Paper I
NG2 expression regulates vascular morphology and function in human brain tumours

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Introduction

A basic concept in tumour biology is that neoplastic growth depends on angiogenesis (Folkman, 1995). Glioblastoma Multi-
forme (GBM), the most common and anaplastic brain tumour in adults, is characterised by florid angiogenesis, increased vascular permeability and focal necrosis (Burger and Kleihues, 1989). Human GBMs are further denoted by diffuse infiltration of tumour cells into the brain parenchyma, where they are initially supported by the pre-existing vasculature through the process of vessel co-option (Holash et al., 1999). Later, as the tumour grows beyond a size rate limited by oxygen and nutrient diffusion, a switch in the balance between inhibitors and stimulators triggers angiogenesis (Folkman et al., 1971). Although the formation of new vessels is a constant finding in all solid tumours, animal experiments have demonstrated that different cancer cell lines implanted at the same site recruit vessels with different microvascular architecture (McDonald and Choyke, 2003). Although these cell lines do not disseminate diffusely into the brain, they are useful in studying angiogenesis in animal models. For example, early studies showed high correlation between angiogenic GBM tumours in vivo and potent angiogenic factors secreted by GBM cell lines in vitro (Klagsbrun et al., 1976). Others have shown that conditioned medium from either GBM tumour explants or established cell lines, contained potent and specific endothelial cell mitogens in vitro and in vivo, and that almost all malignant glioma cell lines express epidermal growth factor receptor (EGFr), of which the majority of GBM tumours have gene amplification or rearrangement (Sang et al., 1989). So, the angiogenesis observed in experimental studies using cell lines xenografted into nude rat brains may be highly informative of the complex communication networks between the tumour–host microenvironment during vascular formation in cancer patients. The exact mechanisms that regulate such networks are still poorly described. Since tumour angiogenesis coincides with poor prognosis, specific mechanisms that regulate vascular morphology may represent novel targets for therapy.

NG2 is a transmembrane proteoglycan that correlates with an aggressive disease course when expressed on tumour cells. It
increases tumour cell proliferation in vitro and promotes angiogenesis in vivo that predisposes animals to poorer survival outcomes by binding angiotatin (Chekenya et al., 2002b; Goretzki et al., 2000). We have previously reported NG2 expression in 13/18 human glioblastomas from patient biopsy material (Chekenya et al., 2002a). NG2 was expressed on both the tumour cells and their associated vasculature. Vessel associated NG2 was localised on both the pericyte and basement membrane components of the tumour vasculature. NG2 is also widely expressed on newly formed blood vessels during wound healing where its expression is restricted to “activated” microvascular pericytes (Ozerdem and Stallcup, 2003; Schlingemann et al., 1990). Pericytes are present at very early stages of angiogenesis, and are thought to stimulate endothelial cell recruitment and guide the formation of capillary tubes via cell–cell interactions or through the release of soluble factors (Fukushi et al., 2004; Nehls et al., 1992; Ozerdem et al., 2001, 2002; Schlingemann et al., 1990). Whether the vessels in NG2 expressing tumours are functionally different from those associated with NG2 negative tumour cells is not known. To address this issue, we used serial MRI and implemented pharmacokinetic models and multispectral data analysis to compare fundamental tumour characteristics such as vascular volume, permeability, tumour growth, peritumoural oedema and necrosis in the NG2 expressing U251 tumours (U251-NG2) and the wild type U251N human GBM tumours (U251-WT) xenografted into immunodeficient nude rat brains over time. Increased permeability is a uniform finding in tumour vasculature which gives rise to increased leakage of contrast agents. Dynamic contrast-enhanced (DCE) MRI, primarily with small-molecular-weight contrast agents (gadolinium diethylenetriamine penta-acetic acid bismethylamide; Gd-DTPA-BMA), has been extensively used to diagnose tumours and to monitor microvascular parameters. However, whilst Gd-DTPA-BMA is useful in distinguishing defective and leaky tumour endothelium from the normal counterparts, it only marginally discriminates the vasculature of distinct tumour types (Daldrup et al., 1998; Su et al., 1998). Therefore, we also used the high-molecular-weight dendritic tracer Gadomer in DCE MRI for the assessment of vascular permeability and blood volumes. We also compared the spatial distribution of Gd-DTPA-BMA and Gadomer in tumour tissue to distinguish between vasculatures of different leakiness. Furthermore, we correlated the MRI data analysis of microvascular parameters as well as tumour growth, vasogenic oedema and necrosis with histological and ultra structural analyses of the tumours. The present findings demonstrate that the tumour vasculature in U251-NG2 tumours is structurally and functionally different from that in U251-WT tumours, suggesting that NG2 expressed by tumour cells may modulate vessel structure during tumour angiogenesis. Since these alterations in microvascular architecture also were accompanied by a more aggressive disease course, NG2 may represent a target for future therapies.

