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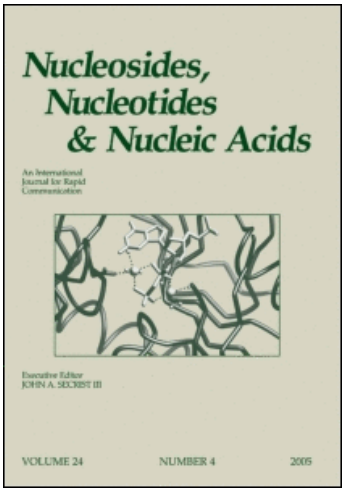

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INCREASED MIGRATION BY STIMULATION OF THYMIDINE PHOSPHORYLASE IN ENDOTHELIAL CELLS OF DIFFERENT ORIGIN

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□ *Thymidine phosphorylase (TP) catalyzes the phosphorylytic cleavage of thymidine to thymine and deoxyribose-1-phosphate. The latter may be involved in the angiogenic stimulation of TP. In the present study, we investigated whether thymidine and deoxyribose (dR) could stimulate angiogenesis in vitro of two types of endothelial cells (isolated from umbilical veins (HUVEC) and endothelial colony forming cells (ECFC)), and whether the stereoisomer L-deoxyribose (L-dR) and the thymidine phosphorylase inhibitor (TPI) could reduce this. Both cell types had a low TP activity. Thymidine increased the migration of both HUVECs and ECFCs, but dR only that of the ECFCs. The invasion was not changed by any of the agents tested. In conclusion, TP may play a role in the migration of HUVECs and ECFCs, but not the invasion.*

Keywords HUVEC; ECFC; migration; invasion; thymidine phosphorylase

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INTRODUCTION

The platelet derived endothelial cell growth factor (PD-ECGF) is also known as thymidine phosphorylase (TP). TP is overexpressed in a wide variety of solid tumors.[1] TP is an angiogenic factor and was shown to stimulate angiogenesis in various studies. In addition, a high TP in cancer cells may change its invasive characteristic.[2] TP converts thymidine (TdR) to thymine and deoxyribose-1-phosphate (dR-1-P). dR-1-P can be further converted to deoxyribose (dR), which is thought to be the compound by which TP acts as an angiogenic stimulator. Angiogenesis is often studied using endothelial cells derived from human umbilical veins (HUVECs), but more recently it was hypothesized that new blood vessels might also be formed from circulating endothelial (progenitor) cells (EPC),[3] rather than from existing blood vessels. EPCs are cells that are thought to originate from the bone marrow, which can differentiate into endothelial cells and are thought to contribute

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to neovascularization.[3,4] In the present study, we used Endothelial Colony Forming Cells (ECFCs)[5,6] as a model for EPCs and determined whether TP expression in the HUVECs and ECFCs was important for their angiogenic potential. Therefore, we determined the TP enzymatic activity of these cells, the migration and invasion capacity after stimulation of TP with TdR, with the reducing sugar dR and the inhibitors L-dR and TPI.

MATERIALS AND METHODS

Cells

Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cords as described previously[7] and were cultured in M199 medium, supplemented with 10% FCS (PAA laboratory GmbH, Germany), 10% human serum (HS) and ECGF. ECFCs were isolated from mononuclear cells that were obtained from the cord blood, diluted 1:3 with PBS (Phosphate buffered saline, B. Braun, Germany), using Ficoll Paque density gradient separation medium (GE Healthcare Bio-sciences AB, Sweden) and Leucosep 50 mL tubes (Greiner Bio-One, Germany). The mononuclear cells were cultured in a concentration of 2.5×10^6 cells per cm^2 in EBM-2 complete medium (Endothelial cell basal medium-2, Lonza, USA) supplemented with 0.1% pen-strep (Lonza, Verviers, Belgium), 10% FCS and EGM-2 SingleQuotes without hydrocortisone and gentamycin/amphotericin-B (Lonza, USA). When the colonies started to appear (after 10–15 days) ECFCs were trypsinized and subcultured into flasks.[8] Subcultured ECFCs showed characteristics of true endothelial cells, such as CD105, CD146, CD31, VE-cadherin, Tie-2, VEGFR1, uptake of AcLDL an UEA binding, and CD133, CD14, and CD45 were absent (data not shown). The concentrations of TdR and dR were very low in M199 and EBM-2 medium. For all experiments with HUVECs and ECFCs, at least 3 different isolations (e.g., donors) were examined for their (angiogenic) response in order to get a reliable result. All cells were maintained in a humidified 5% CO_2 atmosphere at 37°C.

Thymidine Phosphorylase Activity

The TP activity was determined as described previously.[9] The activity was measured using TdR as a substrate and by detecting the conversion to thymine by HPLC.

Migration Assay

Cell migration was determined using the wound healing assay as described previously.[10] Cells were grown until confluence and a scratch wound

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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 agents, diluted in low-serum-medium (5% FCS and 5% HS for HUVECs and 0.1% for ECFCs), corresponding to the cell type used. Wounds were captured at 2.5× magnification with a microscope (TCS 4D, Leica, Jena, Germany), and Q500MC software (Leica) at time points 0 and 6 hours. The wound width was measured and compared with the initial width at the 0 hour time point (set at 0%).

Invasion Assay

The invasion assay was carried out as described previously,[10] using transwell chambers with a fluorescence-blocking 8 μm pore polycarbonate filter insert (#35-1152; HTS Fluoroblock Insert, Falcon, Becton Dickinson Labware, USA) in 24 wells plates (# 35-3504; Falcon, Becton Dickinson). The upper side of the insert was coated overnight at room temperature (RT) with 100 μl matrigel (50 ng/ml in PBS; Sigma, USA). Cells (50 000/insert) were allowed to invade for 8 hours (with or without stimulators or inhibitors in the medium corresponding to the cell type). Thirty minutes before analysis, 5 μM calcein-AM was added to the lower compartment and the amount of fluorescence was measured.

