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CD133⁺ Renal Progenitor Cells Contribute to Tumor Angiogenesis

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In the present study, we tested the hypothesis that resident progenitor cells may contribute to tumor vascularization and growth. CD133⁺ cells were isolated from 30 human renal carcinomas and characterized as renal resident progenitor cells on the basis of the expression of renal embryonic and mesenchymal stem cell markers. CD133⁺ progenitors differentiated into endothelial and epithelial cells as the normal CD133⁺ counterpart present in renal tissue. In the presence of tumor-derived growth factors, these cells were committed to differentiate into endothelial cells able to form vessels *in vivo* in SCID mice. Undifferentiated CD133⁺ progenitors were unable to form tumors when transplanted alone in SCID mice. When co-transplanted with renal carcinoma cells, CD133⁺ progenitors significantly enhanced tumor development and growth. This effect was not attributable to the tumorigenic nature of CD133⁺ progenitor cells because the same results were obtained with CD133⁺ cells from normal kidney. CD133⁺ progenitors contributed to tumor vascularization as the majority of neoformed vessels present within the transplanted tumors were of human origin and derived from the co-transplanted CD133⁺ progenitors. In conclusion, these results indicate the presence of a renal progenitor cell population in renal carcinomas that may differentiate in endothelial cells and favor vascularization and tumor growth. (*Am J Pathol* 2006, 169:2223–2235; DOI: 10.2353/ajpath.2006.060498)

The process of angiogenesis is essential for the growth of tumor and for the development of metastasis.¹ Migration and proliferation of endothelial cells originating from local capillaries because of the local production of proangio-

genic factors is thought to be the primary mechanism for tumor angiogenesis.² In addition, it has been demonstrated that endothelial precursor cells originating from bone marrow may contribute to the endothelial lining of adult neovasculature in both physiological and pathological angiogenesis,^{3–6} including tumor vascularization.⁷ Mobilization of CD34⁺ progenitor cells from the bone marrow is thought to be induced by the tumor itself, namely by tumor-produced vascular endothelial growth factor or angiopoietins, followed by endothelial differentiation and by incorporation of precursors into the tumor vessels.⁷ The extent of the contribution of bone marrow-derived endothelial precursors to tumor vascularization in murine models varied, depending on the experimental setting, from 0 to 90%.^{7–10} However, recent data in humans showed a minimal contribution of bone marrow endothelial cells to tumor vascularization.¹¹ In addition, it has been recently proposed that bone marrow-derived cells may differentiate in tumor vessel pericytes and contribute to tumor angiogenesis by providing growth factors rather than by endothelial differentiation.^{12,13}

Beside bone marrow-derived circulating cells, tissue resident stem cells, such as neural, muscle, and skin stem cells, were shown to differentiate into endothelial cells and to participate to normal tissue vascularization after damage,^{14–17} thus providing a regenerative response. The possibility that tissue-resident normal stem cells may contribute to tumor vascularization and growth has not so far been investigated. We recently identified a population of CD133⁺ resident renal progenitor cells in adult normal human kidney.¹⁸ These cells were capable

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of expansion, self-renewal, and differentiation into endothelial or epithelial cells both *in vitro* and *in vivo*.

In the present study, we investigated the possibility that renal resident progenitor cells may contribute to vascularization, and thus growth, of renal tumors. To test this hypothesis, we investigated the presence of CD133⁺ progenitor cells in 30 human renal tumors, and after purification and characterization we evaluated their angiogenic potential. Moreover, we studied whether cotransplantation of resident CD133⁺ progenitor cells with renal carcinoma cells promoted tumor development, angiogenesis, and growth in SCID mice. We also evaluated the tumorigenic ability of CD133⁺ progenitor cells isolated from tumors in respect to the normal counterpart isolated from normal renal tissue.

Materials and Methods

Renal Cell Carcinomas and Deriving Cell Lines

Studies were performed on 30 renal carcinomas (histological types: 23 clear-cell, four papillary, one chromophobe, and two undetermined renal carcinomas). Patients' ages ranged from 29 to 82 years, and 65% were male. Tumor specimens were finely minced with scissors and then digested by incubation for 1 hour at 37°C in Dulbecco's modified Eagle's medium (DMEM) containing collagenase II (Sigma Chemical Co., St. Louis, MO). After washing in medium plus 10% fetal calf serum (FCS) (Life Technologies, Inc., Grand Island, NY), the cell suspension was forced through a graded series of meshes to separate the cell components from stroma and aggregates. Aliquots of the cell suspension were subjected to fluorescence-activated cell sorting analysis for quantification of CD133⁺ cells. CD133⁺ cells were isolated by magnetic cell sorting, using the MACS system (Miltenyi Biotec, Auburn, CA).¹⁸ CD133⁺ cells were plated onto fibronectin in the presence of a nondifferentiating expansion medium, consisting of 60% DMEM LG (Invitrogen, Paisley, UK), 40% MCDB-201, with 1× insulin-transferrin-selenium, 1× linoleic acid 2-phosphate, 10⁻⁹ mol/L dexamethasone, 10⁻⁴ mol/L ascorbic acid 2-phosphate, 100 U of penicillin, 1000 U of streptomycin, 10 ng/ml epidermal growth factor, and 10 ng/ml platelet-derived growth factor-BB (all from Sigma) and 2% FCS (EuroClone, Wetherby, UK).¹⁹ For cell cloning, single cells were deposited in 96-well plates in the presence of the nondifferentiating expansion medium. The CD133⁻ renal tumor cell fraction, used as control, was plated and maintained in DMEM with 10% FCS. CD133-positive cells from normal renal tissue were obtained as previously described.¹⁸ The K1 renal cancer cell line was previously established and characterized.²⁰

