which is identical to platelet-derived endothelial cell growth factor (Furukawa *et al*., 1992), is an enzyme involved in nucleic acid homeostasis. The level of TP, a 47 kDa subunit homodimer, has been found to be elevated in the plasma of cancer patients (Pauly *et al*., 1977). High TP immunoreactivity has been detected in nonsmall cell lung cancer (O'Byrne *et al*., 2000) and human colorectal cancer (Matsuura *et al*., 1999), both of which showed increased vascular density. TP levels have also been shown to vary during the course of the menstrual cycle (Zhang *et al*., 1997), and in the edges of a gastric ulcer (Kasugai *et al*., 1997). We have recently implicated TP in atherosclerosis, in which macrophages, foam cells and giant cells from aortic and coronary plaques were shown to be immunoreactive for TP, and a correlation was established between the severity of stenosis and plaque microvascular density (Boyle *et al*., 2000). Furthermore, we demonstrated that TP could induce an angiogenic response, and the migration of endothelial cells (Moghaddam *et al*., 1995). Xenografts of MCF-7 cells transfected with TP-cDNA were found to increase the vascular density and also the tumour growth rate *in vivo* (Moghaddam

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et al., 1995). Similarly, Ha-ras-transformed 3T3 cells transfected with TP cDNA formed tumours with an increased vascular density (Ishikawa *et al*., 1989).

Despite the vast amount of data that implicates TP in pathophysiological angiogenesis, the downstream signals and the molecular mechanisms underlying this process remain obscure. In contrast to other angiogenic factors that are secreted to act directly on endothelial cells, TP lacks a signal sequence. This means that TP is not secreted from the carcinoma cells, and therefore should act indirectly on the endothelial cells. Recently, it has been shown that the enzymatic activity of TP is essential for its angiogenic property (Miyadera *et al*., 1995), suggesting that the reaction products could be angiogenic in nature. TP catalyses the reaction:

$$\text{Thymidine} + \text{phosphate} \rightleftharpoons \text{thymine} + \text{2-deoxy-D-ribose 1} - \text{phosphate}$$

The current study was designed to evaluate the effects of TP and the enzymatic products on neovascularisation responses *in vivo* and *in vitro.* Furthermore, we also evaluated the effect of a TP inhibitor, 6-amino-5-chlorouracil. In addition, using human carcinoma cells transfected to overexpress human TP, we have hypothesised a possible mechanism, by which TP may promote neovascularisation in a pathological setting.

Methods

Materials

Human recombinant-thymidine phosphorylase or platelet-derived endothelial cell growth factor (rh PD-ECGF) and human recombinant vascular endothelial growth factor (VEGF) were procured from R&D Systems, USA. A TP inhibitor, 6-amino-5-chlorouracil, was a gift from Dr R. Bicknell, ICRF, Oxford, U.K. while 2-deoxy-D-ribose (2-DDR) and 2-deoxy-L-ribose (2-DLR), 2-deoxy-D-ribose-L-phosphate (2-DDR-1-P), thymine, M199 medium, endothelial cell growth – supplement (ECGS), L-glutamine, heparin, penicillin – streptomycin and amphotericin-B were all procured from Sigma Chemical Co., U.K. Endothelial basal medium and Bullet kit™ were obtained from Clonetics (U.K.). Radioactive ^{133}Xe was obtained from Dupont Pharma, Belgium.

Endothelial cell culture and 'wounding'

Human umbilical vein endothelial cells (HUVECs) were pooled cells (usually from three donors) obtained from Clonetics (U.K.). A 24-well plate, with a Thermanox coverslip (Fisher Scientific, U.K.) in each well, was coated with 0.1% gelatin. Unless otherwise described, the cells were seeded at a density of 3×10^4 cells well^{-1} in M199 media supplemented with 20% FCS. When the cells reached confluence, the medium was refreshed with 1% serum and maintained for 24 h to synchronise the cells.

The confluent monolayer of synchronised cells was scraped with a multichannel wounder (Lauder *et al*., 1998), thereby causing 11 parallel lesions, each 400 μm wide, on the monolayer. Following the injury, coverslips were rinsed in phosphate buffer saline (PBS) to dislodge any cellular debris, and placed in a well containing the drug/peptide or vehicle in M199 medium supplemented with 5% FCS. In experiments involving the inhibitor of TP, the cells were pretreated with the inhibitor for 1 h prior to wounding of the monolayer. At 24 h postinjury, the experiments were stopped by washing the coverslips and fixing in absolute alcohol. Some of the coverslips were fixed immediately after wounding, and served as time zero (T_0) controls.

Quantification of the regeneration of 'wounded' area

Recovery of the denuded area was quantified using a Leica Q500, semiautomated, computerised image analysis system. Images were grabbed using a Nikon Diaphot inverted microscope ($\times 10$) coupled to a JVC CCD. For each coverslip, four fields of view were selected at random. The lesion area of each field of view was measured, and by use of the data for time zero (T_0), the lesion area was converted to give the percentage of regeneration relative to T_0 values.

Cell proliferation assay

The monolayer of endothelial cells were 'wounded' as above and cultured in 5% FCS with vehicle or peptides/drugs. After 24 h of incubation, they were washed in ice-cold PBS, trypsinised and counted with a haemocytometer using the trypan blue exclusion method.

Tube formation assay

An AngioKit™ coculture system (TCS CellWorks Ltd, U.K.) was used to study the tube formation by endothelial cells. The AngioKit was seeded with cells on day 0, and the optimised growth medium was changed on days 3, 5, 7, 10 and 12, as per the manufacturer's instructions. It was then fixed and stained for CD31 (TCS CellWorks, U.K.) on day 14. Suramin (20 μM) and VEGF (2 ng ml^{-1}) were used as negative and positive controls, respectively. Thymine and 2-deoxy-D-ribose-1-phosphate were added on day 4. The appropriate treatments were replenished with each medium change.

Comparison of venule length was conducted using the 'AngioSys' image analysis system (TCS Cellworks). Four images were grabbed per well, from the four quadrants. Experiments were run in quadruplicates, and data were expressed as the mean $\pm$ s.e.m.

Mouse sponge angiogenesis model

Male balb/c mice (20 – 22 g in body weight; Tucks, U.K.) were acclimatised to standard laboratory conditions for 7 days prior to induction in the experiment. On day 0, the animals were anaesthetised with 4% and maintained on 2% isoflurane in a mixture of oxygen (0.8 l min^{-1}) and nitrous oxide (0.6 l min^{-1}). The hair of the dorsum was removed using a hair clipper, and the area was sterilised with 70% alcohol. An incision was made 0.5 cm caudal from the base of the tail, and two bilateral subcutaneous air pockets were created up to the dorsal subscapular region. Two sterile polyether polyurethane sponges (Acer Associates, Bucks, U.K.) (170 mm^3) were inserted into the pockets, localised on the dorsal subscapular region, and the incision was closed with 5/0 Mersilk (Ethicon Ltd, U.K.). The treatment with vehicle or drugs was started 24 h after implantation of the sponges and continued for 10

days. The maximum volume of injection was kept a constant 30 μl, and was administered directly into the sponge using a 30G Precision Glide needle (Becton Dickinson and Co., U.S.A.).

Assay for functional status of the neovasculature

On day 15, following implantation of the sponges, the animals were anaesthetised using a combination of fentanyl citrate – fluanisone (Hypnorm™, Janssen Pharmaceutica, Belgium) and midazolam (Hypnovel™, U.K.), diluted 1 : 1 : 20 in saline. Vascularisation was assessed as a function of blood flow through the implants by injecting ^{133}Xe-containing saline and measuring its clearance from the sponge over a 6-min period. Radioactivity was measured using a microprocessor scalar ratemeter (Nuclear Enterprise, U.K.), linked to a low-energy X-ray/γ-ray NaI-crystal with an Al-entrance window, on an HG-type mount coupled to an NE 5289C preamplifier.

For gross morphological evaluation of the neovascular response, the animals were killed by cervical dislocation and the dorsal skin flap was dissected and everted. Images were captured using a Nikon camera with a macro-lens. Angiogenesis was quantified as the in-growth of vessels into the granuloma tissue, measured in a semiblind manner. All *in vivo* procedures conformed to U.K. Home Office guidelines for handling of animals.

