Identification of a ‘Snapshot’ of Co-Expressed Angiogenic Markers in Laser-Dissected Vessels from Unstable Carotid Plaques with Targeted Arrays

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Key Words

Angiogenesis • Atherosclerosis • Carotid plaques • Laser-capture microdissection

Abstract

Background/Aims: Angiogenesis is a feature of the atherogenic process, with intimal neovascularisation arising from vessels in the adventitia, adjacent to a plaque. Immature, leaky blood vessels from unstable plaques proliferate abnormally and, being poorly invested with smooth muscle cells, may contribute to instability of the plaque by facilitation of inflammatory cell infiltration and haemorrhagic complications. **Methods:** We used laser-capture microdissection to isolate angiogenic areas of the extracellular matrix (containing CD105/flt-1-positive, fragile thin-walled vessels) and non-angiogenic vascular areas (CD105-negative, with smooth muscle cell covering) of complicated endarterectomy plaques, and specifically designed angiogenesis-Taq-Man real-time PCR microarrays to identify gene expression. **Results:** Important pro-angiogenic components, including Notch-3, delta-like-4 (DLL4), Tie-2, angiopoietin-1 (Angio-1) and receptor for advanced glycation end products (RAGE), and one anti-angiogenic factor, endostatin, were up-regulated in these regions. Immunohistochemistry demonstrated localisation within intimal, active (CD105-positive) microves-

sels and co-localisation of Notch-3 and DLL4/Tie-2 and Angio-1 in the same vessels indicating multiple/synergistic signalling mechanisms associated with vessel development. **Conclusion:** These data, although providing only a snapshot of information, demonstrate that plaque vascularisation occurs in the presence of multiple angiogenically active factors. Knowledge of their combined effects could help in the formulation of novel therapeutics designed to stabilise or prevent their formation in the treatment of atherosclerosis.

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Introduction

The common factor of major diseases with an ischemic component, including ischemic cardiopathy, cerebrovascular disease, peripheral vascular disease and sudden death due to ischemic factors, is atherosclerosis. This is defined as a chronic inflammatory process characterised by the formation of focal injuries affecting vascular function and producing thrombotic complication leading to the clinical events responsible for cardio- and cerebrovascular mortality [1]. Angiogenesis is a recognised feature of the atherogenic process, with intimal neovascularisation arising most frequently from the dense network of vessels in the adventitia, adjacent to a

plaque, rather than from the main artery lumen. New blood vessels may have an active role in plaque metabolic activity and actively promote its growth beyond the critical limits of diffusion from the artery lumen [2, 3]. A strong correlation between areas of increased vascularity and intraplaque haemorrhage was first reported by Mofidi et al. [4], using histological staining with anti-CD34 in symptomatic patients following endarterectomy. The same authors recently demonstrated a direct association between plaque angiogenesis and neurological events using computerised tomography [5]. The irregular nature of blood vessel formation has been likened to tumour angiogenesis, and hence the factors responsible for their growth may be different from those seen during normal wound healing [6]. Our previous studies and those of others have suggested that haemorrhagic, leaky blood vessels from unstable carotid plaques proliferate abnormally. These relatively large-calibre, but immature neovessels are poorly invested with smooth muscle cells and possess structural weaknesses which may contribute to instability of the plaque by facilitation of inflammatory cell infiltration and haemorrhagic complications [7]. In a study of coronary artery atherogenesis, from patients subjected to heart transplant, lesions with the highest neovessel content were demonstrated to be of type VI and associated with the highest rate of thrombotic episodes [8].

These processes are in part initiated by hypoxia generated in the plaque, the specific action of growth factors, particularly vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (FGF-2), secreted by vascular and inflammatory cells and other factors such as haemodynamic stress. Immature neovessels may contribute to instability of the plaque by facilitation of inflammatory cell infiltration and haemorrhagic complications. However, our understanding of the mechanisms responsible for induction and maintenance of plaque neovascularisation is incomplete and requires further investigation.

