Angiogenesis (2007) 10:35–45
DOI 10.1007/s10456-006-9061-x

ORIGINAL PAPER

An intimate interplay between precocious, migrating pericytes and endothelial cells governs human fetal brain angiogenesis

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Received: 17 May 2006 / Accepted: 11 December 2006 / Published online: 17 January 2007
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Abstract In order to better understand the process of angiogenesis in the developing human brain, we have examined the spatial relationship and relative contributions of endothelial cells and pericytes, the two primary cell types involved in vessel growth, together with their relation with the vascular basement membrane. Pericytes were immunolocalized through use of the specific markers nerve/glial antigen 2 (NG2) proteoglycan, endosialin (CD248) and the platelet-derived growth factor receptor β (PDGFR-β), while endothelial cells were identified by the pan-endothelial marker CD31 and the blood brain barrier (BBB)-specific markers claudin-5 and glucose transporter isoform 1 (GLUT-1). The quantitative analysis demonstrates that microvessels of the fetal human telencephalon are characterized by a continuous layer of activated/angiogenic NG2 pericytes, which tightly invest endothelial cells and participate in the earliest stages of vessel growth. Immunolabelling with anti-active matrix metalloproteinase-2 (aMMP-2) and anti-collagen type IV antibodies revealed that aMMP-2 producing endothelial cells and pericytes are both associated with the vascular basement membrane during vessel sprouting. Detailed localization of the two vascular cell types during angiogenesis suggests that growing microvessels of the human telencephalon are formed by a pericyte-driven angiogenic process in which the endothelial cells are preceded and guided by migrating pericytes during organization of the growing vessel wall.

Keywords Angiogenesis · Basement membrane · Endosialin · Endothelial cells · Human brain · MMP-2 · NG2 · PDGFR-β · Pericytes

Abbreviations

aMMP-2	Active matrix metalloproteinase-2
BB	Blocking buffer
BBB	Blood–brain barrier
CD31	PECAM-1
CNS	Central nervous system
ECM	Extracellular matrix
GLUT-1	Glucose transporter isoform 1
mAb	Monoclonal antibody
NG2	Nerve/glial antigen 2
pAb	Polyclonal antibody

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PBS	Phosphate-buffered saline
PDGFR-β	Platelet-derived growth factor receptor β
VEGF	Vascular endothelial growth factor

Introduction

During early ontogenesis of the human brain, blood vessels develop according to a specific pattern of angiogenesis [1]. In the fetal telencephalon, the future cerebral hemispheres, vessels initially penetrate radially through the nervous wall, sprouting collaterals that generate progressively denser microvascular networks with subsequent formation of anastomotic tracts [2, 3]. Angiogenesis is still pronounced at mid-gestation in the telencephalon, a period when corticogenesis is in progress and the processes of lamination and neuronal differentiation require a high metabolic rate.

Angiogenesis is known to be a multistep process involving both endothelial cells and pericytes, and it also includes degradation of the existing vascular basement membrane, with subsequent basement membrane re-assembly around newly formed vessels. In the developing brain, the endothelial contribution to vessel growth has been well described. In contrast, the role of pericytes in vessel sprouting, elongation and remodelling, and the nature of the endothelial cell/pericyte/basement membrane relationships during these events, are less well-documented and still under debate. In part, this is due to the fact that immunocytochemical identification of pericytes has been difficult in the absence of reliable markers. In addition, there are divergent ideas about the function of vascular pericytes. The traditional view implicates these cells in the inhibition of endothelial cell proliferation and migration during the late stages of vessel maturation. However, there is also a body of experimental data suggesting that pericytes contribute to the early growth of microvessels by stimulating and guiding endothelial cells [4–13].

This study, which focuses on the interactions between vascular cells and their underlying basement membrane in human telencephalon during cerebral cortex angiogenesis, takes advantage of NG2 and endosialin as newly recognized markers of activated pericytes. The transmembrane proteoglycan NG2 specifically identifies pericytes in angiogenic microvasculature during pre- and postnatal development, as well as in tumours, in granulation tissue and in pathological corneal and retinal angiogenesis [14–19]. NG2 is expressed in microvessels of normal mammalian brain, and is highly up-regulated in brain tumours [15, 20–22]. The ability of NG2 to reveal the angiogenic microvasculature has also been utilized for quantitatively evaluating the extension of the fetal microvascular network involved in the angiogenic process. Endosialin, a transmembrane glycoprotein expressed primarily by stromal fibroblasts, has recently been reported to colocalise with NG2 on pericytes of neoangiogenic tumour vessels [23]. The platelet-derived growth factor receptor β (PDGFR-β), an established early marker of activated pericytes [11, 24], has been additionally utilized to confirm the activated/angiogenic state of the telencephalon microvessels. Microvascular endothelial cells of the early human fetal telencephalon express molecules characteristic of the BBB phenotype, such as GLUT-1, which regulates the entrance of glucose into the brain, and the tight junction proteins occludin and claudin-5 [25–27]. Together with the pan-endothelial marker CD31 (PECAM-1), GLUT-1 and claudin-5 were therefore utilized as BBB-specific endothelial markers.

