Available online at www.sciencedirect.com
ScienceDirect
Microvascular Research 75 (2008) 247–255
Microvascular Research
www.elsevier.com/locate/ymvre

Regular Article

Exploring vascular dysfunction caused by tirapazamine

Lynsey A. Huxham, Alastair H. Kyle, Jennifer H.E. Baker, Krista L. McNicol, Andrew I. Minchinton *

Department of Medical Biophysics, British Columbia Cancer Research Centre, 675 West 10th Ave, Vancouver, British Columbia, Canada V5Z 1L3

Received 14 March 2007; revised 20 July 2007; accepted 24 September 2007
Available online 11 October 2007

Abstract

We have previously reported that the hypoxic cytotoxin tirapazamine causes central vascular dysfunction in HCT-116 xenografts. Here we further extend this finding to SiHa xenografts and SCCVII murine tumors. Within 1 day after treatment with tirapazamine both tumor types develop areas of non-perfused tissue in central regions of tumors. To explore the mechanism by which the hypoxic cytotoxin tirapazamine causes vascular dysfunction we altered the blood oxygen content with carbogen (95% O_2 and 5% CO_2) breathing in tumor bearing mice. Carbogen treatment was able to decrease the number of tumors responding to tirapazamine but was not able to eradicate the vascular dysfunction completely. In complementary *in vitro* studies, immunohistochemical staining of tirapazamine-treated endothelial cells indicated that, unlike the vascular targeting agent (VTA) combretastatin-A-4-phosphate, the vascular effects caused by tirapazamine are not due to microtubule disruption. Another possible mechanism of action for tirapazamine could involve its ability to inhibit nitric oxide synthase (NOS). Studies combining other vascular targeting agents (VTAs) such as the combretastatins have shown a potentiation of vascular disruption in tumors when combined with NOS inhibitors, possibly due to vessel constriction from decreased nitric oxide (NO) levels. We propose the theory that vascular dysfunction caused by tirapazamine may be via NOS inhibition. In support of this hypothesis preliminary experiments showed NOS inhibition with L-NNA (*N*-omega-nitro-L-arginine) increases tumor necrosis, 1 day after administration, in our HCT-116 tumor model.

Keywords: Carbogen; NOS; Tirapazamine; Vasculature; Tubulin; SiHa; HCT-116; SCCVII

Introduction

Solid tumors contain both chronic and acutely hypoxic cells that are less sensitive to the effects of ionizing radiation therapy (Gray et al., 1953; Thomlinson and Gray, 1955; Raleigh et al., 1996). Chronically hypoxic cells are situated far from vasculature and may be quiescent due to a sub-therapeutic dose of drug or they may not respond to chemotherapies that target cycling cells (Jain, 1997, 1998; Huxham et al., 2004; Kyle et al., 2004). Hypoxic cytotoxins were designed to exhibit preferential toxicity to these cells at low oxygen tensions and could therefore be useful in combination with standard therapies (Brown and Lemmon, 1991). However, we have previously shown that the hypoxic cytotoxin tirapazamine causes central vascular dysfunction in HCT-116 tumor xenografts and proposed that this effect was related to its activation in central blood vessels that reside at intermediate levels of oxygen or that have temporarily become hypoxic (Huxham et al., 2006).

Here we examine the occurrence of central vascular dysfunction in SiHa tumor xenografts and SCCVII murine tumors after tirapazamine administration, further suggesting that tirapazamine acts as a vascular targeting agent (VTA).

We have proposed the induction of central vascular dysfunction by tirapazamine may be related to toxicity towards endothelial cells that are at intermediate levels of oxygen or that have temporarily become hypoxic. In order to test this theory here we have administered tirapazamine to mice breathing carbogen, a gas consisting of 95% O_2 and 5% CO_2, which can decrease tumor hypoxia (Raleigh et al., 1999).

VTAs are compounds that selectively target the existing tumor vasculature. In general, all VTAs cause a cessation of

* Corresponding author. Fax: +1 604 675 8049.
E-mail address: aim@bccrc.ca (A.I. Minchinton).

0026-2862/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.mvr.2007.09.006

perfusion in blood vessels, which subsequently progresses to large areas of necrosis in the center of the tumors within 24 h after administration, but a viable rim of tissue remains after treatment. Combretastatin-A-4-phosphate (CA4-P), the most studied VTA, is a microtubule destabilizing drug, which causes central necrosis in tumors (Dark et al., 1997; Tozer et al., 2002; Vincent et al., 2005). Other small molecule VTAs such as DMXAA do not appear to interact with tubulin but instead are thought to act via cytokine induction and disruption of the actin cytoskeleton (Blakey et al., 2002; Baguley, 2003). Other treatment modalities have also been found to cause the same specificity for central tumor vasculature, such as antibodies targeted towards endothelial cells, and photodynamic therapy (Thorpe, 2004; Ran et al., 2005; Chen et al., 2006).

We investigated the ability of tirapazamine to disrupt microtubules in SiHa cells (human cervical squamous carcinoma cells) and HUVECs (human umbilical vein endothelial cells) under oxic (20% oxygen) and hypoxic (0.1% oxygen) conditions in glass slide chamber flasks.

As tirapazamine is a known nitric oxide synthase (NOS) inhibitor, and under hypoxic conditions can be converted by NOS to an active radical form resulting in DNA strand breaks (Garner et al., 1999), we reasoned that NOS could underlie the mechanism of tirapazamine-induced vascular dysfunction. As a preliminary investigation of this hypothesis we tested the ability of the non-specific NOS inhibitor L-NNA (*N*-omega-nitro-L-arginine) to induce vascular dysfunction in our HCT-116 tumor model.

