Chapter 18

Isolation of Endothelial Cells from Human Tumors

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Abstract

Antiangiogenic drugs have been used successfully for the treatment of colorectal cancer (CRC) and several other tumor types. Until recently, viable tumor endothelial cells (TEC) and normal endothelial cells of uninvolved colon tissue (NEC) from the same patient have not been available to optimize treatment strategies in vitro. Here, we describe a protocol for the isolation of TEC and NEC. These cells were isolated at a very high purity via magnetic cell sorting of tissue samples obtained from surgical specimens of patients suffering from CRC. Isolated TEC and NEC expressed CD31, CD105, VE-cadherin, VCAM-1, ICAM-1, and E-selectin, formed capillaries in basal membrane extract, and were able to take up acetylated LDL. They were negative for podoplanin, CD45, CD68, and CK-20, indicating blood vessel endothelial lineage. Expression of vWF was more pronounced in NEC cultures, whereas vWF was absent or only slightly expressed in all TEC cultures in vitro. Lower intracellular concentrations of vWF were also detected in TEC as compared to NEC at the tissue level. The latter finding demonstrated that differential features of TEC and NEC in vivo are stably perpetuated in culture. The isolated endothelial cell cultures may provide a useful in vitro model system to elucidate epigenetic effects on angiogenesis in cancer and to optimize antiangiogenic therapy.

Key words: Endothelial cells, Angiogenesis, Colon, Colorectal carcinoma, Tumor, Collagenase II, MACS, CD31

1. Introduction

The formation of new blood vessels from preexisting vasculature (angiogenesis) is key to progression and metastasis of solid tumors (1–4). Without adequate vascular supply, solid tumors do not grow beyond a size of 1–2 mm. For further progression, angiogenesis is needed to compensate the lack of nutrients and oxygen (5).

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Angiogenesis is initiated by proangiogenic factors, such as vascular endothelial growth factor and basic fibroblast growth factor, which are released by the tumor cells and activate resting endothelial cells (EC) to migrate, proliferate, differentiate, and finally to form new blood vessels (5–7). The rapid proliferation of tumor endothelial cells (TEC) and the persistent proangiogenic stimulation lead to the formation of incomplete and irregular vessels with fenestrations, irregular blood flow, and increased permeability as compared to normal vasculature (2, 8, 9). Evidence exists that TEC are different from normal endothelial cells (NEC) as indicated by the expression of specific "tumor endothelial markers" and cytogenetic abnormalities (10, 11).

Colorectal cancer (CRC) is one of the leading cancers in Western countries with 500,000 deaths worldwide per year (12). Almost a third of all patients suffer from metastatic disease at first diagnosis (13). Nearly 50% of all diagnosed CRC patients will develop metastasis with mostly fatal prognosis in the following (13). For more than a century, surgery has played the major role in curative treatment of CRC. The presently applied treatment of choice for advanced disease is surgery combined with adjuvant chemotherapy (12). Of note, a significant response to antiangiogenic therapy has been observed when patients with advanced disease are treated with the anti-VEGF antibody bevacizumab in combination with chemotherapeutics (14). Since this first report, antiangiogenic strategies are increasingly considered as a relevant therapeutic option for the therapy of CRC. However, further improvement of treatment protocols by drug optimization and patient selection is required. This demands in vitro model systems that allow optimization of antiangiogenic treatment strategies in CRC.

Here, we describe a protocol for the isolation of pure, viable EC from human CRC and healthy colon tissue. These cells may allow optimization of antiangiogenic therapy. In addition, the comparative molecular analysis of TEC and NEC may identify further molecular targets for improved antiangiogenic treatment of CRC (15).

2. Materials

Our group was the first to succeed in the isolation of pure microvascular endothelial cells from CRC (15). Here, we describe a detailed protocol of the isolation process.

