Vasculogenic mimicry in malignant mesothelioma: an experimental and immunohistochemical analysis

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INTRODUCTION

Malignant mesothelioma (MM) is an aggressive malignancy of the serosal membranes lining the pleural, peritoneal and pericardial cavities. Most MMs are attributable to asbestos exposure. Although there are now bans on asbestos in most industrialised countries, the incidence of pleural MM is stable or even increasing, due to long latency between commencement of exposure and diagnosis, and the continued presence of asbestos in the built environment.1–3 Prognosis remains poor, with median survivals of about 12 months, highlighting the importance of novel treatment strategies.4

Adequate blood supply is essential for tumour growth, invasion and metastasis.5 In mesothelioma, microvessel density is independently associated with decreased survival,6–7 and high levels of VEGF-A in serum and pleural effusions are adverse prognostic factors.8,9 Traditionally, tumour vascularisation was thought to occur via angiogenesis by the sprouting of pre-existing host blood vessels to form new vascular channels, but tumour angiogenesis is now thought to involve alternative mechanisms of vascularisation including vasculogenic mimicry (VM). First described in aggressive uveal melanomas,10 some tumour cells re-differentiate to an endothelium-like phenotype to form hollow channels and narrow conduits with a lumen through which blood can flow.11–15 VM has been observed in other tumours,16–23 sometimes associated with decreased survival.19 The occurrence and significance of VM in MM has yet to be evaluated although there exists one report of VM in ‘mesothelial sarcomas’.24

In solid tumours, angiogenesis is stimulated by pro-angiogenic factors in response to hypoxia in the tumour microenvironment. Members of the VEGF family are important regulators of angiogenesis.8,9,25,26 There are several VEGF isoforms.27 VEGF-A and VEGF-B act via their receptors VEGFR1 (Flt-1) and VEGFR2 (Flk-1/KDR).28 MMs secrete VEGF-A and can express VEGFR1, and in some MM cell lines VEGF-A acts directly as an autocrine growth factor in vitro, in a dose-dependent manner.29 This effect may be dependent on expression of VEGFR1.30 Our observations suggest that although classical pathways of angiogenesis contribute to disease progression in MM, alternative mechanisms of cancer progression such as VM may be significant.

MM is a morphologically protean tumour with epithelial, biphasic and sarcomatoid histological subtypes31–34 and, occasionally, heterologous sarcomatous differentiation such as chordoid and osseous differentiation.35 MMs routinely co-express epithelial and mesenchymal markers, such as cytokeratins and thrombomodulin, a glycoprotein expressed by mesothelium, vascular endothelium, synovium and placental syncytiotrophoblast.32,36–40 Sarcomatoid features predict poor prognosis, and the morphology-based MM classification correlates with expression profiles of molecular markers of epithelial-to-mesenchymal transition (EMT).31,41 Based on our clinical observations that rare MMs express specific vascular endothelial markers such as CD31 while maintaining cytokertatin expression and positive expression of mesothelial markers, and coupled with the limited clinical response to anti-angiogenic therapies, we postulated that MM is capable of VM.

Summary

Vasculogenic mimicry, the process in which cancer cells form angiomatoid structures independent of or in addition to host angiogenesis, has been recorded in several otherwise non-endothelial malignant neoplasms. This study describes evidence of routine vascular mimicry by human mesothelioma cell lines in vitro, when the cell lines are cultured alone or co-cultured with human umbilical vascular endothelial cells, with the formation of angiomatoid tubular networks. Vasculogenic mimicry is also supported by immunohistochemical demonstration of human-specific anti-mitochondria antibody labelling of tumour-associated vasculature of human mesothelioma cells xenotransplanted into nude mice, and by evidence of vascular mimicry in some biopsy samples of human malignant mesotheliomas. These studies show mosaic interlacing of endothelial and mesothelial cell lines that co-label or label individually for immunohistochemical demonstration of human-specific vascular endothelial markers such as CD31 while mesothelial markers.

Key words: Mesothelioma; vasculogenic mimicry; angiogenesis; VEGF; CD31; mesothelial markers.

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MATERIALS AND METHODS

Mesothelioma cell cultures

NCI-H226, NCI-H28, NCI-H2052, NCI-2452, MSTO-211H and Met5a cell lines were obtained from the ATCC and used within 2 passages. All cell lines were maintained in complete DMEM (10% fetal calf serum, 50 U/mL penicillin, and 50 μg/mL streptomycin) and incubated at 37°C at 5% CO₂. Human umbilical vein endothelial cells (HUVECs) used as positive controls for tube formation assays were obtained from consenting donors.43 (approved by the Human Research Ethics Committee of the Royal Adelaide Hospital, Adelaide, South Australia); HUVECs were cultured in M199 media supplemented with 20% FCS, 50 U/mL penicillin, 50 μg/mL streptomycin, 1 μM sodium pyruvate, 1% v/v non-essential amino acids and 1% v/v Glutamax.