The angiogenic process requires degradation of the vascular basement membrane, which underlies endothelial cells and pericytes and impedes vessel sprouting [28]. Among the key components of the brain vessel basement membrane, collagen type IV has previously been demonstrated to be involved in the angiogenesis process [29] as well as in the formation of endothelial tight junctions [30, 31]. Since MMP-2 is known to be a primary basement membrane-degrading enzyme during CNS angiogenesis [32–36], documentation of its localization in relation to basement membrane disruption and sites of endothelial cell-pericyte interplay may provide novel insights into the dynamics of vessel sprouting in the developing human CNS.

Materials and methods

Processing of brains

Autopsy specimens of telencephalon were collected from five human fetuses spontaneously aborted at 22 weeks of gestation and with no history of neurological pathologies. The sampling and handling of human specimens conformed to the ethical rules of the Department of Pathology, Medical School, University of Bari, Italy, and approval was gained from the local Ethics Committee of the National Health System in compliance with the principles stated in the Declaration of Helsinki. The fetal age was estimated on the basis of the crown-rump length and/or pregnancy records (counting from the last menstrual period).

The dorso-lateral walls of the telencephalic vesicles (future cortex of cerebral hemispheres) were dissected (≤0.5 cm in thickness), halved, and fixed for 3 h at 4°C by immersion in 2% paraformaldehyde plus 0.2% glutaraldehyde solution. Specimens were then washed in phosphate-buffered saline (PBS, pH 7.6) and stored at 4°C. Halves of the telencephalon samples were embedded in paraffin and serially sectioned (5 μm). Sections were collected on Vectabond™ treated slides (Vector Laboratories Inc.; Burlingame, CA, USA). The remaining blocks of tissue were sectioned at 20 μm using a vibrating microtome (Leica Microsystem; Milan, Italy). Vibratome sections were collected in PBS and stored at 4°C.

Antibodies

Six rabbit polyclonal antibodies (pAbs) raised against intact and recombinant fragments of rat NG2 [37] were tested on sections of paraffin-embedded tissues to identify the reagents that most effectively recognize human NG2 in these types of specimens. After rehydration in PBS, the sections were treated with 3% hydrogen peroxide for 10 min at room temperature (RT) to quench endogenous peroxidase activity, preincubated with blocking buffer (BB; PBS, 1% bovine serum albumin, 2% fetal calf serum) for 30 min at RT, then incubated overnight at 4°C with primary antibodies at various dilutions. The sections were immunostained using the labelled streptavidin-biotin peroxidase method (DAKO LSAB Kit; Dako, Milan, Italy) and the reaction was visualized with the substrate-chromogen 3-amino-9-ethylcarbazole (AEC, Vector). Finally, the sections were counterstained with hematoxylin and coverslipped in aqueous mounting medium (Glycergel, Dako). Of the different antibodies, an affinity-purified pAb against the central NG2/D2 domain was found to give the strongest and most specific signal. The antibodies NG2/D3, NG2/EC, 553 and N143.8 gave weaker signals, while the H28/B5 antibody was non-reactive. The NG2/D2 antiserum was subsequently used to label the vibratome sections at an optimal dilution of 1:50. Other primary antibodies utilized were: the affinity purified mouse monoclonal antibody (mAb) anti-endosialin (CD248, subclone B1/22.4; 1:250 dilution; a gift from Prof. C. M. Isacke, The Breakthrough Breast Cancer Research Centre, London, UK) and the mouse mAb anti-phospho-platelet-derived growth factor receptor β (PDGFR-β) (1:50 dilution; Tyr751; Cell Signalling Technology; Beverly, MA, USA); the endothelial markers mouse mAb anti-CD31 (1:100 dilution; Dako), rabbit pAb anti-GLUT-1 (1:50 dilution; Chemicon; Temecula, CA, USA), and mouse mAb anti-claudin-5 (1:50 dilution; Zymed Lab.; CA, USA); mouse mAb and rabbit pAb anti-collagen type IV (1:50 dilution, Dako; 1:100 dilution, Acris Antibodies, Hiddenhausen, Germany); the rabbit pAb anti-MMP-2 active form (aMMP-2) (1:50 dilution; Chemicon).

Immunohistochemistry and confocal microscopy

Double immunolabellings were performed with the following combinations of antibodies: NG2/CD31, NG2/claudin-5, endosialin/GLUT-1, NG2/endosialin, NG2/collagen IV, endosialin/collagen IV, NG2/PDGFR-β, CD31/collagen IV, aMMP-2/collagen IV. Vibratome sections were collected on polylysine-treated slides (Menzel GmbH, Germany), dehydrated for 10 min at RT, incubated: (1) in BB, for 5 min; (2) in 0.5% Triton X-100 in PBS, for 30 min; (3) in the primary antibodies suitably diluted in BB, overnight at 4°C; (4) in a mixture of the appropriate fluorochrome-conjugated secondary antibodies goat anti-mouse and anti-rabbit IgGs (1:200 dilution in BB, Alexa Fluor™ 488 and 568; Molecular Probes), for 1 h. The sections were washed for 10-min × 3 in PBS between each step, counterstained with TO-PRO-3™ (1:10K dilution; Molecular Probe), mounted in Vectashield (Vector), and finally sealed with nail varnish. Negative control sections were prepared by (a) omitting one of the primary antibodies, (b) pre-adsorbing the primary antibodies with an excess (20 nM) of the pure antigen when available, (c) mismatching the secondary antibodies. In all cases, the results of these negative controls confirmed the specificity of the antibody staining. Sections from all the five examined fetuses were viewed on a Leica TCS SP2 (Leica, Germany) confocal laser scanning microscope using a 63× objective lens with either 1× or 2× zoom factors. Images were acquired according to a sequential scan procedure. Confocal images were taken and analysed at 350-nm intervals through the z-axis of the section. Individual optical planes and multiple serial optical sections were digitally recorded, and stored as TIFF files using Adobe Photoshop software (Adobe System, CA, USA), then representative images from the examined samples were chosen for figure editing.