2.1. Tissue Collection

1. 1× Hank's Balanced Salt Solution (1× HBSS; PAA, Pasching, Austria) supplemented with 1× penicillin/streptomycin (PAA) and 250 µg/mL amphotericin B (PAA).
2. 50 mL Falcon tubes, sterile capped (BD Biosciences, Heidelberg, Germany).

2.2. Generation of a Single Cell Suspension

1. Collagenase II (Biochrom, Berlin, Germany): dissolve 1 g of collagenase II in a suitable volume of 1× HBSS without antibiotics to obtain a final stock solution of 34,200 U/mL; sterile filtered, aliquoted, and stored at –20°C.
2. EBM-2-MV (Lonza, Cologne, Germany).
3. Bovine skin gelatin, type B 1.5% (Sigma-Aldrich, Munich, Germany) dissolved in 1× PBS (Biochrom), stirred for 1 h at 65°C, autoclaved, and sterile filtered; storage at 4°C for 6 weeks.
4. Scalpel No. 23 (Feather Safety Razor, Osaka, Japan) and forceps.
5. Cell strainer 100 μm (BD Biosciences).
6. Syringes, 10 mL (BD Biosciences).
7. Dynal sample mixer, model MX1 (Invitrogen, Karlsruhe, Germany).
8. 15-mL Falcon tubes (BD Biosciences).

2.3. Enrichment of Tumor and Normal Endothelial Cells by MACS

1. CD31 MicroBead kit (Miltenyi Biotec, Bergisch Gladbach, Germany).
2. FcR Blocking Reagent (Miltenyi Biotec).
3. MACS buffer: 1× PBS pH 7.2, 0.5% bovine serum albumin, 2 mM EDTA; sterile filtered and stored at 4°C; degas before use.
4. MACS separation columns (Miltenyi Biotec).
5. MACS preseparation filter (Miltenyi Biotec).
6. MACS separation unit (separator and stand, Miltenyi Biotec).
7. Accutase (PAA).
8. EBM-2-MV (Lonza).
9. 1.5% gelatin type B (Sigma) dissolved in 1× PBS (Biochrom), stirred for 1 h at 65°C, autoclaved, and sterile filtered; storage at 4°C for 6 weeks.
10. 1× PBS (Biochrom).

3. Methods

In the following sections, the methodological procedures are described in detail. Specific tips that may improve the outcome are described in Subheading 4.

3.1. Tissue Collection

1. Cut pieces of 0.5 up to 3 g (depending on tumor size) from the center of the malignancy to be used for the isolation of tumor endothelial cells (TEC). Morphologically visible

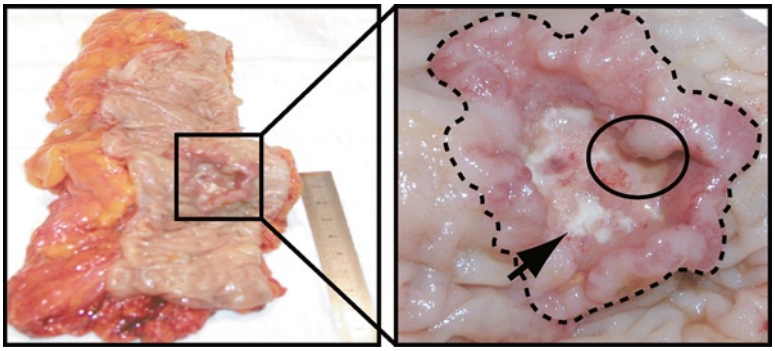

Fig. 1. Viable tumor tissue can be obtained from the center of malignancy by cutting nonhypoxic, nonnecrotic areas from colorectal carcinoma. Pieces of up to 3 g were cut from a nonhypoxic, nonnecrotic area from the central part of the tumor (area of resection marked by *circle*; invasive margin marked by *dashed line*; necrotic area shown by *arrow*). Tissue for the isolation of normal endothelial cells has been removed at a distance of about 10 cm from the tumor margin (*dashed line*). The figure is partly reproduced with permission from Schellerer et al. (15).

hypoxic or necrotic parts of the tumor should be avoided (Fig. 1). Uncompromised healthy colon tissue is harvested at a safety distance of at minimum 10 cm apart from the tumor and is used for the isolation of corresponding normal endothelial cells (NEC). Place the resected specimen immediately in a 50 mL falcon tube with ice-cold HBSS (see Note 1).

