Immunobiology 219 (2014) 308–314

Contents lists available at ScienceDirect

Immunobiology

journal homepage: www.elsevier.com/locate/imbio

Lung cancer neovascularisation: Cellular and molecular interaction between endothelial and lung cancer cells

Sabine Kaessmeyer[a,*], Kanti Bhoola[b,1], Svetlana Baltic[b], Philip Thompson[b,2], Johanna Plendl[a,2]

[a] Institute of Veterinary Anatomy, Department of Veterinary Medicine, Freie Universität Berlin, Koserstraße 20, 14195 Berlin, Germany
[b] Lung Institute of Western Australia and Centre for Asthma, Allergy and Respiratory Research, The University of Western Australia, Nedlands, WA 6009, Australia

ARTICLE INFO

Article history:
Received 14 June 2013
Accepted 14 November 2013
Available online 22 November 2013

Keywords:
CD31
Co-culture
Endothelial cells
Immunocytochemistry
Immunohistochemistry
Lung adenocarcinoma
Lung squamous carcinoma

ABSTRACT

Background: Novel vascular-independent conduits have been observed in some cancers. These have been variously described as vasculogenic mimicry, mosaic vessel formation, vascular co-option and intratumour embryonic-like vasculogenesis. Despite lung cancer being the most common cancer worldwide, there is little information on its neovascularisation or the pathways involved.
Methods: An *in vitro* model involving co-cultures of microvascular lung endothelial cells and squamous or adenocarcinoma lung cancer cells was developed to assess their angiogenic interaction. Cells were incubated and examined by phase contrast microscopy and by immunocytochemistry in both mono- and co-cultures. Cultured cells and lung cancer tissue sections were assessed for new tumour vessel formation, expression of the endothelial marker CD31 and morphology.
Results: Lung tumour cells and endothelial cells interacted morphologically *via* pseudopodia and used alternative pathways to generate new vessels. Co-culturing microvascular endothelial and squamous carcinoma cells led to endothelial cells surrounding tumour cells and the tumour cells being incorporated into vessel walls. Co-culturing endothelial and adenocarcinoma cells resulted in cellular contact and the formation of tumour cell bridges around clusters of endothelial cells. These adencocarcinoma cells became strongly positive for CD31. Tumour tissue section studies supported the *in vitro* findings.
Conclusion: Lung carcinoma cells when co-cultured with lung endothelial cells modify their cellular and molecular features that encourage alternative means of providing blood supply. The mechanisms underpinning these non-angiogenic processes need to be further investigated and should be considered when anti-tumour therapeutic interventions are being considered.

Introduction

The formation of blood vessels is initiated by two distinct processes, namely vasculogenesis and angiogenesis. Vasculogenesis is the *in situ* differentiation of endothelial precursor cells (angioblasts) into endothelial cells (EC), which then re-assemble into primitive vascular plexuses. Angiogenesis generally involves the expansion of a primitive vascular network into a complex one (Hillen and Griffioen, 2007; Patan, 2000; Makanya et al., 2009) by growth of endothelial sprouts from pre-existing blood vessels through migration, proliferation, three-dimensional organisation and tube formation of ECs.

Whereas formation of new blood vessels plays a major role during prenatal development, in adults, blood vessel growth is linked primarily to tissue repair and clinical disorders such as tumour growth (Vacca and Ribatti, 2011). The growth of solid tumours beyond 1–2 mm in diameter requires the induction of new blood vessel formation (Bergers and Benjamin, 2003). It has

Abbreviations: CD, cluster of differentiation; EC, endothelial cells; NSCLC, non-small cell lung cancer; HMVEC-L, human microvascular endothelial cells-lung; rhFGF, recombinant human fibroblast growth factor; VEGF, vascular endothelial growth factor; GA, gentamicin/amphotericin; FBS, fetal bovine serum; H520, human lung squamous cell carcinoma cells 520; ATCC, American Type Culture Collection; H2126, human lung adenocarcinoma cells; DMEM, Dulbecco's modified Eagle medium; PBS, phosphate buffered saline; PFA, paraformaldehyde; TBS, Tris-buffered saline.

* Corresponding author at: Institute of Veterinary Anatomy, Department of Veterinary Medicine, Freie Universität Berlin, Koserstraße 20, D-14195 Berlin, Germany. Tel.: +49 03083853558; fax: +49 03083853480.
E-mail address: sabine.kaessmeyer@fu-berlin.de (S. Kaessmeyer).
[1] Institutes of Anatomy, Histology & Pathology, and Physiology, Universidad Austral de Chile, Valdivia Box 567, Chile
[2] Equal last author.

0171-2985/$ – see front matter © 2013 Elsevier GmbH. All rights reserved.
http://dx.doi.org/10.1016/j.imbio.2013.11.004

been assumed that tumour vascularisation can be explained by angiogenesis. However, in the last decade, novel angiogenesis-independent pathways have been observed in the blood supply of certain tumours. Such alternative processes, found particularly in aggressive tumours, are designated variously as vasculogenic mimicry, mosaic vessel formation, vascular co-option and intra-tumour embryonic-like vasculogenesis (Hillen and Griffioen 2007; Bussolati et al., 2011; Chang et al., 2000; Ackermann et al., 2012).

