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Endothelial cell apoptosis in the context of quantification of angiogenesis in solid human adenocarcinomas: A novel double immunolabelling technique to identify endothelial cell apoptosis

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ABSTRACT

Standardised methods of microvessel density quantification have been published. However, a reliable and reproducible method to visualise endothelial cell apoptosis is lacking, which is a shortcoming in assessing vascular remodelling during angiogenesis. The aim of this study was to validate a newly developed technique to demonstrate endothelial cell apoptosis by double immunolabelling with anti-CD34 and anti-activated caspase-3 in human adenocarcinomas. Double immunolabelling was used to identify apoptotic endothelial cells in six tumours of eight different human adenocarcinomas. Microvessel density and rate of apoptotic endothelial cells were quantified. The technique revealed endothelial cell apoptosis simultaneously with the identification of microvessel density on one slide. These characteristics were reproducible in adenocarcinomas of various sites. In conclusion, apoptotic endothelial cells and microvessel density can now be evaluated simultaneously within one and the same area, allowing a more reliable histological quantification of angiogenesis.

1. Introduction

One of the vital features to ascertain tumour growth is the development of neovessels in the tumour for maintaining tumour vascularity, a process known as tumour angiogenesis. Quantification of tumour microvessels has been acknowledged to have prognostic significance for tumour behaviour in several human solid tumours (reviewed in [1]). The important role of microvessel density (MVD) has been recognised, based on the predictive value of response to anti-cancer therapy and on predicting the probability of metastasis [2–5]. Methods to quantify MVD in solid human tumours as a measure of tumour angiogenesis have been established using specific endothelial cell markers, and objective criteria to standardise the methods of MVD quantification have been published [6]. However, the absence of a reproducible method to visualise endothelial cell apoptosis has been regarded as a shortcoming in assessing remodelling during angiogenesis, as has been put forward in the second international consensus paper on the methodology and criteria of evaluation of quantification of angiogenesis of solid human tumours [6]. In this paper, we present the results and the validation of

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our newly developed technique to demonstrate endothelial cell apoptosis by double immunolabelling with anti-CD34 and anti-activated caspase-3, in a panel of 48 human adenocarcinomas from eight different sites.

2. Materials and methods

All procedures and use of (anonymised) tissue were performed according to recent national guidelines. Archival paraffin blocks of tumours were retrieved from the files of the Department of Pathology and Laboratory Medicine of the University Medical Center Groningen, The Netherlands. Included were adenocarcinomas of colon, stomach, pancreas, lung, breast, kidney, prostate and colorectal liver metastases. For each tumour type, six samples were evaluated. The staining technique is described in Table 1.

Only vital and solid parts of the tumour were selected, avoiding necrotic areas and tumour regions that mainly consisted of stroma. Slides were evaluated in two ways. First, MVD was quantified using the Chalkley point overlap morphometric technique on the tumours, as described previously [6]. Briefly, the Chalkley grid, which consists of a circle and 25 randomly scattered dots, is inserted in the ocular of the microscope. This creates a visual image in which the dots are superimposed on the area of interest in the histological slide (Fig. 1, upper panel right). The tumour is screened at low magnification for vascular hot spots. Subsequently, at 200× magnification the ocular containing the grid is rotated until the maximum number of dots overlap stained microvessels. This number is counted and the mean of three vascular hot spots is recorded. Second, the number of apoptotic endothelial cells in the same hot spot areas was counted in the whole field of the circle of the Chalkley grid.

3. Results

Endothelial cells were invariably and robustly decorated by cytoplasmic staining of CD34 regardless of the primary site of the tumour. However, staining patterns varied from a very regular, well organised microvessel distribution in adenocarcinomas of the lung and kidney to a more erratic and haphazard pattern in adenocarcinomas of the colon and pancreas (Fig. 1). The Chalkley count of MVD showed that lung, kidney and gastric adenocarcinomas contained the highest MVD. Breast, prostate and pancreatic adenocarcinomas ranked intermediate, whereas adenocarcinomas of colon and colorectal liver metastases had the lowest MVD (Fig. 2).