2. From now on, work under a sterile laminar flow for the whole procedure.
3. Wash the specimen by transferring it using sterile forceps from a 50 mL falcon with fresh ice-cold 1× HBSS to a new falcon with 1× HBSS four times consecutively.

3.2. Generation of a Single Cell Suspension

1. Remove the specimen from the falcon tube and place it in a 10-cm cell culture dish. Mince the tissue into approximately 10-mm^3 pieces using a fresh, sterile scalpel (see Note 2).
2. Put the minced tissue into a 15-mL falcon tube filled with 3 mL EBM-2 supplemented with 0.5% FBS (=EBM-2-Low). Put approx. 0.2 g of tissue in one falcon tube (see Note 3).
3. According to the weight of the tissue pieces in each falcon tube, calculate the required amount of collagenase II (50 µl enzyme per 0.1 g tissue; 17,100 U/g; see Note 4).
4. Pipet the calculated amount of collagenase II into each falcon tube and add EBM-2-Low up to a total volume of 5 ml.
5. Put the falcon tubes in the Dynal sample mixer at 37°C for 1 h at 5% CO_2 with the lowest speed available.
6. Put a cell strainer on a 50 mL falcon tube and pour the digested tissue through the strainer. Wash the filter from the inside and outside using 3 mL EBM-2-Low each time.
7. Centrifuge with 500×*g* for 5 min at 20°C and discard the supernatant.

8. Resuspend the cell pellet in 5 mL EBM-2-MV and cultivate the cells until 70–80% confluence in a T-25 cell culture flask precoated with 1.5% gelatin for at least 2 h. Wash the cells twice with 1× PBS the next day and add fresh medium. Afterward renew the medium every second day.

3.3. Enrichment of Tumor and Normal Endothelial Cells by MACS

1. Usually, 5–7 days after initial seeding, the cultures reach 70–80% confluence and the cells can be positively selected for CD31 by MACS (see Note 5 and Fig. 2). Repeat MACS selection until all nonendothelial cells are removed (Fig. 2).

