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Angiogenic effect of naive and 5-fluorouracil resistant colon carcinoma on endothelial cells *in vitro*

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Abstract

Tumour associated neovascularisation is a complex interplay between inhibitory and stimulatory angiogenic factors. Despite intense research in this field, little is known about the interaction between endothelial and chemoresistant cancer cells. For this purpose, we assessed the impact of cellular supernatants of the primary adenocarcinoma cell line CCL228, its lymph node metastasis CCL227, and four subclones resistant to different levels of 5-fluorouracil on the growth of microvascular and macrovascular endothelial cells. The growth of endothelial cells *in vitro* was affected to a moderate degree by supernatants from colon cancer cell lines. This effect was independent of the degree of chemoresistance. The stimulation of endothelial cells by the growth factors VEGF, bFGF, and PD-ECGF in the presence of supernatants from cancer cell lines was generally higher in macrovascular endothelial cells when compared with microvascular cells. The secretion of VEGF from colon cancer cells *in vitro* was inversely related to the degree of chemoresistance with the low chemoresistance phenotype producing VEGF 8.7-fold higher than the high resistance subclone. With a maximal secretion of 1500 pg VEGF/ml cell supernatant, the concentration necessary to directly stimulate the growth of endothelial cells was not achieved. In conclusion, chemoresistance affects the interaction between colon cancer cells and endothelial cells dependant on the endothelial cell type. Although the level of chemoresistance has a profound impact on the production of VEGF by cancer cells with low, intermediate or high resistance, it does not differentially affect growth stimulation or inhibition of endothelial cells *in vitro*.

Keywords: Colon cancer; Endothelial cells; Chemoresistance; Cancer pharmacology

1. Introduction

Angiogenesis is one of the fundamental physiologic processes in mammalians, e.g., during embryogenesis or wound healing. In the development of cancer, tumour associated angiogenesis is a critical process in the growth of the primary tumour as well as for invasion and metastasis [1]. Without vasculature the neoplasm is limited to a few millimetres in size due to the inadequate supply with oxygen and other nutrients. As a consequence, increased angiogenesis is correlated with poor prognosis in cancer patients [2].

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As a strongly regulated process, tumour associated neovascularisation depends upon a complex interplay between inhibitory and stimulatory angiogenic factors. The initiation of vascular development, either physiological or pathological, requires growth factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) or platelet derived endothelial cell growth factor (PD-ECGF) as major angiogenic inducers [3–5]. All these compounds act as pro-angiogenic factors by stimulation of endothelial cell growth and movement [6]. In particular, VEGF and PD-ECGF [7,8] are specific endothelial growth factors, whereas bFGF is a fibroblast growth factor also known to stimulate endothelial cells [9].

Although the basic mechanisms of tumour associated angiogenesis have been the subject of many investigations, little is known about the interaction between endothelial cells and chemoresistant cancer cells. The aim of the present investigation was to evaluate a possible interaction between endothelial cells and colon carcinoma cells *in vitro*. For this purpose, we assessed the impact of cellular supernatants of a primary adenocarcinoma, its lymph node metastasis, and subclones resistant to 5-fluorouracil (FU) on the growth of microvascular and macrovascular endothelial cells under exposure to different endothelial growth factors and their antagonizing antibodies. Here, we show that the growth of endothelial cells *in vitro* was affected to a moderate degree by supernatants from all colon cancer cell lines and that this behaviour was independent of the degree of chemoresistance. The stimulation of endothelial cells by growth factors in the presence of supernatants from cancer cell lines was generally higher in macrovascular endothelial cells when compared with microvascular cells. The secretion of VEGF from colon cancer cells *in vitro* was inversely related to the degree of chemoresistance with the low chemoresistance phenotype producing VEGF by a factor of 8.7 higher than the high resistance subclone.

2. Materials and methods

2.1. Cultivation of tumour cells

Tumour cells CCL228 (SW480) primary adenocarcinoma and CCL227 (SW620) lymph node metastasis from CCL228 (obtained from ATCC, Rockville, MD) were maintained in RPMI 1640 with 2 mM Glutamax I, 10% heat inactivated foetal calf serum, 50 μg/ml gentamycin (all reagents from GIBCO BRL, Paisley, UK) at 37 °C in a humidified atmosphere of 5% CO_2:95% air. FU resistant subclones from CCL227 were generated by continuous exposure of tumour cells to 5-FU as previously described [10]. For the exposure of endothelial cells, the supernatant from cancer cells was collected after 2 and 4 days of cultivation.

2.2. Cultivation of endothelial cells

The endothelial cells HDMEC (human dermal microvascular endothelial cells) and HUVEC (human umbilical vein endothelial cells), both from Promocell (Heidelberg, Germany) were maintained in optimized endothelial cell culture medium (termed V- and MV-medium, Promocell) and incubated at 37 °C in a humidified atmosphere of 5% CO2:95% air.

In incubation studies with tumour cell supernatants, endothelial cells were assayed in a mixture of 50% endothelial cell culture medium (V- or MV-medium) and 50% tumour cell supernatant. As controls, the data reported in this manuscript were calculated referring to endothelial cells growing in 50% endothelial cell culture medium (V- or MV-medium) and 50% tumour cell medium (RPMI 1640 with 10% FCS). In the performed MTT-assays, this signal has been used as 100% reference for calculating the relative growth.

The influence of this approach on the absolute growth of HUVEC and HDMEC was assessed at the begin of the investigation with the following results: HDMEC was hardly influenced when growing 2 days or 4 days in a mixture of 50% endothelial cell culture medium and 50% tumour cell medium (mean growth of HDMEC with regard to 100% MV-medium: 101.4% and 95.7%, respectively; $n = 6$), whereas the proliferation of HUVEC was decreased after 2 days and after 4 days under these conditions (mean growth of HUVEC with regard to 100% V-medium: 73.5% and 67.4%, respectively; $n = 6$).

2.3. Viability assay

For experimental purposes, 5000 endothelial cells per well were seeded in microtiter plates under standard cell culture conditions. After 24 h, supernatant from cancer cells (2 days or 4 days after seeding) was added to the endothelial culture. When using growth factors or neutralizing antibodies, cells were incubated over 72 h at different concentrations. The viability of the endothelial cells was evaluated in triplicate using an MTT-assay (Cell titer non-radioactive cell proliferation assay from Promega, Madison, WI, USA). After the addition of dye solution, the water insoluble crystals were solubilised overnight and the absorbance was monitored at 570 nm with a reference wavelength of 690 nm.