Endothelial cell apoptosis was clearly visible in the double staining technique using anti-activated-caspase-3 and anti-CD34. Apart from the cytoplasmic staining of CD34, perinuclear or nuclear staining by anti-activated-caspase-3 was shown in some microvessels of the tumour. CD34-negative and anti-activated-caspase-3-positive nuclear or perinuclear staining of tumour cells could be discerned in the vicinity of the microvessels.

In general, the rate of apoptotic endothelial cells was rather low. Lung and kidney adenocarcinomas contained the highest numbers of apoptotic endothelial cells, although no significant difference was found between the various tumour types. No significant correlation was found between endothelial cell apoptosis and MVD.

4. Discussion

Assessment of angiogenic activity has been recognised as an important parameter both as a predictive factor for metastasis and to study the effects of anti-angiogenic treatment of malignant tumours [1,7]. Quantification of vascular density within tumours has been performed in several types of malignancies using various methods to estimate angiogenic activity. The second international consensus paper on the methodology and criteria of evaluation of quantification of angiogenesis of solid human tumours recommended several criteria for quantification of angiogenesis [6]. Apart from methods to quantify MVD within the tumour, the consensus paper dealt with the dynamics of angiogenesis by recom-

Table 1 – Immunohistochemical staining protocol for double immunolabelling with anti-cleaved caspase 3 and anti-CD 34 for paraffin-embedded formalin-fixed tissue specimens

Rinse with phosphate-buffered saline (PBS) between each step
After deparaffinising the slides, antigen retrieval is performed by microwave heating (8 min at 700 W in 1 mM ethylene diamine tetra-acetic acid (EDTA) at pH 8)
Endogenous peroxidase blocking: 30 min in 0.3% H_2O_2 in PBS
Blocking of endogenous biotin when necessary using avidin/biotin blocking kit (Vector Laboratories, Burlingame CA, United States of America (USA))
Incubate with anti-cleaved caspase 3 rabbit polyclonal antibody (Cell Signalling Technology, www.cellsignal.com), dilution 1/50 in 1% bovine serum albumin (BSA) for 1 h at room temperature
Peroxidase labelled goat-anti-rabbit antibody (DAKO, Glostrup, Denmark) at a dilution of 1/100 in 1% BSA/PBS + 1% normal human AB serum for 30 min
Incubate with rabbit-anti-goat (DAKO, Glostrup, Denmark) at a dilution of 1/100 in 1% BSA/PBS + 1% normal human AB serum for 30 min followed by diaminobenzidine (DAB) reaction for 10 min
Incubate with anti-CD34 monoclonal antibody (clone QBEND 10, Immunotech, Marseille, France), in a ready to use dilution for 1 h at room temperature
Incubate with alkaline phosphatase conjugated goat-anti-mouse antibody (DAKO, Glostrup, Denmark) dilution 1/50 in 1% BSA/PBS for 30 min at room temperature
Incubate with Chromogen Fast Red substrate (Sigma, St. Louis, MI, USA) for 30 min
Nuclear counterstaining with haematoxylin for 2 min and slides mounted using Kaiser's gelatin