Computerized morphometric analysis

As a preliminary to the morphometric analysis, the histological homogeneity of the telencephalon laminar configuration in the five selected fetuses was examined on Nissl-stained paraffin sections [38] and morphometry was carried out on four 20-μm thick vibratome sections for each fetus collected from areas equivalent to those of the paraffin sections. These sections were

double stained with anti-collagen IV and anti-NG2 antibodies according to the fluorescence immunohistochemistry method described above and examined under a Leica DMI6000B CS microscope (Leica Microsystem) connected to a CCD/KAI-2000 video camera (SPOT Insight Color, Diagnostic Instruments, Mich., USA). Morphometry was performed on the selected sections with the image analysis system VIDAS 2.5 (Kontron Elektronik GmbH; Eching, Germany) by a macro (i.e. a semiautomatic sequence of commands) developed in our laboratory to define and execute image processing and measurement. Briefly, 90 randomly chosen fields for each group of four sections – total examined fields $n = 450$ – were acquired at 40× magnification (field area 29,867 μm^2); on each field, the vessel profiles were digitalized alternatively from collagen IV and NG2 fluorescence immunosignals, discriminated by a thresholding modality, transformed in a binary image, and submitted to an automatic measurement to obtain the vessel area (μm^2), and the percentage of the vessel area on the area of the field (%). These values were calculated and expressed as means and standard deviations (SD) for each of the five examined fetuses. The mean differences between fetuses were assessed by analysis of variance followed by the Bonferroni test and *t*-test ($P \leq 0.05$) and, because no statistically significant differences were established, the results were pooled together as total mean and percentage of vessel area for both NG2 and collagen IV microvessels. On the same sections, growing microvessels ($n = 60$) were identified by NG2 and the diameter (μm) at their initial and terminal segment was measured together with the diameter of the vessel of origin; the results were expressed as mean value ± SD.

Results

Identification of endothelial cells, pericytes, and basement membrane in the microvasculature of the telencephalon

Anti-NG2 and anti-endosialin antibodies allowed reliable identification of microvascular pericytes in double immunoreactions performed with the endothelial markers (CD31, claudin-5, GLUT-1) and collagen IV (Fig. 1). CD31, claudin-5 and GLUT-1 specifically outlined the endothelial cells of the radial telencephalic microvessels and their collaterals (Fig. 1A–C). CD31 and GLUT-1 reactivities were almost continuously distributed along the endothelial lining (Fig. 1A, C), whereas claudin-5 staining was punctate or linear, and appeared to be mainly restricted to junctional profiles of the endothelial cells (Fig. 1B). NG2 and endosialin specifically stained only the pericyte layer that was tightly apposed to the endothelial lining (Fig. 1A, C). On NG2 and endosialin double staining the two markers colocalised on pericytes, however, the staining pattern did not perfectly overlap: NG2 expression was continuous whereas endosialin labelling was interrupted by unstained gaps (Fig. 1D–F; see also A–C). The pericyte nature of the cells labelled by the anti-NG2 and anti-endosialin was also revealed by NG2/collagen IV and endosialin/collagen IV staining. The results showed the NG2- and endosialin-reactive pericytes embedded in a collagen IV basement membrane (Fig. 1G–N). NG2 colocalised with the thick, continuous collagen IV basement membrane (Fig. 1G–I), whereas the endosialin discontinuous pattern corresponded to the pericyte body and their finger-like processes (Fig. 1L–N).

Computer-aided morphometry, which aimed to evaluate the activated/angiogenic state of the telencephalon microvasculature labelled by NG2/collagen IV demonstrated that the mean value of the area covered by collagen IV microvessels (1,592.65 ± 923.31 μm^2; $n = 450$) corresponded to 5.32% of the field area (29,867 μm^2), whereas the mean value of the area covered by NG2 microvessels (1,404.99 ± 882.45 μm^2; $n = 450$) corresponded to 4.70%, indicating that a very large proportion (88%) of the telencephalon microvessels is provided with NG2 pericytes.

Distribution patterns of endothelial cells, pericytes, and basement membrane in growing, immature microvessels of the telencephalon

Double immunoreactions performed with NG2 and endosialin, the endothelial markers CD31 and claudin-5, and collagen type IV, also allowed analysis of growing, immature microvessels (Fig. 2). These were recognized as vascular branches of various length stemming from the main vascular trunks of the telencephalon (i.e. radial vessels and their collaterals) and terminating either freely within the neuropil (Fig. 2A, B; E, F) or by anastomosis with neighbouring microvessels (Fig. 2C, D, G, H). Unlike the vessels of origin characterized by a mean diameter of 7.94 μm ± 1.19 ($n = 60$), the growing microvessels showed a large point of origin with a diameter of 3.20 μm ± 0.63 ($n = 60$) and progressively diminished in calibre, frequently terminating with a thin, solid tip of 1.50 μm ± 0.35 ($n = 60$). These immature vessels were composed of NG2- and endosialin-reactive pericytes. The NG2 labelling extended in uninterrupted fashion from the initial to the final portion of the microvessel