Methods

Cell culture

The human glioblastoma (GBM) cell line U251N (American Type Culture Collection, Rockville, MD) was transfected with the NG2 cDNA as previously described (Chekenya et al., 2002b). The cells were propagated in Dulbecco’s Modified Eagle’s (DMEM) medium supplemented with geneticin disulphate (800 g/ml) (Sigma, Dorset, United Kingdom) as described previously (Chekenya et al., 2002b). Multicellular spheroids were made by seeding 3 × 10⁴ cells in 10 ml DMEM into 75 cm² flasks base coated with agar as earlier described (Yuhas et al., 1977).

Animals

Nineteen, 6–7 week old immunodeficient nude rats (Han: mu/ mu Rowett Nude), weighing approximately 30 g, of both sexes were bred in an isolation facility at 25°C (55% relative humidity) in a specific pathogen free environment and animal husbandry protocols were maintained as previously described (Chekenya et al., 2002b). Both sexes were used in order to rule out hormonal effects on tumour growth. In accordance with ethical guidelines, this enabled us to reduce the total number of animals bred for the experiments. All animal procedures were performed in accordance with protocols approved by The National Animal Research Authority (Oslo, Norway). The maximum follow-up time was 82 days for both groups (n = 10, U251-NG2 and n = 9, U251-WT).

Intracranial implantation

The animals were anaesthetised before surgical procedures with subcutaneous injections of fentanyl/flumison/midazolam as previously described (Thorsen et al., 2003). The rats were immobilised in a stereotaxic frame (David Kopf Instruments model 900, Tujunga, CA) and a burr hole was made 1.5 mm to the right of the sagittal suture and 0.5 mm posterior to the Bregma. 10 µl of phosphate buffered saline (PBS) containing 15 spheroids was injected into the forebrain to a depth of 1.5 mm from the brain surface. Closure was effected with 3.0 ethilon suture. The animals were sacrificed by CO₂ inhalation and decapitation when neurological signs became evident. The brains were then removed and fixed in 4% paraformaldehyde (PFA).

Histological and immunohistochemical evaluations

After fixation, the tumours were coronally cut in half exactly and the two blocks were processed separately for paraaffin-wax embedding following standard procedures. With the cut edge facing the microtome, 5 µm thick tissue sections were cut in planes corresponding to those utilised in the MRI evaluations, and placed on poly-l-lysine coated glass slides. Tissue for histopathological examination was stained with Harris Haematoxylin and Eosin (H&E; Merck, Darmstadt, Germany) and examined with a Nikon Eclipse (E600) light microscope. Images were captured with a Nikon DXM1200 digital camera (Nikon Corporation, Tokyo, Japan). Indirect immunohistochemistry was performed using the EnVisionSystem, horse-radish peroxidase (HRP) and 3’-diaminobenzidine (DAB) (Dako, Glostrup, Denmark) method, as described in the manufacturer’s protocol. Primary antibodies used to label formalin fixed, paraaffin embedded tissue were: rabbit anti-human von Willebrandt Factor (vWF, diluted 1/500 in TBS; Dako), anti-human Vimentin (diluted 1/500 in TBS, Dako), rabbit anti-rat NG2 (diluted 1/500 in TBS), which was a generous gift from W.B. Stallcup, The Burnham Institute, San Diego, CA. Epitope retrieval was performed with Proteinase K (Dako) for vWF and citrate buffer (pH 6.5) for NG2 and Vimentin. Tissue sections immunostained using DAB and Fast Red were visualised and analysed using the image analysis system LUCIA, version 4.21 (Laboratory Imaging Ltd., Prague, The Check Republic). For immunocyto-
T1-maps were created from five T1-images with consecutive TR times. The interslice distance is shown in brackets. Post-Gd-DTPA-BMA T1 3 407/13 256/C2 DCE 3 122.5/5 64/C2 T1 3 100/8.8 64/C2 T1 3 300/8.8 64/C2 T1 3 500/8.8 64/C2 T1 3 1500/8.8 64/C2 T1 3 2000/8.8 64/C2 4 cm. The animals were continuously anaesthetised with 1–2% isoflurane in 70/30% N\textsubscript{2}/O\textsubscript{2} through a facemask. Table 1

![Table 1](image)

