Pathol. Oncol. Res.
DOI 10.1007/s12253-008-9140-y

ORIGINAL PAPER

Hypoxia Generates a More Invasive Phenotype of Tumour Cells: An *In Vivo* Experimental Setup Based on the Chorioallantoic Membrane

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Received: 3 September 2008 / Accepted: 27 November 2008

Abstract Of all processes involved in carcinogenesis, local invasion and the formation of metastases are the clinically most relevant but the scientifically least well understood at their molecular level. Recent experimental progress has identified that tumour hypoxia not only induces tumour angiogenesis, but also modulates the expression of several genes that have been implicated in tumour invasion and metastasis. Here we developed an in vivo model to understand a number of molecular pathways and cellular mechanisms for tumour invasion in hypoxia. For this purpose fertilized chicken eggs were incubated for 10 days in normoxic conditions. Subsequently colon carcinoma cells (SW-480) were placed on the chorioallantoic membrane. During the following 6 days the eggs were incubated either in normoxic conditions or in stepwise decreasing hypoxic conditions. SW-480 colon carcinoma cells did not invade the epithelial layer in normoxic conditions. In contrast an invasion through the epithelial layer in to the mesoderm was already seen after 3 days when incubated in hypoxic conditions. The chorioallantoic membrane assay described in this paper allows investigating tumour invasion and its cellular mechanisms under defined hypoxic conditions.

Keywords Chorioallantoic membrane · Hypoxia · Tumour invasion · Tumor progression

Abbreviations
CAM Chorioallantoic membrane
BD Breeding day

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Introduction

A tumour cell's abilities to invade adjacent tissues and disseminate into distant organs have been considered the biological hallmarks of malignancy for a long time [1–3].

About 90% of all cancers originate from epithelial tissue. The most apparent morphological change occurring during the transition from a benign tumour to a malignant and metastatic one is a change from highly differentiated, epithelial morphology to a migratory and invasive phenotype

Published online: 12 December 2008

of tumour cells. Metastatic tumour cells break through the basal lamina barrier and invade the mesenchymal layer. This process is called the "epithelial mesenchymal transition (EMT)" and it is a prime subject of cancer research [3–5].

Recent experimental findings identified that tumour hypoxia not only induces tumour angiogenesis, but also modulates the expression of several genes that have been implicated in tumour invasion and metastasis [6, 7].

To understand the molecular pathways and cellular mechanisms of EMT and tumour invasion in conditions of hypoxia an *in vivo* model is needed. The following paper introduces such a feasible method based on the chorioallantoic membrane (CAM) assay.

Material and Methods

Eggs and Preparation

Fertilized chicken eggs (White Leghorn, 50–54 g, Charles River Wiga, Sulzfeld, Germany) were disinfected with a sterile gauze saturated with 70% of ethanol and incubated for 9 days under conditions of 37°C temperature, 80% humidity and normoxia (20.5% O_2) in an upright position with the blunt pole on top (Fig. 1). After 9 days the eggs were prepared as follows: first a 2 mm hole was milled carefully into the egg shell on the tip of the blunt pole using a graving instrument (Proxon Micromat System®, Niersbach, Germany). The dust generated during milling was removed with a moist sterile swab. The outer shell membrane was not ruptured. Then an anatomical forceps was used for perforation of the outer shell membrane to open up the air cell. Subsequently the egg shell was broken according to the topography of the air cell. Thus a circled window of 2–3 cm could be opened up (Fig. 1b). Using a stereomicroscope the inner shell membrane was humidified with 0.5 ml of Ringers' solution. This procedure allows removing the inner shell membrane with micro tweezers without destroying the epithelial layer and the vascular architecture of the CAM (Fig. 1c). The window was closed with cover slip, which was fixed with scotch tape. The eggs were then incubated for another day so as to allow possible micro lesions to recover. On the 10th breeding day (BD) the eggs were inoculated with 106 tumour cells (SW480) dropped as a suspension in isotonic NaCl solution on the CAM (Fig. 1d). Afterwards the window was again closed with cover slip and tape. Now samples were divided up into two groups of different breeding conditions. The first, normoxic group (nor-group, $n=10$) was incubated in a 20.5% oxygen atmosphere. The second, hypoxic group (hyp-group, $n=10$) was incubated in an atmosphere of decreasing oxygen levels from 18% O_2 (on 10th BD) over 15% O_2 (on 11th BD) to 13% O_2 (12th BD). This procedure allows the chick embryo, and the tumour cells too, to adjust to the changing breeding conditions down to levels of hypoxia similar to that of tumour cells spreading in tissue. The tumour nodules on the CAM were harvested either on the 13th BD ($n=5$) or on the 16th BD ($n=5$). In total we looked at 20 samples divided into four groups of five samples each: two groups with different breeding conditions (nor-group and hyp-group) each subdivided into two different groups of incubation time [3 days or 6 days (D): nor-group-3D ($n=5$) or nor-group 6D ($n=5$), hyp-group-3D ($n=5$), hyp-group-6D ($n=5$)]. After the planned incubation intervals individual tumor growth and spread were documented by photographs (Fig. 1e) (0,6×, 1,2× and 2,5×) (Photo-camera, Cannon® Tokyo, Japan) and subsequently the CAM was harvested and fixed in formaldehyde (4 vol%, 24 h)