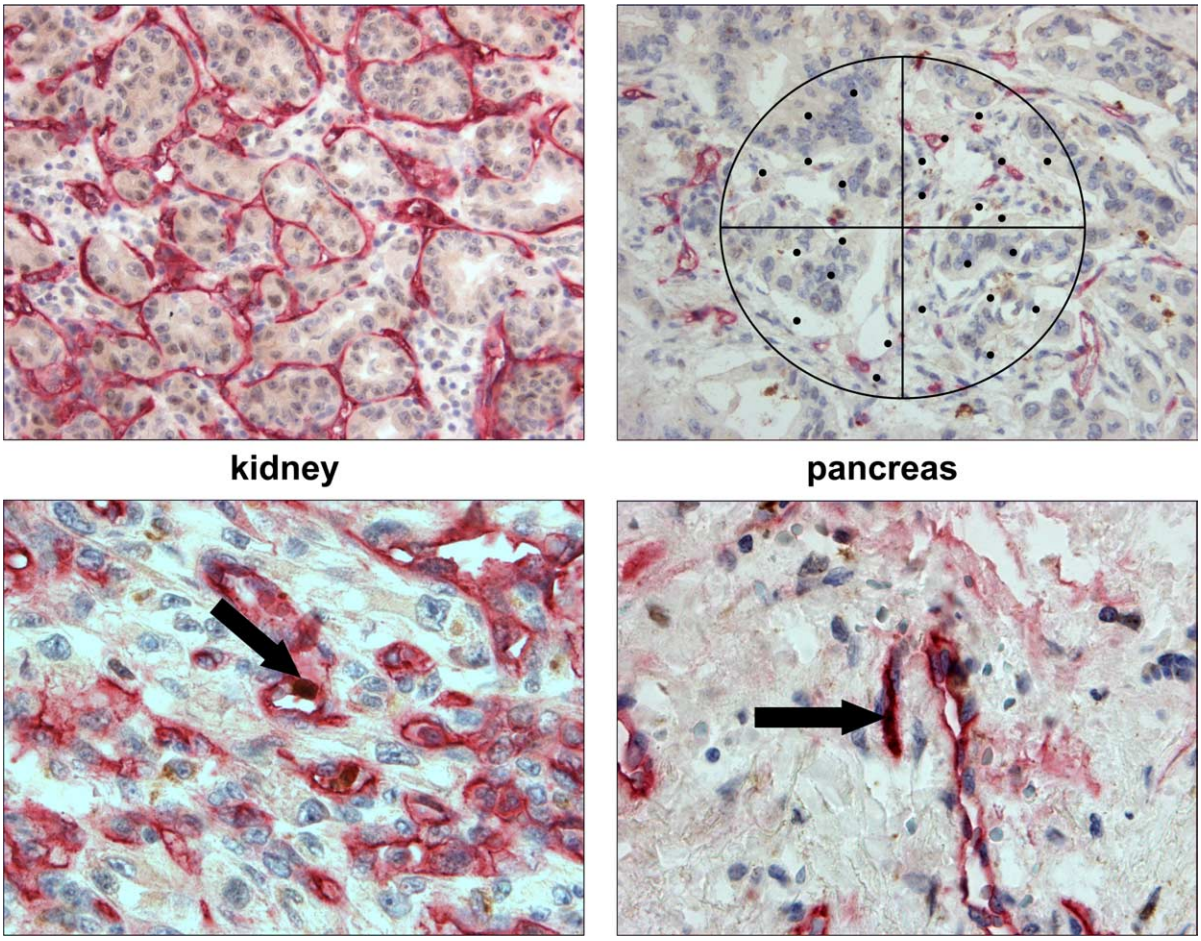

Fig. 1 – *Upper panel*: overall pattern of tumour microvessels as visualised by staining with CD34 in adenocarcinoma of kidney and pancreas. The right image demonstrates the Chalkley grid superimposed. *Lower panel:* endothelial cell apoptosis within tumour microvessels as shown by double immunolabelling with anti-CD34 and anti-activated caspase 3 (*arrows*) in adenocarcinoma of kidney and pancreas.