Cell Differentiation

Epithelial differentiation was obtained in the presence of fibroblast growth factor-4 (10 ng/ml) and hepatocyte growth factor (20 ng/ml) (Sigma).¹⁹ Endothelial differentiation was obtained by culturing the cells in EBM medium (Cambrex Bio Science, Baltimore, MD) with vascular endothelial

growth factor (10 ng/ml) (Sigma) and 10% FCS on endothelial cell attachment factor (Sigma).²¹ For osteogenic differentiation, cells were incubated in α -minimal essential medium (α -MEM) supplemented with 10% FCS, 10% horse serum, 100 U/ml penicillin, 100 μ g/ml streptomycin, 12 mmol/L L-glutamine, 20 mmol/L β -glycerol phosphate (Sigma), 50 ng/ml thyroxine (Sigma), 1 nmol/L dexamethasone (Sigma), and 0.5 μ mol/L ascorbate 2-phosphate (Sigma).²² The media was changed two times per week for 3 weeks. The cells were fixed with 10% formalin for 20 minutes at room temperature and stained with Alizarin Red, pH 4.1 (Sigma) for 20 minutes at room temperature. For adipogenic differentiation, the cultures were incubated in α -MEM supplemented with 10% FCS, 10% HS, 100 U/ml penicillin, 100 μ g/ml streptomycin, 12 mmol/L L-glutamine, 5 μ g/ml insulin (Sigma), 50 μ mol/L indomethacin (Sigma), 1 $\times$ 10⁻⁶ mol/L dexamethasone, and 0.5 μ mol/L 3-isobutyl-1-methylxanthine (Sigma).²² The medium was changed two times per week for 3 weeks. The cells were fixed with 10% formalin for 20 minutes at room temperature and stained with 0.5% Oil Red O (Sigma) in methanol (Sigma) for 20 minutes at room temperature.

For experiments using the tumor cell-conditioned medium, K1 tumor cells were grown until subconfluence in a T75 flask and incubated overnight with 10 ml of DMEM plus 0.1% BSA. The conditioned medium was then removed and added, at 10% concentration, to DMEM plus 10% FCS for CD133⁺ cell stimulation. CD133⁺ cells cultured in DMEM plus 10% FCS in the absence of tumor-derived conditioned medium were unable to grow and died after 2 to 3 days.

Immunofluorescence and Immunohistochemistry

Cytofluorimetric analysis was performed using the following antibodies, all fluorescein isothiocyanate or phycoerythrin conjugated: anti-CD133 monoclonal antibody (mAb) (Miltenyi Biotec), anti-CD44, anti-CD31, anti-CD29, and anti-CD105 mAbs (DakoCytomation, Copenhagen, Denmark), anti-KDR mAb (R&D Systems, Minneapolis, MN); anti-CD34, anti-CD45, anti-CD73, anti-CD90, anti-CD117, and anti-vascular endothelial (VE)-cadherin mAbs (Becton, Dickinson and Company, San Jose, CA). Isotype-matched fluorescein isothiocyanate- or phycoerythrin-conjugated control mouse IgG was from DakoCytomation. For cytofluorimetric analysis, cells were incubated for 30 minutes at 4°C with the appropriate antibody (Ab) or with the irrelevant control in phosphate-buffered saline (PBS) containing 2% heat-inactivated human serum. Where a second step reagent was needed, cells were stained by the addition of fluorescein isothiocyanate-conjugated goat anti-mouse or anti-rabbit IgG and incubated for further 30 minutes at 4°C. Cells were analyzed on a FACScan (Becton, Dickinson and Company). Ten thousand cells were analyzed in each experimental point. Indirect immunofluorescence was performed on cells cultured on chamber slides, fixed in 4% paraformaldehyde containing 2% sucrose and, when needed, permeabilized with HEPES-Triton X-100 buff-

er.¹⁸ The following antibodies were used: rabbit anti-von Willebrand factor (vWF), mouse anti-E cadherin, and mouse anti-VE cadherin (DakoCytomation), rabbit anti-pan-cytokeratin (Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-vimentin (Sigma), and rabbit anti-Pax2 (Covance, Princeton, NJ). Control mouse, rabbit, or goat nonimmune immunoglobulins were used as controls. Fluorescein isothiocyanate-conjugated anti-mouse or anti-rabbit IgG (DakoCytomation) was used as secondary antibodies. Sections from paraffin-embedded blocks of human renal carcinomas or Matrigel plugs were collected onto poly-L-lysine-coated slides. Endogenous peroxidase activity was blocked with 6% H₂O₂ for 8 minutes at room temperature. Anti-CD133 mAb (Miltenyi), anti-mouse β_2 -microglobulin, or anti-HLA class I polyclonal Ab (Santa Cruz Biotechnology) or anti-vWF polyclonal antibody or anti-fluorescein/Oregon Green polyclonal Ab (Molecular Probes, Leiden, The Netherlands), used to detect cells labeled with fluorescent dye carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes),²³ were applied to slides overnight at 4°C. Horseradish peroxidase-labeled anti-rabbit or anti-mouse Envision polymers (DakoCytomation) were incubated for 30 minutes. The reaction product was developed using 3,3'-diaminobenzidine. Omission of the primary Ab or substitution with an unrelated rabbit serum or mouse IgG served as negative control. The percentage of vessels positive for mouse β_2 -microglobulin or human HLA class I was counted in four nonsequential sections for each experiment at $\times 100$ magnification.