The imaging sequences included in the protocol

<table>
<thead>
<tr>
<th>Sequence</th>
<th>FOV [cm]</th>
<th>TR/TE [ms]</th>
<th>Acquisition matrix</th>
<th>NEX</th>
<th>Turbo factor</th>
<th>Slice thickness [mm]</th>
<th>No. of slices</th>
</tr>
</thead>
<tbody>
<tr>
<td>T2 (RARE)</td>
<td>3</td>
<td>6000/60</td>
<td>128 × 128</td>
<td>4</td>
<td>8</td>
<td>1 (1.2)</td>
<td>11</td>
</tr>
<tr>
<td>FLAIR</td>
<td>3</td>
<td>2500/60</td>
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<td>2</td>
<td>1 (1.2)</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>3</td>
<td>2000/8.8</td>
<td>64 × 64</td>
<td>3</td>
<td>(3.5)</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>3</td>
<td>1500/8.8</td>
<td>64 × 64</td>
<td>3</td>
<td>(3.5)</td>
<td>3</td>
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<tr>
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<td>500/8.8</td>
<td>64 × 64</td>
<td>3</td>
<td>(3.5)</td>
<td>3</td>
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<tr>
<td>T1</td>
<td>3</td>
<td>300/8.8</td>
<td>64 × 64</td>
<td>3</td>
<td>(3.5)</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>3</td>
<td>100/8.8</td>
<td>64 × 64</td>
<td>3</td>
<td>(3.5)</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>DCE</td>
<td>3</td>
<td>122.5/5</td>
<td>64 × 64</td>
<td>3</td>
<td>(3.5)</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Post-Gd-DTPA-BMA T1</td>
<td>3</td>
<td>407/13</td>
<td>256 × 256</td>
<td>4</td>
<td>1 (1.2)</td>
<td>11</td>
<td></td>
</tr>
</tbody>
</table>

T1-maps were created from five T1-images with consecutive TR times. The interslice distance is shown in brackets.
general assumption that the increase in $T_1$ relaxation rate is proportional to the total tissue concentration ($C_T$) of Gd-based tracer as previously described (Donahue et al., 1997). Microvascular parameters were assessed using two different pharmacokinetic models in order to evaluate whether our results were biologically dependent or model-dependent. The dynamic MRI data were analysed using the model developed by Su et al. (1994) and the model developed by Tofts et al. (Tofts and Kermode, 1991; Tofts, 1997; Tofts et al., 1999). In Su’s model, the time course of the calculated $C_T$ can be described by the microvascular parameters fractional blood volume, $D_0$ [mM], which is related to the local blood volume, and $D_1$ [mM min$^{-1}$] which expresses the permeability or leakage from blood vessels to tissue. Finally, $C_T(t)$ is also determined by $K_2$ [min$^{-1}$], which expresses the elimination of tracer from tissue back to blood. In Toft’s model, the contribution of tracer in the plasma to the total tissue concentration is neglected and fractional blood volume is therefore not calculated. Here, permeability is defined as $K_{\text{trans}}$ [min$^{-1}$], which is dependent on vessel permeability and vessel surface. The elimination rate or wash-out of tracer from the tissue is defined as $k_{\text{ep}} = K_{\text{trans}} / v_e$ [min$^{-1}$], where $v_e$ is the extravascular extracellular space (EES).

All image processing and analyses were performed in Matlab, v. 6.5 (Mathworks, Inc, Natick, MA). The images from all channels were re-sampled to a 256 x 256 matrix using bi-cubic interpolation without any filtering of the images. Regions of interest (ROIs) for computation of permeability parameters for individual animals were drawn a priori on an image from the DCE-sequence, using a frame with maximum signal enhancement and a slice covering the centre of the tumour. Voxels with delayed or no low contrast enhancement were excluded from the ROI, and the microvascular parameters were estimated on a pixel-by-pixel basis and parametric maps were generated. Solid tumour tissue, peritumoural oedema and necrosis were manually delineated and volumes estimated using colour encoded (RGB) multispectral images computed from geometrically corresponding post-Gd-DTPA-BMA T1-weighted, T2-weighted and FLAIR images. The amount of red in the RGB image was proportional to the SI in the T2-channel, and the amounts of green and blue were proportional to the SI in the FLAIR and post-Gd-DTPA-BMA T1-channel, respectively. Thus, voxels with high SI in more than one channel got a mixed colour. The volume of interest in each slice was then calculated as the number of pixels within a ROI determined by the colour encoding and multiplied with the voxel size of 0.0244 mm$^3$ (corrected for interslice gap). Three-dimensional scatter plots derived from the multispectral images were generated to show the different features of the tumour correlated to the SI in the three channels, where blue pixels represent solid tumour tissue, red pixels oedema, green pixels CSF, and black pixels necrotic tissue. The scatter plots were also used as a control for the delineation of the different tumour characteristics in the image. The tumour volume doubling (Vd) time for each group was determined as $V_d = \frac{t_1 \log_2}{\log(V_2 / V_1)}$ (Nakajima et al., 1998), from multispectral RGB images at each time-point, where, $t$ is the time between each scanning, $V_2$ and $V_1$ are the solid tumour mean volumes for each group at the second time-point and at the first time-point, respectively. Colour encoded images generated from a post-Gd-DTPA-BMA T1-weighted image (encoded red) and a single frame from the DCE sequence (encoded green) were used for visualisation of spatial contrast enhancing patterns obtained with Gd-
DTPA-BMA and Gadomer, respectively. Images from the two channels used for comparison of contrast enhancement pattern represented corresponding slices and time-points after injection of the contrast agents.