In disease situations, abnormally behaving cells are surrounded by heterogeneous tissue elements, such that the areas of interest (diseased cells) may constitute <5% of the volume of a tissue biopsy specimen. Previous studies using conventional technology have employed microarrays to identify general changes in gene and protein regulation in biopsies from normal and abnormal regions [9–11], but have failed to discover cell-specific changes, and, in particular, those associated with angiogenesis. We hypothesise that even within active regions of remodelling, angiogenic and non-angiogenic areas may co-ex-

ist, as we have demonstrated in our pilot studies by observation of microvessels expressing CD105+/CD105– in complicated carotid arterial plaques [12]. Laser capture microdissection (LCM) can be used to isolate microvessels in evolving lesions. This technique, when combined with the recently introduced cDNA pre-amplification technology and quantitative real-time PCR TaqMan low-density arrays (LDA; Applied Biosystems, Foster City, Calif., USA), provides a powerful and sensitive tool for identification of genetic changes associated with blood vessel activation. TaqMan-LDA have been successfully used to identify gene de-regulation in aggressive human melanoma cell lines [13]. We hypothesised that specifically designed TaqMan arrays combined with real-time PCR could be used to identify the genetic fingerprints of angiogenic neovessels and surrounding inflammatory micro-areas in complicated unstable plaque regions.

Patients and Methods

Patients

Human carotid (n = 30) plaques were taken from patients following endarterectomy and classified according to Stary et al. [14]. Samples were obtained from the Tissue Bank of the Cardiovascular Investigation Centre, Sant Pau Hospital, Barcelona, Spain, with full ethical approval from the University Hospital of Bellvitge. Arterial plaques were dissected into 2-mm cross-sectional pieces and alternative sections reserved for LCM (prepared in OCT) or immunohistochemistry (IHC) and fixed in 10% buffered saline prior to paraffin embedding. Only plaques demonstrating loci of microvessels and RNA (obtained from whole sections; 6 µm) with RNA Integrity Numbers (RIN) >5.0 were considered for further study (tables 1, 2).

Immunohistochemistry

Frozen sections were labelled to identify endothelial cells (ECs) using antibodies directed against CD31, and the specific markers of microvessel angiogenesis Flt-1 (VEGF receptor-1 [15]) and CD105 [16]. Haematoxylin was used to counterstain the nuclei. Slides were incubated in 0.5% v/v H₂O₂ in methanol for 30 min, with normal serum for 20 min and then with a primary antibody (diluted in normal serum) for 30 min, followed by 30-min incubation with biotinylated secondary antibody (diluted 1:50) and finally with ABC complex (diluted 1:50) for 30 min at room temperature. Staining was completed after incubation with DAB substrate chromogen solution for 3–10 min. Slides were counterstained with haematoxylin, dehydrated, cleared and mounted in DPX. Negative controls were prepared to ascertain the specificity of the antibodies by omission of the primary antibody and replacement with PBS or non-immune serum. Dissected tissue areas were placed into categories of either: active content of microvessels with many Flt-1- and/or CD105-positive ECs or inactive with few vessels expressing these markers. In the second part of the study, antibodies to proteins related to several of the novel genes identified as de-regulated from the TaqMan microarrays

Table 1. Clinical details of patients/specimens used in the study

Patient No.	Age years	Sex	Dyslipidaemic	Symptomatic	Histology of plaque
216 ¹	65	M	yes	no	C, F, H
231 ¹	74	M	yes	yes	C, U
260	69	M	no	yes	C, U
280 ¹	72	M	yes	yes	C, U, H
284	60	M	yes	yes	C, U
305 ¹	53	M	no	yes	C, U
391	64	M	yes	yes	C, F
431	72	M	yes	yes	C, U
361 ¹	66	M	no	no	C, H, U
390 ¹	74	M	yes	no	C, F
371 ¹	75	M	yes	yes	C, U

C = Complicated; F = fibrous; H = haemorrhagic; U = ulcerated. All patients had >80% stenosis.

¹ Samples used for laser capture.

were obtained, and expression and localisation within endarterectomy specimens were determined by histology and IHC as described previously [10]. Antibodies purchased from Abcam (Cambridge, UK) were directed against angiopoietin (Angio)-1, Tie-2, endostatin, Notch-3, delta-like-4 (DLL4) and receptor for advanced glycation end products (RAGE; 1:100). For double staining, the first primary antibody was developed in DAB and the secondary antibody fluorescently labelled with FITC (CD105; green) in a consecutive staining process.