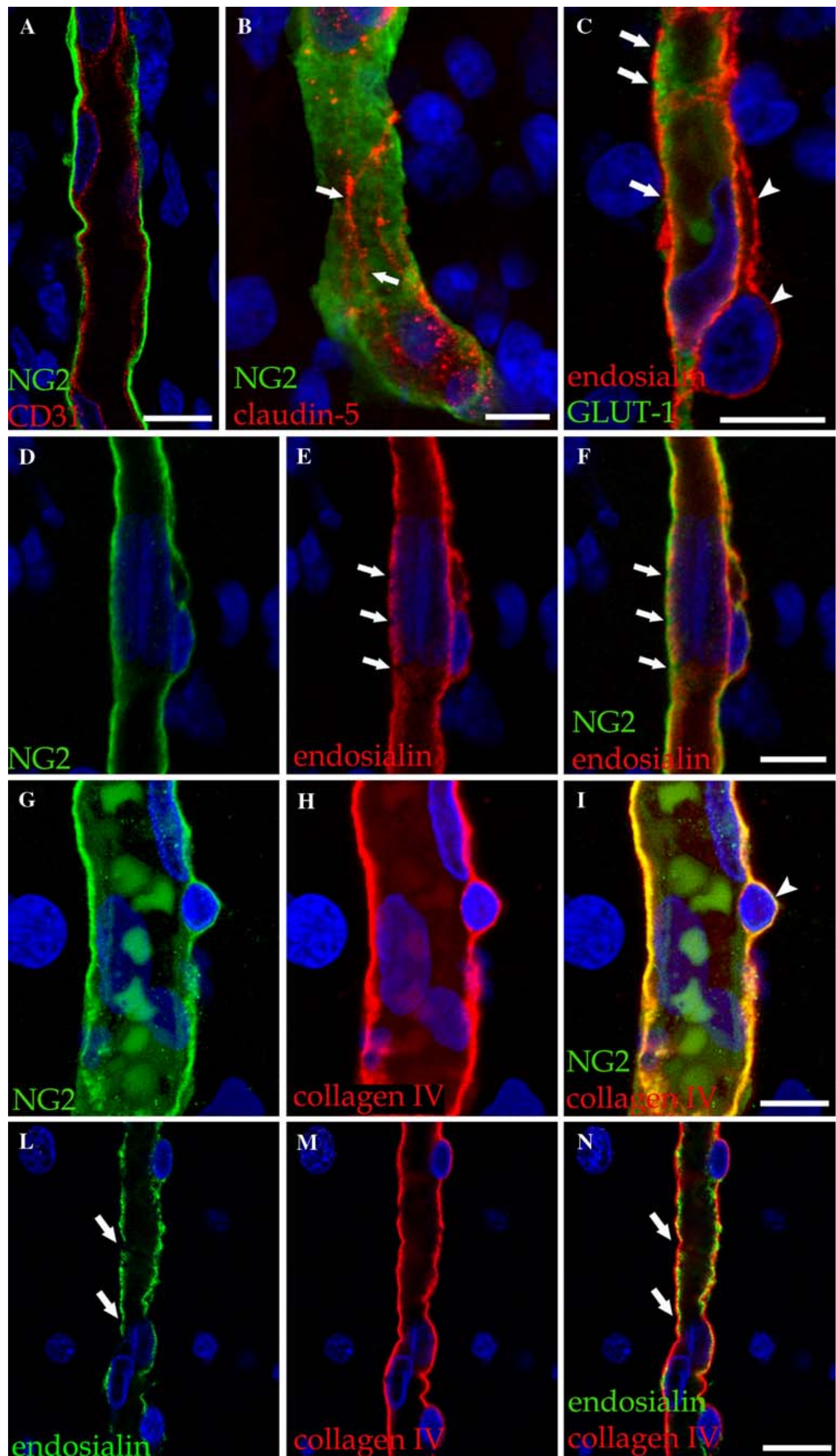
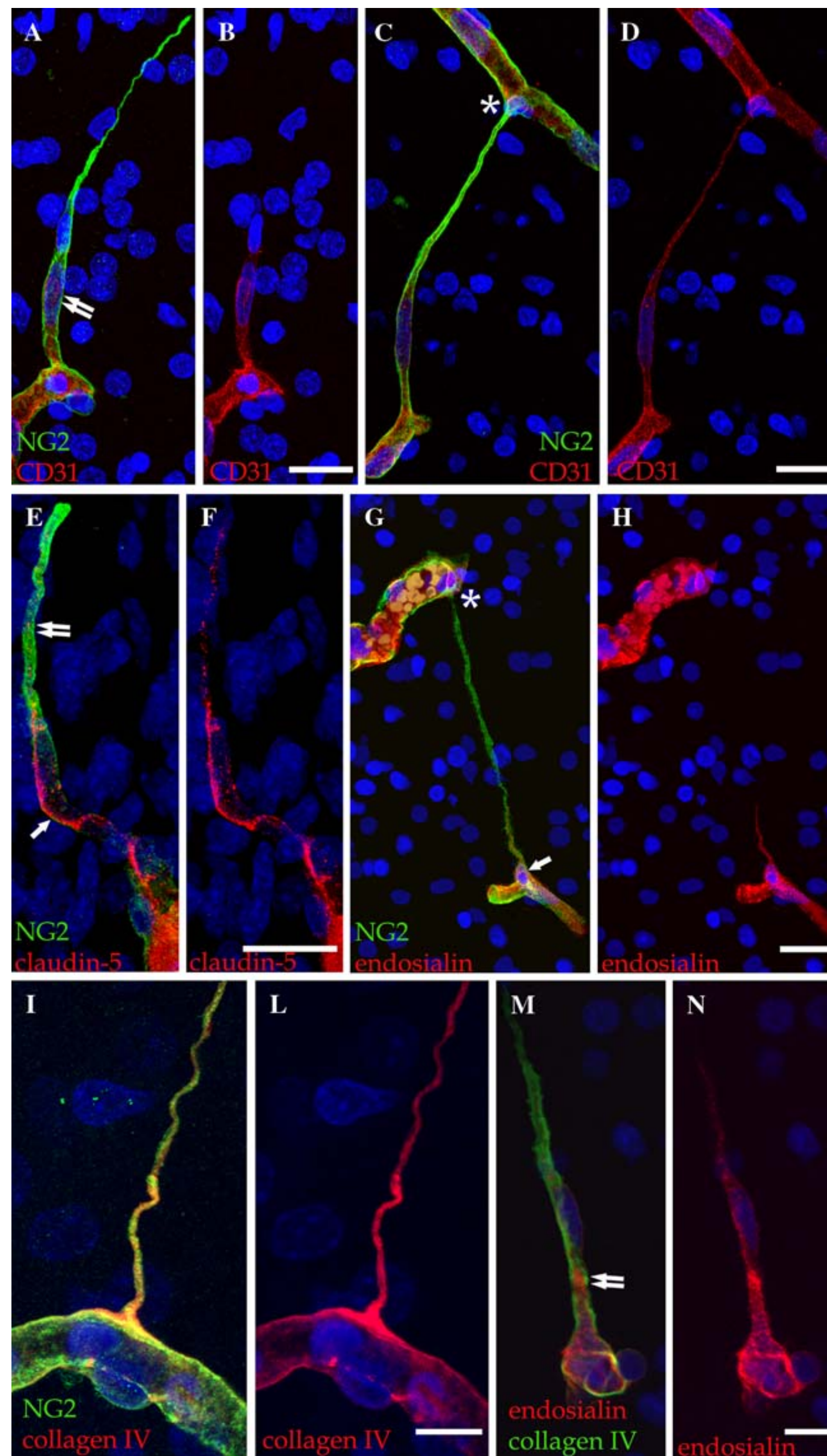