Statistical analysis

Image data were analysed by non-parametric Mann–Whitney U tests using Graphpad Instat software version 3.05 (Graphpad Inc, San Diego, CA). The Kaplan–Meier (Kaplan and Meier, 1959) method and the log-rank test (Mantel and Haenszel, 1959) were used to generate and analyse survival curves.

Results

NG2 regulates vascular morphology

NG2 expressing tumours, U251-NG2, and the NG2 negative controls, U251-WT, were examined histologically to assess NG2's

Fig. 2. NG2 regulates vascular morphology. (A) H&E stained histological section of a highly vascular U251-NG2 tumour showing stagnant blood lakes, necrosis with picnotic nuclei (black arrowheads), magnification 200×, scale bar 60 μm. (B) Histological section of U251-WT tumour showing less blood vessels and little necrosis, magnification 200×, scale bar 60 μm. vWF immunostaining of U251-NG2 (C) and U251-WT (D) tumour showing a highly abnormal vasculature in the U251-NG2 compared to the U251-WT tumours, magnification 200×, scale bar 60 μm. (E) Transmission electron microscopic image of U251-NG2 tumour showing large, dilated thin-walled vessels lacking pericyte coverage, magnification 1500×, scale bar 10 μm. (F) Transmission electron microscopic image of a U251-WT tumour showing small capillary-like vessels with distinct basement membranes and pericyte coverage, magnification 2200×, scale bar 10 μm. (G) Higher mean ± SEM MVD counts of vWF positive endothelial cells in U251-NG2 compared to U251-WT tumours, Mann–Whitney U test, \( P = 0.001 \). (H) Higher mean ± SEM MVA in the U251-NG2 compared to the U251-WT tumours, Mann–Whitney U test, \( P = 0.001 \). Quantified as described in Methods.
role during tumour vessel formation. This comparison showed that NG2 expression caused a dramatic change in the tumour microvascular architecture as the vascular elements became both more numerous and structurally deranged. U251-NG2 tumours contained large areas with vascular ‘lakes’ composed of tightly packed erythrocytes within dilated thin-walled vessels, Figs. 2A and E. In contrast, the U251-WT tumours exhibited a more homogeneous histological architecture with small, tightly packed tumour cells. The tumour core appeared hypovascularised and the endothelial cells were mostly associated with basement membranes (Figs. 2B and F). Immunolabelling for the vascular marker vWF displayed irregular and markedly dilated vessels in the U251-NG2 tumours with numerous endothelial cell proliferations, Fig. 2C.

Table 2
Estimated microvascular parameters of U251-NG2 and U251-WT tumours

<table>
<thead>
<tr>
<th>Vascular parameter</th>
<th>U251-NG2 5 weeks (n = 8)</th>
<th>U251-NG2 7 weeks (n = 8)</th>
<th>U251-WT 7 weeks (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( D_0 ) [mM]</td>
<td>0.0288 (0.0015)</td>
<td>0.0338 (0.0019)</td>
<td>0.0238 (0.0015), *P = 0.0121</td>
</tr>
<tr>
<td>( D_1 ) [mM min(^{-1})]</td>
<td>0.0560 (0.0043)</td>
<td>0.0628 (0.0019)</td>
<td>0.0488 (0.0019), *P = 0.0061</td>
</tr>
<tr>
<td>( K_2 ) [min(^{-1})]</td>
<td>0.1432 (0.0021)</td>
<td>0.0505 (0.0011)</td>
<td>0.0572 (0.0009), *P = 0.163</td>
</tr>
<tr>
<td>( K_{\text{trans}} ) [min(^{-1})]</td>
<td>0.0076 (0.0008)</td>
<td>0.0085 (0.0009)</td>
<td>0.0059 (0.0007), *P = 0.006</td>
</tr>
<tr>
<td>( k_{\text{ep}} ) [min(^{-1})]</td>
<td>0.0936 (0.0017)</td>
<td>0.0483 (0.0015)</td>
<td>0.0560 (0.0057), *P = 0.527</td>
</tr>
</tbody>
</table>

\( D_0 \) represents fractional blood volume, \( D_1 \) and \( K_2 \) express blood–tissue permeability and elimination rate, respectively, using Su’s model. With Tofts’ model, \( K_{\text{trans}} \) expresses blood–tissue permeability and \( k_{\text{ep}} \) the elimination rate of tracer from tissue. The results are expressed as means (±SEM). Results were considered statistically significant at \( P < 0.05 \) using Mann–Whitney \( U \) test.

\( ^a \) Compared to U251-NG2 tumours at 7 weeks.

The vessels in the U251-WT tumours were smaller and less dilated, Fig. 2D. Ultrastructural examination of the NG2 tumours revealed abnormally thin-walled endothelial cells that resembled embryonic post-capillary venules, Fig. 2E. There were no obvious gap junctions and the basement membrane was very thin and disordered. There were areas of variable extension lacking pericyte coverage. These features were in striking contrast to the endothelium of the U251-WT tumours, which exhibited thicker blood vessels with a more regular endothelial lining, Fig. 2F.