Laser Capture Microdissection

Serial 6-µm frozen sections were taken from carotid endarterectomy specimens (see table 1 for clinical details). Broad areas were scanned by double labelling with CD105 and UEA1 (*Ulex europaeus* agglutinin I) to estimate regions containing high concentrations of CD105-positive vessels (fig. 1a, b). Suitable tissue samples were then treated as described. Sections were taken in groups of four. The first stained for CD105, the second prepared on a matrix (polyethylene naphthalate membrane)-coated slide for laser capture and stained only with haematoxylin prior to laser capture (to reveal morphology), the third stained with anti-CD31 and the fourth with anti-Flt-1. Photomicrographs of each stained section were taken and each area analysed by an experienced pathologist. Specific areas rich in positive or negative microvessels were marked and LCM was performed on the middle section using a Robot Microbeam laser microscope (PALM Microlaser Technologies, Bernried, Germany). The focused, pulsed laser provides enough energy to melt the thermoplastic film at the precise location. The laser diameter was set to 15 µm and small (<200 µm) CD105-positive/-negative microvessel-rich areas were selected with close reference to the stained serial sections. The captured areas were collected on the cap of a microcentrifuge tube and homogenised in RLT buffer (RNeasy MicroKit; Qiagen, Hilden, Germany). If the laser did not succeed in releasing the tissue completely, the resultant marked area was carefully removed by an experienced dissection microscopist

Table 2. Expression of angiogenic markers in neointimal vessels of carotid endarterectomy specimens (samples chosen on the basis of presenting with sufficient serial sections containing CD105-positive vessels)

Patient ref.	NOTCH3	DLL4	Tie-2	Angio-1	RAGE	Endostatin
216	++	+++	+++	++	+++	++
231	++	++	+++	+++	–	–
361	++	+	+	++	–	+
280	+++	++	+	+	+	++
390	++	++	++	++	+	++
371	++	+	+	+++	+	+
305	+++	++	+++	++	++	++

– = No staining; + = staining in 20–50% of CD105-positive vessels; ++ = staining in >50% of vessels; +++ = staining in the majority of vessels.

and placed directly into RLT buffer. At least five groups of sections were treated like this and the laser-cut areas pooled to ensure sufficient numbers of cells were obtained for RNA analysis. Selected microvessels/cells were extracted and RNA purified using the Qiagen RNeasy Micro-Kit. The quantity and quality of RNA was assessed using an Agilent Technologies 2100 bioanalysis and microchip system (fig. 1c–e). The cDNA obtained from 1 ng of total RNA was pre-amplified using the TaqMan Applied Biosystems PreAmp Master Mix Kit, and equality of gene amplification tested using the housekeeping control β-glucuronidase (GUS) and measuring relative expression of CD31, CD105 and Flt-1 (fig. 2a, b). Confirmation was made that the samples were CD31/CD105/Flt-1-enriched using TaqMan real-time PCR (fig. 2c, d) showing correlation between CD105 expression and Flt-1 indicating both were associated with active samples (Spearman rank: $p = 0.025$; $\rho = 0.742$). A final selection was made of six control and six active regions with RIN ≥ 6.0 .

TaqMan Arrays

The custom TaqMan-LDA chosen was a 48 TaqMan gene expression assay (Applied Biosystems) preconfigured in a 384-well format and spotted on a microfluidic card (2 replicates/assay). Chosen genes are listed in the Appendix; if the data generated (Cts) differed >0.35 SD, the result was discarded. The real-time RT-PCR amplifications were run on an ABI Prism® 7900HT sequence detection system (Applied Biosystems) with a TaqMan low-density array upgrade. Five to 22 ng of cDNA was combined with TaqMan Universal PCR Master Mix, and real-time PCR carried out according to the manufacturer's instructions. In order to identify the angiogenic factors and intracellular signalling mechanisms involved, we have designed an LDA containing 46 candidate markers derived from published data on wound recovery, tumour angiogenesis, angiogenesis in other diseases including stroke and diabetic retinopathy, and from our own and other microarray studies on atherosclerosis. Genes were chosen for the array based on published profiling studies of ac-

Color version available online

Fig. 1. Immunostaining, identification and separation of angiogenic microvessels. **a** Low-power photomicrograph showing an area of neointima with a high concentration of CD105-positive blood vessels [taken from endarterectomy specimen 305 (online version: yellow insert); arrows; $\times 10$]. **b** Higher power of the same sample showing a clearly labelled, compact group of blood vessels (arrows; $\times 100$). **c** Example of CD105-positive immature and irregularly shaped vessels chosen as positive sample areas. **d** Bio-analysis of laser-cut areas was carried out using an Agilent Technologies 2100 analyser and only samples without evidence of RNA degradation and $RIN \geq 6.0$ were chosen for pre-amplification. Arrows and peaks show 18S and 28S ribosomal RNA.

tivated ECs, in vivo/in vitro analysis of tumour/disease-activated blood vessels, and our own published [10] and unpublished microarray data (see Appendix). Paired control and CD105-positive samples were chosen for comparison to eliminate inter-plate variation, and each pair had similar RIN and the same loading cDNA concentration (see statistical analysis for further information).