Harvesting of primary MM cells from pleural effusion fluids

Twelve MM pleural effusion samples (with corresponding confirmatory biopsies), originating from eight patients (6 male, 2 female; 7 epithelioid and 1 sarcomatoid MM) were tested after diagnostic procedures were finalised (approved by Southern Adelaide Clinical Human Research Ethics Committee). Four benign reactive effusions were also tested. Samples were centrifuged at 500 x g for 10 min at 25°C, and the cell pellet was cultured in complete DMEM. Supernatants were stored at −80°C. Purity of samples (≥80%) was confirmed by positive immunohistochemistry for the mesothelial cell marker calretinin and with the epithelial antibody CAM5.2. Diagnosis of corresponding biopsies was confirmed as above.

Tube formation assay

Bidi jisidoe angiogenesis slides (Bidi, Germany) were coated with 10 μL growth factor nutrient-reduced Matrigel (BD Biosciences, USA), and allowed to polymerise for 30 min at 37°C. Cells were seeded in triplicate (1.6 x 10⁵ cells/well) in complete DMEM and incubated at 37°C. For MM and HUVEC co-culture, 8 x 10⁴ cells from each cell type were combined and subsequently seeded at 1.6 x 10⁵ cells/well onto Matrigel. Photos were taken at 1 h and 6 h on an Olympus IX71 Fluorescence Inverted Microscope (Olympus, Japan), and ‘stitched’ together using Adobe Photoshop (Adobe, USA).

Immunofluorescent staining of tube formation assays

Cultures were washed twice with PBS, and fixed for 30 min at 25°C with 10% buffered formalin. Blocking was performed using 10% normal goat serum for 90 min, followed by addition of 1:100 primary antibody (Calretinin, 18-0211, Zymed, USA; CD31, J7/70A, M0823, Dako, Denmark) in 10% normal goat serum. After 2 h incubation, wells were washed and incubated with secondary antibody for 2 h. Nuclei were counterstained with Hoechst 33342 (Bio-Rad, USA) for 30 min at 25°C.

Animal model

A heterotopic xenograft mouse model of MM was used to evaluate the origin of the vasculature in MM. Briefly, 1 x 10⁶ NCI-H226 cells in PBS were injected subcutaneously into the hind flank of BALB/C nude mice (n = 6). Tumours were grown to 100 mm³ and animals euthanised by CO₂ exposure (approved by Flinders University and Southern Adelaide Local Health Network Animal Welfare Committee).

Immunohistochemistry

All diagnostic immunohistochemical studies were performed by a clinical laboratory participating in a quality assurance program (QAP), using National Association of Testing Authorities (NATA)-approved procedures and with appropriate controls. For cell block preparation from cultured cells and effusions, cell pellets were resuspended in 1–2 drops of sheep plasma. One drop of thrombin was added and the mixture was allowed to set before immersion in 10% formalin. Graded concentrations of ethanol and isopropanol were applied before embedding in paraffin. Paraffin sections were cut at 4 μm, deparaffinised and quenched with 1% H₂O₂. Cell block sections originating from cultured cells then underwent immunohistochemistry under the same conditions as clinical samples. For the human mitochondria immunohistochemistry, sections underwent EDTA retrieval and block with 10% normal goat serum (Sigma), before overnight incubation with 1:750 primary antibody MAB1273B (clone 113-1; Merck Millipore, USA).