Fig. 1 **(A–N)** Confocal images of microvessels in 22-week human telencephalon. **(A–C)** Double immunolabelling with NG2/CD31, NG2/claudin-5, and endosialin/GLUT-1. CD31 (**A,** red), claudin-5 (**B,** red), and GLUT-1 (**C,** green) reactive endothelial cells are clearly distinguished from NG2 (**A**, **B**; green) and endosialin (**C**, red) labelled pericytes. The NG2 sleeve completely covers the endothelial lining (**A**, **B**), which in **B** is revealed by the junction-specific localization of claudin-5 (arrows). In **C,** the endosialin staining outlines a pericyte cell body (arrowheads) and appears discontinuous along the opposite vessel front (arrows). (**D**–**F**) NG2 (**D**) and endosialin (**E**) double immunolabelling; the merged image (**F**) reveals the incomplete overlap of the two markers and several endosialin-negative gaps (arrows; see also arrows in **E**). (**G**–**N**) NG2/collagen IV (**G**–**I**) and endosialin/collagen IV (**L**–**N**) double immunolabellings. NG2 and collagen IV stainings completely colocalise (merged image, **I**) and outline a pericyte cell body (**I**, arrowhead); the endosialin and collagen IV staining patterns do not completely overlap (merged image, **N;** arrows), due to endosialin discontinuity (**L**, arrows). Scale bars: 15 µm in (**A**); 10 µm in (**B**, **C**, **F**, **I**); 20 µm in (**N**)



(Fig. 2A, C, E, G, I), whereas the endosialin labelling was mainly observed in the initial segment of the microvessel (Fig. 2H, N). These growing NG2 microvessels contained CD31 and claudin-5 endothelial cells; the endothelial cells appeared in the initial segment of the vessels while they were not always seen to extend for the whole length of the vessel, which often appeared distally formed only by NG2 pericytes (Fig. 2A, B, C–F). The claudin-5 endothelial staining was linear and junctional in the vessel initial segments, but thereafter became punctate and cytoplasmic (Fig. 2E, F). We considered the progressive, proximo-distal change in the claudin-5 staining pattern to be an indicator of the direction of vessel growth. The newly formed