The microvascular density (MVD) and the area fraction representing vascular elements (MVA) were estimated using computer-assisted imaging analysis. Quantification of MVD

Fig. 3. NG2 regulates vascular function. In panel A, post-Gd-DTPA-BMA T1-weighted image showing high and homogeneous gadolinium contrast enhancement throughout the U251-WT tumour. (B) A temporally and geometrically corresponding image from the DCE sequence of U251-WT tumour showing low Gadomer contrast enhancement in the tumour rim and no enhancement centrally in the tumour. (C) Computed colour encoded images where Gd-DTPA-BMA (red) and Gadomer (green) indicating higher leakage of small-molecular-weight contrast agent Gd-DTPA-BMA, seen as a predominance of the red colour in the RGB image. (D) Post-Gd-DTPA-BMA T1-weighted image showing high gadolinium contrast enhancement throughout the U251-NG2 tumour. (E) DCE image of U251-NG2 tumour showing high Gadomer contrast enhancement peripherally in tumour. (F) Colour encoded image where Gd-DTPA-BMA (red) and Gadomer (green) indicate high leakage of both Gd-DTPA-BMA and Gadomer contrast agents peripherally (seen as yellow in RGB image). Central parts of the tumour show a higher vessel leakiness of small molecular substances (seen as orange in RGB image). Higher vasogenic oedema in U251-NG2 tumour bearing animals compared to U251-WT controls at (G) 5 week time-point, Mann–Whitney \( U \) test, \( P = 0.040 \) and (H) 7 weeks time-point, Mann–Whitney \( U \) test, \( P = 0.018 \).
counts of vWF stained tissue sections revealed higher vessel counts in the U251-NG2 tumours compared to the U251-WT controls. Similarly, the microvascular area was significantly higher in the U251-NG2 compared to the U251-WT tumours, Mann–Whitney U test, $P = 0.0010$. These MVD and MVA counts are consistent with those obtained using other passaged tumours in nude rat brains (data not shown).

**NG2 regulates vascular function**

To clarify whether these alterations in vascular morphology were also accompanied by functional changes, we estimated microvascular parameters such as permeability, blood volume and elimination rate of tracer. There was an increase in blood–tissue permeability ($D_1$, $K_{trans}$) and fractional blood volume ($D_0$) between the two time-points (i.e. 5 and 7 weeks) in the U251-NG2 tumours (see Table 2). However, we found a decreased wash-out of tracer from tumour tissue in NG2 expressing tumours at the second time-point compared to 5 weeks. Analyses of longitudinal microvascular changes in the U251-WT tumours were not performed due to the small tumour volumes at 5 weeks. There was higher vessel leakiness ($P = 0.006$) and fractional blood volume ($P = 0.0121$) in U251-NG2 compared to U251-WT tumours at 7 weeks. In contrast, there was no significant difference in the contrast elimination rates ($K_2$, $k_{ep}$), between the U251-WT and U251-NG2 tumours, $P = 0.163$ and $P = 0.527$, respectively (Table 2). The differences in blood fraction and blood–tissue permeability also persisted when comparing tumours of similar sizes, but here, U251-NG2 tumours revealed a higher elimination rate of tracer from tumour tissue than the U251-WT tumours.

Comparison of contrast enhancement patterns using two contrast agents with different molecular weights (i.e. Gd-DTPA-BMA and Gadomer) revealed differences in their spatial distribution in U251-NG2 and U251-WT tumours of the same size. Both tumours had a more clearly delineated edge with Gd-DTPA-BMA, Figs. 3A and D. Larger regions within the solid tumour tissue in the parental tumours had little or no enhancement with Gadomer, Fig. 3B, and colour encoded images revealed more prominent Gd-DTPA-BMA permeability compared to Gadomer in U251-WT tumours, indicated by the predominance of the red channel, Fig. 3C. In contrast, U251-NG2 tumours revealed both high ring enhancement with Gd-GTNA, Fig. 3D, and larger regions within the solid tumour tissue with Gadomer contrast enhancement, Fig.

![Fig. 4. NG2 expression increases tumour growth. Indirect immunocytochemical staining for NG2 (green) in U251-WT (A) and U251-NG2 cells (D). Propidium iodide (red) was used as nuclear counterstain, magnification 630x, scale bar 60 µm. Composite H&E stained histological section from an entire rat brain showing a homogenous small U251-WT tumour (B) and a large highly necrotic U251-NG2 tumour (E), magnification 40x. (C) Immunohistochemical staining shows that NG2 is expressed only on oligodendrocyte precursor-like cells in the normal brain of U251-WT tumour bearing animals (white arrowheads, magnification 100x, scale bar 50 µm) (F) Double immunohistochemistry for NG2 (DAB, brown) and pan-human vimentin (Fast Red), showing that the tumour cells are of human origin and express NG2 in U251-NG2 tumours. Mitotic figures are seen. Haematoxylin nuclear counterstain (blue), magnification 200x, scale bar 60 µm. High volumes of U251-NG2 tumours compared to U251-WT determined by MRI at 5 weeks time-point (G), but not statistically significantly different, Mann–Whitney U test, $P = 0.065$. (H) Significant difference between U251-NG2 and U251-WT tumour volumes at 7 weeks, Mann–Whitney U test, $P = 0.019$. (I) Necrosis was not present in the U251-WT tumours and was only apparent in U251-NG2 tumours after 7 weeks. Data represent MRI determined mean volumes ± SEM [mm³].
3E. The multichannel colour encoded images also indicated that the U251-NG2 tumours had greater co-localisation of Gd-DTPA-BMA and Gadomer vessel leakiness (indicated in yellow) as seen in Fig. 3F.