Statistical Analysis

cDNA samples were initially paired for TaqMan analysis to reduce inter-plate variation. Samples were chosen based on the RIN and the amount of cDNA loaded. If relative quantity (RQ) values between duplicates differed >0.1 , the sample was discarded. General differences in gene expression between individual pairs (CD105 positive and CD105 negative) were considered if the RQ difference was ≥ 0.3 (approximately equivalent to a 3.0-fold difference). When 5 of 6 samples showed differences, statistical analysis was carried out. Using GUS as a housekeeping control, non-parametric testing was carried out comparing expression between all 5 CD105-positive and -negative samples (Mann-Whitney U test), and Spearman rank correlation of both CD105 and Flt-1 expression with the genes of interest. Genes chosen for further analysis had to satisfy at least two of the following criteria: (1) 5 of 6 paired samples had RQ differences >0.5 ; (2) Mann-Whitney U test showed a significant difference between the two sets of 6 samples ($p < 0.05$), or (3) Spearman rank correlation showed a significant correlation between CD105/Flt-1 expression and expression of the gene of interest.

Results

Selection of Areas of Concentrated Microvessels and Pre-Amplification of RNA Demonstrating Equality of Multiplication of Key Genes

An example of an endarterectomy specimen with foci of CD105-positive blood vessels is shown in figure 1a, b, with CD105-positive immature and irregularly shaped vessels often with a significant inflammatory component chosen as positive sample areas (fig. 1c, d) and an area also containing more mature vessels with smooth muscle cell coating and lack of inflammatory cell infiltration, which were almost always CD105 negative (fig. 1e). Extraction of RNA showed good purity with $RIN > 5.0$ and S18 and S28 peaks (fig. 1f).

Selected Immunopositive CD105-Positive Tissue Samples Were Enriched with CD105 and Flt-1 Gene Expression

As described in Patients and Methods, our pilot study pre-amplification analysis demonstrated that Flt-1, CD105 and CD31 amplified to a similar extent and within acceptable limits with ΔCt lower than the cut-off point

Fig. 2. Pre-amplification confirmation of gene expression in CD105/Flt-1-positive and -negative regions. **a, b** Non-significant differences in ΔC_t s between pre-amplified test genes and the endogenous control, thereby indicating no bias in gene amplification. **c** Significant correlation between CD105 and Flt-1 expression in all the tested samples indicated reliability of the markers

to discriminate between active and inactive vessels ($p < 0.05$). **d** Significant differences in gene expression of CD105 and Flt-1 in the positively selected samples compared with the CD105-negative samples indicated the sampling/IHC was accurate ($p < 0.01$).

of 1.5 and no significant differences in Ct of any of the post-amplified genes (fig. 2a, b). A strong correlation was also seen between the ratios of CD105/GUS and Flt-1/GUS in all samples indicating accurate sampling of angiogenic and non-angiogenic regions (Spearman rank: $p < 0.001$; fig. 2c). Micro-areas chosen by laser capture on the basis of CD105/Flt-1 IHC demonstrated a notable increase in their gene expression (fig. 2d; Mann-Whitney: $p = 0.10$; non-significant due to a higher dispersion of the data in CD105-positive samples). No significant difference in CD31 expression was seen between paired samples (data not included).

TaqMan Microfluidity Card Comparison of Samples Revealed Significant Differences in the Expression of Key Angiogenic Genes Correlating with CD105 and Flt-1 Gene Expression

Of the 47 genes tested, 7 pro-angiogenic genes were significantly up-regulated in regions containing CD105/Flt-1-positive vessels [Tie-2, heparin-binding epidermal growth factor (EGF)-like growth factor, RAGE, Angio-1, KDR, Notch-3 and DLL4], and one anti-angiogenic gene, endostatin. Figure 3 shows mean expression of each gene in the 6 CD105-positive (online version in blue; given a value of 1.0) and negative (online version: red) paired

Fig. 3. TaqMan microfluidity card results show relative expression of de-regulated angiogenic genes in CD105-/Flt-1-positive (online version: blue) and -negative (online version: red) samples. In each case, the positive paired sample was given an arbitrary value of 1.0. * $p < 0.05$; ** $p < 0.01$. Expression of the genes Tie-2, Angio-1, Notch-3, DLL4, endostatin and RAGE significantly correlated with CD105-positive microvessels.

samples. Protein expression and localisation of RAGE, Angio-1, Tie-2, DLL4 and Notch-3 as well as the anti-angiogenic protein endostatin were determined in 7 complicated endarterectomy specimens, containing foci of microvessels shown in tables 1 and 2.