For confocal microscopy analysis, sections from cryostatic or paraffin-embedded sample of Matrigel plugs were stained for CD133 antigen, HLA class I polyclonal or HLA class I mAb (BioLegend, San Diego, CA), polyclonal anti-mouse β_2 -microglobulin Ab (Santa Cruz Biotechnology), anti-fluorescein/Oregon Green polyclonal Ab, and anti-human CD31 (Becton, Dickinson and Company) or anti-mouse CD31 (Abcam, Cambridge, UK) mAbs. Alexa Fluor 488 or Texas Red goat anti-rabbit IgG and Alexa Fluor 488 or Texas Red anti-mouse IgG (all from Molecular Probes) were used as secondary antibody. Confocal microscopy analysis was performed using a Zeiss LSM 5 Pascal model confocal microscope (Carl Zeiss Int., Oberkochen, Germany). Hoechst 33258 dye (Sigma) was added for nuclear staining.

Fluorescence in Situ Hybridization

Fluorescence *in situ* hybridization was performed using the Vysion kit for the detection of human chromosome 17 (Vysis Inc., Downers Grove, IL). SG CEP 17 DNA probe hybridizes to the centromere (band region 17p11.1-q11.1, locus D17Z1) of human chromosome 17. *In situ* hybridization was performed on 5- μ m sections according to the manufacturer's guidelines. In brief, the sections were deparaffinized, dehydrated in 100% ethanol, and dried at 45 to 50°C for 2 to 5 minutes. Slides were then subjected to protease digestion for 10 to 20 minutes at 38°C, denatured (72°C for 5 minutes), and hybridized (37°C) with prewarmed probes (CEP17 Spectrum Green;

Vysis Inc.) overnight (16 to 18 hours) in HYBrite hybridization system (Vysis Inc.). They were then washed with posthybridization wash buffer (2 $\times$ standard saline citrate, 0.3% Nonidet P-40) at 72°C and counterstained with 4,6-diamidino-2-phenylindole, mounted, maintained in darkness for 15 minutes at 4°C, and then observed with a confocal microscope.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) for Pax-2

Total RNA was extracted from cells with guanidinium thiocyanate/phenol/chloroform, precipitated with isopropyl alcohol, and washed with 70% ethanol. Two μ g of RNA were reverse-transcribed using oligo(dT) primers and 15 U of reverse transcriptase enzyme (Eppendorf, Hamburg, Germany). Two μ l of cDNA were amplified with forward (5'-ATGGATATGCACTGCAAAGCAGA-3') and reverse (5'-CGTGCTGGGAACAATGGTGTG-3') primers, with TaqDNA polymerase (Invitrogen), as described.²⁴ Reactions were performed for 30 cycles at a melting temperature of 52°C and analyzed with an ethidium bromide-stained 1.5% agarose gel.

Scanning Electron Microscopy

Samples were postfixed in 2.5% glutaraldehyde, dehydrated in alcohol, dried, and coated with gold by sputter coating. The specimens were examined in a scanning T300 electron microscope (JEOL, Tokyo, Japan). Images were obtained via secondary electron at a working distance of 15 to 25 mm and at an accelerating voltage of 20 to 25 kV.

In Vivo Angiogenic and Tumorigenic Potential of CD133⁺ Renal Progenitor Cells

To evaluate angiogenesis, endothelial committed tumor-derived CD133⁺ cells (1×10^6 cells) were implanted subcutaneously into SCID mice (Charles River, Jackson Laboratories, Bar Harbor, ME) within growth-factor depleted Matrigel basement membrane (Becton, Dickinson and Company), as described.¹⁸ To evaluate progenitor cell tumorigenesis, undifferentiated tumor-derived CD133⁺ cells (1×10^6 cells) were transplanted subcutaneously into SCID mice within Matrigel basement membrane, as described.²⁵ Cells were harvested using trypsin-ethylenediaminetetraacetic acid, washed with PBS, counted in a microcytometer chamber, and resuspended in 250 μ l of DMEM. Cells were chilled on ice, added to 250 μ l of Matrigel at 4°C, and injected subcutaneously into the left back of SCID mice via a 26-gauge needle using a 1-ml syringe. To evaluate the involvement of CD133⁺ cells in tumor engraftment, CD133⁺ or CD133⁻ human cells were transplanted subcutaneously into SCID mice in combination with renal carcinoma cells (K1) obtained from a renal clear carcinoma, at different ratio (1×10^4 CD133⁺ cells/ 1×10^6 K1 cells, 1:100 ratio; or 1×10^6 CD133⁺ cells/ 1×10^4 K1 cells, 100:1 ratio) and com-

pared with injection of K1 cells alone (1×10^4 to 1×10^6). After 7 days (angiogenesis experiments) or 3 or 8 weeks (tumor detection), mice were sacrificed and Matrigel plugs recovered and processed for histology. In selected experiments, CD133⁺ cells (1×10^5) were labeled with CFSE, formed tumors were digested in Matrigel-digesting solution (Becton, Dickinson and Company) plus collagenase II (Sigma), and the recovered cells were analyzed by cytofluorimetric analysis.