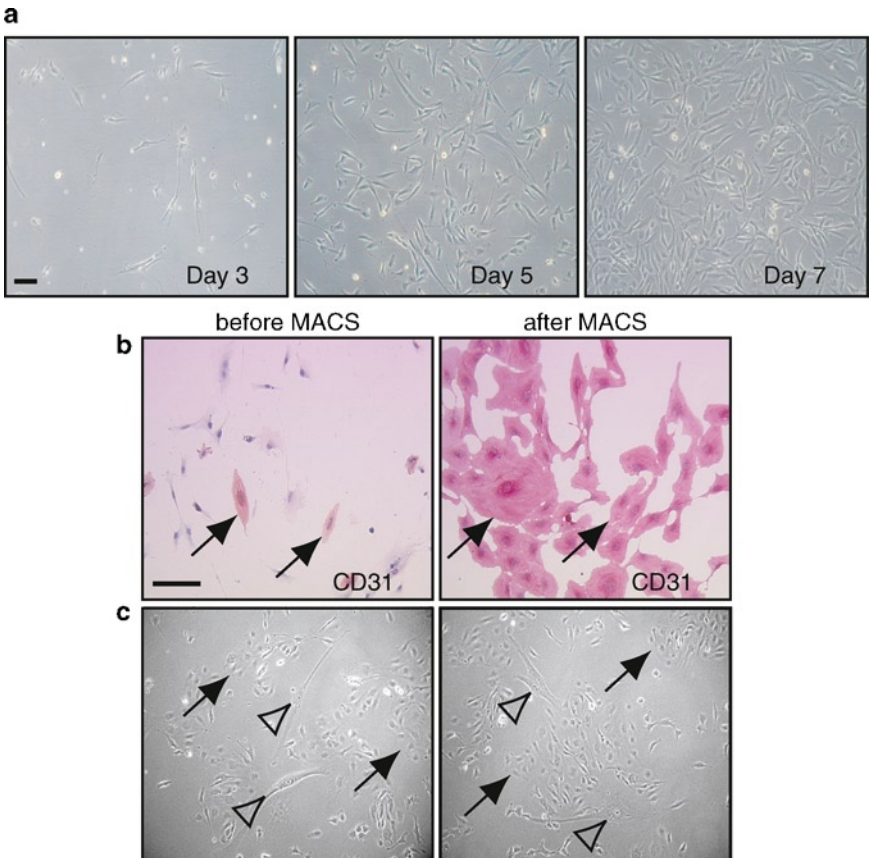

Fig. 2. Tumor endothelial cells can be enriched from a mixture of cells arising after initial cultivation of digested colorectal carcinoma tissue using CD31-MACS. (**a**) Tissues were enzymatically digested by Collagenase II and mechanically dispersed. The resulting single cell suspension was seeded in gelatin-coated culture flasks. After 5–7 days phenotypically different cells arose. The scale bar corresponds to 100 μm. (**b**) Endothelial cells isolated from healthy colon and colorectal cancers were positively selected using CD31-MicroBeads. The yield of endothelial cells was monitored by immunocytochemical staining of the CD31 surface antigen before and after MACS. Positive staining is visualized by *pink color* (*arrows*). Counterstaining was performed with hematoxylin (*blue color*). Scale bar corresponds to 100 μm. (**c**) Clones of endothelial cells can be observed arising between nonendothelial cells after some enrichment rounds of the endothelial cells by CD31-MACS (*arrows*, endothelial cells; *arrowheads*, nonendothelial cells). The figure is partly reproduced with permission from Schellerer et al. (15).