Tumour Cells

SW480 human colorectal adenocarcinoma tumour cell line (ATCC® Number: CCL-228^{TM} were cultured in Dulbecco's modified Eagle's medium (DMEM®, PAA Laboratories

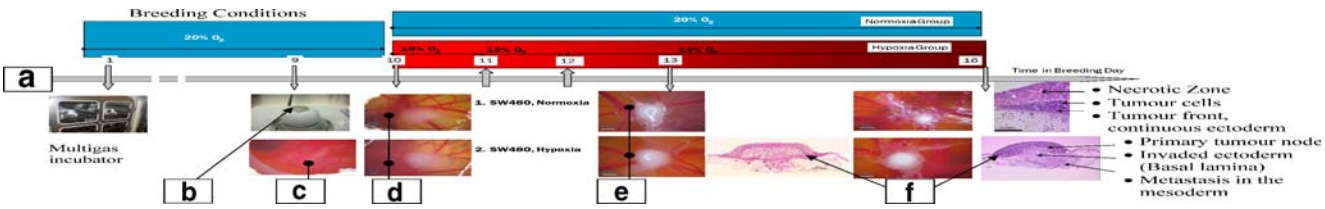

Fig. 1 Chorioallantoic Model Assay for investigating tumour invasion in hypoxia. Work flow in breeding days (BD) (**a**). During the first 10 BD the eggs were incubated in an upright position in normoxic conditions (20.5% O_2) and were opened on the blunt pole on the 9th BD (**b**). To get the chorioallantoic membrane out, the inner shell membrane was removed (**c**). Tumour cells 10^6 (SW-480) were seeded as a suspension on the 10th BD (**d**). Next the eggs were incubated either in normoxic or in stepwise hypoxic conditions. Tumour nodules occurred on the 13th BD (**e**). Histomorphological and immunohistochemical evaluations were performed after paraffin embedding and slicing vertically to the CAM's surface (**f**)

