Histology far away from Flatland:
3D roller-coasting into grade-dependent angiogenetic patterns in oligodendrogliomas

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Abstract

Angiogenesis plays a key role in tumour progression, and undergoes structural changes associated to tumour biology itself. Although vessel density can be easily evaluated in brain tumours using a traditional immunohistochemical approach, other parameters of conceptual/biological interest, such as the complex patterns of vascular growth, cannot be fully understood using a traditional bi-dimensional evaluation. We use here surgical specimens derived from oligodendrogliomas as a model for a novel elucidative 3D reconstruction of the grade-dependent vascular arborisation in brain tumours.

Key words: 3D • oligodendroglioma • angiogenesis

Angiogenesis, the process of formation of new capillaries from pre-existing blood vessels, is required for embryogenesis, normal physiological growth, repair, and expansion of neoplastic tissues [1].

The optimization of tumour oxygenation and the extensive coverage of the tumour area by vascular structures mainly base on two different morphologically recognizable angiogenic models, which are sprouting/branching or alternatively intussusception [2, 3]. In the process of sprouting and branching, vessels are formed by extensions of existing vessels. Intussusception is instead characterized by formation of transluminal pillars, which appear as holes and growth finally resulting in partitioning of the vessel lumen [4].

The vessel neo-formation in tumours varies accordingly to different factors, including tumour aggressiveness, and involves various molecules regulating the balance between the ‘on’ and the ‘off’ of the angiogenic switch [5, 6]. Brain tumours represent an ideal model in which variations in lesion malignancy are accompanied by specific changes in the vascular neo-formed component: such structural specificity is so strict to become in many cases a relevant diagnostic tool (i.e., the presence of glomeruloid vessels as a hallmark of high grade-III or IV- astrocytomas versus grade II tumours) [7]. Also, in oligodendroglial tumours the vascular network is a useful diagnostic-grading instrument: grade II oligodendrogliomas are characterized by branching capillary sized blood vessels with a ‘chicken wire’ pattern [8], whereas grade III tumours display glioblastoma-like features with glomeruloid vessels [9]. Such difference can be noticed in conventional haematoxylin and eosin staining and becomes very evident following an immunohistochemical staining of blood vessels (using an antibody directed towards an endothelial antigen, such as CD34). However, the traditional histological approach allows a ‘limited’ bi-dimensional vision: the aim of our investigation was to expand
simple 2D vision with a 3D reconstruction of oligodendroglioma vascular network in order to obtain a novel objective model closer to the biological implications of neoangiogenesis in brain tumours. To achieve this goal, two patients with oligodendrogliomas grade II and III respectively (according to the WHO classification of brain tumours), were selected from the files of the Pathology Department at the University of Turin. Several (up to 40) serial sections, 3 μm thick, were cut from formalin-fixed, paraffin-embedded blocks and underwent an immunohistochemical reaction performed in an automated immunostainer (Ventana BenchMark AutoStainer, Ventana Medical Systems, Tucson, AZ, USA) using a CD34 monoclonal antibody (clone RB END/10, pre-diluted, Ventana-Diapath, Tucson, AZ, USA). Additionally, two other cases of oligodendroglioma grade II and III underwent a more limited 3D reconstruction (on 20 serial sections only), in order to reconfirm the results observed in the more extended tri-dimensional study of the two previous models. Images of the serial sections were acquired by the system Slide (Olympus, Hamburg, Germany, http://www.olympus.com). The reconstruction of areas of interest was performed by Amira 4.0, advanced 3D visualization and volume modeling software (TGS Template Graphics Software, http://www.tgs.com). In order to have the studied structures unchanged through sections, we previously included anthracotic lymphonode in four points of paraffin embedded sample as fiducial tissue markers [10]. Then, we could manually align serial sections by matching these reference points with the software tools. Finally, AMIRA allowed to segment automatically the regions of interest which were interpolated and finally 3D reconstructed (Fig. 1).

Immunostaining of grade II oligodendroglioma showed a high microvessel density mainly characterized by a branching architecture. Furthermore, only few vessels had a clear visible lumen, since the vessel diameter in the majority of vascular structures was very small. As an alternative, an hypothetical change in the vessel diameter within its own path could not be excluded, but was not verifiable in 2D. An additional feature typical of grade II oligodendrogliomas (and lacking in grade III tumours) was the pronounced focal positivity to CD34 in apparently single isolated cells.

The 3D reconstruction of oligodendroglioma grade II was instrumental in comprehending both these patterns: in fact, numerous and delicate vessels were inter-connected each other so as to assume a clear-
that may generate new vessels in a few hours, and the reduced requirement for cell migration, proliferation and basal membrane production [14]. Thus, in respect to the grade II oligodendroglioma, the angiogenic process of the anaplastic form can be characterized as a stable system able to expand very rapidly at low metabolic need. In the additional cases of oligodendroglioma grade II and III studied with a more limited 3D reconstruction (up to 20 serial sections) we reconfirmed the grade-related specificity of the vascular network described above.

Comparing our 3D reconstruction with that described in glioblastoma multiforme and cerebral white, neocortical grey matter and cerebellar cortex of a normal human brain [15], it can be noticed that the vascular pattern in low-grade oligodendroglioma is very similar to that described in the normal brain tissue, whereas in the anaplastic variant of oligodendroglioma it looks similar to glioblastoma. One difference between our oligodendroglioma 3D reconstruction and that of normal brain is that in the latter the small structures referring to CD34 single cell positivity, probably due to recent new-formed ‘in progress’ vessels, are absent. The prognostic significance of vessel density and vessel morphometry in oligodendroglioma has been debated in the past with controversial generated hypotheses [8, 16, 17]. Probably, it can be necessary to integrate information derived from the calculation of microvessels density or expression of vascular endothelial growth factor.

**Fig. 1** Progressive 3D vessel reconstruction. Forty serial sections of a grade II oligodendroglioma were stained using an anti-CD34 antibody (A) in order to recognize the vascular structures. A sequential (B–L) automated analysis and reconstruction of the different layers allowed a final 3D informative image of the complex vascular system unrolling within the tumour.
(VEGF) with data related to the structure of vascular system to reliably predict therapy efficacy and patient's survival, as suggested. As an example, in our 3D model, the area occupied by isolated small vascular structures functionally excluded was 1.3% of the total examined area in grade II oligodendrogliomas, and 0.5% in grade III tumours. Such percentage corresponded to 56.7% of the total vascular surface within the grade II tumours versus 22% in grade III oligodendrogliomas: the implication of these data in affecting therapy effectiveness probably deserves further consideration, and possibly opens new scenarios on the correlation between brain tumour grade and angiogenesis and its real biological significance.

In conclusion, we have shown here a novel 3D vision of grade-dependent vascular changes in oligodendrogliomas. This 3D reconstruction amplifies the angiogenic differences related to tumour grade partly
evident using a traditional bi-dimensional approach and allows to concretely elucidate the occurring variation in spatial distribution of the blood vessels and to comprehend some features otherwise not clearly identifiable in 2D. Specifically, in the present model, 3D approach highlighted two distinct vascular patterns and their strict dependence to tumour aggressiveness, with a sprouting/branching process proper of a slow growing grade II tumour, and an intussuceptive process, proper of rapidly growing tumours, activated by the requirement of a rapidly expanding vascular compartment. Altogether, these data provide supportive evidence that metabolic requirement and/or production of angiogenic factors proper of differently aggressive tumours dictate alternative angiogenetic processes.

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