Wounding experiments with human TP cDNA-transfected cells

The cell lines EV11 and 2T10 were transfectants of the human bladder carcinoma cell line, RT112, generated according to the method described by Brown *et al*. (2000). Briefly, the human bladder carcinoma cell line RT112 was transfected with two pBABE vectors (one empty and the other containing full-length cDNA for human TP) incorporated into retroviral particles. EV11 contained the empty vector clone, while the 2T10 cells were transfected to overexpress human TP. Both the cell lines were grown in DMEM, with 10% FCS and glutamine, and selection pressure maintained using 1 μg ml^{-1} puromycin. Experiments were performed in the absence of puromycin.

For the wounding experiments, the cells were grown to confluence on Thermanox coverslips, and mechanically wounded as described earlier. The total recovery of the monolayer at 24 h postinjury was quantified using the protocol described earlier.

In simultaneously run experiments, the cells were harvested by trypsinisation at identical time points, and counted using trypan blue exclusion with a haemocytometer.

Western analysis for haemeoxygenase-1 (HO-1)

Cell monolayers, before and at different points after wounding, were washed in ice-cold PBS before applying SDS – PAGE sample buffer (50 μl of 3 × strength; 1 × sample buffer: 4% (w v^{-1}) sodium dodecyl sulphate, 5% (v v^{-1}) glycerol, 60 mM Tris and 0.01% (w v^{-1}) bromophenol blue; pH 6.8) to each test well under reducing conditions (50 mM 2-mercaptoethanol). Following dissolution of cellular protein by rapid mixing, the well contents were transferred to a separate tube and combined with two further washings of the well with deionised water (50 μl). Samples were vortexed, centrifuged at 10,000 × g for 2 min and heated at 95°C for 5 min. Equivalent amounts of protein per sample were electrophoretically resolved on 10% polyacrylamide gels. Following electrophoretic transfer onto nitrocellulose (0.22 μm), the membrane was washed briefly in Tris-buffered saline and saturated overnight in TBS supplemented with 0.1% (v v^{-1}) Tween 20 and 5% (w v^{-1}) dried milk. All primary incubations were for 1 h at 22°C in TBS containing 0.1% (v v^{-1}) Tween 20 (TBST) followed by washing five times for 10 min each in TBST. The antibody directed to HO-1 (used at a 1 : 200 dilution) was obtained from Transduction Laboratories, U.S.A. A monoclonal antibody against constitutive $Gq_{\alpha 11}$ (Sigma, U.K.) was used at a 1 : 800 dilution to assess equal protein loading. Membranes were incubated for 1 h at 22°C with a 1 : 2000 dilution of the appropriate horseradish peroxidase-conjugated secondary antibody in TBST containing 5% (w v^{-1}) dried milk. Excess antibody was removed by washing as above and immunocomplexes were visualised using enhanced chemiluminescence detection, according to the manufacturer's instructions (Amersham Life Science, U.K.).

Statistics

All data shown were calculated from a minimum of three independent experiments. Replicates were run in each experiment. Values are expressed as mean ± s.e.m. Statistical analysis was carried out using Student's t-test, or using one-way analysis of variance followed by Newman Keuls *post hoc* test (Graphpad Prism 3, U.S.A.). $P<0.05$ was fixed as the level of significance.

Results

Recovery response of a mechanically injured monolayer of HUVECs in the presence of TP

A confluent monolayer of synchronised human umbilical vein endothelial cells was injured using a multichannel wounder as described. The total area wounded at T_0 ranged between 50 and 54%. As shown in Figure 1, a basal regeneration of the wounded monolayer was observed in the presence of 5% serum. The addition of TP induced a concentration-dependent regeneration of the wounded monolayer of HUVECs, comparable to that observed following the addition of VEGF (Figure 1). A plateau phase, as seen in the TP concentration – effect curve, was not observed with VEGF over the concentration range investigated (Figure G).

Figure 1h, shows the effect of TP and $VEGF_{165}$ on the proliferation of HUVECs, following a mechanical injury to a confluent monolayer. As compared with the vehicle-treated control, TP did not increase the cell proliferation at any of the concentrations used. In contrast, $VEGF_{165}$ induced a dose-dependent increase in the proliferation of cells, as quantified by cell counts at the end of 24 h of incubation with the growth factor.

Tube formation assay

We studied the effects of the products, thymine and 2-DDR-1-P phosphate on the formation of endothelial tubes in a

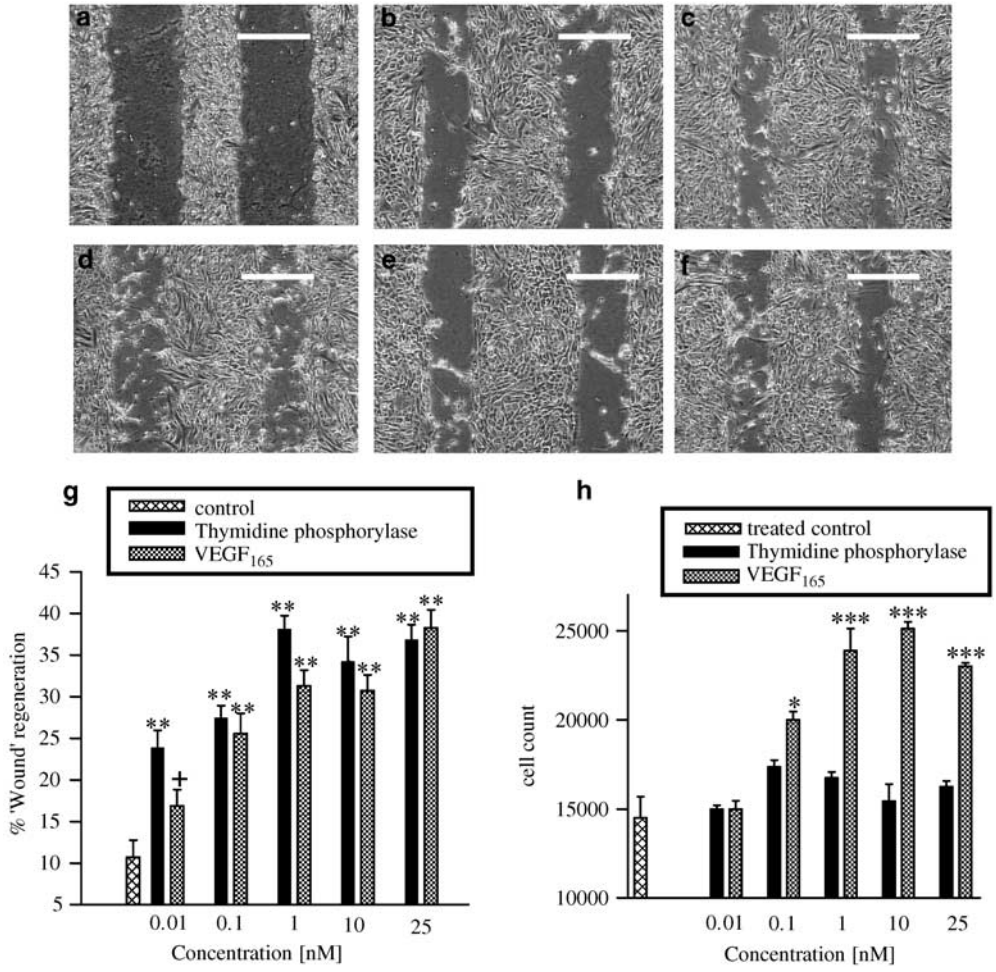

Figure 1 The effect of thymidine phosphorylase and $VEGF_{165}$ on HUVECs monolayer regeneration, following a mechanical injury. The top panel of phase contrast micrographs show: (a) the total denudation at the time of injury (T_0); (b) basal regrowth in the presence of vehicle (5% FCS); (c) regeneration in the presence of VEGF (1 nM); (d) regeneration in the presence of thymidine phosphorylase (TP) (1 nM); (e) preincubation for 1 h and the continuous presence of TP inhibitor, reverts the TP-induced regeneration to basal level; (f) TP inhibitor has no effect on the $VEGF_{165}$-induced recovery. Images were captured with a Nikon Diaphot inverted microscope at $\times 4$ objective, coupled to a CCD (JVC), and grabbed using a Q500 Leica software. The calibration bar represents 400 μm. The graphs show the concentration – effect curve of TP and $VEGF_{165}$, on (g) the recovery of a 'wounded' area, and (h) the proliferation of endothelial cell. A synchronised monolayer of HUVECs was injured using a multichannel wounder, producing 11 linear lesions, and transferred to fresh media supplemented with 5% FCS, with appropriate treatments. They were incubated for a further 24 h, following which, they were either fixed and image analysed, or trypsinised and counted using a haemocytometer. Data expressed are mean $\pm$ s.e.m. of at least four separate experiments with quadruplicate wells in each. In the wound recovery experiment, data are shown as percentage of T_0 values. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs vehicle-treated controls, $+P<0.05$ vs corresponding TP-treated group.