IHC Confirmed Expression of Notch-3, DLL4, Tie-2, Angio-1 and RAGE in Neointimal Vessels

Analysis of protein localisation in 7 endarterectomy specimens demonstrated strong staining of these molecules in neointimal microvessels and co-localisation with CD105-positive vessels (fig. 4a–j; patient 305). Analysis of adjacent sections suggested co-localisation of Notch-3 with DLL4 in angiogenic vessels and Tie-2 with Angio-1 (fig. 4a–j). In many of the vessels, all of the pro-angiogenic proteins were expressed together. RAGE was expressed in fewer vessels (approximately 50% on average per specimen) and in 5/7 specimens. Expression of these proteins was predominant in small, irregularly shaped and often in-patent microvessels with thin walls enclosed often by a single layer of ECs with no smooth muscle cell or pericyte support. Non-angiogenic (CD105-negative) vessels from the same sections stained negative for these proteins (fig. 4k–r; patient 305). Protein expression was almost totally specific to blood vessels, with smooth muscle cells, macrophages and other inflammatory cells negatively stained in all sections examined.

Specifically, figure 4 shows CD105-stained endarterectomy sections (fig. 4a–j; patient 305; $\times 40$) revealing an area rich in positively stained microvessels and immu-

nostaining in the same region (online version: red box) of microvessels for CD105, DLL4, endostatin and Notch-3, respectively, in adjacent serial sections from the same specimen (fig. 4a–d; $\times 100$; arrows). Figure 4 (f, g) shows a specific blood vessel staining positive for CD105 (fig. 4f) and also for Tie-2 in the adjacent section (fig. 4g; $\times 100$; arrows; online version: blue box), and similar positive staining (fig. 4h–j; $\times 40$; arrows) in the same region of active blood vessels for Tie-2 (fig. 4h), Angio-1 (fig. 4i) and RAGE (fig. 4j). Endostatin and RAGE stained only a proportion of the CD105-positive vessels, whilst the other proteins were expressed in the majority of vessels. Figure 4k–r shows a different area of the same section with adjacent sections demonstrating the presence of CD31-positive (fig. 4k, l) but CD105-negative vessels [fig. 4m ($\times 20$), n ($\times 40$); arrows]. Figure 4o–r reveals negative staining for DLL4, Notch-3, Tie-2 and Angio-1, respectively, in these vessels ($\times 40$; arrows). Endostatin and RAGE were also not identified by immunostaining in these areas (data not shown).

These pro-angiogenic proteins were co-expressed in many of the CD105-positive vessels (fig. 5), which shows Notch-3, Tie-2, Angio-1 and DLL4 staining (DAB; fig. 5b, d, f; online version: brown) in CD105-positive vessels (FITC; fig. 5a, c, e; online version: green) and combined layering to show co-localisation (fig. 5g, h).

Endostatin Was Expressed by Some Neointimal CD105-Positive Vessels

Endostatin was expressed in many CD105-positive intimal microvessels in 6/7 plaques (fig. 4; patient 305), sug-

Fig. 4. IHC demonstration of protein association with CD105-positive neovessels. **a–j** CD105-stained endarterectomy section (sample 305; $\times 40$) showing an area rich in positively stained microvessels. **a–d** Immunostaining in the same region (online version: red box) of microvessels for CD105, DLL4, endostatin and Notch-3, respectively, in adjacent serial sections from the same specimen ($\times 100$; arrows). **f, g** A specific blood vessel staining positive for CD105 (**f**) and also for Tie-2 in the adjacent section [$\times 100$ (online version: blue box); arrows]. **h–j** Similar positive staining (arrows) in the same region of active blood vessels for Tie-2 (**h**), Angio-1 (**i**) and RAGE (**j**). $\times 100$. Endostatin and RAGE stained only a proportion of the CD105-positive vessels, whilst the other proteins were expressed in the majority of vessels.

gesting that even within closely grouped intimal micro-regions, different processes of cellular control are in operation. Smooth muscle and inflammatory cells were negatively stained for endostatin in all sections examined.