Human MM biopsy diagnosis

Of the five biopsy cases reported in this study, two were identified among 18 in-house MM samples submitted for histological subtype investigated at the Flinders Medical Centre (FMC) over a 15-month interval in 2015–2016; three cases represented referrals among a greater number of cases for most of which no paraffin embedded tissue was received. Diagnosis was established by light microscopy and positive immunohistochemical (IHC) labelling for epithelioid MM markers (CK5/6, calretinin, WT1, D2-40, thrombomodulin and HBME-1),2,44 to exclude an epithelioid haemangiendothelioma (EHE) or an epithelioid angiosarcoma (ASA).34,44 Supported by radiological demonstration that the tumour was serosa-based (pleural or, in one case, peritoneal), with no imaging evidence of any extraserosal tumour.34,44 One referred biopsy case was also studied by immunohistochemistry for glycoporphin A as a marker for erythrocyte cell membranes.48 Another referral case was immunolabelled for TLE-1 (Transducin-like enhancer of split-1) and Erythroblast transformation specific related gene product (ERG; a marker for endothelial cells and some prostatic carcinomas).49,50 Although TLE-1 labelling is demonstrable in about 90% of synovial sarcomas or more, it is also recorded in benign schwannian and solitary fibrous tumours, and about 37% of non-synovial sarcomas that include malignant peripheral nerve sheath cell tumours.51–53 Matsuyama et al.54 found TLE-1 expression in >25% of cells in 20 of 29 mesotheliomas of all histological subtypes (about 69%), and those authors54 concluded that TLE-1 expression had no or only limited value in the distinction between mesothelioma and synovial sarcoma. Some ‘early’ papers on VM included staining with periodic acid–Schiff (PAS) (plus CD31 immunohistochemistry55) or immunohistochemistry for laminin; however, PAS can stain epithelial, mesothelial or vascular basement membranes, and the same consideration applies to laminin immunohistochemistry, so that such markers do not per se distinguish between mesothelial and endothelial differentiation.56 Therefore, we have concentrated on CD31 positivity in combination with strong cytokeratin expression and mesothelial cell markers in step sections of the same areas of the same mesotheliomas.

RESULTS

Mesothelioma cell lines and MM primary cells form tube-like interconnected networks in vitro under permissive conditions

All MM cell lines tested (NCI-H226, NCI-H28, NCI-H2052, NCI-2452 and MSTO-211H) formed tube-like structures after 6 h incubation in vitro (Fig. 1B–F) when cultured on reconstituted basement membrane (Matrigel) without growth factors. Likewise, all 12 primary MM cell samples obtained from pleural effusion fluids showed tube formation (Fig. 1G, representative of the seven epithelioid MMNs tested; Fig. 1H the single sarcomatoid MM). The tubes formed by the MM cells were comparable to positive control tubes formed by bonta fide vascular cells, HUVECs, under the same conditions (Fig. 1I). In contrast, mesothelial cells obtained from reactive effusions and the normal mesothelial Met5a cell line (Fig. 1A) did not form these networks under the same conditions. The tube-like structures did not label for CD31. Calretinin and cytokeratin expression were maintained in the cultured cells, including the single sarcomatoid MM.

Mesothelioma primary cells and HUVECs form interconnected networks in vitro

Primary MM cells incubated together with HUVECs formed interconnected networks consisting of both cell types, indicated by labelling of the mesothelial component for calretinin. There were no isolated islands of either cell type present, suggesting that vascular endothelial cells and MM tumour cells are capable of forming vascular mosaic networks (Fig. 1J).
MM cell lines and primary cells undergo vasculogenic mimicry in vitro. Cells were seeded (1.6 × 10^5 cells/well) onto Matrigel and incubated for 6 h. For co-culture of HUVECs and MM, cells were labelled in situ with the anti-mesothelial antibody calretinin (red), and counterstained with Hoechst (blue). Photos were taken using an Olympus IX71 inverted fluorescence microscope fitted with 4x objective. The vasculogenic mimicry potential of MM cell lines was comparable to that of HUVECs, in contrast to the benign control cell line Met5a which did not exhibit tube formation under the same conditions. Co-cultured HUVEC and MM formed tube-like structures consisting of a mosaic of both cell types. (A) Met5a showing absence of tubal networks. (B) NCI-H28. (C) NCI-H226. (D) NCI-H2052. (E) NCI-H2452. (F) MSTO-211H. (G) Primary MM cells (epithelioid subtype). (H) Primary MM cells (sarcomatoid subtype). (I) HUVECs. (J) Primary MM cells co-cultured with HUVECs, labelled with the mesothelial marker calretinin (red), counterstained with Hoechst nuclear stain (blue).
The vasculature in a heterotopic MM xenograft model is partially of human origin

Immunohistochemistry for human-specific mitochondria in sections of heterotopic xenografted MM in BALB/C nude mice confirmed that some vessels were of mouse origin, being lined with mouse endothelial cells, but labelling with the human mitochondria-specific antibody MAB1273B showed labelling of cells lining vessels containing red blood cells at the periphery of the tumour, indicative of VM in vivo (Fig. 2). Labelling for CD31 was apparent in all vascular structures, irrespective of human or mouse origin.