coculture setting. A defined mixture of HUVECs and fibroblasts were plated, and allowed to grow according to the manufacturer's instructions. As shown in Figure 2, VEGF induced a significant increase in the formation of capillary-like structures, while suramin inhibited it. Interestingly, thymine was found to significantly inhibit the formation of the endothelial capillaries at a concentration of 10^{-4} M. Interestingly, this inhibition was reversed by 2-DDR-1-P in a concentration-dependent manner. As shown in Figure 2g, 2-DDR-1-P induced a biphasic stimulation of tube formation in the presence of a lower concentration of thymine (10^{-5} M), and exhibited a linear concentration – effect curve when coadministered with a higher concentration.

Effect of 2-deoxy ribose on endothelial monolayer regeneration, and proliferation

The intracellular dephosphorylation of 2-DDR-1-P results in the formation and release of 2-DDR. We therefore looked at the effects of 2-DDR on endothelial cells following the multichannel injury to a confluent monolayer. The levo-isomer, 2-DLR, was used as the negative control.

As shown in Figure 3, a greater recovery of the wounded area by the endothelial cells was observed in the presence of 2-DDR (10^{-5} M) as compared with vehicle-treated group. The treatment with 2-DDR induced a concentration-dependent regeneration of the wound, with a peak effect observed between 10^{-5} and 10^{-4} M. A positive trend of wound-

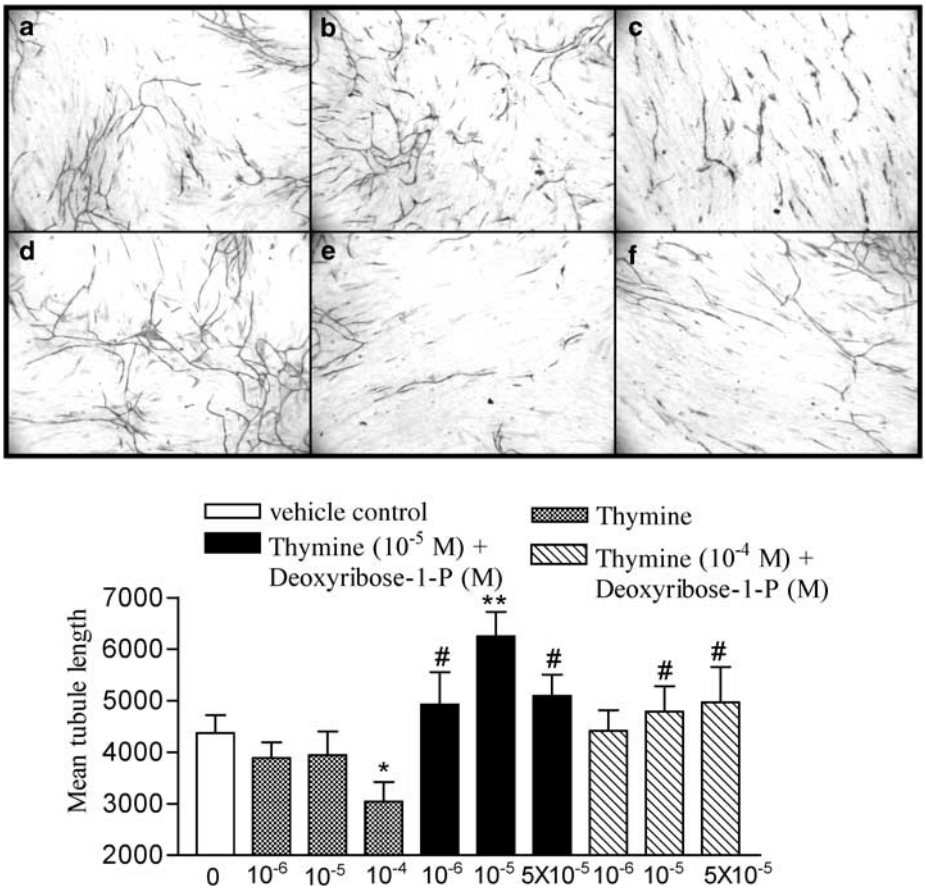

Figure 2 Effect of 2-deoxy-D-ribose-1-phosphate (2-DDR-1-P) and thymine on tube formation by endothelial cells in a coculture system. Photomicrographs depict: (a) vehicle-treated cells, (b) VEGF-induced tubulogenesis, (c) suramin-induced inhibition of tubulogenesis, (d) tube formation induced by 2-DDR-1-P (10^{-6} M), (e) inhibition of tubulogenesis by thymine (10^{-4} M), and (f) reversal of thymine-inhibition by 2-DDR-1-P (10^{-4} M). A coculture of endothelial cells and fibroblasts (Angiokit) was used to study the tube formation by endothelial cells. The AngioKit was seeded with cells on day 0, and the optimised growth medium was changed on days 3, 5, 7, 10 and 12. It was then fixed, and stained for CD31, on day 14. Suramin (20 μM) and VEGF (2 ng ml^{-1}) were used as negative and positive controls, respectively. Thymine and 2-DDR-1-P were added on day 4. The appropriate treatments were replenished with each medium change. Graph (g) shows the comparison of venule length following different treatments, as measured using a 'AngioSys' image analysis system. Four images were grabbed per well, from the four quadrants. Experiments were run in quadruplicate, and data were expressed as the mean $\pm$ s.e.m. $^*P<0.05$, $^{**}P<0.01$ vs vehicle control; $\#P<0.05$ vs matched thymine concentration.

recovery by endothelial cells was also observed following treatment with 2-DLR, with a peak effect at 10^{-4} M. However, the total wound recovery was significantly higher in the 2-DDR-treated cells as compared with equimolar 2-DLR treatment. Concentrations greater than 10^{-4} M of both sugars however had a supramaximal effect, resulting in a bell-shaped concentration – effect curve (Figure 3e).

Figure 3f shows the effects of increasing concentrations of 2-DDR and 2-DLR on cell proliferation. Neither 2-DDR nor 2-DLR induced any statistically significant increase in the number of cells as compared to basal treatment.

Effect of coadministration of TP and 2-DDR

Figure 4, shows the effect of coadministration of TP and 2-DDR on the recovery by a wounded layer of HUVECs. A submaximal concentration of 100 pM for TP was selected, and was combined with increasing concentrations ($10^{-7}-10^{-3}$ M) of 2-DDR or 2-DLR. A biphasic concentration – effect response was observed with 2-DDR alone. Coadministration of TP resulted in a leftward parallel shift of the 2-DDR concentration – effect curve. A similar effect was not observed in the group incubated with TP and 2-DLR.

Effect of coadministration of 2-DDR and 2-DLR.

Figure 5, shows the effects of 2-DDR, 2-DLR and mixture of the two optical isomers, in a 1 : 1 ratio, on the recovery of the injured monolayer of endothelial cells. 2-DDR and 2-DLR induced a concentration-dependent biphasic response as reported earlier. However, the mixture of optical isomers induced a response that was less than that induced by a equimolar concentration of 2-DDR but more than that of 2-DLR. Concentrations higher than 10^{-4} M induced loss of cells in all the treatment groups.