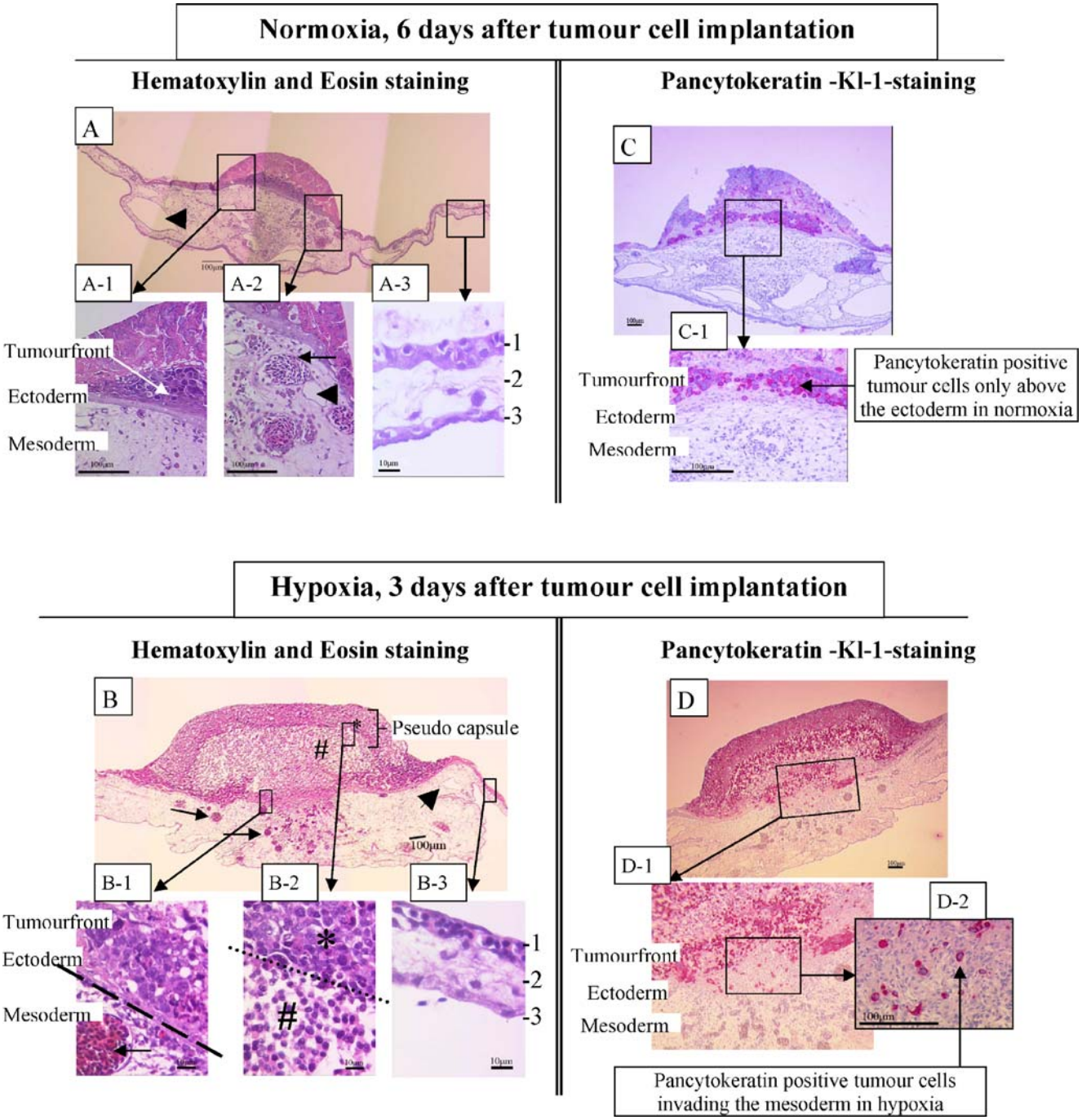

Fig. 2 Hematoxylin and Eosin staining (a, b) and pancytokeratin-Kl-1-staining (c, d) of the tumour nodule grown on the Chorioallantoic Membrane (CAM). Normal histomorpholgy of the CAM (Pic. A-3 and B-3) consists of three layers (1. ectoderm, 2. mesoderm, 3. endoderm) in both breeding conditions (normoxia or hypoxia) without apparent differences. This is presented next to the tumour nodules. A thickening of the CAM, especially of the mesoderm [vessels (arrows), lymph vessels (arrows head)] underneath the tumour nodule as induced by the tumour cells (up to 500 µm, (a, A-1, b, B-1)) in both breeding conditions could be seen. Three different phenotypes (b, B-2) of tumour cells could be distinguished in hypoxia. First: In the apex cell to cell contacts seem to be pronounced (*). The cells are closing in to build up a "pseudo capsule" (B-2, interrupted line). The cells are small and the core plasma relation is balanced. Second: In the middle part the cells are loosely configured (#). Cell to cell contact is sparse. Third: In the tumour front, especially in the centre of progression, necrosis with cell detritus was regularly seen next to infiltration of vital tumour cells through the ectoderm (D-2). Even after 6 days of tumour cell implantation no tumour cell could be detected in the mesoderm in normoxic breeding conditions (**c**, C-1)

GmbH, Pasching, Austria) supplemented with 10% fetal bovine serum. Tumour cells were cultivated in an environment of 21.5% O_2 and 8% CO_2 up to a confluence rate of 80%. For inoculation on the CAM tumour cells were trypsinized (PAA Laboratories GmbH, Pasching, Austria) and washed with phosphate buffered saline (PBS) (2× centrifuge: 20°C, 5 min, 500 RCF). A suspension of 13–15 µl representing 10^6 cells was inoculated on the CAM.

Paraffin Embedding

Each tumour nodule was fixed in 4% formaldehyde for 24 h. For the paraffin embedding the tissue samples were dehydrated in an increasing amount of alcohol. Then the planar portion of CAM with the tumour nodule on it was embedded in paraffin in an upright position for later sections vertical to the CAM's surface. All further investigations were performed on 5 µm tissue slices.