Some biopsies of human MMs show clinical evidence of vascular mimicry

As depicted in Fig. 3–6 and discussed in the corresponding legends, we found that in some biopsy samples of MM, some cytokeratin-positive spindle and/or epithelioid cells formed vascular channels containing red blood cells, and the cells participating in vessel formation showed co-labelling for CD31 and cytokeratins, suggesting that VM is a significant feature in some MMs.

DISCUSSION

It was long thought that the microvasculature in malignant neoplasms represents proliferation of host micro-vessels driven by the secretion of angiogenic factors by the neoplastic cell population. A more complex picture has now emerged, beginning with the first report on VM by Maniotis et al.10 in 1999. VM has now been recorded in a variety of human malignant neoplasms, including uveal and cutaneous melanoma,22 some breast carcinomas,20,58 hepatocellular carcinoma,21,23 gastric adenocarcinoma16 and, especially, MM, as demonstrated by: (1) the expression of a human mitochondrial marker in vascular structures when a standard human MM cell line (NCI-H226) was xenotransplanted into a nude mouse; and (3) a few conventional biopsy samples of human MM, although VM seems to be unrecognisable in most ‘routine’ biopsy cases.

It is important to note in this context that the formation of interconnecting networks occurred spontaneously when MM cells were cultured on reconstituted basement membrane (Matrigel) without growth factors, i.e., without added VEGF. Unlike conventional culture, this matrix simply allows three-dimensional structures to form, but does not contain pro-angiogenic growth factors. However, MM cells themselves can secrete VEGF. It is unlikely that VM in MM coincides with epithelial to mesenchymal transition31 (previously shown to correlate with sarcomatoid change), because all primary MM cells tested were capable of VM in vitro, and only one sample was sarcomatoid in type (sarcomatoid MMs are notoriously paucicellular in effusion fluid). Rather, this may be an expression of the known pluripotency of mesothelial cells. This suggests that the positive labelling of tumour cells for endothelial markers in the biopsies represents an ‘extreme’ degree of endothelial differentiation in recognisable tumour cells, whereas in the majority of cases, those cells that have transitioned to vascular structures are simply not recognised as being neoplastic in character. The presence of human-derived vessels in a standard heterotopic xenograft MM model, where a MM tumour cell line (i.e., no possible contamination by human vascular cells) was injected also supports the notion that this may not be uncommon in MM. The labelling of all vessels for CD31 in the animal model also suggests that in biopsy samples VM may easily be overlooked, unless non-vascular tumour cells also express CD31. Labelling for CD31 is not necessary for MM cells to acquire a vascular phenotype, because the tube-like formations in vitro did not label for CD31.

The majority (67–93%) of human MM samples label for thrombomodulin as well as cytokeratins,32,36–40 and this may be an indication of the ability to differentiate into vascular structures. Rare MMs also show labelling of tumour cells for CD31,49 and as discussed above, it is impossible to ascertain by CD31 labelling alone whether vessels are of tumour cell origin or stromal-derived microvasculature. Two ‘conventional’ mesothelial markers—thrombomodulin (CD141) and D2–40—are also endothelial markers,39,70,71 suggesting some plasticity in differentiation. There has been a previous report of vasculogenic mimicry in ‘mesothelial sarcomas’,24 but this is not standard nomenclature, and that study did not specify the criteria used to make that diagnosis.24

Clearly, a major differential diagnostic consideration in the biopsy cases that we illustrate is MM with VM versus...
pseudomesotheliomatous \textit{ASa} \textsuperscript{72} or \textit{EHE}. \textsuperscript{72} Serosal endothelial sarcomas are rare and reported cases of pleural \textit{ASa} were defined by positive labelling for endothelial markers\textsuperscript{45,72–74} and negative mesothelial markers.\textsuperscript{5,47} Some reports on serosal \textit{ASa}/\textit{EHE} do not include any data on labelling for mesothelial markers,\textsuperscript{45,73–76} two other publications recorded a negative result for the only mesothelial marker used, namely calretinin\textsuperscript{46} or HBME-1.\textsuperscript{45} Labelling for vimentin and cytokeratins may not reliably discriminate between \textit{MM} and endothelial sarcomas.\textsuperscript{45} For these reasons, we have used multiple (six) \textit{MM} markers—\textit{CK}5/6, calretinin, \textit{WT}1, \textit{D2-40}, HBME-1 and thrombomodulin—and we have sought...
multifactorial evidence for VM in MM (i.e., cell culture of MM, and human mitochondrial immunohistochemistry in a MM xenograft). We consider those five clinical tumours to represent MM, based on co-expression of multiple mesothelial markers, areas of tubulopapillary architecture, and spindle-cell sarcomatoid areas in some tumours. The concept that MM can show specific patterns of mesenchymal differentiation (including heterologous sarcomatoid differentiation) may in part explain the readiness for differentiation as vessels under permissive conditions in vitro. The known pluripotency of normal mesothelial cells has resulted in their use for tissue engineering, including replacement of neural crest derived corneal endothelium. Our study has found that evidence of vascular differentiation in biopsies of MM varies greatly and Attanoos et al. found no CD31 expression in 92 mesotheliomas investigated, but all primary MM cells tested as part of our study were capable of VM in vitro. Terada et al. suggested their case of pericardial ASa might have represented a peculiar MM with abnormal differentiation along an angioblastic pathway, and in 2012 Klabatsa et al. reported a diffuse pleural mesothelioma with epithelioid and angiosarcomatous components, which may correspond to our clinical cases: angioblastic differentiation may be analogous to heterologous osteochondroid differentiation in the sarcomatoid component of some MMs. Although CD31 can label macrophages, we consider the co-labelling for cytokeratins (and other more specific mesothelial markers in step sections the same cell clusters in our biopsy cases and labelling for ERG in one case) negates this explanation for the CD31 positivity.

