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Tumor-Associated Endothelial Cells with Cytogenetic Abnormalities

Kyoko Hida,[1,2] Yasuhiro Hida,[1,2] Dhara N. Amin,[1,2] Alan F. Flint,[4] Dipak Panigrahy,[1,2] Cynthia C. Morton,[5] and Michael Klagsbrun[1,2,3]

[1]Vascular Biology Program, [2]Department of Surgery, [3]Department of Pathology, and [4]Division of Genetics, Children's Hospital and Harvard Medical School; and [5]Dana-Farber/Harvard Cancer Center Cytogenetics Core, Departments of Obstetrics, Gynecology and Reproductive Biology, and Pathology, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts

ABSTRACT

Tumor angiogenesis is necessary for solid tumor progression and metastasis. Tumor blood vessels have been shown to differ from normal counterparts, for example, by changes in morphology. An important concept in tumor angiogenesis is that tumor endothelial cells are assumed to be genetically normal, although these endothelial cells are structurally and functionally abnormal. However, we hypothesized that given the phenotypic differences between tumor and normal blood vessels, there may be genotypic alterations as well. Mouse endothelial cells were isolated from two different human tumor xenografts, melanoma and liposarcoma, and from two normal endothelial cell counterparts, skin and adipose. Tumor-associated endothelial cells expressed typical endothelial cell markers, such as CD31. They had relatively large, heterogeneous nuclei. Unexpectedly, tumor endothelial cells were cytogenetically abnormal. Fluorescence *in situ* hybridization (FISH) analysis showed that freshly isolated uncultured tumor endothelial cells were aneuploid and had abnormal multiple centrosomes. The degree of aneuploidy was exacerbated by passage in culture. Multicolor FISH indicated that the structural chromosomal aberrations in tumor endothelial cells were heterogeneous, indicating that the cytogenetic alterations were not clonal. There was no evidence of human tumor-derived chromosomal material in the mouse tumor endothelial cells. In marked contrast, freshly isolated normal skin and adipose endothelial cells were diploid, had normal centrosomes, and remained cytogenetically stable in culture even up to 20 passages. FISH analysis of tumor sections also showed endothelial cell aneuploidy. We conclude that tumor endothelial cells can acquire cytogenetic abnormalities while in the tumor microenvironment.

INTRODUCTION

Tumor angiogenesis is necessary for solid tumor progression and metastasis (1). In tumor angiogenesis, tumors are invaded by normal host blood vessels (2) or co-opt pre-existing normal host blood vessels (3). The importance of tumor angiogenesis has been shown in transgenic RIP-Tag2 mice in which a temporal switch to the angiogenic phenotype is accompanied by rapid tumor progression (4). Tumor blood vessels differ from normal counterparts, for example, by changes in morphology (5), altered blood flow (6), enhanced leakiness (7), structural abnormalities in basement membrane (8), and abnormal pericytes (9).

There have been attempts recently to analyze tumor-associated endothelial cells at the molecular and biological level. Serial analysis of gene expression comparing gene expression patterns of endothelial cells derived from blood vessels of normal and malignant human colorectal tissues revealed that 46 transcripts that predominate in endothelium were elevated in tumor-associated endothelial cells (10). Some of these, designated as tumor endothelial markers (TEMs), were associated with the endothelial cell surface (11). There are only a few reports of tumor endothelial cells being cultured, for example, human renal carcinoma tumor endothelial cells (12) and murine lung carcinoma endothelial cells (13).

An important concept in tumor angiogenesis is that tumor blood vessels contain genetically normal and stable endothelial cells unlike tumor cells, which typically display genetic instability. We hypothesized that given the phenotypic differences between tumor and normal blood vessels, there may be genotypic alterations as well. Accordingly, we isolated two populations of pure murine endothelial cells from two human tumor xenografts and from two normal counterparts. In this report we show that these tumor endothelial cells are aneuploid using both conventional karyotype analysis of cultured cells and fluorescence *in situ* hybridization (FISH) analysis of freshly isolated uncultured cells and that their centrosomes are abnormal as well. Under identical conditions normal endothelial cells are near diploid, have normal centrosomes, and remain stable in culture. These studies demonstrate that in contrast to normal endothelial cells, tumor endothelial cells are cytogenetically abnormal and suggest that the tumor microenvironment contributes to these aberrations.

