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Microflow of fluorescently labelled red blood cells in tumours expressing single isoforms of VEGF and their response to vascular targeting agents

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Abstract: In this work we studied the functional differences between the microcirculation of murine tumours that express only single isoforms of vascular endothelial growth factor-A (VEGF), namely VEGF120 and VEGF188, and the effect of VEGF receptor tyrosine kinase (VEGF-R TK) inhibition on their functional response to the vascular disrupting agent, combretastatin A-4 phosphate (CA-4-P), using measurement of red blood cell (RBC) velocity by a 'keyhole' tracking algorithm. RBC velocities in VEGF188 tumours were unaffected by chronic treatment with a VEGF-R tyrosine kinase inhibitor, SU5416, whereas RBC velocities in VEGF120 tumours were significantly increased compared to control VEGF120 tumours. This effect was accompanied by a reduced tumour vascularisation. Pre-treatment of VEGF120 tumours with SU5416 made them much more resistant to CA-4-P treatment, with a RBC velocity response that was very similar to that of the more mature vasculature of the VEGF188 tumours. This study shows that vascular normalization following anti-angiogenic treatment with a VEGF-R tyrosine kinase inhibitor reduced the response of a previously sensitive tumour line to CA-4-P.

Keywords: Red Blood Cell Velocity, Microcirculation, Tumour Vasculature, Keyhole Tracking Model

1. Introduction

Vascular endothelial growth factor-A (commonly known as VEGF[1]) is the predominant protein involved in normal and tumour angiogenesis, a process crucial for the supply of oxygen, nutrients and growth factors to tumours [2]. The VEGF gene undergoes alternative splicing to produce multiple functional isoforms, such as 121, 165 and 189 in human [3] and 120, 164, and 188 in the mouse [4]. Fibrosarcoma cell lines have been developed to express single isoforms of VEGF and used to study their roles in tumour vascularisation. Tumours expressing only VEGF120 are well vascularised, but present with immature blood vessels that are fragile and leaky and the tumours are prone to haemorrhage, particularly when grown in the dorsal skin-flap window chamber model, where vessels struggle to penetrate beyond the tumour edge [5]. VEGF188 expressing tumours have a lower vascular volume than VEGF120 tumours, but the blood vessels are much more mature, showing good levels of pericyte coverage and less haemorrhage [5].

Vascular disrupting agents (VDAs) are a group of compounds that selectively target tumour vasculature and cause a devastating collapse of blood flow in solid tumours, culminating in tumour cell death. It is thought that VDAs preferentially target immature tumour blood vessels, with most evidence derived from VDAs that are tubulin-binding and endothelial cytoskeleton disrupting [6]. Disodium combretastatin A-4 3-*O*-phosphate (CA-4-P) is the lead tubulin-binding VDA and studies have shown that VEGF120 tumours that present with immature vessels showed reduced vascular and growth response to CA-4-P, compared with VEGF188 tumours that possess a more mature vascular network [5].

The VEGF receptor family of proteins belong to the protein tyrosine kinases that regulate multiple cellular processes that contribute to tumour development and progression, including cell proliferation, differentiation and cell survival [7, 8], and many human tumours exhibit aberrant tyrosine kinase activity that drives their growth. Tyrosine kinase inhibitors therefore represent attractive anti-tumour or anti-vascular targets in the clinic [7, 9]. Several multi-target tyrosine kinase inhibitors that include targeting VEGF receptors have reached clinical practice or are in clinical trial [10, 11]. Under some dosing strategies, treatment with VEGFR-2 receptor antibodies or small molecule inhibitors can result in tumour vascular 'normalisation', whereby vascular morphology is altered and blood flow is improved over a limited time-frame [12-14]. However, very little is known about how chronic tyrosine kinase inhibitors affect the function and morphology of the surviving blood vessels in tumours, and how this affects subsequent treatment stratagems. We sought to investigate the effects of tyrosine kinase receptor inhibition on the development of tumours expressing single isoforms of VEGF, and their subsequent response to CA-4-P treatment.