Effect of a TP inhibitor on the TP- and 2-DDR-induced regeneration of a monolayer of HUVECs

The cells were pretreated for 1 h with a TP inhibitor, 6-amino-5-chlorouracil, before the monolayer was wounded using

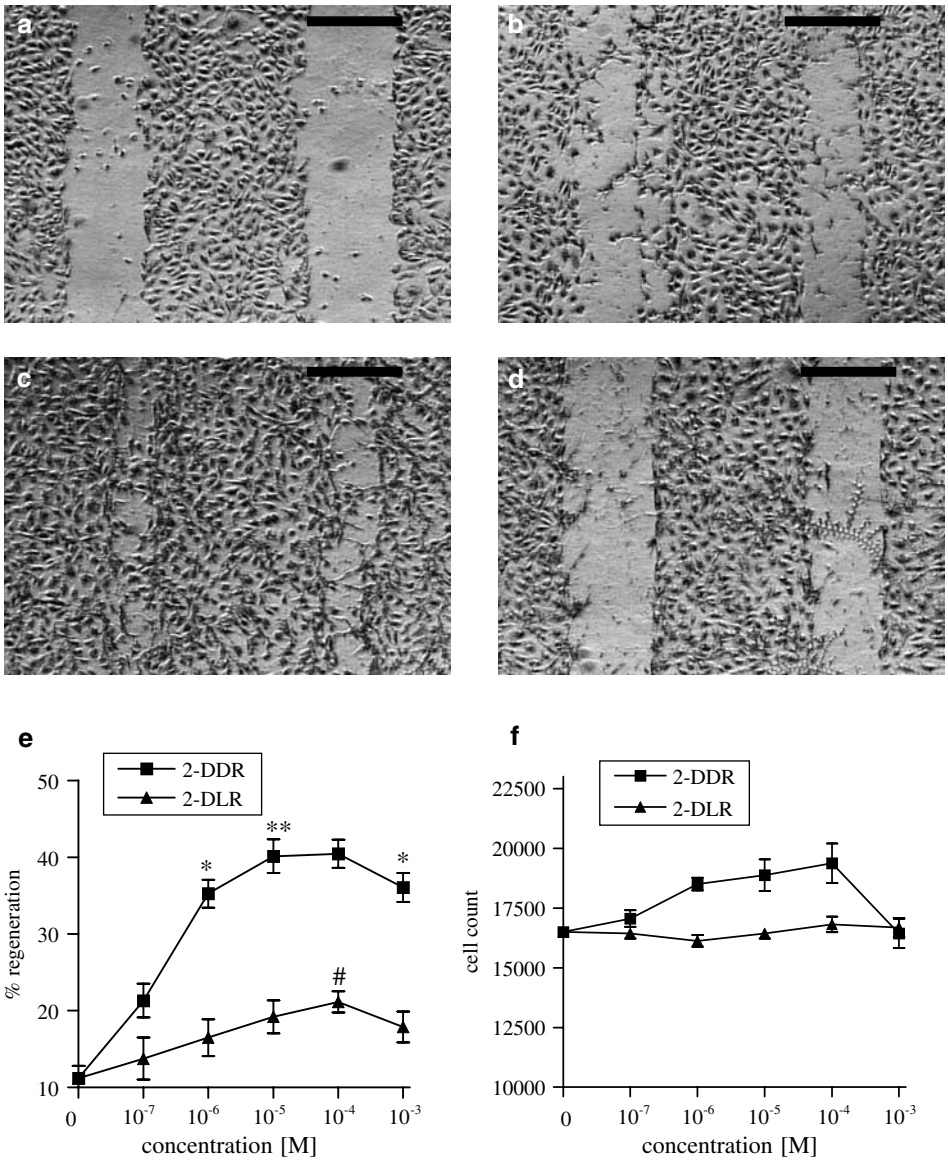

Figure 3 Effect of 2-deoxy-D-ribose and 2-deoxy-L-ribose on the proliferation of endothelial cell, and regeneration following a mechanical injury. Cells were grown and injured as described in Methods, and transferred to fresh media supplemented with 5% FCS, with and without the sugars. They were incubated for a further 24 h following which, they were either fixed and image analysed, or trypsinised and counted using a haemocytometer. Nomarsky images were captured using a Nikon Diaphot ($\times 4$ objective) as described in Methods. Photomicrographs depict (a) the total wounded area at T_0, and the recovery in (b) vehicle-treated cells, (c) 2-deoxy-D-ribose (10^{-5} M)-treated cells and (d) 2-deoxy-L-ribose (10^{-5} M)-treated cells, 24 h postinjury. The graphs show the concentration – effect curves depicting (e) wound recovery and (f) cell count, following treatment with increasing concentrations of the sugars. Data expressed are mean $\pm$ s.e.m. of at least four separate experiments with duplicate/quadruplicate wells in each. In the wound recovery experiment, data are shown as percentage of 0 h values. $^*P<0.001$, $\#P<0.05$ vs vehicle-treated control. The bar represents 400 μm.

the multichannel wounder. The drug was then maintained for the entire duration of the study. As shown in Figure 6a, the inhibitor of TP did not exert any inhibitory effect on the basal cell proliferation at any of the concentrations used. However, the inhibitor of TP blocked the TP-induced monolayer regeneration without altering the basal or VEGF-induced response (Figure 6b).

In vivo *angiogenesis*

As shown in Figure 7, the administration of TP induced a dose-dependent neovascular response in the sponge implant. At a dose of 0.5 and 1 pmol, TP induced a 131 ± 14 and $179 \pm 4\%$ increase in the vessel counts, respectively, as compared to basal vessel count ($100 \pm 6\%$), respectively (Figure 7d), consistent with the trend observed in the clearance of ^{133}Xe (Figure 7e). Table 1 shows the effect of TP treatment on the $t_{1/2}$ of clearance of ^{133}Xe from the sponges. A faster clearance denotes enhanced neovascularisation and blood flow.

The administration of 2-DDR induced a strong angiogenic effect in the sponge implant. The dose for DDR (2×10^{-9} mol $\sim 10^{-5}$ M) was selected from the *in vitro* studies, as this concentration had induced the maximal monolayer recovery. As shown in Figure 7d, the angiogenic effect in terms of vessel counts was similar in both TP-treated (1 pmol) and DDR-treated sponges. The total ^{133}Xe cleared was also significantly

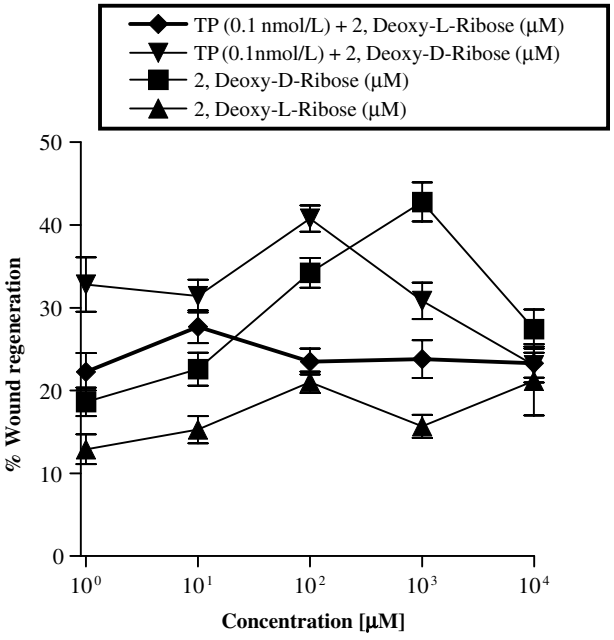

Figure 4 Effect of thymidine phosphorylase, on the 2-deoxy-D-ribose- and 2-deoxy-L-ribose-induced regeneration of the monolayer, postinjury. Cells were plated at a density of 20,000 cells/well, in a 24-well plate containing a coverslip in each well. They were cultured to confluence in 20% FCS-supplemented media and then transferred to media with 1% FCS for 24 h. The monolayer was then lesioned using a multichannel wounder (described in Methods), and transferred to fresh media supplemented with 5% FCS, with and without the sugars. They were incubated for a further 24 h, following which, they were fixed and image-analysed. Data expressed are mean ± s.e.m. of at least four separate experiments with quadruplicate wells in each. Data are shown as percentage of 0 h values.

higher in the DDR-treated group than in the vehicle-treated group.