Immunohistochemistry

Immunostaining was performed on 5 µm sections of paraffin embedded tissues. To detect the tumour cells themselves, we utilized pancytokeratin -Kl-1-staining, performed as described in the protocol (1:200, IgG1 (mouse), Immunotech®, Marseille, France). Hematoxylin and Eosin staining was used to analyze classic histological criteria such as invasion and penetration through the epithelial layer or nucleus plasma relation of the cells.

Results

Survival experiments showed no difference between the eggs in normoxia (76%, (64/85)) and stepwise hypoxia as above (75%, (59/80)) during all procedures. However a daily decrease of the oxygen supply from 20% to 15% to

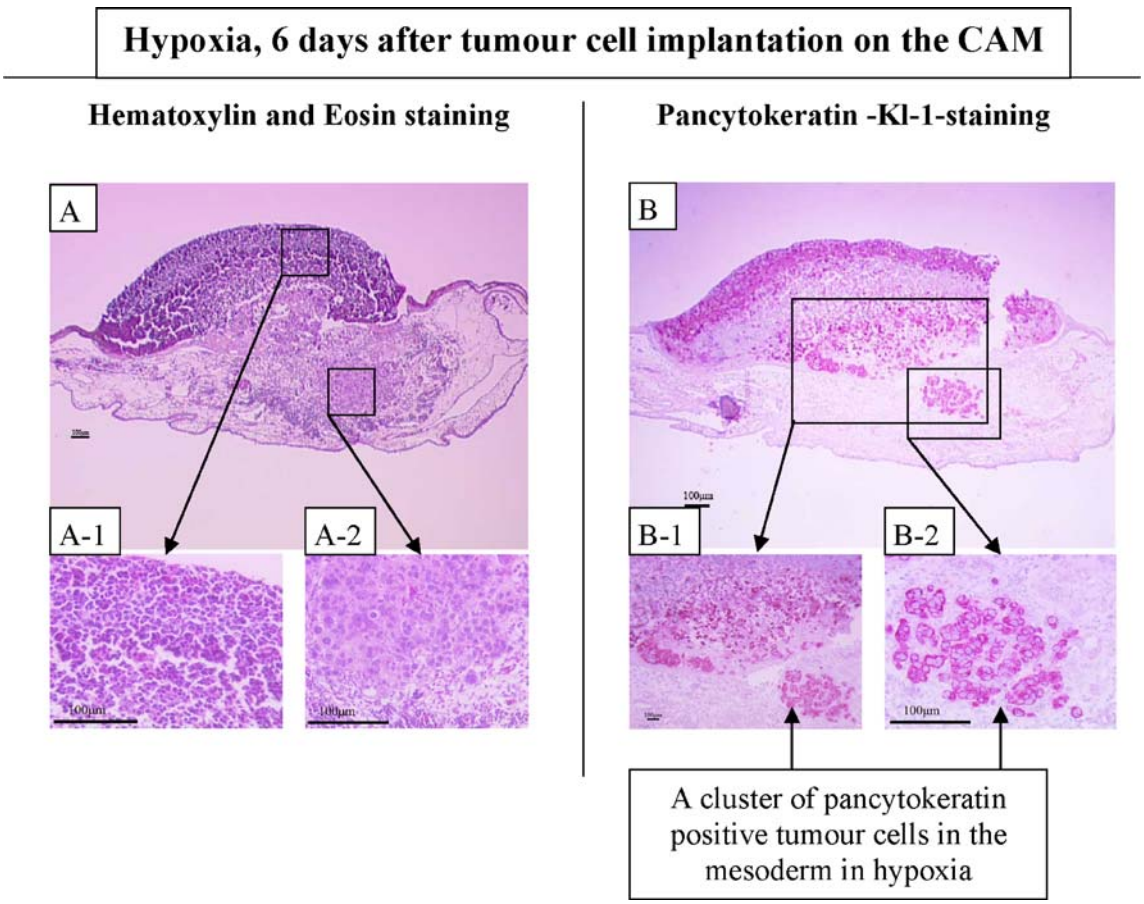

Fig. 3 "Satellite metastasis" in the mesoderm was built up after 6 days of tumour cell implantation on the CAM. CAM is negative for immunostaining pancytokeratin-Kl-1. Only human tumour cells SW-480 are positive. The depth and the kind of invasion in the mesoderm by the tumour cells are easily detectable (**b**, B-1, B-2). Clusters of tumour cells in the mesoderm (A-2, B-2) show an increased core plasma relationship. Cell to cell contacts seem to be pronounced again (A-1 vs. A-2)