Discussion

When the balance between pro- and anti-angiogenic factors is altered in favour of cellular activation, diseases which have an important element associated with tissue expansion, e.g. solid tumours and atherosclerosis, can

Fig. 4. **k–n** A different area of the same section with adjacent sections demonstrating the presence of CD31-positive (**k**, **l**; arrows) but CD105-negative vessels (**m**, **n**; arrows). **k**, **m** $\times 20$. **l**, **n** $\times 40$. **o–r** Negative staining for DLL4, Notch-3, Tie-2 and Angio-1, respectively, in these vessels ($\times 40$; arrows). Endostatin and RAGE were also not identified by immunostaining in these areas (data not shown).

benefit through enhanced vascularity. Angiogenesis is a complex process requiring modulation of multiple proteins which affect cell growth following intracellular signalling through intracellular signal transduction pathways. Identification of the cellular components responsible for promotion of neointimal blood vessel proliferation in complicated unstable atherosclerotic lesions will help in defining future therapies for controlling plaque development. In this work, for the first time to our knowledge, we have successfully isolated microregions of angiogenic and quiescent microvessels from carotid endarterectomy specimens obtained at transplant from patients with high-grade plaques and compared the expression of the key angiogenic genes using real-time PCR and TaqMan microfluidity cards. From nanogram quantities of material, we have identified up-regulation of 7 genes with key roles in the modulation of angiogenesis. Confirmation of their expression was made by IHC on dissected samples, and we have shown specific association of Tie-2, Angio-1, RAGE, Notch-3 and DLL4 in neointimal CD105-positive blood vessels.

LCM and nano-scale amplification of RNA allows the isolation of specific micro-sized microvessel-containing cellular areas of interest from heterogeneous tissue components [17]. Blood vessels from glioblastoma multiforme, isolated by LCM, underwent cDNA pre-amplification and microarray analysis revealing up-regulation of insulin-like growth factor binding protein-7 and SPARC compared with normal brain vessels [18]. Similarly, Roy et al. [19] compared global gene expression between blood vessels isolated by laser capture from normal skin and identified de-regulated genes from those within chronically wounded tissue. In our work, because we needed to discriminate between active and quiescent vessels, we labelled serial sections staining in groups of four; the first was stained with anti-CD105, the second was used for laser capture, and the third and fourth stained with anti-CD31 and anti-Flt-1 antibodies. We confirmed that the areas chosen contained enriched markers of EC activation using RNA pre-amplification technology and housekeeping controls. This showed that the relative amplification of genes was similar. Real-time PCR analysis of our microfluidity card data showed significant correlation in expression between de-regulated angiogenic genes and our markers of EC activation (CD105 and Flt-1) confirming the validity of our methodology. During our investigations, detailed analysis of RNA preservation was performed at different stages of our modified protocols. Our unpublished data demonstrate limitations of techniques employing visualisation of tissue components

with rapid immunological staining techniques. We found that exposure of frozen, high-quality tissue sections to biological stains or even PBS for ≥ 30 min severely reduced the quality and RIN of the RNA making it unsuitable for further analysis. Only treatment with UEA1 in sterile water retained RNA quality over this time, in agreement with the work of Roy et al. [19].

One of the main aims of this work was to identify expression of angiogenic and anti-angiogenic factors produced in microregions of neointimal plaques in association with active microvessels. For this reason, in these experiments, we carefully dissected concentrated areas of vessels including closely associated extracellular matrix encompassing any inflammatory components (fig. 1 gives an example of this selection). In this way, we were able to gain an insight into the local micro-environment to which the growing vessels were subjected. Expression or synthesis of genes directly by ECs would feature as the main constituent due to their high relative concentration (identified by histology in all samples); however, any component consisting of secreted factors from inflammatory infiltrates would also be seen giving us an overall view of the composition of these micro-hotspots. In relation to other studies of gene and protein expression associated with plaque angiogenesis or, to be more accurate, identified components of unstable plaque tissue associated with vascular regions, our results confirm potentially important roles of VEGF pathway signalling, which operates via the main VEGF receptor, VEGFR2/KDR, and results in promotion of branching morphogenesis via $\alpha_5\beta_3$ integrin signalling [20] as well as RAGE expression, with Angio-Tie signalling pathways acting as further possible contributors to vessel stabilisation and anti-angiogenic signalling through endostatin (see below), whilst the study also identified angiogenic factors not seen before in this type of lesion, such as HBEGF, DLL4 and Notch-3.