Surprisingly, the administration of the levo-isomer, 2-DLR (2×10^{-9} mol $\sim 10^{-5}$ M), also induced strong neovascularisation in this model. The increase in the vessel count, or in the total ^{133}Xe cleared at the end of 6 min from the 2-DLR-treated sponges, was similar to the 2-DDR-treated group. Table 1 shows the effect of treatment with 2-DDR and 2-DLR on the $t_{1/2}$ clearance of ^{133}Xe from the sponge implants.

Figure 7f, shows the effect of the TP inhibitor on TP- and 2-DDR-induced angiogenesis *in vivo*. The administration of TP inhibitor (2×10^{-9} mol) alone did not exert any effect on the basal neovascularisation, as quantified by the number of vessels entering the sponge implant, but inhibited the TP-induced angiogenesis. At a similar concentration, it failed to exert any inhibitory effect on the 2-DDR-induced neovascularisation.

Figure 7g shows the effect of different treatments on the body weight of the animals. No significant difference was observed between the control group and any of the treated animals.

Recovery response of a monolayer of human TP cDNA-transfected carcinoma cells and wild-type carcinoma cells following a mechanical injury

The human bladder carcinoma cell line, RT112, was transfected to over-express human TP. Immunoblotting for TP demonstrated a strong expression of the protein in 2T10 cells carrying the gene, in contrast to EV11 cells that carried the empty vector (data not shown). The two cell types were grown to confluence and wounded as described earlier. The total recovery of the wound area at 24 h was quantified and expressed relative to the initial area injured. As shown in Figure 8, a greater regeneration was observed with 2T10 cells as compared to the EV11 cell line. Although there was a significant proliferation of cells, there was no significant difference in the number of EV11 and 2T10 cells at 24 h.

Effect of mechanical injury on the expression of HO-1 protein in TP-overexpressing carcinoma cells and wild-type carcinoma cells

HO-1 is an inducible enzyme, and is a good marker for oxidative stress in cells. The expression of HO-1 in 2T10 and EV11 cells was studied using Western blotting, both before wounding the cells and at different time points after injuring the confluent monolayer with the mechanical wounder. As shown in Figure 9a, the expression of HO-1 was identical in both the cell types before the induction of any mechanical stress.

Figure 9b demonstrates the postinjury expression of HO-1, as a function of time, in EV11 and 2T10 cells. The levels of HO-1 in both cell types were identical immediately after wounding, or at T_0. However, a significant increase in the levels of HO-1 was detected in the 2T10 cells by 3 h postwounding, whereas in EV11 cells it was still at the basal level. At 10 h post-treatment, an induction of HO-1 was detected in the EV11 cells, while the early expression of HO-1 in 2T10 cells had subsided to basal level. A second wave of HO-1 induction was observed in 2T10 cells at 24 h postinjury, which was not evident in the EV11 cells.

Discussion

TP or platelet-derived endothelial cell growth factor (PD-ECGF) was reported as an endothelial cell growth mitogen based on radiolabelled-thymidine uptake data (Miyazono & Takaku, 1991). It was subsequently shown that the increase in thymidine uptake was an artefact as the addition of TP/PD-ECGF to cell media caused a depletion of thymidine and resulted in the subsequent cellular uptake of ^{3}H-thymidine. The findings of the current study showed that the administration of TP did not induce a proliferative effect following a mechanical injury to the cell monolayer, consistent with earlier observations. In contrast, the known endothelial mitogen, VEGF, induced a strong concentration-dependent increase in the number of cells. Using actinomycin-D and taxol, we had earlier reported that endothelial cells mount an extensive repair process involving both migration and proliferation components (Lauder *et al*., 1998). However in the present study, the total monolayer recovery by the wounded endothelium was similar at equimolar concentrations of TP and VEGF, despite the fact that VEGF but not TP induced a proliferative response. This suggested that TP could mount a strong angiogenic response by promoting endothelial cell migration. Indeed, endothelial cell migration, but not proliferation, has been suggested to be essential for angiogenesis (Sholley *et al*., 1984).

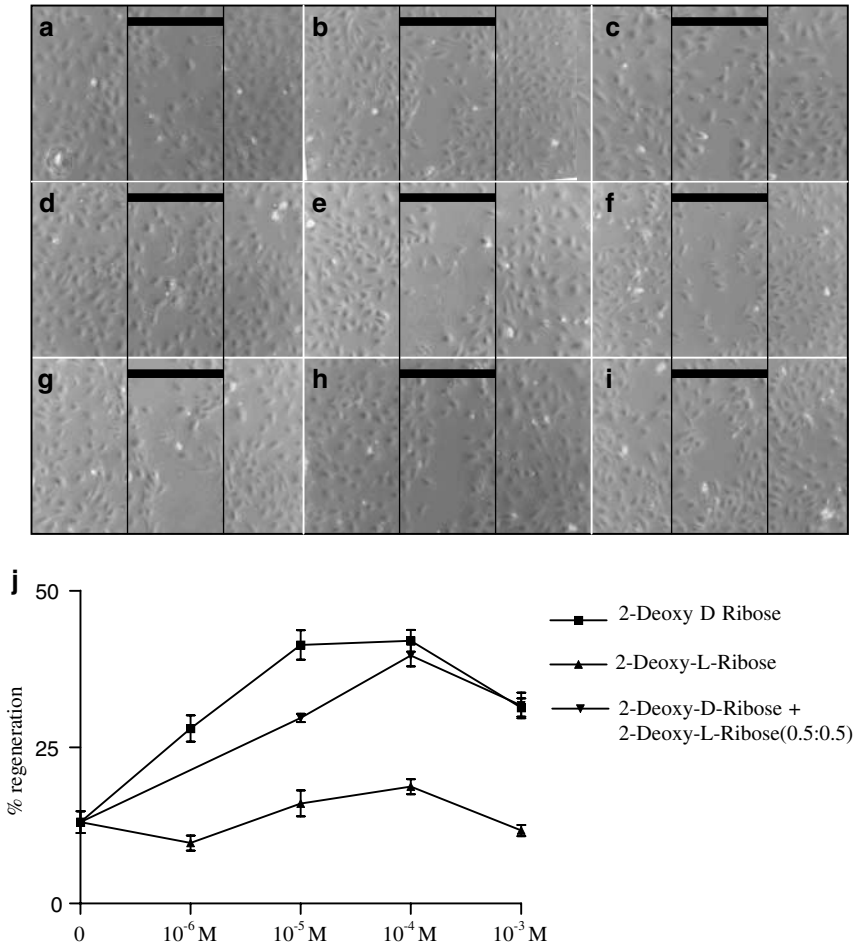

Figure. 5 Effect of combination of 2-deoxy-D-ribose and 2-deoxy-L-ribose in a 1 : 1 ratio on the regeneration of a injured monolayer of endothelial cells. HUVECs were grown to confluence in endothelial growth medium (Clonetics, U.S.A.), and synchronised in 0.5% serum. The confluent monolayer was injured as described in the Methods section, and allowed to recover in EGM supplemented with 5% fetal bovine serum for 24 h in the presence of different concentrations of ribose sugars and their combinations. The cells were fixed at 24 h, and image analysed using an Olympus inverted microscope using a $\times 10$ objective. The photomicrographs depict the total recovery following treatment with (a) vehicle, (b) 2-DDR (10^{-6} M), (c) 2-DDR (10^{-5} M), (d) 2-DDR (10^{-4} M), (e) 2-DLR (10^{-6} M), (f) 2-DLR (10^{-5} M), (g) 2-DLR (10^{-4} M), (h) 2-DDR + 2-DLR (10^{-5} M), (I) 2-DDR + 2-DLR (10^{-4} M). The graph (j) represents the total recovery at 24 h, expressed as a percentage of the initial area denuded. Data represents mean $\pm$ s.e.m. from three separate experiments with duplicate wells in each.