Vasogenic oedema surrounding the solid tumour mass affected larger regions of the brain parenchyma in animals harbouring the rapidly growing U251-NG2 tumours than the more slowly growing U251-WT tumours at the initial scanning time-point, Fig. 3G, Mann–Whitney U test, $P = 0.040$. The vasogenic oedema was also more prominent in the U251-NG2 tumour bearing animals at the second time-point, Fig. 3H, Mann–Whitney U test, $P = 0.018$. When comparing the contribution of vasogenic oedema with the total lesion volume in animals with equivalent lesion volumes in the two groups (i.e. U251-NG2 at 5 weeks, and U251-WT at 7 weeks), peritumoural oedema contributed to 54% of the total affected brain region in the U251-NG2 tumour bearing animals and 30% in the U251-WT animals.

NG2 increases tumour growth

To study the effect of NG2 expression on tumour growth, human glioblastoma tumour spheroids derived from U251-WT (Fig. 4A) and U251-NG2 (Fig. 4D) tumour cells were implanted intracerebrally into immunodeficient nude rats and followed longitudinally using MRI. A histological comparison was done between the U251-WT (Fig. 4B) and the U251-NG2 tumours (Fig. 4E). The U251-WT tumours were highly cellular and homogeneous whereas the U251-NG2 tumours displayed numerous atypical cells with extensive areas of necrosis (Fig. 4E). Double labelling for NG2 with a pan-human anti-vimentin antibody confirmed the NG2 transgene expression in vivo by the human U251 tumour cells, Fig. 4F. In contrast, no NG2 expression was detected in the U251-WT tumours (data not shown). However, NG2 was expressed by oligodendrocyte precursor-like cells (Fig. 4C) in the normal brain.

Overexpression of NG2 in the glioblastoma cells accelerated both the development and growth of the U251-NG2 tumours compared to the U251-WT controls. All U251-NG2 tumour bearing animals developed visible tumours already at the first MRI time-point, with a mean solid tumour volume of $9 \pm 4.4 \, \text{mm}^3$ (mean ± SEM), Fig. 4G. In contrast, only four animals bearing the U251-WT tumours had MR visible tumours after 5 weeks, with a mean tumour volume of $2 \pm 0.8 \, \text{mm}^3$ (Fig. 4G). By the second scanning time-point, the U251-NG2 tumours had affected large regions of the hemisphere and caused a midline shift. The mean tumour volume in the NG2 group was $99.5 \pm 38.2 \, \text{mm}^3$ (Fig. 4H), with a mean tumour volume doubling time of 3.9 days. In contrast, estimation of the solid U251-WT tumour tissue revealed a significantly slower growth rate, with the mean tumour volume being $18.5 \pm 8.9 \, \text{mm}^3$ (Fig. 4H), Mann–Whitney $U$ test, $P = 0.019$, and a mean tumour volume doubling time of 4.5 days. Four animals receiving the U251-NG2 tumours developed central...
NG2 expression in vivo predicts shorter survival outcomes. (A) Kaplan–Meier cumulative survival analyses for U251-NG2 and U251-WT recipient animals (n = 19 animals analysed), log rank $t_{13,45}$, df = 1; $P < 0.0002$. Immunohistochemistry for Ki67 in (B) U251-WT (magnification 400×, scale bar 60 μm), and (C) U251-NG2 tumours (magnification 400×, scale bar 60 μm), showing proliferating tumour cells. (D) % Ki67 labelling index (mean ± SEM) in the U251-NG2 compared to the U251-WT tumours, unpaired Student’s t test, $t_{4,31}$, df = 4, $P = 0.0125$. Quantified as described in Methods.

necrotic foci during the scanning periods whilst none of the U251-WT tumours depicted MRI visible necrotic regions at any of the two time-points, Fig. 4I. Multispectral MRI analyses of solid tumour tissue, oedema and necrosis were estimated by using the multichannel colour-encoding method, see Figs. 5A–D.