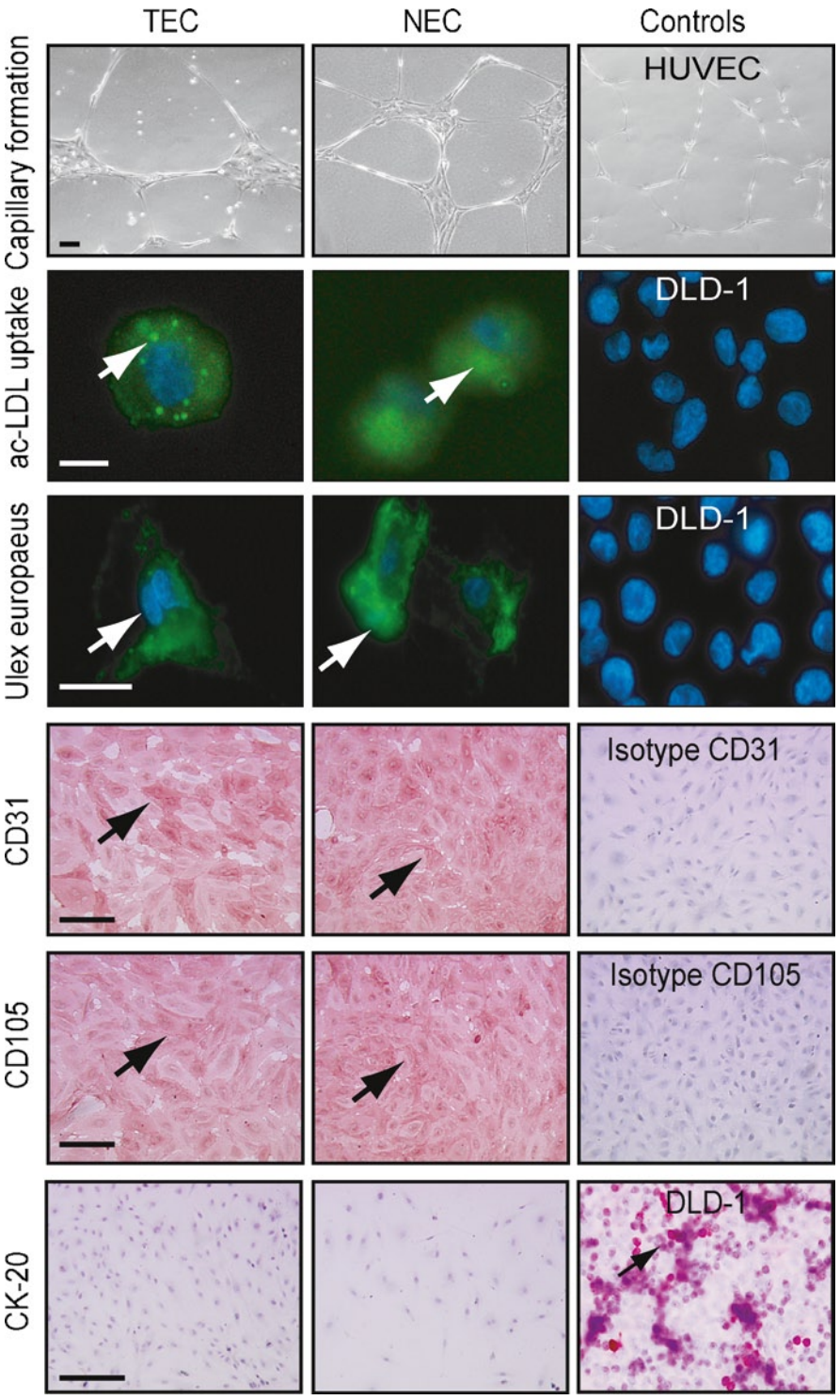

Fig. 3. The isolated tumor and normal endothelial cell cultures show endothelial cell lineage. Pure endothelial cell populations (>93%) of tumor (TEC) and normal endothelial cells (NEC) were characterized by different assays. For capillary formation, TEC and NEC were seeded onto an extract of extracellular basement membrane and were stimulated with angiogenic growth factors. HUVEC were used as a positive control. For the uptake of acetylated low-density lipoprotein (ac-LDL), TEC and NEC were incubated with Alexa488-labeled acetylated LDL (*green fluorescent signal*, *arrows*) for 1 h and were counterstained by DAPI (*blue fluorescent signal*). TEC and NEC cultures were incubated with FITC-conjugated *Ulex europaeus*-lectin for 1 h and the signal (*green*, *arrow*) was visualized using epifluorescence microscopy.

2. Wash the cells with 1× PBS and add 1–2 mL accutase until cells are completely detached; rinse the dish with EBM-2-MV and collect the cells in a falcon tube.
3. Follow the manufacturer's instructions for a positive selection for endothelial cells using the CD31 MicroBead Kit as detailed below (see Note 6).
4. Put a preseparation filter on a 15-mL falcon tube and prewet it using 0.5 mL EBM-2-MV; from now on, work all the time on ice and use precooled solutions!
5. Centrifuge the cells at 4°C for 3 min with 300 × *g* and discard the supernatant.
6. Dissolve the pellet in 60 µl MACS-buffer, add 20 µL FcR blocking reagent, vortex briefly, and add 20 µL CD31 MicroBeads (see Note 7); incubation for 15 min at 4°C.
7. Add 1 mL MACS buffer to the cells and centrifuge at 4°C for 3 min with 300 × *g*; discard the supernatant.
8. During this centrifugation step, fix a suitable column (see Note 7) in the MACS separator and prewet the column with 0.5 mL MACS buffer.
9. Dissolve the pellet after centrifugation in 1 mL MACS buffer and pipet 2× 0.5 mL of the cell suspension on the column.
10. Wash the column with 3× 0.5 mL MACS buffer.
11. Elute the cells from the first column using the stamp with 1 mL MACS buffer on a second (prewet before with 0.5 mL MACS buffer) column.
12. Wash the column two times with 0.5 mL MACS buffer and once with 0.5 mL EBM-2-MV
13. Elute the cells using the stamp with 1 mL EBM-2-MV in a suitable cell culture dish ($1–2 \times 10^4$ cells per cm^2) precoated with 1.5% gelatin (see Note 8).
14. Cultivate the cells until confluence with EBM-2-MV medium renewal every second day.
15. In case of significant (>10%) contamination by nonendothelial cells, perform additional cycles of magnetic cell sorting (see Note 9). Contamination with nonendothelial cells can be analyzed by staining an aliquot of the cells for CD31, and subsequent immunocytochemical or FACS analysis (Figs. 2b and 3).