Vasculogenic mimicry, first described by Maniotis et al. (1999) in uveal melanoma, is the formation of a complete capillary-like network that is comprised only of tumour cells instead of vascular ECs. In tumours such as colon carcinoma, mosaic vessels have been described, in which, attached to the luminal surface of the vascular channels are both ECs as well as non-ECs; the latter, lacking expression of specific endothelial cell markers (Chang et al., 2000; Ackermann et al., 2012). Tumour cells that grow along pre-existing vessels but without evoking an angiogenic response, referred to as vessel co-option, have been found in cerebral glioblastoma, breast adenocarcinoma, and in melanomas (Bartha and Rieger 2006; Holash et al., 1999). Finally, *de novo* generation of tumour vessels can arise from the differentiation of stem and progenitor cells of hematopoietic origin or from those resident in tissues and participate in tumour progression (Bussolati et al., 2011).

Lung cancer is the leading cause of cancer worldwide (Kimman et al., 2012) and non-small cell lung cancers (NSCLC; subtypes squamous cell carcinoma and adenocarcinoma), account for approximately 80–90% of lung cancers (Yano et al., 2011). Despite this, there is a lack of information on their neovascularisation, especially with respect to alternative cellular processes for vessel formation (McClelland et al., 2007; Passalidou et al., 2002). Tumour neovascularisation is a complex process based upon a sequence of interactions between tumour cells and ECs (Levine et al., 2001; Witz, 2009). *In vitro* culture models allow analysis of each step involved in vascular growth including the interaction between endothelial and tumour cells. As such an *in vitro* lung cancer vascular model should help identify the specific steps and mechanisms involved (Auerbach et al., 2003; Kaessmeyer and Plendl, 2009; Kassmeyer et al., 2009; De Spiegelaere et al., 2012; Sievers et al., 2011; Bahramsoltani et al., 2009).

The aim of the current study was to develop an *in vitro* model to investigate the vascular and molecular interactions that occur between lung endothelial and lung carcinoma cells thereby providing evidence for recruitment of ECs and the induction of new blood vessel formation by lung carcinomas. A morphological study was designed to monitor co-cultures of microvascular lung ECs with two types of NSCLC.

Materials and methods

The study was approved by the Human Research Ethics Committee of Sir Charles Gairdner Hospital Nedlands Western Australia, Australia.

Cell culture

Cells and culture media

Lung derived normal human microvascular ECs (HMVEC-L, Lonza, Walkersville Inc., Walkersville, USA) were incubated in basic EC culture medium (EGM-2-MV; Lonza) supplemented according to the supplier's instructions with rhFGF, VEGF, vitamin C, GA-1000, hydrocortisone and FBS. Human lung squamous cell carcinoma cells (H520, ATCC) and human lung adenocarcinoma cells (H2126, ATCC) were cultured in DMEM with 10% FBS, 1% L-glutamine and 1% penicillin/streptomycin (10,000 U/ml) (all from Sigma–Aldrich, Taufkirchen, Germany; and referred subsequently as 'tumour cell medium'). Cells were incubated in a humidified atmosphere (37 °C, 5% CO_2) and the medium was replaced every 2–3 days.

Table 1
Experimental design for culture and co-culture time-points: tumour cells, adapted to culture medium of endothelial cells, were added at a concentration of 2×10^4 cells to the lung endothelial cells (4×10^4 cells seeded).

Human lung microvascular endothelial cells (HMVEC-LV) Time in culture	Duration of co-culture of H520 tumour cells and HMVEC-LV	Duration of co-culture of AC H2126 tumour cells and HMVEC-LV
28 Days	24 h	24 h
28 Days	7 Days	7 Days
28 Days	14 Days	14 Days
28 Days	32 Days	32 Days

Priming of lung tumour cells

Adaptation (priming) of the tumour mono-cell cultures to the HMVEC-L cell medium was carried out over a 48 hour period in steps that sequentially involved mixing fresh tumour cell medium with 25%, 50%, 75% and finally 100% of HMVEC-L cell medium.

Endothelial cell culture

HMVEC-L cells were seeded in 24-well plates from Iwaki (Tokyo, Japan) onto round glass cover slips (12 mm diameter, Roth, Karlsruhe, Germany) which were gelatine coated (Difco Laboratories, Detroit, USA, 1.5% in PBS). Optimal seeding density of 4×10^4 cells per well resulted in their adherence and formation of an even monolayer.

Direct co-culture design (Fig. 1)

The primary co-culture design allowed HMVEC-L cells to be incubated with the primed lung H520 or H2126 tumour cells (each trypsinised, seeding density: 2×10^4) for varying times. On the day of co-culturing, the HMVEC-L cells had already been in culture for 28 days (Stage 3, Table 2). The precise time schedules for the co-cultures are shown in Table 1. At the end of each co-culture incubation period, cells were rinsed, fixed and labelled with CD31 for subsequent immunocytochemistry.

Phase contrast microscopy of cultured cells

Light micrographs were taken with a Zeiss Axiovert 25 inverted microscope (Zeiss MicroImaging GmbH, Jena, Germany) and a 000610 video camera INTEQ (INTEQ, Berlin, Germany) using Zeiss Axiovision image-processing software.

Lung tissue samples

Archival lung carcinoma specimens were obtained from the Lung Institute of Western Australia and PathWest Inc. The tissue samples were fixed in 4% PFA, paraffin embedded, and cut into 5 μm sections. Multiple tissue sections of resected normal lung, adenocarcinoma (1 female, 1 male) and squamous cell carcinoma (2 males) were stained by immunohistochemistry as described below.

Immunohistochemistry

Antigen retrieval was achieved by heating tissue slides in citrate buffer at 95 °C in a water bath and blocking of endogenous peroxidase activity with TBS-buffer with 0.6% (v/v) H_2O_2, followed by washing in TBS-buffer plus 0.01% polysorbate 20 (Tween20, Sigma–Aldrich). The slides were pre-incubated with 20% (v/v) donkey IgG serum in buffer A, and finally incubated with CD31 overnight at 4 °C (see Table 3).