10% rendered only five of 18 (27%) eggs alive after 4 days of breeding. We found out that a gradual lowering of oxygen levels as described here produces more accurate results with the least possible deranging of the physiology of the CAM. In fact, there were no morphological differences seen on the CAM in greater distance from tumour nodule itself under both breeding conditions (Fig. 2:A-3, B-3). Thickness of CAM next to the tumour nodule was 55±5 μm. We saw a thickening of the CAM, especially of the mesoderm (vessels, lymph vessels) underneath the tumour nodule as induced by the tumour cells (up to 500 μm, Fig. 2:A,B) in both breeding conditions.

Tumour cells cultivated in hypoxia regularly show three different phenotypes, which build up three layers (Fig. 2:b, B-2). From top to button: 1."pseudo capsule". The cells are close together. The cells are small and the core plasma relation is balanced. 2. In the middle part the cells are loosely configured. Cell to cell contact is sparse and the cells form "pseudo-vessel-like" lines. 3. The tumour front (Fig. 2:D-2,D-3). CAM is negative for immunostaining pancytokeratin-Kl-1. Only human tumour cells SW-480 are positive (Fig. 2:c,d). So the depth and the kind of invasion in the mesoderm by the tumour cells are easily detectable. The invasion of individual tumor cells (Fig. 2:d,D-3) after 3 days of incubation or cluster formation of tumor cells (Fig. 3:b,B-2) after 6 days of incubation could be demonstrated in hypoxia. In normoxia even after 6 days of incubation the tumor cells were vital but did not invade the mesoderm (Fig. 2:A-1,C-1).

Discussion

An in vivo experiment is presented here to investigate the effect of oxygen on the invasion capacity of the SW480 colon carcinoma tumour cell line. The stepwise daily decrease of the oxygen supply (20% to 18% to 15% to 13% O_2) gives tumour cells and chick embryos sufficient time to adapt to hypoxia. No morphological changes were seen in greater distance from tumor nodules as demonstrated in Fig. 2A-3 and B-3. The thickness of the CAM in this region as described in the literature [8] was 55±5 μm. Underneath the tumor nodule in both breeding conditions especially the mesoderm gets thicker. An increase of vessels and lymph vessels could be observed (Fig. 2:a,b). There is a paracrine interaction of human tumor cells with avian CAM. It has already been shown, that human cytokines like vascular growth factor VEGF [9], Interferon IFN-α [10] and IFN-β [11] interact with the CAM locally and increase or respectively decrease vascular density. The next step will be to find out which of the secreted proteins are responsible for the thickening and invasion of the mesoderm.

Next is to point out that tumour cells cultivated in hypoxia show a different histopathological design with three layers (Fig. 2:b). The meaning of these three layers is unclear. It is to suspect that the region of the tumor nodule is subject to a gradient of oxygen. Tumor cells develop different phenotypes in the same nodule. The cells close in to build up a "pseudo capsule" in the first layer (Fig. 2:b, B-2). Second: In the middle part the cells are loosely configured. Cell to cell contact is sparse and the cells form "pseudo-vessel-like" lines. Perhaps this allows nutrients to reach the cells in the apex. Third: In the tumour front, especially in the centre of progression, necrosis with cell detritus was regularly seen next to infiltration of vital tumour cells through the ectoderm (Fig. 2:D-2,D-3). After 3 days few tumor cells could be observed in the mesoderm. After 6 days tumor cells grew in clusters (Fig. 3:A-2). The development of metastases is characteristic of the malignant progression of tumour cells [1]. The process during which tumour cells gradually acquire all necessary properties to do so is summarized as a "cascade of the metastasis". A core component in the starting phase of this cascade is a contact inhibition between tumour cells followed by the invasion into neighboring structures [3, 5]. An environment of hypoxia—as it is present in any kind of solid tumor—seems to foster cell heterogeneity thus increasing the possibility that some of these cells will transform into a phenotype prior to metastasis [7]. In conclusion, we could show that hypoxia generates more invasive phenotypes of tumour cells on the CAM-assay. Further investigations will aim at describing cellular mechanisms under defined hypoxic conditions *in vivo* leading to more aggressive tumour growth.

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