◀ **Fig. 2** (**A–N**) Confocal images of growing microvessels in 22-week human telencephalon. (**A–D**) NG2/CD31 double immunolabelling. (**A**) The merged image of an immature microvessel labelled by both NG2 and CD31 (double arrow); the NG2 pericyte tip moves toward the surrounding neuropil and the CD31 endothelium occupies only the initial microvessel segment (compare with **B**, CD31 single channel; red). (**C**) An immature NG2/CD31 labelled microvessel anastomoses with an adjacent, patent microvessel (asterisk). A CD31-reactive endothelial core fills the entire NG2 labelled microvessel (**D**, CD31 single channel; red). (**E, F**) NG2/claudin-5 double immunolabelling. The progressive endothelial invasion of an NG2 labelled microvessel is demonstrated by the claudin-5 staining, whose pattern appears junctional at the vessel initial segment (**E**, arrow) and finely punctuate distally (**E**, double arrow), as better shown by claudin-5 single channel (**F**, red). (**G, H**) NG2/endosialin labelling. (**G**) An immature microvessel labelled by NG2 (green) from its origin (arrow) to its connecting end (asterisk) and by endosialin (red) only in its initial segment (compare with **H**, endosialin single channel; red). (**I–N**) NG2/collagen IV and endosialin/collagen IV labelling. (**I, L**) Along an immature microvessel, NG2 (green) and collagen IV (red) staining patterns overlap; (**M, N**) A collagen IV labelled microvessel (green) is only partially decorated by endosialin labelling (red), which is confined to the initial vessel segment. Scale bars: 20 μm in (**B**, **D**, **F**); 30 μm in (**H**); 10 μm in (**L**, **N**)

microvessels contained a basement membrane in which collagen IV reactivity colocalised with that of NG2 and endosialin (Fig. 2I–N).

The activated/angiogenic state of the telencephalon vasculature was confirmed by NG2/PDGFR-β labelling. In the main vessel trunks, the NG2 reactive pericytes were also labelled by PDGFR-β, which showed a punctate staining pattern mainly localized along the endothelial front of pericytes, where it partly colocalised with NG2 (Fig. 3A–C). The growing microvessels showed an intense immunolabelling for the PDGF β receptor, which was revealed together with NG2 along the whole vessel length up to its tip (Fig. 3D–F).

Vascular cell-basement membrane relationships at vascular sprouting points

Other aspects of early angiogenesis were studied by co-detection of collagen IV/CD31, NG2/claudin-5 and aMMP-2/collagen IV (Fig. 4A–G). Thin endothelial processes, hereafter referred as filopodia, were seen to arise from CD31 or claudin-5 stained endothelial cells at the tip of growing vessels. These filopodia penetrated the collagen IV basement membrane and the layer of NG2 pericytes and radiated into the surrounding neuropil (Fig. 4A–C). At sprouting points, revealed by degradation of the collagen IV basement membrane, aMMP-2 positive endothelial cells and pericytes were seen to disengage from the vessel of origin and form a vascular bud (Fig. 4F). Interestingly, endothelial cells and pericytes expressing the active form of MMP-2 were observed in both parental and growing microvessels, and are possibly related to vascular remodelling (Fig. 4D, E, G).

Discussion

We have identified angiogenic pericytes in developing telencephalon microvessels on the basis of their

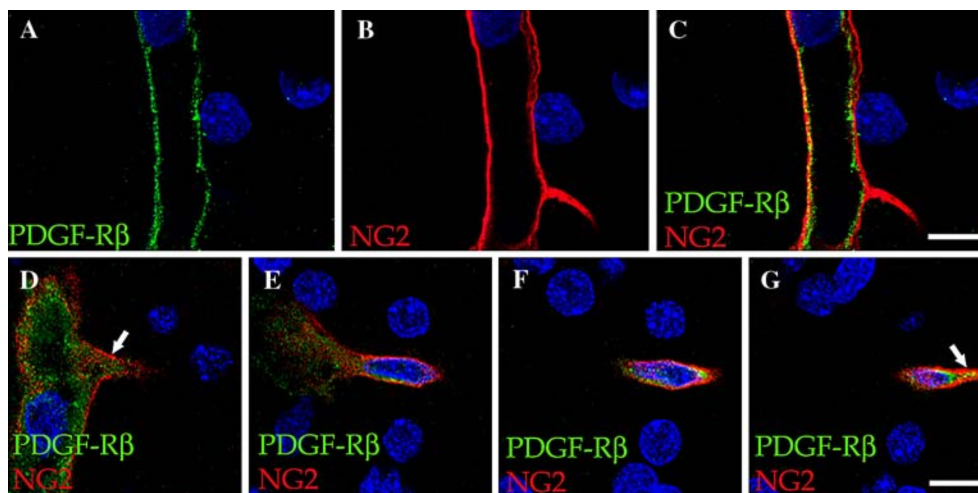
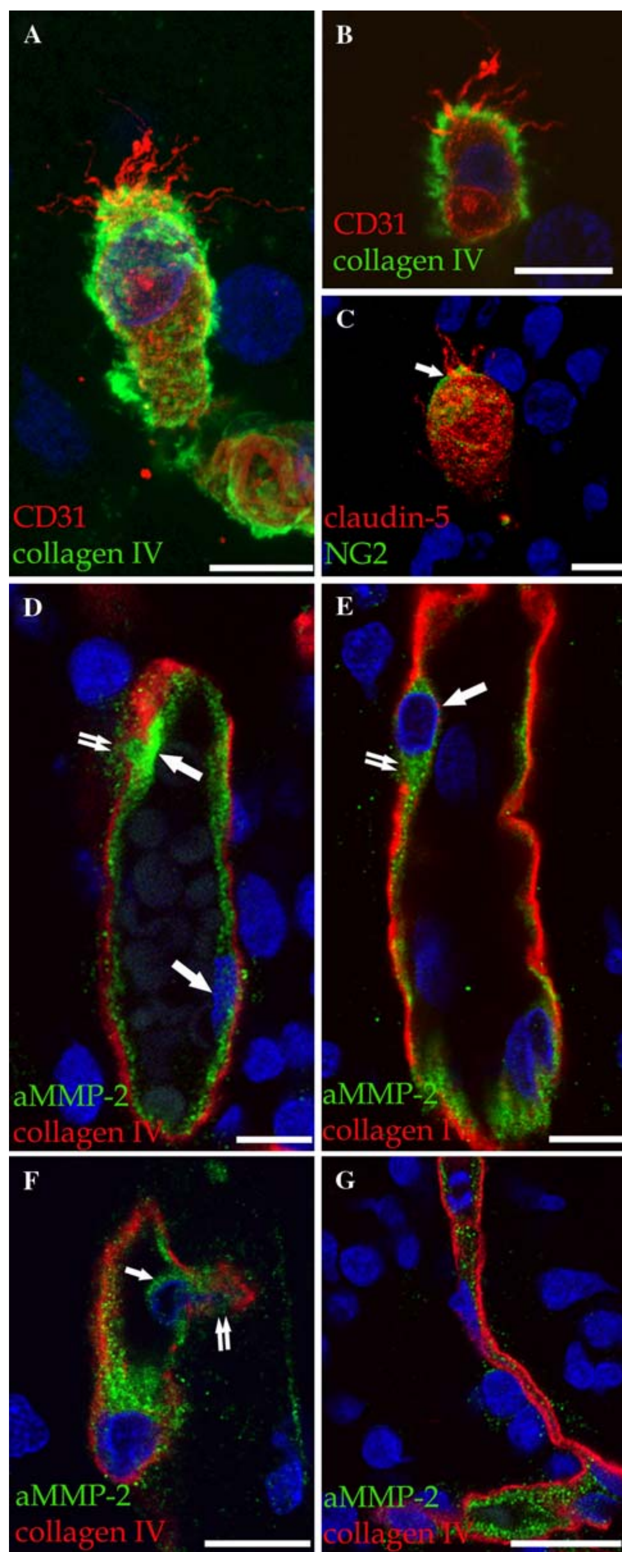