Furthermore, the U251-NG2 tumours were significantly more tumourigenic compared to the U251-WT tumours. All NG2-tumour bearing animals developed intracranial tumours (100%) compared to 5 out of 9 (55%) animals harbouring the U251-WT tumours. To investigate whether these effects of NG2 might expedite mortality, the survival outcomes for 19 animals were analysed. The animals receiving the U251-NG2 tumours (n = 10) had a significantly shorter survival, with a median survival of 59.5 days, compared with the control animals (n = 9), with a median survival of 82 days; log rank $t_{13,45}$, df = 1; $P < 0.0002$, Fig. 6A. In line with these observations, Ki67 labelling confirmed that the NG2 positive tumours had a higher proliferative rate than their NG2 negative counterparts (Figs. 6B–D).

Discussion

The present study demonstrates that NG2 expressing tumour cells recruited host-derived neovasculature and regulated its morphology and function differently than the NG2 negative control tumours. These vascular changes coincided with a higher tumour take and a more rapid disease course suggesting that NG2 is a key regulator in the cross-talk between the tumour and its host microenvironment. Previously, we have shown that NG2 is expressed on both tumour cells and their associated vessels in biopsy tissue from glioma patients (Chekenya et al., 2002a), and that its expression increases with tumour grade. Thus, NG2 expression may provide a mechanism for stromal activation promoting tumour progression, leading towards a more aggressive disease course.

Consistent with previous studies (Chekenya et al., 2002b), histological analyses revealed that the U251-NG2 tumours were associated with increased microvessel density. However, using electron microscopy, we also found that the vascular elements had larger endothelial gaps, disrupted basement membranes and less pericyte coverage. Thus, NG2 not only mediates quantitative changes in the tumour vasculature, but also massive structural changes. Moreover, the functional implications were assessed using dynamic contrast-enhanced MRI that only measures perfused and functional vasculature. This technique provides a valuable supplement to MVD counts that do not distinguish between functional and non-functional vessels. The dynamic contrast-enhanced MRI analyses showed a higher blood supply and vessel permeability for high-molecular-weight contrast agents in the NG2 positive tumours. Therefore, our findings demonstrate that the U251-NG2 tumours are supplied by more functional vessels with bigger defects, as illustrated by the increased extravasation of Gadomer. This was also corroborated by large areas of intratumoural haemorrhage present in the tissue sections indicating increased vessel leakage (Liwnicz et al., 1987; Van den Brenk et al., 1977) that is associated with ongoing angiogenesis and vascular remodelling (Hashizume et al., 2000).

Since U251-NG2 tumour bearing animals had a shorter survival, the increased permeability seen in this group might indicate a more aggressive and malignant phenotype. Several other groups have also reported a correlation between vessel leakiness and pathological grade using dynamic contrast-enhanced MRI in animal models (van Dijke et al., 1996) and in humans (Daldrup et al., 1998; Gossman et al., 2000; Roberts et al., 2000). In contrast, Gd-DTPA-BMA enhanced more homogeneously in both tumours, due to a more rapid leakage and diffusion of the smaller particles. Thus, high- or intermediate-molecular-weight contrast agents may provide a more reliable estimate of the fractional blood volume and differences in permeability between tumours (Buckley et al., 2004). Our findings are in this respect consistent with reports from other studies (Roberts, 1997; Su et al., 1999). Furthermore, since the biodistribution of a drug within the tumour bed depends on microvascular perfusion, permeability and diffusion (Jain, 1989), the distribution studies of Gadomer in solid tumour tissue may predict the efficacy of a given therapeutic agent. Gd-DTPA-BMA on the other hand, seems more suitable for the delineation and visualisation of the viable tumour tissue.

The elimination rate of Gadomer from the tissue in the U251-NG2 was not distinguishable from that of the U251-WT tumours at 7 weeks. U251-NG2 tumours showed a decrease in wash-out rate of tracer from 5 to 7 weeks within the NG2 positive tumour group. Contrast media elimination from necrotic tissue is relatively slow (Matsubayashi et al., 2000) and may explain this finding, since histological and multispectral MRI analyses both revealed that large necrotic regions had developed in these tumours by the 7th week.

Although the U251-NG2 tumours were highly vascularised, their structurally irregular, large and frequently collapsing thin-
walled vessels are most likely due to an inability to match the rapid growth of the neoplastic cells. Although T1-weighted imaging with Gd-DTPA-BMA is used clinically for the detection of necrosis and delineation from viable tumour, the lack of enhancement does not always indicate necrosis. Therefore, we combined post-Gd-DTPA-BMA T1-weighted, T2-weighted and FLAIR sequences to limit the risk of false positive necrosis. Multisequence imaging and analyses of necrotic areas using colour encoded images correlated well with the tissue histology, and were also effective in visualising and distinguishing other fundamental tumour features such as oedema and entrapment of CSF. The U251-NG2 tumour bearing animals developed a more extensive vasogenic oedema in the surrounding brain parenchyma than animals with NG2 negative tumours, which may be explained by a disruption of the blood–brain barrier seen in the NG2 tumours. In addition, recent studies have shown that NG2 accelerates u-PA-dependent proteolysis of plasminogen to plasmin (Goretzki et al., 2000) that is involved in the degradation of the ECM and basement membranes. The break-down of these structures is accompanied by the sequestering of fluid into the interstitial tissue (Davies, 2002), which may further contribute to the vasogenic oedema.