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Statistical Analysis

Potential differences between treated and untreated controls were evaluated using the two-tailed Student's *t*-test for paired data. Changes were considered significantly different when $p < 0.05$.

RESULTS AND DISCUSSION

HUVECs had a comparably low TP activity as ECFCs (5.1 ± 0.4 and 5.4 ± 0.6 nmol/million cells/h, respectively), compared to most tested cancer cell lines (varying from 0.6 to 70 nmol/million cells/h).[11] This is in agreement with previously reported data for ECFCs and HUVECs.[12] In the latter study, TP was found to be a key regulator in angiogenesis induced by ECFCs, both in vitro and in vivo.

ECFCs are currently being studied in cardiovascular diseases, but because of their capacity to invade into tumor tissues, they also have potential as a target for anti-angiogenic agents in cancer. HUVECs are a widely accepted model to study angiogenesis and are used mostly. HUVECs had a higher migration after 6 hours than ECFCs (Figure 1). The migration of HUVECs was increased by TdR and inhibited by TPI and L-dR, although this increase and inhibition were not significant.

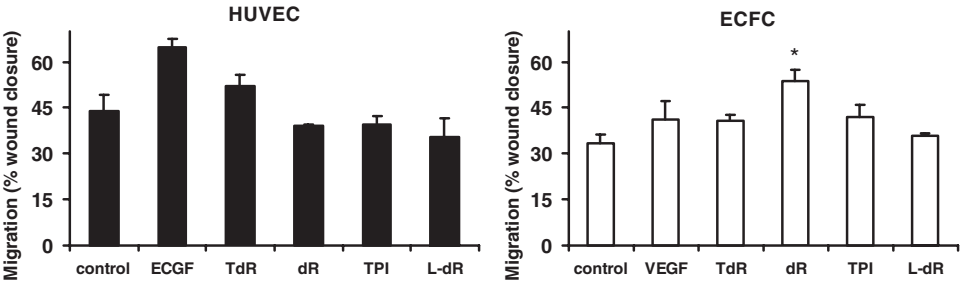

FIGURE 1 Migration of HUVECs and ECFCs after 6 hours exposure to 100 μM TdR, 100 μM dR, 50 μM L-dR, or 10 μM TPI. Controls represent the medium with the low% serum, to which TdR, dR, L-dR, and TPI were added. All values represent means of at least 4 independent experiments ± SEM, $^*p < 0.05$.

Since in HUVECs dR did not stimulate the migration at all, this may indicate that dR might not be the most important factor of TP-mediated HUVEC-migration. dR-1-P formed from TdR can also be isomerized to deoxyribose-5-phosphate, which can subsequently be converted to glyceraldehyde-3-phosphate and enter the pentose phosphate pathway or glycolysis.[13,14] It is not clear whether the pentose phosphate pathway/glycolysis pathway only play a role in capturing dR-1-P, rather then play a role in angiogenesis. It is possible that HUVECS and ECFC respond differently to TdR derived sugars. Hence, in HUVECs, dR-1-P entering the pentose phosphate pathway might play a role in angiogenesis rather than dR-mediated angiogenesis. On the other hand, ECFC migration was increased to some extent by dR (Figure 1), which was in a concentration dependent manner (data not shown). For these ECFCs, dR might be an important angiogenic factor, since it stimulated the migration.[12] It is possible that dR is used for advanced glycation end products (AGE), via Schiff base and/or Maillard reactions.[15] dR itself or AGEs may therefore stimulate the cell migration of ECFCs. This indicates that in the two endothelial cell types TdR-derived sugars enter different pathways.

HUVECs and ECFCs had a comparable level of invasion. The invasion was neither stimulated nor inhibited after exposure to TdR, dR, L-dR, or TPI (Figure 2). dR has previously been reported to be a good stimulator

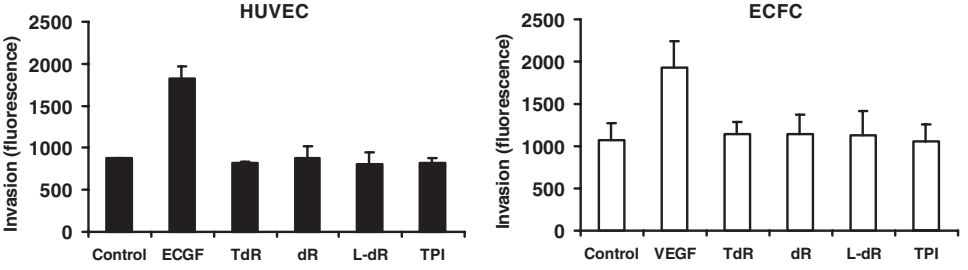

FIGURE 2 Invasion of HUVECs and ECFCs after 7 hours exposure to 100 μM TdR, 100 μM dR, 50 μM L-dR, or 10 μM TPI. Controls represent the medium with the low% serum, to which TdR, dR, L-dR, and TPI were added. All values represent means of at least 3 independent experiments ± SEM.

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of migration and invasion of endothelial cells, including HUVECs.[16] This stimulation was mediated by activation of intracellular kinases, including the focal adhesion kinase and p70/s6k.[17,18] Besides dR, other factors may also be involved in TP mediated angiogenesis, such as secretion of IL-8 or VEGF.[9,19]

In conclusion, these data indicate that the migration of HUVECs and ECFCs can be stimulated by TdR, the substrate for TP and the product dR (for ECFCs). The invasion was not affected by TdR or dR. This low level of stimulation may be related to the low intrinsic TP expression of these cell types.

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