Fig. 3 (**A–F**) Confocal images of microvessels in 22-week human telencephalon double immunolabelled with NG2 and PDGFR-β. (**A–C**) PDGFR-β (**A**, green) and NG2 (**B**, red) stain the pericyte sleeve of a radial vessel. The NG2 continuous labelling and PDGFR-β punctate pattern partly colocalize (merged image, **C**). (**D–G**) four sequential stacks of optical planes (merged images) showing a NG2/PDGFR-β growing vessel from its sprouting point (**A**, arrow) to its invading tip (**D**, arrow); the two markers are seen to concentrate and colocalize in (**C, D**). Scale bars: 10 μm in (**A–C**); 15 μm in (**D–G**)



◀ **Fig. 4** (**A–G**) Confocal images of vessel sprouting points in 22-week human telencephalon. (**A–C**) CD31/collagen IV and claudin 5/NG2 double immunolabelling. CD31 (red in **A,** merged image, and in **B**, single optical plane of **A**) and claudin-5 (red in **C**) reveal endothelial filopodia, which are seen to extend beyond the limits of both the collagen IV basement membrane (green in **A** and **B**) and the NG2 pericyte layer (green in **C**, arrow). (**D–G**) aMMP-2/collagen IV double labelling. Endothelial cells (**D**, arrows) and a pericyte (**E**, arrow) are strongly reactive for the active form of MMP-2; enzyme reactivity appears at points where collagen IV labelling is uneven or absent (**D**, **E;** double arrow); (**F**) an aMMP-2 labelled vessel sprout (arrow) characterized on its growing front by signs of collagen IV loss (double arrow); (**G**) aMMP-2 marks an immature microvessel revealed by its collagen IV basement membrane. Scale bars: 10 μm in (**A–F**); 20 μm in (**G**)

expression of the NG2 proteoglycan, the surface glycoprotein endosialin and the receptor β of the platelet-derived growth factor (PDGFR-β). Each marker showed a very similar staining pattern and a comparable cell distribution in all the five examined samples. Our results with NG2 in human brain angiogenesis are consistent with previous studies on pericyte-dependent angiogenesis using animal models of both normal and pathological neovascularization [15–19]. In addition, the computer-aided quantitative analysis carried out on NG2/collagen IV labelled sections clearly demonstrates that at mid-gestation a significant proportion (88%) of the telencephalon vasculature is provided with NG2 angiogenic pericytes.