Multisequence imaging and multispectral analysis proved to be a powerful tool in characterising the tumour phenotypes. However, when acquiring a high number of sequences, there is trade-off between acquisition time and image quality. The spatial resolution of DCE images was not ideal (i.e. 0.47 × 0.47 × 3 mm), but increasing the spatial resolution would affect the temporal resolution of the dynamic sequence using the imaging system available for this study. Moreover, increased spatial resolution would further increase the total imaging time (here 70 min), which we wanted to minimise in order to ensure that the animals survived the scanning sessions. Although, the temporal resolution of the dynamic contrast-enhanced imaging was 16.7 s in the first 30 frames where leakage of Gadomer from vessels to tumour tissue dominated, the transfer of the contrast agent is dependent on both diffusion and convection. These processes are relatively slow compared to the blood flow. Furthermore, the microvascular parameters were estimated by Gauss–Newton non-linear least-squares data fitting on a pixel-by-pixel basis. Since this showed a good line fit, it would indicate that our temporal resolution was sufficient. Moreover, the temporal resolution used in this study is in keeping with other permeability studies of small animals (Verhoye et al., 2002; Su et al., 1998).

Gd-DTPA-BMA was injected approximately 40 min after injection of Gadomer. Visual inspection of images acquired towards the end of the dynamic sequence showed wash-out of Gadomer from tumour tissue. Further post-processing of the images, by measurement of SI and tracer concentration over time, revealed that the signal intensity had almost reached the baseline values in most regions of the tumour, where a very low tissue concentration was found. The influence of Gadomer on the SI in the SE T1-weighted and on the distribution of Gd-DTPA-BMA was therefore negligible, ruling out possible interference of Gadomer on the SI in the SE T1-weighted image and tissue distribution of Gd-DTPA-BMA. Furthermore, since it was critical to minimise the time elapsed between the sequences in order to avoid animal mortality due to prolonged general anaesthesia, the wash-out of Gadomer was not empirically confirmed by a pre-Gd-DTPA-BMA SE T1-weighted sequence.

We observed a 55% tumour take in the control group, compared to 100% in the U251-NG2 tumours, which also showed higher proliferative rate as indicated by higher Ki67 labelling indices and shorter in vivo tumour volume doubling times. These results correlate well with previous findings that demonstrated that NG2 expression increased the proliferation of melanoma and glioma cells in vitro and their tumourigenicity and metastatic potential in vivo (Burg et al., 1998; Chkenya et al., 1999, 2002b). The increased tumourigenicity is likely due to induction of tumour angiogenesis. Indeed, recent studies show that proteolytically cleaved and soluble forms of the NG2 proteoglycan induce endothelial cell migration, promote the assembly of capillary networks and stimulate angiogenesis in vivo by binding to galectin-3 and α3β1 integrin receptors on endothelial cells (Fukushi et al., 2004). It has also been previously reported that NG2 interacts with angiotatin and neutralises the latter’s ability to inhibit endothelial cell proliferation (Goretzki et al., 2000), emphasising the role for NG2 in promoting angiogenesis through pericyte-endothelial cell cross-talk in the host microenvironment.

Since NG2 is expressed on the cell surface, its involvement in tumour-host interactions makes it an attractive candidate for targeted therapies. Because NG2 regulates the transition from small, poorly vascularised tumours to large, highly vascular and aggressive tumours, we and others postulate that it may be an amenable therapeutic target (Chekenya and Pilkington, 2002; Grako et al., 1999; Ozerdem, 2004; Ozerdem and Stallecup, 2004; Winkler et al., 2004).

The fact that NG2 is present on the tumour cells as well as on the host-derived neovasculature, would also allow for simultaneously targeting both the malignant and stromal cell compartments within the tumour. Since stromal cells are non-transformed, they are less likely to develop drug resistance, which is a leading cause of treatment failure. Finally, our findings demonstrate that dynamic contrast-enhanced MRI is a valuable and reliable tool to study functional vascular dynamics in brain tumours non-invasively in vivo. DCE is so far the imaging technique for evaluating tumours clinically with respect to their state of functional microcirculation, as positron emission tomography is better at resolving larger vessel structures (Padhani, 2002). Two different pharmacokinetic models both confirmed the differences in permeability between the two groups, ruling out that our findings were model-dependent and making our findings more robust. The spatial pattern of contrast enhancement with Gadomer in solid tumour tissue might indicate the biodistribution of blood-bourne molecules used in therapeutic strategies, and thus is useful for treatment planning. In addition, serial multispectral MRI may give valuable information about other fundamental tumour features such as the development and progression of necrosis or oedema, which may be prognostically relevant.

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