Under normal conditions endothelial cells remain in a quiescent state, but become highly proliferative during angiogenesis (Folkman & Klagsbrun, 1987). The current *in vitro* model of studying the effects of the molecules on the regeneration response of endothelial cell monolayer, following a 'wound', therefore most closely resembled the clinical setting. We used medium containing 5% serum to study the wound regeneration, as TP has been shown to stimulate the migration of endothelial cells only in the presence of serum or thymidine (Moghaddam *et al*., 1995). This suggests that the enzymatic activity of TP is essential for its angiogenic effects. Indeed, site directed mutagenesis studies, involving Lys115→Glu, Leu148→Arg and Arg202→Ser, have emphasised the importance of TP enzymatic activity for its angiogenic potential (Miyadera *et al*., 1995). It could be possible that the exogenous TP was metabolising thymidine in the serum, resulting in breakdown products that induced chemokinesis of the cells in the current study. Interestingly, using an established endothelial cell-fibroblast coculture tube formation angiogenesis assay, we observed that thymine could exert an antiangiogenic effect, which could be reversed by the addition of 2-DDR-1-P. However, during TP over-activity, the concentration of 2-DDR is elevated in plasma instead of 2-DDR-1-P. In the current study, we therefore evaluated the angiogenic potential of 2-DDR. We observed that 2-DDR induced a concentration-dependent migration of endothelial cells that was significantly greater than that induced by the enantiomer 2-DLR. This was similar to the observations of Brown & Bicknell (1998) using a Boyden chamber. Interestingly, in the present study, concentrations of 2-DDR greater than 10^{-4} M produced a supramaximal response.

The administration of TP induced a strong dose-dependent neovascular response into the sponges. This was consistent with our earlier findings that TP could induce angiogenesis in a rat sponge model, and increase the blood flow into skin grafts (Moghaddam *et al*., 1995). In the current study, we further demonstrate that 2-DDR can induce a strong angiogenic effect in the *in vivo* sponge granuloma model. Surprisingly, unlike the *in vitro* studies, the administration of 2-DLR into the sponge induced an almost similar angiogenic response as 2-DDR. The

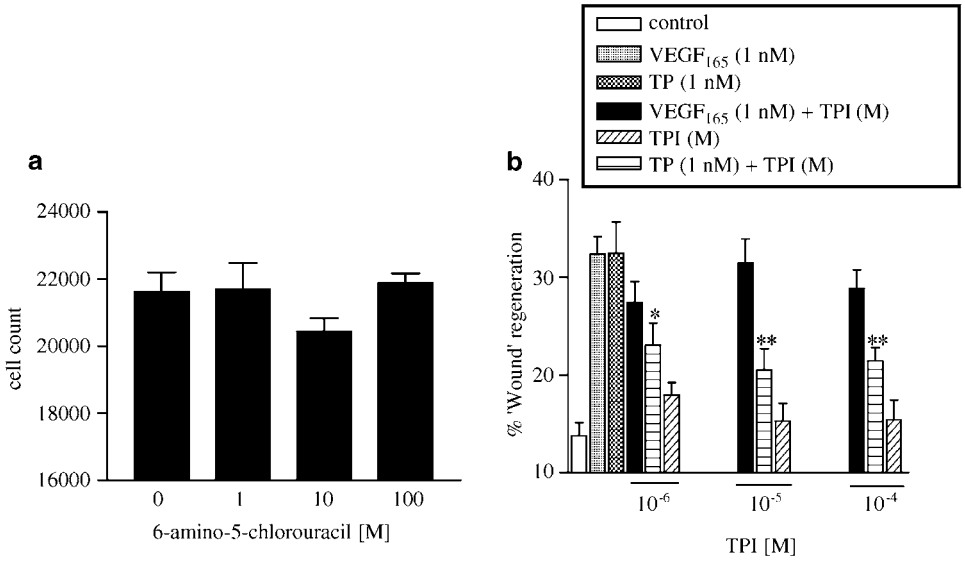

Figure 6 Effect of a thymidine phosphorylase inhibitor (TP), 6-amino-5-chlorouracil, on (a) the proliferation of endothelial cell, and on (b) the thymidine phosphorylase- and $VEGF_{165}$-induced regeneration of the monolayer, postinjury. Cells were plated at a density of 20,000 cells well^{-1}, in a 24-well plate containing a coverslip in each well. They were cultured to confluence in 20% FCS-supplemented media and then transferred to media with 1% FCS for 24 h. The TP inhibitor was added 1 h prior to 'wounding'. The monolayer was then lesioned using a multichannel wounder, and transferred to fresh media supplemented with 5% FCS, with and without the compounds. They were incubated for a further 24 h, following which, they were either fixed and image analysed, or trypsinised and counted using a haemocytometer. Data expressed are mean $\pm$ s.e.m. of at least three separate experiments with duplicate/quadruplicate wells in each. In the wound recovery experiment, data are shown as percentage of T_0 values. $^*P<0.05$, $^{**}P<0.01$ vs thymidine phosphorylase-treated controls.

inability of 2-DLR to induce a similar response *in vitro* suggests that the ability of 2-DDR to induce endothelial cells migration is highly stereospecific. However, Brown and Bicknell (1998) reported that using 2-deoxy-D[3H]-ribose did not reveal any specific binding on endothelial cells, indicating that the effects of 2-DDR could be nonreceptor-mediated. Another hypothesis can be that 2-DDR could possibly enter the cells, and subsequently get phosphorylated to 2-deoxy-D-ribose 5-phosphate, which is then cleaved to acetaldehyde and glyceraldehyde (Brown & Bicknell, 1998). These molecules can then enter the glycolytic pathway; therefore, generating two molecules of ATP for every molecule of 2-DDR. In contrast, 2-DLR does not enter glycolysis. Indeed, it has been demonstrated that endothelial cells migrate towards sugars that are accessible sources of energy (Vogel *et al*., 1993). In addition, a recent paper has reported that cancer cells express a higher level of glucose transporter proteins (Glut-1), and Glut-1 was associated with an increase in the expression of matrix metalloproteases and invasion (Ito *et al*., 2002). A good test would be to block the enzymes catalysing these changes, and the only inhibitor available would be disulfiram that blocks the enzyme ribose kinase, and indeed a recent paper has demonstrated an antiangiogenic activity for disulfiram although that was linked to the inhibition of superoxide dismutase (Marikovsky *et al*., 2002). However, further work is essential to test this hypothesis, but the supramaximal effect seen with higher concentrations of 2-DDR could also be explained on this basis, as at such concentrations 2-DDR would inhibit glycolysis (reviewed by Brown & Bicknell, 1998). Furthermore, elevated sugar levels activate protein kinase C in endothelial cells (Tesfamariam *et al*., 1991), and a PKC inhibitor has been shown to inhibit the migration of endothelial cells (Rattan *et al*., 1996).

In the present study, a faster and greater expression of HO-1, was observed in the cells transfected with the human TP-gene, following a mechanical stress. Under normal physiological conditions, HO-2 is the major isoform found in the mammalian tissue, approximately in a 2 : 1 ratio to HO-1. However, HO-1 has been shown to be transcriptionally activated, and exert a protective role against oxidative stress through the generation of carbon monoxide (CO) (Choi and Alam, 1996). Interestingly, the transfection of rabbit coronary microvessel endothelial cells with human HO-1 gene was shown to promote blood vessel formation (Deramaudt *et al*., 1998). Furthermore, in a recent study, Brown *et al*. (2000) reported that the TP-mediated generation of reducing sugars leads to oxidative stress, and the subsequent release of angiogenic factors, such as VEGF, IL8 and MMP-1 from tumour cells (Brown *et al*., 2000).

The overexpression of HO-1 or the exogenous administration of CO was shown to prevent the apoptosis of endothelial cells. In addition, endothelial cells that expressed HO-1 were found to inhibit apoptosis of endothelial cells not expressing HO-1, suggesting that CO acts as an intercellular signal. Indeed in a separate study, TP and 2-DDR were found to exert an antiapoptotic effect in KB cells (Kitazono *et al*., 1998). These reports increase the implications of the findings of the current study, as the expression of HO-1 by TP/2-DDR-induced oxidative stress in a tumour could result in the stabilisation of a neovasculature through a CO-dependent antiapoptotic effect on the newly recruited endothelial cells. Although further experiments using a coculture system are necessary to prove this hypothesis, it is further supported by the observation that an elevated level of TP is associated with a lower tumour apoptotic index (Matsuura *et al*., 1999).