Results

Isolation and Characterization of CD133⁺ Renal Progenitor Cells in Renal Carcinomas

We investigated by cytofluorimetric analysis the presence of a cell population expressing the CD133 stem cell marker in cells dissociated from tissue specimens of 30 renal carcinomas (23 clear cell, four papillary, one chromophobe, and two undetermined renal carcinomas). All carcinomas displayed a CD34⁻/CD133⁺ cell population with a percentage ranging from 0.25 to 1.87% of the total cells (Figure 1A; $0.90 \pm 0.17\%$). This percentage was not different from that detected in normal renal tissue.¹⁸ No correlation among the number of CD34⁻/CD133⁺ cells and tumor stage or grade or patient age or sex was observed (not shown). CD34⁺/CD133⁺ cells were undetectable by cytometric analysis. By immunohistochemistry, CD133⁺ single cells or CD133⁺ small aggregates were found sparsely within the renal tumor tissue and in proximity of tumor vessels (Figure 1C).

CD133⁺ cell isolates were obtained from all tumors, characterized after magnetic isolation and cultured in nondifferentiating medium.¹⁹ All CD133⁺ cell isolates did not express the hemopoietic marker CD34 (Figure 1B). Moreover, selected experiments performed depleting the CD34⁺ cells before magnetic sorting of CD133⁺ cells did not affect the percentage of CD133⁺ cells obtained from renal carcinomas or their characteristics. CD34⁻/CD133⁺ cells derived from renal carcinomas (RC-CD133⁺) were characterized in comparison with circulating hematopoietic CD34⁺/CD133⁺ cells, with bone marrow-derived mesenchymal stem cells, and with CD34⁻/CD133⁺ renal progenitor cells that we previously described in normal human kidney.¹⁸ RC-CD133⁺ did not express CD45 and CD14 (Figure 1B), suggesting that they did not derive from hematopoietic CD34⁺/CD133⁺ cells. In addition, at variance with circulating endothelial progenitors, the RC-CD133⁺ isolates did not express the endothelial markers KDR (Figure 1B). RC-CD133⁺ cells expressed the mesenchymal stem cell markers CD44, CD29, and CD73,²² but, at variance with bone marrow-derived mesenchymal stem cells, they did not express CD105. Moreover, bone marrow-derived mesenchymal stem cells do not usually express CD133.^{26–28} RC-CD133⁺ cells expressed the renal embryonic marker Pax-2, which is absent both in mesenchymal and hematopoietic stem cells (Figure 1, B and E) and is present in CD133⁺ cells derived from normal human renal tissue.¹⁸ No differences were observed among isolates from dif-

ferent histological tumor types. At variance with hematopoietic CD34⁺/CD133⁺ cells, RC-CD133⁺ cells grew in adhesion in nondifferentiating expansion medium for up to seven to nine passages without changes in their phenotype or marker expression.

RC-CD133⁺ cells and clones differentiated into both endothelial and epithelial cells in the presence of specific growth factors. No differences were observed in RC-CD133⁺ cell lines obtained from different patients, with respect to the different age or sex. Epithelial differentiation by hepatocyte growth factor and fibroblast growth factor-4 was demonstrated by expression of cytokeratin, vimentin, E-cadherin (Figure 2), as well as of the renal markers alkaline phosphatase and Na/Cl co-transporter (not shown). Endothelial differentiation induced by vascular endothelial growth factor was demonstrated by vWF expression (Figure 2), as well as by a panel of endothelial markers (CD31, CD105, VE-cadherin, KDR) detected by cytofluorimetric analysis (not shown). At variance with bone marrow-derived mesenchymal stem cells, RC-CD133⁺ cells were unable to undergo adipogenic or osteogenic differentiation (Figure 2). The ability of RC-CD133⁺ isolates and clones to differentiate into epithelial or endothelial cells in the presence of specific growth factors was similar with that of renal CD133⁺ progenitor cells derived from normal kidney.¹⁸ In the same differentiating conditions, the hematopoietic CD34⁺/CD133⁺ cells did not adhere onto plastic and did not differentiate into epithelial cells, whereas, as previously described,²⁹ they were able to undergo endothelial differentiation when cultured with endothelial differentiating medium (not shown). These results altogether suggest that RC-CD133⁺ cells were different from hematopoietic CD34⁺/CD133⁺ cells and from bone marrow-derived mesenchymal stem cells for the expression of several surface markers and differentiation properties while sharing the same phenotype and functions of resident renal progenitors detected in the normal adult human kidney.

Tumor-Derived Growth Factors Induced Endothelial Differentiation of RC-CD133⁺ Progenitor Cells

RC-CD133⁺ progenitor cells were grown with conditioned medium derived from renal tumor cells. In this condition, RC-CD133⁺ cells lost the CD133 antigen after 3 days of culture (not shown) and acquired expression of endothelial-specific markers, such as KDR, CD105, VE-cadherin, and CD31 (Figure 3A). Complete endothelial maturation, as detected by the expression of all of the endothelial markers, was reached after 10 to 15 days of culture with the tumor-conditioned medium. At this time, PAX-2 expression was still maintained (data not shown). When injected subcutaneously in SCID mice, the endothelial differentiated cells organized *in vivo* into functional vessels, connected with the mouse vasculature as they contained blood cells (Figure 3B). The human nature of these vessels was indicated by the expression of HLA-class I antigen (Figure 3C).