MATERIALS AND METHODS

Antibodies. The following antibodies were purchased: rat antimouse CD31 antibody and fluorescein isothiocyanate (FITC)-antimouse CD31 antibody (PharMingen, Boston, MA); FITC-Bandeirea Simplicifolia Lectin 1-B4 (BS1-B4; Vector Laboratories, Burlingame, CA); FITC-goat antirat antibody (Sigma-Aldrich, St. Louis, MO); Cy3-goat antirabbit IgG (Amersham Bioscience, Buckinghamshire, England); normal rat IgG and normal rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA); and antipericentrin antibody (Covance, Berkeley, CA). Polyclonal rabbit antivascular endothelial growth factor receptor-2 antibody (T014) was kindly provided by Dr. Rolf Brekken (University of Texas-Southwestern Medical Center, Dallas, TX; ref. 14).

Isolation of Endothelial Cells. Endothelial cells were isolated using a magnetic cell sorting system (MACS, Miltenyi Biotec, Auburn, CA). Tumor endothelial cells were isolated from melanoma and liposarcoma xenografts in nude mice (8–10 weeks old, Charles River, Boston, MA). Human A375SM cells (10^6 cells/mouse), obtained from Dr. Isaiah J Fidler (M.D. Anderson Cancer Center Houston, TX; ref. 15), were injected into the dorsal lateral flanks of female nude mice subcutaneously. A human liposarcoma was obtained with patient consent from surgical resection, implanted as pieces (1 mm^3) subcutaneously into nude mice, and tumors were passaged in mice (16). All of the animal procedures were performed in compliance with Boston Children's Hospital guidelines, and protocols were approved by the Institutional Animal Care and Use Committee. When tumors reached ~1 cm in diameter, they were excised. For normal endothelial cells, the skin on the back of female mice and epididymal fat pads from male mice were excised. Excised tissue was minced and digested with collagenase II (Worthington, Freehold, NJ). Blood cells were removed by a single sucrose step-gradient centrifugation with Histopaque 1077 (Sigma-Aldrich), and cell suspensions were filtered. Endothelial cells were isolated using MACS according to the manufacturer's instructions using FITC-anti-CD31 antibody. CD31-positive cells were sorted and plated onto 1.5% gelatin-coated culture plates and grown in EGM-2MV (Clonetics, Walkersville, MD) and 10% fetal calf serum. Diphtheria toxin (500 ng/mL; Calbiochem, San Diego, CA) was added to tumor endothelial cell

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Requests for reprints: Michael Klagsbrun, Children's Hospital, 300 Longwood Avenue, Boston, MA 02115. Phone: 617-919-2157; Fax: 617-730-0233; E-mail address: michael.klagsbrun@childrens.harvard.edu.

cultures to kill human tumor cells that might overgrow the culture (17) and to normal endothelial cell as well so that all of the endothelial cells were consistently treated in the same manner. However, diphtheria toxin treatment was not needed for freshly isolated uncultured endothelial cells. After 24 hours, dead cells were aspirated. After subculture for ~2 weeks, endothelial cell were purified by a second MACS using FITC-BS1-B4 to eliminate contaminating stromal cells and were cultured in EGM-2MV. All of the endothelial cells were split at a 1:3 ratio. The purity of endothelial cells was confirmed by flow cytometric analysis of cell surface protein with FITC-BS1-B4.

Flow Cytometric Analysis of Cell Surface Protein. Endothelial cell monolayers were detached with trypsin-EDTA and incubated with FITC-BS1-B4 for 20 minutes at 4°C. A minimum of 10,000 cells per samples were analyzed on a FACSVantageSE flow cytometer (BD, San Jose, CA).