Materials and methods

Cells

SiHa, human cervical squamous carcinoma cells, and HCT-116, human colorectal carcinoma cells, were purchased from American Type Culture Collection. SCCVII, murine squamous carcinoma cells, were obtained in 1983 from Dr. M. Horsman (Stanford University). All cells were maintained *in vitro* at 37.5°C with 5% CO_2/5% O_2 in minimum essential media (MEM; Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Burlington, ON, Canada) or 10% bovine growth serum (BGS; Hyclone). HCT-116 cells grown for implantation were maintained according to procedure but supplemented with 50 U/ml penicillin/50 μg/ml streptomycin sulfate (Gibco) during expansion.

Human umbilical vein endothelial cells (HUVEC) were a gift from Dr. A. Karsan (BCCRC). HUVECs were maintained in MCDB 131 media (Gibco) supplemented with 10% FBS (Hyclone), 5% BGS, 20 μg/ml endothelial cell growth supplement (ECGS; BD Biosciences, Mississauga, ON, Canada), 15 U/ml heparin (Sigma-Aldrich, St. Louis, MO), 10 mM L-glutamine (Sigma-Aldrich), 50 U/ml penicillin/50 μg/ml streptomycin sulfate (Gibco). Cells were passaged every 7 days and media were changed every 2–3 days. Cells were used between passages 1 and 4.

Mice and tumors

Female NOD/SCID and C3H/HeN mice were bred and maintained in our institutional animal facility in accordance with the Canadian Council on Animal Care guidelines. The experiments described in this paper were approved by the Animal Care Committee of the University of British Columbia. Mice were allowed free access to standard laboratory rodent food and water. HCT-116 cells (50 μl of 1.6×10^8 cells/ml) were implanted from *in vitro* subcutaneously (s.c.) into the sacral region of female NOD/SCID mice. The mice used were between 8 and 20 weeks of age and ranged in weight from 20 to 28 g. The weight of the excised tumors was 84±35 mg (mean±SD). SiHa tumor xenografts were maintained in female NOD/SCID mice and cells (50 μl of 20×10^6 cells/ml) were implanted s.c. into the sacral region of mice 11–13 weeks of age, weighing between 22 and 26 g. The mean weight of the excised tumors was 85±31 mg. SCCVII tumors were maintained in female C3H/Hen mice and cells (50 μl of 4×10^6 cells/ml) were implanted s.c. into the sacral region of mice 10 or 52–56 weeks of age, weighing between 26 and 46 g. The mean weight of the excised tumors was 121±46 mg.

Treatment

Mice were treated when their tumor volumes reached 100 mm^3 as measured using calipers and the formula $V=\pi/6$ (l×h×w).

Tirapazamine was synthesized as described previously (Huxham et al., 2006) and was administered at 60 mg/kg (0.34 mmol kg^{-1}) using a 1.25 mg/ml solution in saline. Tumors were excised on days 1–4 after drug administration.

Carbogen (95% O_2 and 5% CO_2) was delivered to a modified cage through a water warming apparatus at 5 l/min. Mice were exposed to carbogen flow for 10 min before tirapazamine injection. After injection the mice were left breathing carbogen for 3 h, constituting approximately 5 half lives of tirapazamine (half life ~35 min, 60 mg/kg). Mice were then returned to air breathing for the remaining 21 h before tumor excision.

L-NNA (*N*-omega-nitro-L-arginine) was dissolved in saline and administered i.p. at doses 20, 60 and 180 mg/kg and the tumors excised 1 day later.

As with previously reported experiments (Huxham et al., 2006) all mice were administered (i.p.) 5-bromo-2-deoxyuridine (BrdUrd) at 1500 mg/kg and pimonidazole at 60 mg/kg 2 h before excision. As a marker of blood vessel perfusion, all mice were administered (i.v.) 70 μl of 0.6 mg/ml carbocyanine (DiOC7(3)) 5 min prior to sacrifice. After excision, tumors were frozen at −20°C on an aluminum block, covered in embedding medium (O.C.T.) and stored at −80°C until sectioning.

Immunohistochemistry

This procedure has been reported elsewhere (Huxham et al., 2006) but briefly tumor cryosections (10 μm thick) were cut with a Cryostar HM560 (Microm International GmbH, Walldorf, Germany) air dried and imaged for carbocyanine. Slides were fixed in acetone–methanol before staining. Vasculature was stained using an antibody to PECAM/CD31 and bound pimonidazole was detected using Hypoxyprobe™-1 Mab1. After the slides were imaged for vasculature and pimonidazole they were stained for BrdUrd incorporation using a monoclonal mouse anti-BrdUrd (clone BU33). Slides were then counterstained with hematoxylin, dehydrated and mounted using Permount (Fisher Scientific, Fair Lawn, NJ, USA) before imaging. Image acquisition is the same as that reported previously (Kyle et al., 2007).

Image analysis

Image analysis has previously been described but will be briefly explained here (Huxham et al., 2006). Using the NIH-Image software application images from each tumor section were overlaid and staining artifacts were removed. The total number of pixels for necrotic or non-perfused tissue was obtained and the proportions of necrotic and of non-perfused tissue were then calculated by dividing the number of pixels that were necrotic or non-perfused by the total number of pixels for each tumor. Perfused regions are areas of viable tissue where vasculature is labeled with both CD31 and carbocyanine, as well as BrdUrd. Non-perfused areas stain with hematoxylin, are not necrotic and have CD31 staining, but no carbocyanine and no BrdUrd labeling. Necrotic areas show no staining. Percentage values are reported as mean±standard deviation.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 4. Nonparametric equations were used in all instances and a P value<0.05 was considered significant. A Kruskal–Wallis test was used to find significance within a group of samples and a Dunn's multiple comparison test was used to find the