Figure 1. Isolation and characterization of CD133⁺ progenitor cells from human renal carcinomas. **A:** Flow cytometric analysis showed that ~1% of total cells extracted from renal carcinomas express CD133 but not CD34 antigen (right dot plot; left dot plot = isotypic control). **B:** Flow cytometric analysis of CD133 immunomagnetic sorted cells showing expression of CD133, CD44, CD29, and CD73 but not of CD14, CD45, CD34, or KDR. All cell preparations ($n = 30$) were examined with similar results. **C:** Micrograph representative of immunohistochemical detection of CD133⁺ progenitor cells within tissue sections of renal carcinomas. Cells expressing membrane CD133 are visible as single cells or as small cell aggregates near vessels (arrows). **D:** Micrograph representative of a section stained with the isotypic control instead of the anti-CD133 mAb. **E:** Micrographs representative of Pax-2 nuclear staining of isolated and cultured RC-CD133⁺ cells. **F:** Pax-2 mRNA expression evaluated by RT-PCR in RC-CD133⁺ cells (lane 1). CD133⁺/CD34⁺ cells from peripheral blood (lane 2) and bone marrow-derived mesenchymal stem cells (lane 4) were negative. Cloned Pax-2 was used as positive control (lane 3). Original magnifications, $\times 250$.

Involvement of RC-CD133⁺ Cells in Tumor Growth and Angiogenesis

RC-CD133⁺ cells injected subcutaneously within Matrigel in SCID mice did not form tumors after 6 months (Table 1), suggesting that they are not tumor-initiating

cells. Co-transplantation of endothelial and tumor cells was previously shown to contribute to tumor growth and angiogenesis in mice.³⁰ To evaluate the possible role of RC-CD133⁺ progenitor cells, an established renal tumor cell line (K1) was subcutaneously injected in Matrigel, in the presence or absence of nondifferentiated RC-

Figure 2. Differentiation of tumor-derived CD133⁺ progenitor cells. Representative immunofluorescence micrographs of differentiation markers in RC-CD133⁺ cells cultured in epithelial, endothelial, adipogenic, and osteogenic differentiation medium (see Materials and Methods). Cytoplasmic expression of cytokeratin and vimentin and surface expression of E-cadherin was observed in epithelial differentiated cells, which were negative for vWF (original magnifications, $\times 400$). vWF expression, in the classical cytoplasmic punctuate pattern, was detected in endothelial differentiated cells, which were negative for cytokeratin (original magnifications, $\times 400$). Adipogenic and osteogenic differentiation was not observed in RC-CD133⁺ progenitor cells. Adipogenic differentiation of bone marrow-derived mesenchymal stem cells (BM-MSCs) was detected by the presence of fat droplets stained by Oil Red. Osteogenic differentiation was detected by positive staining for calcium deposits using Alizarin Red (original magnifications, $\times 250$).

CD133⁺ progenitor cells (1:100 RC-CD133⁺/K1 cell ratio), in the right and left abdominal region of SCID mice (Figure 4). The ratio of 1:100 CD133⁺/K1 cells was chosen because this was similar to that observed in the tumor specimens. The incidence and size of tumors in SCID mice co-transplanted with RC-CD133⁺ progenitor cells and K1 renal carcinoma cells was significantly different from that of mice transplanted with K1 renal carcinoma cells alone (Table 1). After 3 weeks, 1×10^6 K1 renal carcinoma cells in the absence of RC-CD133⁺ cells induced only two tumors of 10 mice injected. The tumors

formed in these two mice presented small neoplastic nodules with few vessels. The vessels were mainly of murine origin, as inferred by the negativity for the human HLA-class I antigen and the positivity for the mouse β_2 -microglobulin (Figure 4, H-L). In contrast, when injected in the presence of 1×10^4 RC-CD133⁺ cells, 1×10^6 K1 renal carcinoma cells formed tumors in all mice (10 of 10) after 3 weeks (Figure 4, Table 1). The promoting effect of CD133⁺ progenitor cells on tumor growth was also observed using CD133⁺ progenitor cells derived from normal renal tissue (Table 1). The incidence and

Figure 3. Endothelial differentiation of RC-CD133⁺ progenitor cells by the tumor-conditioned medium. **A:** Representative cytofluorimetric evaluation of endothelial marker expression by RC-CD133⁺ cells before and after culture in the presence of tumor-conditioned medium. After 10 days of culture, cells lost CD133 expression and acquired the expression of the endothelial markers CD31, KDR, VE-cadherin, and CD105. Dark areas, tested antibodies; white areas, isotypic controls. Ten cell preparations were studied with similar results. Micrograph: representative immunofluorescence staining showing the surface expression of VE-cadherin by RC-CD133⁺ cells cultured for 10 days in the presence of tumor-conditioned medium. VE-cadherin was absent in nondifferentiated cells (day 0). **B** and **C:** *In vivo* differentiation of RC-CD133⁺ progenitor cells in functional vessels. CD133⁺ progenitor cells (1×10^6) cultured for 15 days with tumor-conditioned medium were incorporated at 4°C within Matrigel and subcutaneously injected in SCID mice ($n = 8$). Mice were sacrificed after 7 days, and the Matrigel plugs were excised and processed for histology and immunohistochemistry. **B:** Scanning electron microscopy performed on a freeze-hatched Matrigel plug, showing representative vessels containing red blood cells. **C:** The human nature of endothelial cells lining the vessels is indicated by positive immunohistochemical staining for human HLA class I antigen showing that vessels (arrows) formed in Matrigel derived from the implanted human progenitor cells. Original magnifications: $\times 1000$ (**B**); $\times 400$ (**C**).

size of tumors in control SCID mice co-transplanted with tumor-derived CD133[−] cell fraction and K1 renal carcinoma cells was not significantly different from that of mice transplanted with K1 renal carcinoma cells alone (Table 1).