Immunochemistry. For CD31 and VEGFR2 staining, endothelial cells were plated on gelatin-coated (1.5%) culture chambers and allowed to reach confluence. Monolayers were washed twice and fixed in 100% ice-cold acetone for 10 minutes. Monolayers were blocked in PBS containing 2% goat serum and 5% sheep serum for 1 hour at room temperature. Monolayers were then incubated with first antibody (anti-CD31 or anti-VEGFR-2) at room temperature for 2 hours and with secondary FITC-conjugated antirat or Cy3-conjugated antirabbit IgG for 1 hour at room temperature. After three washes, monolayers were counterstained with Hoechst 33258 (Sigma-Aldrich) and mounted with Vectashield (Vector Laboratories). For FISH and centrosome analysis, uncultured or cultured endothelial cells were cytospun onto glass slides by Cytospin4 (Thermo Shandon, Pittsburgh, PA). Cells were immunostained for CD31 as described above and for centrosomes as described previously (18). Nuclei were stained with Hoechst 33258 (Sigma-Aldrich). Normal IgG was used as a negative control. For *in situ* analysis, frozen sections (10 μM thick) were prepared from tumors grown subcutaneously as described above. Frozen sections of skin from nude mice were also obtained. The frozen sections were fixed in acetone and then in acetone/chloroform;1:1 v/v and stained for CD31 as described above.

Reverse Transcription-PCR. Reverse transcription-PCR (RT-PCR) was carried out as described previously with modification (10). All of the PCR reactions were run for 30 cycles using mouse-specific primers for endothelial cell markers. For human heparin-binding epidermal growth factor-like growth factor (HB-EGF), the reaction was 35 cycles with human-specific HB-EGF primers. GAPDH primers were common for human and mouse. The primers are as follows: CD31: 5′-CACAGATAAGCCCACCAGAG-3′, 5′-TGACAACCACCGCAATG-3′; VEGFR-1: 5′-CTCTGTGGGTTTGCCTGGCGATTTTCT-3′, 5′-GCGGATCACCACAGTTTTGTTCTTGTT-3′; VEGFR-2: 5′-AATTGTACAGCGAGAACAGAG-3′, 5′-ACAGGGATTCGGACTTG-3′; Tie1: 5′-CTCACTGCCCTCCTGACTGG-3′, 5′-CGATGTACTTGGATATAGGC-3′; Tie2: 5′-CCTTCCTACCTGCTACTT-3′, 5′-CCACTACACCTTTCTTTACA-3′; NRP1: 5′-GAAAGAGGGAAATAAAGCCA-3′, 5′-TCCCACCCTGAATGATGACA-3′; NRP2: 5′-AGACGTGGCATTTGGGCATT-3′, 5′-ATCCACGTGCTCCGGAGCCA-3′; TEM1: 5′-TGTGCGTGAAACAGCCTTCA-3′, 5′-GTGCCATCAAAGTCCTCCCA-3′; TEM5: 5′-CGCTCCTATCCGCTCAACA-3′, 5′-CTACCTGGGCTGGGCTCTTC-3′; TEM7: 5′-TCTCAGATTGGCTTCAACTG-3′, 5′-GCTTGGGCGTCTCTCA-3′; TEM8: 5′-AAGTCGTGATGGGCAGAA-3′, 5′-TCGGGAAAGAGAATAGAGCA-3′; GAPDH: 5′-CCAGCCTCGTCCCGTAGACA-3′, 5′-CTGGTCCTCAGTGTAGCCCAAGATG-3′; and human HB-EGF: 5′-AACAAGGAGGAGCACGGGAAAAGA-3′, 5′-CCTATGGTACCTGAACATGAGAAG-3′.

Nuclei Size Measurement. Cells were plated onto culture slides and stained with Hoechst 33258. The nuclear area was measured in ~150 to 200 cells per each cell line using the IP Lab software (Scanalytics, Fairfax, VA). Nuclei of aggregated cells were eliminated from the analysis.

Karyotype and Multicolor-FISH. Karyotype analysis of cultured endothelial cell was carried out by GTG-banding as described (19). Four to five karyotypes in each cell line were analyzed, and 20 metaspread cells were counted. M-FISH was performed using SKY paint probe mixtures for the mouse (Applied Spectral Imaging, Santa Clara, CA) according to the manufacturer's protocol.