2. Methods

All experiments were conducted in accordance with the United Kingdom Home Office Animals (Scientific Procedures) Act 1986 and with local ethical approval. CA-4-P was kindly provided by Professor GR Pettit, Arizona State University.

2.1 VEGF isoform cell lines and tumours

Development of cell lines is described previously [5], but briefly, primary mouse embryo

fibroblasts expressing only single isoforms of VEGF (120, 164 or 188) or all isoforms were isolated from 13.5 days post-coitum (dpc) embryos produced by heterozygous breeding pairs of single VEGF isoform-expressing mice on a Swiss background. Fibroblast cultures were genotyped, as described [15], to identify wild-type samples and those homozygous for the $Vegfa^{120}$, $Vegfa^{164}$ or $Vegfa^{188}$ allele. Fibroblasts were immortalised and oncogenically transformed by retroviral transduction with SV40 and HRAS (*h*-ras) [16, 17]. In this study we used only VEGF120 and 188 tumours as they present with the widest functional and morphological diversity for comparison.

2.2 Window Chambers

All surgery was carried out on male severe combined immunodeficiency (SCID) mice (12-16 week-old, 28-32g) under general anaesthesia using intraperitoneal (i.p) injection of fentanyl citrate (0.8mg•kg^{-1}) and fluanisone (10mg•kg^{-1}; Hypnorm, Janssen Animal Health) and midazolam (5mg•kg^{-1}; Hypnovel, Roche, Welwyn Garden City, UK). Animals were kept warm during surgical procedure, and aseptic technique was used throughout. Surgical procedures are similar to those described previously [18]. Briefly, animals were shaved, depilated and a 15 mm diameter circular area of dorsal skin was removed, as well as the subcutaneous (s.c.) fat and connective tissue of the opposing skin layer, leaving the exposed panniculus muscle. An aluminium window chamber (total weight ~2g), designed to hold two parallel glass windows, was implanted onto the dorsal skin. A tumour fragment (~0.5 mm in diameter, either VEGF120 or 188) from a donor animal was implanted onto the exposed panniculus muscle and the chamber was closed with a glass window to provide a depth of ~200 µm for tumour growth. Sub-cutaneous dextrose saline and intraperitoneal buprenorphine (Vetergesic) were used to aid recovery and animals were kept in a warm room, 28-30 $^{\circ}$C, until the day of experiment. Animals were grouped for treatment and received either the VEGF-R tyrosine kinase inhibitor, SU5416 (50 mg•kg^{-1}, at a concentration of 25 mg•ml^{-1}

subcutaneously) or vehicle control (50 µl of DMSO per mouse) as used previously [19] starting at day 3 after implant, and then given every 4th day until further experimentation.

2.3 Red Blood Cell labelling

Donor red blood cells (RBC) were obtained via cardiac puncture from donor male SCID mice and labelled with the fluorescent dye 1,1', di-octadecyl-3,3,3'3'tetramethylindocarbocyanine perchlorate (DiI) (Molecular Probes, Cambridge Biosciences, UK) for the measurement of RBC velocity in the tumour vasculature. The labelling method has been published previously [20], but briefly the RBCs were separated from blood plasma and white blood cells by centrifugation and the isolated and washed RBCs incubated with DiI for 30 mins. 25 µg of DiI was used per 50 µl of packed RBCs. After incubation the RBCs were washed and resuspended in phosphate buffered saline (50 $\mu l \cdot ml^{-1}$ RBC-DiI complex). The labelled cells were kept for 3 days at $4^{\circ}C$.

2.4 Intravital Microscopy

Intravital microscopy permits the observation of RBC movement in microvessels of small animals under both normal and pathological conditions [21]. The analysis of RBC velocities is used in many areas of research, to measure the responsiveness to vasoactive drugs [22] or response to vascular disrupting agents [23]. Approximately 7-11 days after surgery, when control or SU5416 treated tumours reached ~3.0 mm in diameter, mice were given 0.2 ml (i.v.) of the RBC-DiI complex and treated with a single dose of the vascular disrupting agent, CA-4-P ($30 mg \cdot kg^{-1}$, iv at a concentration of $3\ mg \cdot ml^{-1}$ in 0.9 % NaCl). Tumours were monitored immediately before and at 24 hrs after treatment using a Nikon Eclipse E600FN fluorescence microscope with modified stage, under epi-fluorescence illumination (100 W mercury arc lamp). Videos were taken for 60s at each time point

for analysis of RBC velocity. Fluorescence was set up to excite at 550nm and detect the emissions at 565nm from the labelled RBCs using a custom-made fluorescence cube (Nikon, UK).