By microscopic observation, the K1 tumors, thus formed by co-injection of K1 and RC-CD133⁺ progenitor cells, were characterized by vigorous vascularization, in which several vessels, formed within or in proximity of the tumor (Figure 4), were of human origin. Vessels derived from mice were detected both by staining for mouse CD31 and β_2 -microglobulin ($13 \pm 6\%$). Vessels derived from mice, detected by mouse β_2 -microglobulin, were mainly located around the engrafted tumors and only few

of them were detectable within the tumor (Figure 4, B and C). As shown in Figure 4D, the engrafted tumors expressed the human HLA-class I antigen. Neoformed vessels, positive for human HLA-class I and human CD31 represented the $85 \pm 4\%$ of vessels within the tumors and only the $10 \pm 2\%$ of vessels around the tumors (Figure 4, D–F). vWF staining confirmed the presence of an extensive network of vessels within the implanted tumors (Figure 4G). All isotypic controls, used for both immunohistochemistry and immunofluorescence were negative (not shown). As shown in Figure 5, A and B, the human HLA-class I antigen was co-expressed with the endothelial marker CD31 both in neoformed vessels within the implanted tumors and in some peripheral vessels, con-

Table 1. Increase of *in Vivo* Growth of K1 Tumors by Co-Transplantation with CD133⁺ Cells

Injected cells	Ratio	Tumor size (cm)	Incidence
10 ⁶ K1 RCCs		0.3 ± 0.05	2/10
10 ⁶ RC-CD133 ⁺ cells			0/5
10 ⁶ K1 RCCs + 10 ⁴ RC-CD133 ⁺ cells	1:100	0.92 ± 0.28*	10/10
10 ⁶ K1 RCCs + 10 ⁴ renal-CD133 ⁺ cells	1:100	0.86 ± 0.21*	4/4
10 ⁶ K1 RCCs + 10 ⁴ RC-CD133 ⁻ cells	1:100		0/4
10 ⁵ K1 RCCs			0/4
10 ⁵ K1 RCCs + 10 ⁶ RC-CD133 ⁺ cells	10:1	0.55 ± 0.11*	3/4

To evaluate the role of CD133⁺ cells in tumor growth, K1 renal carcinoma cells (RCCs) (1×10^6 or 1×10^5) with or without CD133⁻ or CD133⁺ cells (1×10^4 or 1×10^6) obtained from different renal carcinomas or from normal renal tissue were subcutaneously injected in the right or left abdomen of SCID mice. To evaluate the tumorigenic ability of CD133⁺ cells, 1×10^6 CD133⁺ cells were subcutaneously injected within diluted Matrigel in SCID mice. Mice were sacrificed after 3 weeks (K1 cells with or without CD133⁺ or CD133⁻ cells) or 2 months (RC-CD133⁺ cells alone). Data represent the mean ± SE. Mann-Whitney comparison test was performed among K1 alone and K1 + CD133⁺ cells; * $P < 0.001$.

firming the human nature of the vascular structures detected by the anti-human CD31 Ab. Anti-human CD31 Ab did not stain murine vessels. Figure 5C shows that human CD31 did not co-localize with the mouse β_2 -microglobulin whereas vessels at the periphery of tumor were co-stained by mouse β_2 -microglobulin and mouse CD31 (Figure 5D). The CD133 marker was detected only in very few cells within the tumor or within the tumor vessels (Figure 5B, inset) suggesting that during differentiation progenitor cells lost the CD133 stem cell antigen. Therefore, to evaluate whether tumor-associated vessels were derived from differentiated CD133⁺ progenitor cells, we detected these cells using labeling of human chromosome 17 by fluorescence *in situ* hybridization. This marker was selected because in preliminary experiments we found deletion of chromosome 17 in the K1 cell line (not shown). Chromosome 17-positive cells were detectable in some vessels and in single cells within the tumor (Figure 5E) but not in the surrounding murine tissue (Figure 5F). The human nature of the tumor formed by K1 cells plus CD133 progenitors was confirmed by expression of human chromosome X (Figure 5G), which was detectable both in tumor cells and in several intratumor vessels.

To evaluate the effective *in vivo* differentiation of RC-CD133⁺ progenitors, these cells were labeled with CFSE, a fluorescent vital dye, before injection, recovered from the tumor after 2 weeks and analyzed by cytofluorimetric analysis for endothelial marker expression. As seen in Figure 6, most of the CFSE-fluorescent cells acquired endothelial markers (CD31, $98 \pm 5\%$; CD105, $52 \pm 18\%$; and KDR, $79 \pm 12\%$; $n = 4$ experiments) (Figure 6, A–D) that were absent in undifferentiated cells before injection (Figure 3). When examined by immunohistochemistry and immunofluorescence, CFSE-positive cells were detected in several vessels within and around the tumor and in a few isolated cells (Figure 6, E–G). To investigate the potency of RC-CD133⁺ cells to promote tumor development, we also performed co-transplantation experiments of RC-CD133⁺ cells (10^6) in a ratio of 10:1 with K1 cells (10^5). The presence of RC-CD133⁺ cells allowed development of K1 tumors in three of four mice whereas no tumor development was observed when the same amount of K1 cells was injected alone (Table 1).