We consider that VM is a property of MM: we are unable to estimate with any reasonable degree of precision the proportion of MMs that display immunohistochemical evidence of VM in the form of CD31 expression in human biopsy.
tissue (perhaps because it is unrecognisable as such in most biopsy samples), but the in vitro findings in our study point to VM as a routine occurrence in MM, with the capacity for anastomosis/mosaicism with host vasculature (Fig. 7), and VM was detectable not only in our MMs with sarcomatoid differentiation, but also in epithelioid MMs. We consider that CD31 labelling in otherwise typical MM samples may simply be an IHC marker for hybrid mesothelial/endothelial differentiation that is part of the pluripotency of MM (with epithelial to mesenchymal transition). Such EMT is at least latent in up to about 50% or more of epithelioid mesotheliomas, and it occurs in about 75% of biphasic MMs and about 85% of sarcomatoid MMs, as demonstrated by vimentin-cytokeratin co-synthesis: in their study of 326 sarcomatoid and desmoplastic MMs, Klebe et al. detected vimentin expression in 101/111 cases (91%), cytokeratins in 161/280 (93%) and calretinin expression in 12/39 cases (31%). Alpha-smooth muscle actin (SMA) and desmin expression is detectable by immunohistochemistry in about 10% of biphasic MMs (and in a greater proportion of reactive mesothelial hyperplasias, up to about 85%).

The exact mechanisms for such EMT in MM appear to be complex and are incompletely understood. Fassina et al. studied N-cadherin, vimentin, SMA, Snail, Slug, Twist, ZEB1, ZEB2, S100A4, MMP2, and MMP9 from epithelioid to biphasic and sarcomatoid MM cell lines by immunohistochemistry and qRT-PCR, and in situ hybridisation. They found ectopic expression of miR-205 (a repressor of ZEB1 and ZEB2 expression) in a commercially available mesothelial cell line and in epithelioid and biphasic MM cell lines and it induced a significant reduction of ZEB1 and ZEB2, with inhibition of migration and invasion. In particular, miR-205 was significantly down-regulated in biphasic and sarcomatoid MMs, correlating with a mesenchymal phenotype and more aggressive behaviour. De Reynies et al. investigated 38 primary pleural MM cell cultures by transcriptomic microarrays (discovery + extension series; n = 67), and 108 frozen pleural MMs by qRT-PCR for gene mutations that included BAP1, CDKN2A, CDKN2B, NF2 and TP53; EMT markers were studied at mRNA and proteonomic levels. From these studies they divided their MMs into two groups: C1 and C2. All sarcomatoid/desmoplastic MMs fell into C2,
whereas the epithelioid and biphasic MMs fell into both C1 and C2, partly correlating with histological subtyping. Genes up-regulated during EMT (such as the adhesion molecules \textit{POSTN} and \textit{VCAN} or the transcription factors \textit{SANI2}, \textit{TCF4} and \textit{HMGA2}) showed higher expression in C2 than C1, whereas genes down-regulated during EMT such as \textit{CDH1} and \textit{CDH3} showed lower expression in C2 than C1. The prognosis for C2 was worse than for C1. In a further prospective study of VM in MMs, we hope to explore further the EMT pathways implicated in MMs with VM, by immunohistochemistry, and proteonomic and metabolomic analysis, together with studies for cancer stem cells in MM.

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