2.5 Velocity Analysis

RBC velocities were calculated from data recorded using a x20 objective with the same region of interest used at each time point. Velocities were calculated using a keyhole tracking algorithm performed in Matlab, as described previously [24].

Briefly, the tracking algorithm consisted of three main steps. Firstly, *Pre-processing* transformed the videos into a sequence of binary images that contained segmented foreground objects (RBCs). The number of operations and time required to process was reduced by averaging data from contiguous pixels. Intensity inhomogeneity was corrected [25] and artefacts were removed with a mean image of the temporal sequence. The centroids of the RBCs were determined together with the distances that separated them from neighbours, if any. Secondly, *Tracking* linked the objects in contiguous frames to form the tracks by means of a 'keyhole model', which predicted the most probable landing position of a 'child' RBC at time $t+1$ from the position of the 'parent' at times $t-1$ and t. Two regions of probability where the RBC is most likely to land were defined: a narrow wedge (60° wide) oriented towards the predicted landing position, and a truncated circle (300°) that complements the wedge; together they resemble a keyhole. All segmented RBCs were examined for possible parent-child relationships and, where these occurred, they formed a series of tracks of different lengths. The third step, *Post-processing,* removed links in tracks that could have resulted from noise, and joined sections that were considered to be split sections from a single track. The same keyhole model was used analysing backward movement i.e. the grandchild (t+2) and the child (t+1) were used to generate a keyhole at time (t). If the RBC of a previous time point was found to

land inside the keyhole, it remained as part of the track, otherwise it was removed. Finally outliers were removed: those tracks whose average velocity exceed 3 times the standard deviation from the mean average velocity of the whole distribution were discarded.

Velocity was calculated at $\mu m \bullet s^{-1}$ from the number of video frames taken for each red cell to travel between two points of measured distance. The average was weighted according to the length of each track to avoid biasing from small tracks. Fig. 1a shows a sample fluorescence image where the bright spots describe RBCs and coloured lines describe the tracks that were detected at that frame (the tracks spanned several frames). Fig. 1b shows all the tracks detected for a video sequence, with time as the vertical axis, and Fig 1c shows the detected RBCs as white blobs circled in red. This process enables rapid and automated calculation of velocity for many red blood cells within a particular time-frame.

2.6 Statistics

Statistical analysis was performed using SPSS version 11.0.2 for the Apple Macintosh. Data are presented as mean ± standard error of the mean. Student's unpaired *t*-test was used to test for significant differences between two groups. Differences were considered significant at $P<0.05$.

3. Results

As expected, repeat administration of the VEGF-R tyrosine kinase inhibitor, SU5416, during tumour growth inhibited vascularisation of both VEGF120 and VEGF188 tumours, as illustrated in Fig. 2. SU5416 also significantly increased red blood cell (RBC) velocity in the VEGF120 tumours compared with vehicle-treated controls (Fig. 3a). However, there was no significant effect of

SU5416 in VEGF188 tumours, where RBC velocity was already high (Fig. 3b). Comparison of Figs. 3a and 3b shows that SU5416 pre-treatment increased RBC velocity in the VEGF120 tumours to similar levels as in the vehicle pre-treated VEGF188 tumours. Differences in response to CA-4-P ± SU5416 were also found between the tumour types (Figs. 3a,b). Specifically, without SU5416 pre-treatment, RBC velocity in VEGF120 tumours was significantly decreased 24h following a single dose of CA-4-P, whereas RBC velocity in VEGF188 tumours was unaffected at this time, consistent with our previous findings [5]. However, SU5416 pre-treatment significantly reduced the effect of CA-4-P in the VEGF120 tumours, with a less than 30% reduction in RBC velocity that was not significantly different from the pre-CA-4-P time point (P = 0.25; Fig. 3a). In contrast, SU5416 pre-treatment did not affect response to CA-4-P of the already resistant VEGF188 tumours (Fig. 3b).