Fig. 3. (continued) Counterstaining was performed with DAPI (*blue fluorescence*). Negative controls of ac-LDL and *U. europaeus* binding have been performed with the colorectal carcinoma cell line DLD-1. Immunocytochemical stainig of TEC and NEC was performed using CD31, CD105 and CK-20 antibodies. Positive staining is visualized by *pink colour* (*arrows*). Counterstaining was performed with hematoxylin (*blue colour*). For CD31 and CD105 isotype staining controls are displayed. DLD-1 cells were stained as a positive control for the CK-20 immunostaining. The *scale bars* correspond to 100 µm (capillary formation, CD31, CD105), 50 µm (CK-20), 10 µm (ac-LDL - uptake, *U. europaeus*). The figure is partly reproduced with permission from Schellerer et al. (15).

16. The first confluent T-25 flask of pure TEC/NEC is designated as passage 0 (see Notes 10 and 11). One passage is defined by a split ratio of 1:4.

4. Notes

1. The elapsed time from resection of the specimen to the start of the isolation procedure is critical for the success of isolation. Keep this time as short as possible. In our laboratory, this does not take more than 30 min.
2. Avoid quenching, pressing, or tearing of the specimen while cutting. This affects the viability of the endothelial cells. Try to use the scalpel like a rocking tool.
3. Weigh the falcon tubes filled with EBM-2-Low before putting the tissue pieces inside and note the weight. Weigh the falcon tubes again after putting the tissue pieces inside and calculate the difference in weight.
4. The protocol was developed for the digestion of colorectal carcinoma. If you intend to use tissue from another tumor, you might have to standardize newly the concentration of the collagenase to be used to obtain a single cell suspension.
5. A MACS separation column with suitable size has to be selected for the procedure. Therefore, count an aliquot of the cells, calculate the total cell number, and choose your column according to the guidelines of Miltenyi Biotec (http://www.miltenyibiotec.com).
6. A MACS separation unit is chosen according to the size of the column and the number of columns that you intend to use simultaneously. Instructions for the selection of a suitable MACS separator can be found on the home page of Miltenyi Biotec (http://www.miltenyibiotec.com).
7. Here, the volumes/concentrations are given for a MS separation column and up to 1×10^7 cells.
8. If you want to make use of the negative cell fraction, keep the flow-through, centrifuge and cultivate the CD31-negative cells.
9. A CD31 antibody (clone JC70A, Dako, Hamburg, Germany) in combination with a mouse APAAP detection system (Dako) is used to evaluate the purity of the cultures by immunocytochemistry in our laboratory (Figs. 2b and 3).
10. The initially established CD31-positive cell cultures may also be characterized for additional endothelial cell markers to

show endothelial cell origin. We used in addition CD105, vWF, VE-cadherin, ICAM-1, VCAM-1, E-selectin, CD45, CD68, CK-20, and podoplanin. Moreover, the cells were analyzed for their ability to form capillary structures, bind *Ulex europaeus*, and take up acetylated-LDL (Fig. 3) (15).

11. In general, viable tumor or normal endothelial cells can be obtained from approx. 30% of the specimens undergoing the isolation and selection procedure.

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