Discussion

In the present article, we describe a population of CD34⁻/CD133⁺ resident progenitor cells in renal carcinomas that differentiated into endothelial cells in the presence of tumor-derived growth factors and promoted tumor development and angiogenesis. The AC133 epitope, a glycosylated antigen of the CD133 molecule, has been used to identify and isolate stem cells from various sources, including the hematopoietic system and the central nervous system.^{31–36} Endothelial progenitor cells also express CD34⁺/CD133⁺ in association with endothelial markers such as KDR.^{3,4} Moreover, the discovery of alternative tissue-specific promoters may suggest the presence of different isoforms of CD133.³⁷ The CD34⁻/CD133⁺ cells obtained from renal carcinomas were different from bone marrow-derived CD34⁺/CD133⁺ cells and mesenchymal stem cells for the expression of several surface markers and differentiation properties. At variance with hematopoietic CD34⁺/CD133⁺ cells, they differentiated in epithelial cells and, at variance of bone marrow-derived mesenchymal cells, they did not differentiate into adipocytes or osteogenic cells. The expression of Pax-2, a developmental renal marker,³⁸ and the ability to differentiate into epithelial expressing renal markers and in endothelial cells suggests, as for the progenitor population isolated from adult kidney,^{18,39} a renal origin. Alternatively, these cells may represent a bone marrow-derived population that has homed to the renal tumor through the circulation and has been resident long enough to have lost blood cell lineage markers.

In human brain and prostate tumors, the CD133 marker identified a tumor stem cell population that was necessary for the proliferation and self-renewal of the tumor in culture and that was able to differentiate into tumor cells.^{40–43} In contrast, the CD133⁺ population that we isolated from renal carcinomas was not a tumor-initiating cell population because it was unable to induce tumors in SCID mice. Therefore, these cells, at variance of those derived from brain and prostate tumors, cannot be identified as cancer stem cells. It can be hypothesized that the CD133⁺ population that we isolated from renal carcinomas represented a normal renal stem cell popu-

Figure 4. Role of tumor-derived CD133⁺ progenitor cells in growth and vascularization of transplanted tumors. Tumor-derived CD133⁺ progenitor cells (1×10^4 cells) were co-transplanted with K1 renal carcinoma cells (1×10^6 cells) in a ratio 1:100 within diluted Matrigel by subcutaneous injection in the left abdominal region of SCID mice. In the right abdominal region, the K1 renal carcinoma cells alone (1×10^6 cells) were injected. **A:** Representative micrograph showing the macroscopic appearance of tumors formed in experiments of co-transplantation of K1 and CD133⁺ stem cells (**left circle**). The left inset shows the low-power microscopic examination of the tissue stained with H&E. No tumor developed when K1 cells were transplanted alone (**right circle and inset**). **B and C:** Representative micrographs of a section of a tumor induced by co-transplantation of K1 and CD133⁺ progenitor cells stained by immunoperoxidase with mouse β_2 -microglobulin Ab. Numerous neoformed vessels derived from mouse were detectable around the tumor and very few within (**arrow**). **D–F:** Representative micrographs of a tumor section stained by immunoperoxidase with anti-human HLA class I antigen Ab. HLA class I antigen was expressed by tumor cells and endothelial cells lining capillaries both in the periphery (**arrows; D and E, inset**) and within the tumor (**arrows, D–F**) indicating an human origin of the positive vessels. **G:** Representative micrograph of a tumor section stained with anti-vWF Ab, showing an extensive vascular network within the tumor. Data are representative of 10 mice per experimental group. **H and I:** Representative micrographs of a section of a tumor induced by transplantation of K1 cells alone stained by immunoperoxidase with anti-human HLA class I antigen Ab. HLA class I antigen was expressed by tumor cells but not by endothelial cells lining capillaries (**I, arrow**). **J and K:** Representative micrographs of a tumor section stained by immunoperoxidase with mouse β_2 -microglobulin Ab. Vessels derived from mouse were stained around and within the tumor (**K, arrows**). **L:** Representative micrographs showing co-localization of mouse β_2 -microglobulin (red) and mouse CD31 (green) within the implanted K1 tumor seen by confocal microscopy. Data are representative of the two mice that developed K1 tumor. Original magnifications: $\times 40$ (**A, B, D**); $\times 150$ (**C, H, I**); $\times 400$ (**E, G, J, K**); $\times 600$ (**E, inset; F**); $\times 630$ (**L**).

lation migrated within the tumor because of local factor production or survived after tumor invasion of the renal parenchyma. Indeed, CD133⁺ renal progenitor cells have been detected sparsely in normal renal tissue both in the cortex, within the interstitial space, and inside the glomeruli as well as in the medulla (Ref. 18 and our unpublished data). This is supported by the recent finding of up-regulation of the stem cell marker CD133 in normal renal tissue in the proximity of carcinomas.⁴⁴