4. Conclusions

Treatment with CA-4-P caused significant reduction in RBC velocity, with no recovery over 24 hours, in VEGF120 tumours, whereas VEGF188 tumours were much more resistant to the effects, in line with previous data [5]. However, pre-treatment with the tyrosine kinase VEGF receptor inhibitor, SU5416, modified the vasculature of VEGF120 tumours, such that RBC velocity was increased and response to CA-4-P was reduced. SU5416 had little or no effect on RBC velocity of VEGF188 tumours or their response to CA-4-P at the studied time points.

These data support the notion that expression of VEGF188 in tumours confers a level of protection from the effects of CA-4-P. Previous results have shown that these tumours have a more mature stable vasculature, which likely contributes to this protection [5]. The immature blood vessels in VEGF120 tumours are rapidly broken down. Tyrosine receptor kinase inhibition with SU5416

significantly increased RBC velocity in individual vessels of VEGF120 tumours, which was accompanied by decreased vascularisation. SU5416 and similar agents were designed to block tumour angiogenesis by interfering with VEGF-mediated effects such as endothelial proliferation and migration. However, the current results support existing evidence that a sub-optimal anti-angiogenic effect can result in a rarefied tumour vascular bed, which nevertheless is efficiently perfused and capable of sustaining tumour growth (vascular normalization) [14]. In addition, we show that pre-treatment with the tyrosine receptor inhibitor reduced the efficacy of a vascular disrupting agent (VDA), which is designed to target the established tumour vasculature. It is impossible at this stage to determine what changes have taken place to incur this protection from the vascular disrupting effects of CA-4-P. Further studies are required to investigate the vascular morphological changes that take place as a result of tyrosine kinase inhibition. It is possible that there is an increase in pericyte coverage of these vessels, which would imply improved support and resistance against CA-4-P treatment. At the time points studied, there was no difference between the treatment groups of VEGF188 tumours and their response to CA-4-P. Further analysis of the overall time course and vascular morphological studies would inform on the mechanisms by which the reported functional changes in tumour vasculature and response to CA-4-P were brought about.

Analysis of RBC velocities in tumours, using the novel algorithm described, is a useful tool in measuring functional responses to vascular-targeted treatments. The number of cells tracked with the algorithm vastly exceeds those that could be tracked manually and therefore the measurements extracted are statistically more reliable. Further analytical developments to interrogate the 3D tracks identified by the algorithm could provide a wealth of further information describing the movement of the RBC through the vasculature such as orientation, length of tracks or geometry of functional vessels.

In summary, by using a functional vascular end-point, namely RBC velocity, this study shows that vascular normalization following anti-angiogenic treatment with a VEGF-R tyrosine kinase inhibitor can have unexpected consequences for subsequent treatment outcome.

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Figure Captions

Fig 1 Tracks obtained from a video sequence. (a) A single frame showing the intensity of the RBCs and 8 tracks detected at this frame. (b) All the tracks detected in the video as a 3D plot with time as the z-axis. Colour of the tracks denotes the velocity. (c) A single frame with many detected RBCs (white blobs circled in red) with their corresponding coloured tracks.

Fig 2 Transmitted light images of VEGF120 and VEGF188 tumours following treatment with SU5416 or drug vehicle. See text for details. Note the reduction in vascularisation in the SU5416-treated tumours.

Fig. 3 Quantitative analysis of RBC velocities of tumours grown in the dorsal skin-flap window chamber. Independent t-test indicates a significant effect of CA-4-P in VEGF120 tumours at 24 hours after treatment compared with control (time 0), when there was no SU5416 pre-treatment but no effect of CA-4-P if VEGF120 tumours were pre-treated with the tyrosine kinase receptor inhibitor SU5416 (a). No significant effect of CA-4-P was detected in VEGF188 tumours, at 24 hours after treatment, regardless of whether tumours were pre-treated with SU5416 (b). * represents significance at $P < 0.05$ between 0 and 24 hrs.