Notch signalling has been demonstrated to induce arterial endothelial differentiation, and recent studies suggest that Notch signalling is activated downstream of VEGF signalling and negatively regulates VEGF-induced angiogenesis and suppresses aberrant vascular branching morphogenesis [21]. In normal circumstances, expression of its ligand, DLL4, is restricted to embryological vessels and is associated with the leading edge of growing vascular networks, where it is one of the major controllers of directional angiogenesis and vessel stabilisation and maturation [22, 23]. Notch and DLL4 signalling is regulated by VEGF and inhibition results in the production of functionally defective vasculature [24, 25]. In our study, we showed up-regulation of gene expression and concur-

Fig. 5. Co-localization of Tie-2 (**a, b**), DLL4 (**c, d**), Notch-3 (**e, f**) and Angio-1 (**g, h**) with CD105-positive blood vessels (arrows) in a region of plaque neointima rich in microvessels from specimen 305. **a, c, e, g** $\times 40$. **b, d, f, h** $\times 100$. Images were layered using Microsoft Photoshop. Online version: CD105 staining is shown as green FITC (**a, c, e, g**) and test proteins developed using DAB (**b, d, f, h**).

rent co-localisation of DLL4 and Notch-3 proteins in CD105-positive neointimal microvessels from complicated carotid plaques, suggesting that actively growing neovessels are re-expressing these embryologically derived genes in an attempt to regulate formation of stable vessels, which could be less prone to leakage and rupture. Further studies would be required to compare Notch-3-

/DLL4-positive and -negative vessels to determine any differences in their structure and patency and also to study expression of other isoforms of Notch in these lesions. Since inhibition of Notch signalling has been shown to increase the total number of vessels in solid tumour models but reduce the patency and vascular function of the remaining vessels (resulting in 'chaotic' vessels [26,

27]), it may have similar effects on neointimal vessels and supplementation could be considered as part of a therapeutic programme of normalisation and stabilisation of existing vasculature promoting plaque stability.

In our study, we identified increased gene expression of Tie-2 and its ligand Angio-1 and went on to show co-localisation of Tie-2 with CD105-positive active neointimal microvessels in carotid plaques. Tie-2 is a tyrosine kinase receptor expressed predominantly on ECs, which has been shown to activate intracellular signal transduction through AKT and ERK1/2 following ligand binding of Angio-1 resulting in angiogenesis both *in vitro* and *in vivo* [28]. In relation to tumour neovascularisation, expression of Tie-2 was concentrated in vascular hot spots at the leading edge of invading tissue [29]. Furthermore, Angio-1 is an anti-inflammatory cytokine which can reduce neovessel leakage and vascular permeability partly via inhibition of cell membrane adhesion molecule expression [30]. Recently, in a study by Post et al. [31], increased expression of Angio-2 was reported, with the Angio-2/Angio-1 ratio in the vasculature of unstable carotid plaques being in favour of Ang-2, although they did not examine the expression of Tie receptors. An increase in Tie-2 together with increased availability of Angio-1 may enhance neointimal vascularisation and promote the formation of stable vessels less prone to haemorrhage.

One other pro-angiogenic gene we found to be up-regulated, but which we have not studied in detail, was RAGE. Advanced glycation end-products (AGEs) are associated with chronic tissue exposure to high glucose levels and, in particular, a characteristic finding in developing atherosclerotic lesions, particularly in patients with diabetes. The receptor for AGEs (RAGE) has been demonstrated in increased amounts in atherosclerotic regions of diabetic rabbits [32]. Activation of this receptor by AGEs (produced particularly in diabetic patients in response to exposure to chronically high glucose levels) is associated with VEGF release and initiation of angiogenesis in vascular ECs *in vitro*, and increased expression of AGEs in the plaque tissue of diabetic patients may contribute to vascular complications and thrombosis [33, 34].

During intimal plaque development, the balance between the angiogenic and anti-angiogenic factors may become altered with increased production of growth factors and cytokines together with a reduction in negative modulators resulting in adventitial vessel angiogenesis at the site proximal to internal vascular damage and plaque growth. In this study, however, we observed increased endostatin expression in some intimal neovessels. Collagen XVIII and its proteolytically released endostatin frag-

ment are abundant extracellular matrix-associated molecules found in the vascular basement membranes and the walls of major blood vessels. Expression of collagen/endostatin is associated with inhibition of angiogenesis in the vascular wall, and it has been shown that apolipoprotein E-/collagen XVIII-knockout animals developed significantly more vasa vasorum than wild-type animals [35]. It is possible that anti-angiogenic mechanisms are also operating in the intimal vascular regions in an attempt to re-balance the ratio of positive and negative factors and turn off the angiogenic switch. At this point, we can only speculate as to the overall effects of the presence of both pro- and anti-angiogenic factors on microvessel formation, proliferation and maturation. Further experiments such as Matrigel implant models may be able to determine the effects of introducing a mixture of factors such as those described in this study and examining in detail the formation and structure of vessels over time.