The observation that CD133⁺ cells cultured in the presence of the tumor supernatant differentiated in endothelial cells supports the role of the tumor in the endothelial commitment of these cells. The *in vivo* angiogenic

experiments in Matrigel demonstrate that CD133⁺-derived endothelial cells are able to form functional vessels expressing human HLA class I connected with the mouse vasculature. These results suggest a possible contribution of these cells in the neoformed vessels in tumors. This hypothesis was tested in co-transplantation experiments of CD133 and K1 tumor cells. We found that tumor-derived CD133⁺ progenitor cells co-transplanted with renal tumor cells significantly enhanced tumor engraftment, growth, and vascularization. Tissue resident stem cells have been shown to participate in tissue regeneration and to contribute to endothelium formation in several organs such as in the heart,⁴⁵ the nervous system,¹⁷ and

Figure 5. Contribution of tumor-derived CD133⁺ progenitor cells to vessels formed within the co-implanted K1 renal carcinoma cells. **A** and **B**: Representative micrographs showing co-localization of human HLA class I and human CD31 in vessels surrounding (**A**) or within (**B**) the implanted tumor seen by confocal microscopy. **B, inset**: Representative micrographs showing co-localization of human CD133 and human CD31 in a vessel within the implanted tumor. **C**: Representative micrographs showing the absence of co-localization of mouse β_2 -microglobulin and human CD31 in vessels within the implanted tumor seen by confocal microscopy. **D**: Representative micrographs showing co-localization of mouse β_2 -microglobulin and mouse CD31 in a vessel within the implanted tumor. Few cells of murine origin were detected around the vessel. **E–G**: Representative micrographs showing that positivity for human chromosome 17 is detectable in cells underlining vessels (V) as well as in isolated cells within the implanted tumor (**E**) by fluorescence *in situ* hybridization analysis. The red autofluorescence of erythrocytes was exploited to show a patent vessel (V). Chromosome 17 was used to identify the injected progenitors because deletion of this chromosome was previously found in K1 renal cells. **F**: Murine surrounding tissues and vessels (V) were negative. **G**: Human chromosome X, which identifies all human cells, was positive in tumor and vessels. Data are representative of six experiments with similar results. Original magnifications, $\times 600$.

the muscle.^{46,47} Hypoxia and tissue damage are considered the initiating events for stem cell activation and differentiation.^{48,49} The enhanced engraftment of tumors

observed in the experiments of co-transplantation with CD133⁺ progenitor cells suggests that these cells may produce a growth factor environment that favors tumor

Figure 6. *In vivo* endothelial differentiation of tumor-derived CD133⁺ progenitor cells co-implanted with K1 renal carcinoma cells. CD133⁺ progenitor cells were labeled with the fluorescent dye CSFE before the co-transplantation experiments and 2 weeks after the tumors were digested, and the recovered cells were analyzed for the expression of endothelial markers. **A:** Representative cytofluorimetric analysis of CSFE fluorescent cells. **B–D:** Expression by CSFE-positive cells of CD31, CD105, and KDR that were negative in nondifferentiated CD133⁺ progenitor cells. **E** and **F:** Representative micrographs showing positive cells in some vessels within and around the tumor by immunohistochemical staining of CSFE. **G:** Representative micrographs showing co-localization of human CD31- and CSFE-positive cells in a vessel (**arrow**) but not in isolated cells detected within the tumor (**arrowheads**). Data are representative of four experiments with similar results. Original magnifications: ×40 (**E**); ×250 (**F**); ×400 (**F**, **inset**); ×600 (**G**).

growth and vascularization. In addition, we provide evidence that the neofomed vessels present within the implanted tumor and in less extent in its proximity were at least in part of human origin and derived from CD133⁺ progenitors. These properties cannot be accounted to

the tumorigenic nature of tumor-derived CD133⁺ progenitor cells because the same results were obtained with normal renal-derived CD133⁺ cells. Therefore, it can be suggested that the CD133⁺ progenitors present in the tumor represent the expansion of a population normally

present in the kidney that may contribute from the inside to tumor neovascularization and growth.

It has been proposed that endothelial cells within tumors may derive from endothelial cells recruited from local capillaries, from dedifferentiated tumor cells or from circulating stem cells.^{7,50,51} Despite several experimental models in which the contribution of EPC has been clearly demonstrated, only few data were available in naturally occurring human tumors. The contribution of bone marrow-derived cells to human tumor vasculature has been assessed in a study analyzing six patients with six tumors developing 15 months after bone marrow transplantation from donors of opposite sex.¹¹ In this study, the bone marrow-derived cells identified by co-expression of human chromosomes and endothelial markers varied from 1 up to 12%, depending on the tumor type. Moreover, in a study of lung cancer, the presence of CD133⁺ cells has been reported.⁸ Although we did not identify CD133⁺/CD34⁺ cells in renal carcinomas, we cannot exclude their role in tumor angiogenesis and progression. It is conceivable that endothelial progenitor cells recruited from the circulation by the tumor may rapidly differentiate, thus losing progenitor markers, at variance of CD133 resident renal progenitors. Our results add a new possible source for the endothelial cell population, ie, tissue resident progenitor cells. These results are in agreement with several studies showing that tumor microenvironment^{52,53} and resident stromal and endothelial cells^{54–56} are critical for tumor implantation and growth. In conclusion, our data suggest that at least a part of the endothelial cells within renal carcinomas may derive from normal resident progenitors and that these cells may contribute to vascularization and tumor growth.

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