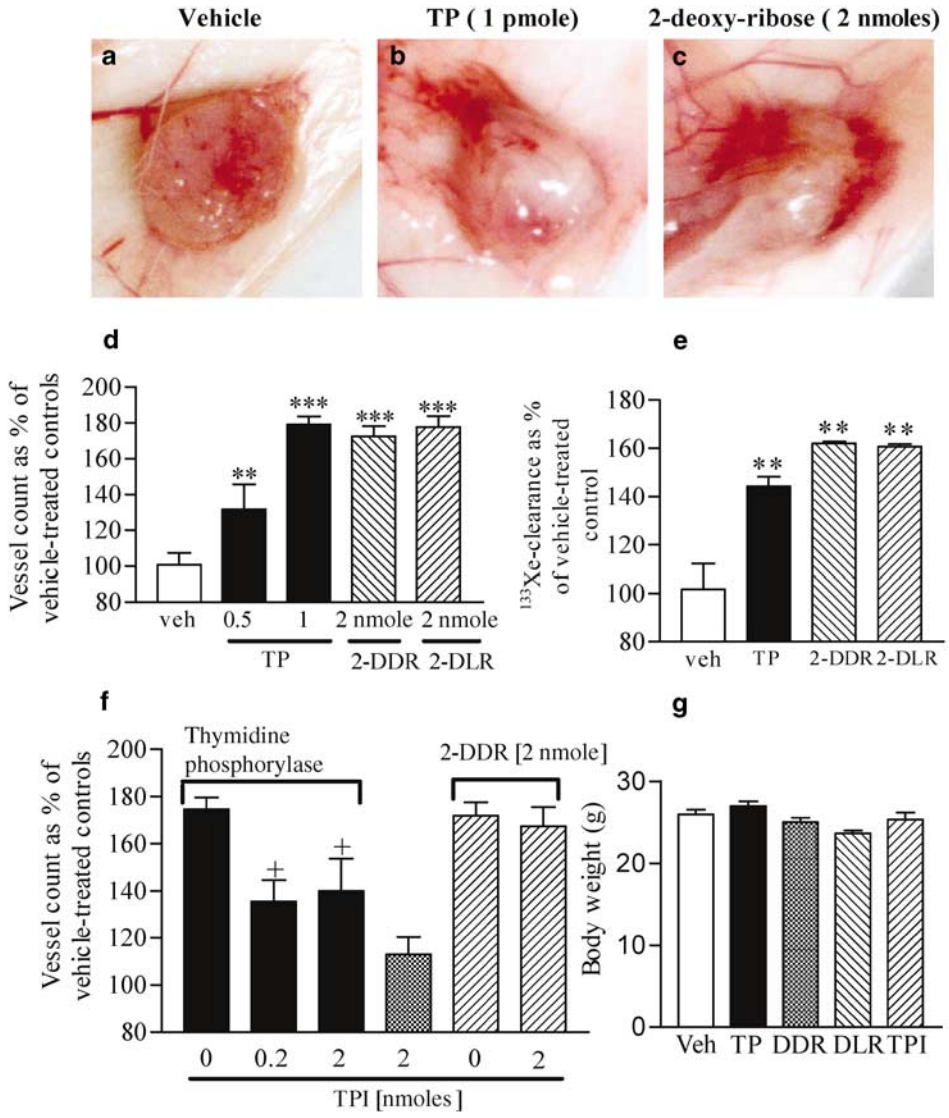

Figure 7 Effect of thymidine phosphorylase (TP) and sugars, 2 deoxy-D-ribose (2-DDR) and deoxy-L-ribose, in a sponge granuloma model of angiogenesis. Pictomicrographs depict (a) neovascularisation in a vehicle-treated sponge, (b) TP (1 pmol/sponge for 10 days)-treated sponge increased the angiogenic response, and (c) a 2-deoxy-D-ribose (2 nmol/sponge $\times$ 10 days)-treated sponge implant. The graphs show the angiogenesis in the sponge implant, as quantified by (d) vessel counts, and (e) measuring the total 6 min ^{133}Xe-clearance, indicating that the treatment with TP, 2-DDR and 2-DLR promoted a strong angiogenic response. Graph (f) shows the effects of a thymidine phosphorylase inhibitor on the angiogenesis induced by TP, and 2-DDR in the sponge granuloma model of angiogenesis, while (g) depicts the body weight of the mice following different treatments. Results were calculated from a minimum of two experiments with at least three – four replicates per test group. Data are expressed as mean $\pm$ s.e.m. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ vs vehicle-treated controls. $+P<0.05$ vs TP-treated controls.

Intriguingly, these findings raise questions regarding the possibility of an angiogenic phenotype being induced by oxidative stress inside the endothelial cells, which could result either from thymidine entering the cells and its subsequent breakdown to 2-DDR, or from elevated levels of 2-DDR following exogenous addition. This argument however fails to explain the inability of 2-DLR to induce a significant angiogenic response *in vitro*. Intriguingly, a recent study has reported that 2-DDR could be an optimal target to block the tumorigenic potential of TP, and that 2-DLR could inhibit the effects of 2-DDR *in vitro* and TP *in vivo* (Uchimiya *et al*., 2002). To test these ambiguities, we designed a simple experiment where the effects of 2-DDR and 2-DLR on wound recovery were compared with a combination of the two optical isomers in a 1 : 1 ratio. We observed that 2-DLR failed to contribute to the recovery induced by 2-DDR, suggesting that the energy component associated with 2-DDR may be a key contributor in the phenotype. Interestingly, both 2-DDR and 2-DLR, and their combination, induced endothelial cell loss at concentrations higher than 10^{-4} M. This observation could possibly be used to explain the antiangiogenic activity of 2-DLR reported by Uchimiya *et al*.; however, further studies are essential to evaluate the mechanism of action.

In the present study, TP overexpressing human carcinoma cells exhibited a faster recovery of the wounded monolayer. The proliferation of both the TP-transfected and the wild-type cells was however identical, suggesting that the

TP-expressing carcinoma cells had a higher migratory capacity as compared with the cells transfected with the empty vector. This could explain earlier observations that TP-expressing tumour cells were more invasive, and TP was often upregulated at the infiltrating tumour edge (Currie *et al*., 2001). Specimens from gastric carcinoma patients also revealed that TP was expressed in the invasive edges of the tumours, and that TP-positive differentiated adenocarcinomas invaded more deeply with poor prognosis (Shimaoka *et al*., 2000).

Table 1 Effect of thymidine phosphorylase (TP), 2-deoxy-D-ribose and 2-deoxy-L-ribose on the neovascularisation into the sponge-implants, as quantified by the $T_{1/2}$ clearance of ^{133}Xe from the sponge-implants

Treatment	$T_{1/2}$ *(s)*	*s.e.m.*	R^2
Vehicle	264.00	15.14	0.98
TP	105.30	10.06	0.82
Deoxy-D-ribose	101.40	10.66	0.85
Deoxy-L-ribose	76.05	3.47	0.98

Sterile sponges were implanted subcutaneously on the dorsum of the mouse under anaesthesia. The vehicle or drug treatments were administered into the sponge, using a 30 G precision glide needle, starting 24 h after implantation, and continued for 10 days. On day 15, the mice were anaesthetised, and functionality of the neovasculature was quantified by measuring the clearance of injected ^{133}Xe from the sponge, over a period of 360 s. The $T_{1/2}$ clearance was calculated by nonlinear regression curve fitting in a one-phase exponential decay equation.

To conclude, the current study clearly demonstrated the enhanced migration of both carcinoma cells and endothelial cells in a TP-enriched microenvironment, an effect possibly mediated by ribose sugars. Furthermore, the release of TP-generated 2-DDR could potentially create a gradient of oxidative stress-induced angiogenic factors from a carcinoma, leading to endothelial chemotaxis, and exert an antiapoptotic effect. In addition, 2-DDR could possibly exert a chemokinetic effect by integrating into the metabolic machinery of the endothelial cell, and ultimately resulting in the cohesive development of a tumour vasculature (schematic in Figure 10). The increased migration of TP-overexpressing carcinoma cells could also signal a potential for increased metastasis. Indeed, an inhibitor of TP, alone or in combination with chemotherapeutic agents, has been shown to exert an antimetastatic activity in mice bearing an experimental liver metastasis of Co-3 colon cancer (Miyadera *et al*., 1998). This was similar to the findings of the current study, where a TP inhibitor could block TP-induced angiogenesis *in vivo*.