Endosialin has been demonstrated in tumour vasculature, where it colocalises with NG2 in pericytes [23, 39]. A recent work has described the expression of endosialin in stromal fibroblasts and periendothelial cells in mouse embryonic and adult tissues, suggesting that endosialin staining is identical to NG2 staining [40]. Our results during brain angiogenesis demonstrate for the first time that NG2 and endosialin colocalize in pericytes, and that endosialin can be considered a specific marker of brain pericytes. Nevertheless, differences were observed in NG2 and endosialin staining patterns along microvascular walls. NG2 staining was continuous, whereas endosialin appeared interrupted by unstained gaps. At present, it is not clear if this difference is due to shedding of the NG2 proteoglycan from the cell surface [41]. In the context of the angiogenic process, shed NG2 could be specifically sequestered by basement membrane components or by other molecules of the extracellular matrix (ECM) that serve as adapter molecules at the basement membrane-interstitial ECM interface. Although previously undetected in cortical vessels [42], one such candidate for NG2 sequestration may be collagen type VI, which is known to be a primary integrin-independent ligand of NG2 [43], displays a marked affinity for collagen type IV and is localized to microfilamentous structures immediately adjacent to vascular basement membrane [44]. Unlike NG2, the

discontinuous staining pattern of endosialin possibly depends upon its localization on the pericyte membrane, thus reflecting the morphology of the pericyte finger-like processes. While we have found that NG2 and endosialin are strongly expressed by angiogenic pericytes of the developing telencephalon, they are absent from these cells in the adult normal tissues including adult human brain [23, 45; our unpublished observations]. These data highlight the remarkable similarities between the molecular features of nascent angiogenic brain microvessels and those of developing vessels within tumour lesions [46, 47].

The finding that NG2 and PDGFR-β are co-expressed in pericytes associated with a large part of the telencephalon vasculature, including the vascular sproutings, further emphasizes the angiogenic/activated state of pericytes and substantiates their involvement in early angiogenesis.

To our knowledge, growing microvessels similar to those observed herein have not previously been described during normal human brain vascularization. These vessels appeared to originate from pre-existing radial vessels or their collaterals, were composed entirely of NG2 pericytes, were first occupied by endothelial cells only in their initial segment, and thereafter they appeared colonised by endothelial cells throughout their entire extension. Endothelium-free pericyte tubules have been previously reported in tumour and retinal neovascularization, as well as in an experimental model of corneal angiogenesis [16, 17]. It seems conceivable that the endothelium-free telencephalic microvessels represent an early stage of vessel growth, and that endothelial cells from the parental vessels are recruited and guided by pioneering pericytes through pericyte-derived factors, such as VEGF [4, 8, 26, 48, 49]. Alternatively, the NG2 proteoglycan expressed on pioneering pericytes may be directly responsible for the recruitment and stimulation of endothelial cells, in accordance with its importance in mediating pericyte/endothelial interaction [9, 18]. The possible primary role of NG2 is also supported by the comparison of NG2 and endosialin expressions during early vessel growth: endosialin was present together with NG2 in pericytes of the initial portion of the growing microvessel, whereas it was absent in NG2 pioneering pericytes moving toward the surrounding neuropil.

We have previously shown that in developing human brain MMP-2 expression correlates with the initial phases of vessel sprouting that involves degradation of the vascular basement membrane [35]. In this context, among the early angiogenic events that we observed is the emergence of endothelial filopodia from the tip of the sprouting vessel. These endothelial processes passed through both the collagen IV-containing basement membrane and the pericytic layer to reach the surrounding neuropil. Extension of endothelial cell filopodia has previously been described during CNS vascularization and in the early postnatal retina, where this process seems to be dependent upon VEGF stimulation [50–52]. During the vascularization process in the developing human brain, VEGF expression has been found in neurons, endothelial cells and pericytes and may be responsible for both paracrine and autocrine stimulation of endothelial cells [26]. Following filopodial invasion of the basement membrane, a subsequent step leading to vessel sprouting through disassembly of the basement membrane may be provided by the action of MMP-2. This proteinase, released by endothelial cells and pericytes allows their migration from the vascular wall and emanation of sprouts at the basement membrane break points. The successive step is represented by the growth of pericyte-made, immature microvessels that invade the nervous tissue and are subsequently colonised by endothelial cells, which finally build a lumen-provided new vessel. The observation that vascular cells reactive for aMMP-2 were also seen associated with a continuous collagen IV labelling agrees with the concept that proteinases, besides disassembling the basement membrane, may trigger its partial modification and/or formation of proteolytic fragments. Both these events can regulate endothelial cell functions during angiogenesis and vascular morphogenesis [53, 54].

The BBB markers GLUT-1 and claudin-5 we used were valuable not only for identifying endothelial cells from pericytes but also for acquiring information about BBB developmental events in the human telencephalon. The expression of both these molecules in the endothelial cells of the growing microvessels revealed their BBB phenotype, in contrast with the concept that newly formed endothelia lack established BBB markers [10, 55]. However, it is noteworthy that the claudin-5 staining we observed in the growing microvessels did not appear restricted to the endothelial junctional areas, as found in mature microvessels [27].

This study demonstrates for the first time in vivo the angiogenic potential of human telencephalic microvessels and confirms that the sprouting of nascent vessels is controlled by an intimate interplay between migrating pericytes and endothelial cells. In particular, our results highlight a guiding role for pioneering pericytes, which initiate the angiogenic process and subsequently recruit endothelial cells to organize the microvessel wall and form an integral new vessel.

Acknowledgements This work was funded by Ministero dell'Istruzione Università e Ricerca (MIUR; FIRB 2001) and Fondazione Cassa di Risparmio di Puglia (FCRP 2005) to DV. We thank Clare M. Isacke and John R. MacFayden, The Breakthrough Breast Cancer Research Centre, Institute of Cancer Research, London, for the generous gift of endosialin antibody. We gratefully acknowledge Mary V. C. Pragnell, BA for linguistic help, and Marisa Ambrosi, Antonella Grano, and Loredana Lorusso for their excellent technical assistance.

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