mending to assess the proliferative activity of endothelial cells in relation to tumour cell proliferation, the pericyte coverage index of the vessels and the fraction of endothelial cell apoptosis. All of these issues have been established as pivotal steps in angiogenesis in which the balance of endothelial cell proliferation and apoptosis is a major determinant [8–10]. Recently, anti-angiogenesis treatment was put in a more optimistic perspective and it is to be expected that this type of treatment in combination with conventional chemotherapy will become increasingly important [11]. Since anti-angiogenesis treatment has as its main target the endothelial cells of tumour vasculature, it is extremely important to obtain reliable and reproducible methods to quantify endothelial cell apoptosis in tumour specimens. Also, pericytes play a role in the increased leakiness of vessels, which is characteristic of tumour vessels. In experiments with co-cultures of endothelial cells and pericytes, it was demonstrated that intact endothelial cells protect pericytes from apoptosis [12]. Methods to show the pericyte coverage of vessels and endothelial cell proliferation have already been established [6]. Studying endothelial cell apoptosis, both in vivo and in vitro, is especially important for the development of new anti-angiogenic drugs [13,14]. Until now visualisation of apoptosis of endothelial cells was performed using the terminal transferase-mediated dUTP nick end-labelling (TUNEL) method. It has, however, been demonstrated that both in necrotic and autolytic tissue DNA ends are generated which stain positive with the TUNEL method [15]. In in vitro and in vivo experiments, in which a vital staining technique with propidium iodide was followed by fixation and TUNEL staining, it was demonstrated that up to 40% of apoptotic cells were TUNEL-negative [16]. This low sensitivity makes the TUNEL staining method unreliable to identify apoptotic cells.

In the present study, we demonstrated the results of our novel double immunolabelling staining technique to show apoptosis in endothelial cells in human solid tumours. We analysed angiogenic characteristics of eight different types of adenocarcinomas of various origins. Our results showed that MVD was highly variable among the various types of tumours.

As has been recommended by the consensus paper, endothelial cell apoptosis should be evaluated together with

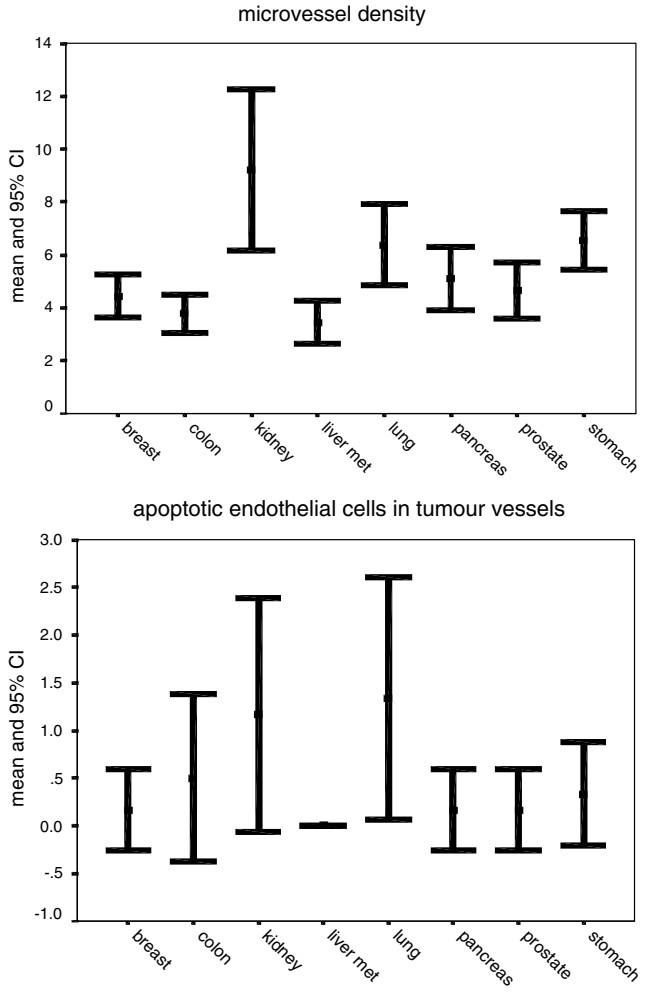

Fig. 2 – Mean and 95% confidence interval (95% CI) of microvessel density, number of apoptotic endothelial cells in tumour vessels (*n* = 6 for each tumour type; liver-met: colorectal liver metastasis).

endothelial cell proliferation because these two processes determine angiogenesis.

Studies comparing the ratio endothelial cell proliferation/endothelial cell apoptosis in tumour specimens before and after anti-angiogenic treatment are needed in order to analyse the importance of this ratio for effect measurement of this type of treatment.

Conflict of interest statement

None declared.

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