In relation to the overall process responsible for proliferation and maturation of neointimal vessels, what appears to be of critical importance is the time of expression and the number and type of angiogenic molecules effective in the vicinity of the developing microvessels, which may vary dependent on the surrounding matrix. The 'normalisation' process involves development of a vascular bed correctly differentiated into macro- and microvasculature, with microvessels having structurally defined inter-endothelial junctions, basement membrane and pericyte coverage [36]. In other studies, we have shown (using the same technology and identical TaqMan microarrays) that the hypoxic environment associated with neovascularisation of stroked brain tissue induces expression of an overlapping, but certainly not identical group of angiogenic factors [37]. In this case, matrix metalloproteinase-2 and Tie-2 were also overexpressed whilst hepatocyte growth factor- α , c-kit and monocyte chemotactic protein-1 were only increased in stroke neovessels, whilst RAGE, Notch-3 and DLL4 were only seen up-regulated in this study. The importance of understanding the concerted expression of multiple factors in modulating angiogenesis in disease is demonstrated by other published work. For example, the process of angiogenesis in tumours is often magnified by increased expression of VEGF at the same time as other factors, including FGF-2, platelet-derived growth factor and Angio-1. Fernando et al. [38] showed that concurrent expression of an angiogenesis inhibitor such as endostatin or thrombospondin-1 was not sufficient to prevent vessel growth in the presence of multiple up-regulated angiogenic factors. The presence of RAGE reduced VEGF-mediated ves-

self formation in insulin-deficient diabetic mice [39], whilst tumour vessel development in the presence of endostatin and Angio-1 produces stabilised functional vasculature due to Angio-1 blocking of VEGF-induced vascular leakage, and preservation of inter-endothelial junctions by endostatin [36]. Similarly, signalling pathways downstream of Angio-1/Tie-2 activation in ethanol or cyclic strain-treated ECs appear to require Notch and DLL4 [40, 41], and VEGF also has the capacity to activate the Notch pathway, in this case, helping to maintain a balance between excessive angiogenesis and vessel maturation [42].

These studies and others demonstrate that the rate at which new microvessels are formed and/or are able to mature is probably governed by the number and concentration of relevant factors expressed. This may have important consequences in relation to attempts to induce therapeutic angiogenesis for the production of mature intimal vessels less prone to leakage and rupture, and the process may be site specific. Future studies should aim to examine the effects of modulating these factors in terms of ratio and concentration with a view to optimising stable re-vascularisation in vivo. To this end, our ongoing follow-up work is designed to identify the correct composition of angiogenic/anti-angiogenic factors required for optimisation of vessel formation and maturation using a variety of 3-dimensional in vitro and in vivo models of vascularisation.

In summary, here we have used LCM combined with nano-scale RNA extraction and targeted gene arrays to identify the genetic fingerprints of newly activated angiogenic neovessels and surrounding inflammatory microareas in complicated unstable plaque regions. Our data suggest a complicated activation and maturation process which would require targeting of multiple proteins for effective modulation. Weak neovascular beds in plaque intima as well as activated adventitial blood vessels are options for targeted drug therapy, but – due to the requirement of stable/constituent expression – would require administration for example via infusion of nanoparticle

suspensions using catheters or implant-based drug delivery employing antagonists or siRNA directed at pro-angiogenic molecules such as those defined here [43]. Nanoparticles could then be used for effective delivery of drugs to target sites with a preferential aim to prevent initial development of neointimal vascular sites by blocking adventitial blood vessel activation in patients with low-grade plaques. Existing unstable intimal vessels could be targeted with combinational perivascular gene transfer therapy to stabilise them and prevent further development. Inhibition of angiogenesis might be an important target for prevention of development of active, unstable plaque lesions.

Appendix

Unpublished Microarray Data

Kallistatin; transforming growth factor- β ; platelet-derived growth factor; hepatocyte growth factor; EGF; Angio-1; CD13; CD44; c-kit; monocyte chemotactic protein 1; interleukin-8; granular colony-stimulating factor; hypoxia-inducing factor-1; thymosin β 4; matrix metalloproteinases 1, 2, 3 and 9; RAGE; integrin V; receptor for hyaluronan-mediated motility; Tie-1 and Tie-2; tumour necrosis factor- α ; cyclo-oxygenase-2; heparin-binding EGF-like growth factor; PR39; regulated upon activation normal T-cell expressed and secreted; thrombospondin 1; T-cadherin; DLL4; C-reactive protein; Toll-like receptors 2, and 4; tissue inhibitor of metalloproteinase-1; inhibitor of the differentiation 1/3, and hyaluronidase-1.

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