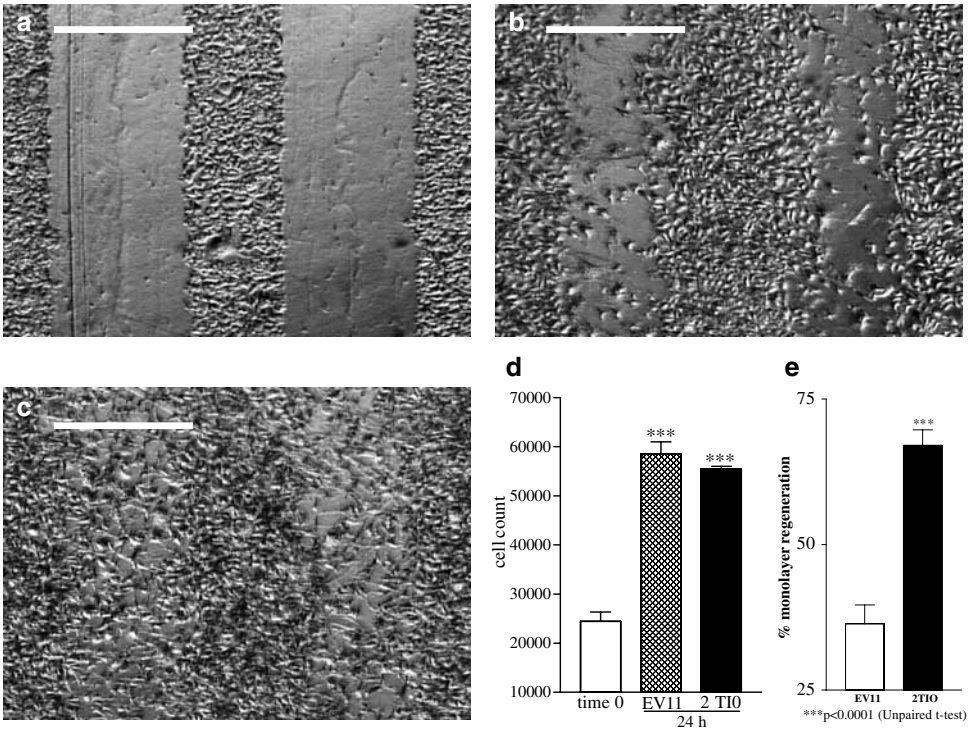

Figure 8 Response of TP-transfected and wild-type human bladder carcinoma cells to mechanical denudation of the confluent monolayer. The cell line RT112 was transfected with human TP cDNA to generate the 2T10 cell line. EV11 was generated as the empty vector control transfectant. The cells were grown to confluence on Thermanox coverslips in medium supplemented with 10% FCS and glutamine. They were then wounded using a multichannel mechanical wounder, and transferred to fresh medium. They were incubated for a further 24 h, after which they were either fixed and image-analysed, or trypsinised and counted using a haemocytometer. Nomarsky images were captured using a Nikon Diaphot ($\times 4$ objective) as described in Methods. Photomicrographs depicting (a) a wounded area at time 0 where the cells were fixed immediately after wounding, wound recovery in (b) regeneration by EV11 cells, (c) regrowth of 2T10 cells, 24 h postinjury. Graphs depict (e) cell count and (f) wound recovery, following incubation for 24 h post-injury. Data expressed are mean $\pm$ s.e.m. of at least three separate experiments with duplicate wells in each. In the wound recovery experiment, data are shown as percentage of 0 h values. ***$P<0.001$.

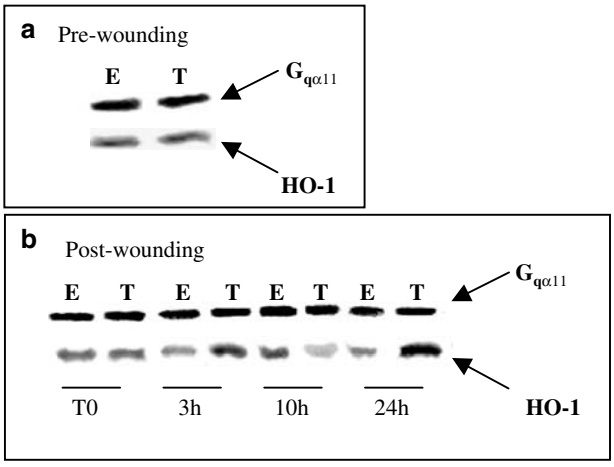

Figure 9 Level of hemeoxygenase 1 (HO-1) at different time points following mechanical injury to a confluent monolayer of human bladder carcinoma cells. The cell line RT112 was transfected with human TP cDNA to generate the 2T10 (T) cell line. EV11 (E) was generated as the empty vector control transfectant. Cells were grown to confluence on Thermanox coverslips, and then wounded using a multichannel mechanical wounder, and transferred to fresh medium. At fixed time intervals, the cells were lysed in buffer and samples were separated on a 10% SDS – PAGE. Haemeoxygenase was detected using a goat polyclonal antibody raised against a peptide mapping at the carboxy terminus of HO-1 of human origin. A concomitant control for $G_{\alpha q11}$was run to establish the uniformity of protein loading. Block (a) shows the levels of HO-1 prior to the induction of the injury, while (b) shows the time course of the expression of HO-1 in the cells after injury.

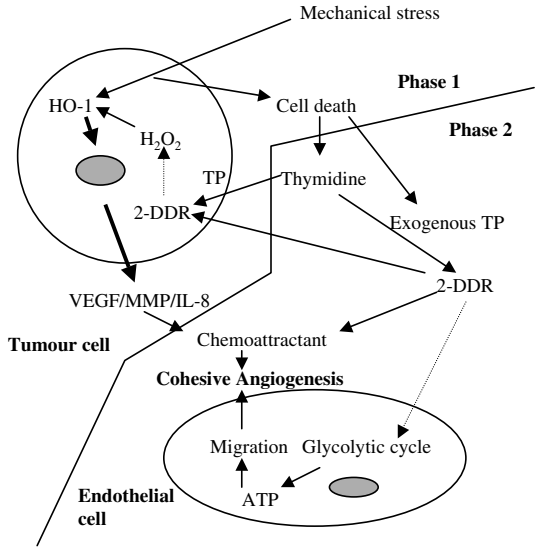

Figure 10 A proposed mechanism for the angiogenic effects of thymidine phosphorylase. Mechanical stress can lead to cell death (normal inside a solid tumour), and result in the release of thymidine in the microenvironment. Thymidine can enter live carcinoma cells, and is broken down by TP to 2-DDR-1-P, which can be dephosphorylated to 2-DDR. Inside a tumour cell (phase 1), reducing sugars can undergo rearrangement reactions, leading to the generation of free radicals. The concomitant upregulation of HO-1 has been implicated in increasing the expression of VEGF, MMPs and IL-8. These can then act on the host endothelial cells to induce an angiogenic effect *in vivo*. Furthermore, CO released would stabilise the neo-vessels through an antiapoptotic effect. Additionally, as seen in phase 2, the TP released from injured cells, or added exogenously, can act on thymidine and lead to the generation of 2-DDR, which is a chemotactic/chemokinetic factor, and promote angiogenesis. The 2-DDR that enters the cell can also be incorporated into the glycolytic machinery, and generate energy that can further serve towards a migratory phenotype. This model explains why both 2-DDR and 2-DLR could exert angiogenic effects *in vivo*, but only 2-DDR was active *in vitro*. Thin arrows indicate possible links elucidated in this study, while hashed arrows are putative links. The thicker arrows indicate pathways established by other studies (Brown *et al*., 2000).

Furthermore, the TP-induced migration of endothelial cells was almost completely blocked by the TP inhibitor. This suggests that TP inhibitors can be useful in exerting an antimetastatic effect in TP-expressing carcinomas, and in inhibiting tumour angiogenesis. In addition, the elucidation of downstream signal molecules of TP allows a multitarget approach in the management of pathological conditions characterised by the elevated levels of the enzyme.

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