FISH. After immunostaining of CD31, slides were fixed for 45 minutes using Histochoice (AMRESCO, Solon, OH) as described (20). FISH was performed using a Texas red-conjugated bacterial artificial chromosome mapped to mouse chromosome 17 band region E4 (RP23–10B20). The selection of the chromosome 17 probe was based on its known ability to provide a robust signal by FISH. The efficiency of the probe was evaluated in mouse embryonic stem cells: FISH analysis showed that 95% of nuclei had two copies and 5% had one copy of chromosome 17. Nuclei were counterstained with DAPI II (Vysis, Downers Grove, IL). Hybridizations signals were counted under the microscope in 100 interphase nuclei. For *in situ* analysis, frozen sections described above were fixed in a acetone/chloroform as described, stained with CD31 followed by FISH with a mouse chromosome 17 probe.

RESULTS

Isolation and Characterization of Tumor and Normal Endothelial Cell Counterparts. Mouse endothelial cells were isolated from two different human tumor xenografts grown subcutaneously. One tumor arose from implantation of highly metastatic human melanoma cells (15). The other tumor was a human liposarcoma derived from a patient and passaged through mice continually but never cultured (16). Normal endothelial cell counterparts were isolated from mouse skin and adipose tissue. The melanoma endothelial cells, liposarcoma endothelial cells, skin endothelial cells, and adipose endothelial cells were purified using FITC-conjugated anti-CD31 antibody and anti-FITC microbeads. Before subculture, diphtheria toxin was used to kill any contaminating human tumor cells because they express human HB-EGF, the diphtheria toxin receptor (17). Diphtheria toxin does not interact with mouse heparin-binding-EGF so that mouse endothelial cell survive this treatment. Normal endothelial cells were also treated with diphtheria toxin for consistency. The isolated tumor and normal endothelial cells were 97.3% to 99.7% pure (Fig. 1, *A–D*). All four of the endothelial cell cultures (passages 11 to 15) were CD31 (Fig. 1, *E–H*) and VEGFR-2 (Fig. 1, *I–L*) positive as shown by immunostaining. Several endothelial cell markers were screened by RT-PCR (Fig. 1*M*). Both tumor endothelial cell types (Fig. 1 *M, lanes 1* and *2*) expressed endothelial cell markers such as CD31, VEGFR-1, VEGFR-2, neuropilin (NRP) 1, NRP2, and various TEMs (10, 11), as did normal endothelial cells (Fig. 1 *M, lanes 3* and *4*). There were some notable differences between tumor and normal endothelial cells. For example, Tie1 and Tie2 were down-regulated in both tumor endothelial cell lines, whereas neither of the normal endothelial cell lines expressed TEM7. Human melanoma cells of whose tumors are the source of melanoma endothelial cells expressed human HB-EGF (Fig. 1 *M, lane 5*). However, no human HB-EGF was detected in any of the mouse endothelial cell lines, showing that the endothelial cell cultures were not contaminated with human tumor cells.

Tumor endothelial cell were characterized further (data not shown). They maintained CD31 and VEGFR-2 expression for at least 25 passages, grew faster, and had a lower serum requirement but did not grow in soft agar. They were much more responsive to the mitogenic effects of fibroblast growth factor-2 (FGF-2) and epidermal growth factor (EGF).

Tumor Endothelial Cells Have an Abnormal Karyotype. While culturing endothelial cells, it was noticed that the nuclei of tumor endothelial cells were larger than those of normal endothelial cells. The relatively larger sizes of tumor endothelial cell nuclei are shown visually in Fig. 4. Nuclear size was analyzed quantitatively (Fig. 2*A*). On average, tumor endothelial cell nuclei were 1.3- to 1.5-fold larger and were also much more heterogeneous in size distribution than normal endothelial cell nuclei. The heterogeneity and enlarged size of tumor endothelial cell nuclei suggested possible nuclear abnormalities. Therefore, the karyotypes of tumor and normal endothelial cells were analyzed by GTG-banding (Fig. 2*B*). Representative images are shown here. Both melanoma endothelial cells and liposarcoma endothelial cells (passages 14; Fig. 2*B, panels a* and *b*) had complex abnormal karyotypes. They were aneuploid in 15 of 20 melanoma endothelial cells (75%) and 16 of 20 liposarcoma endothelial cells

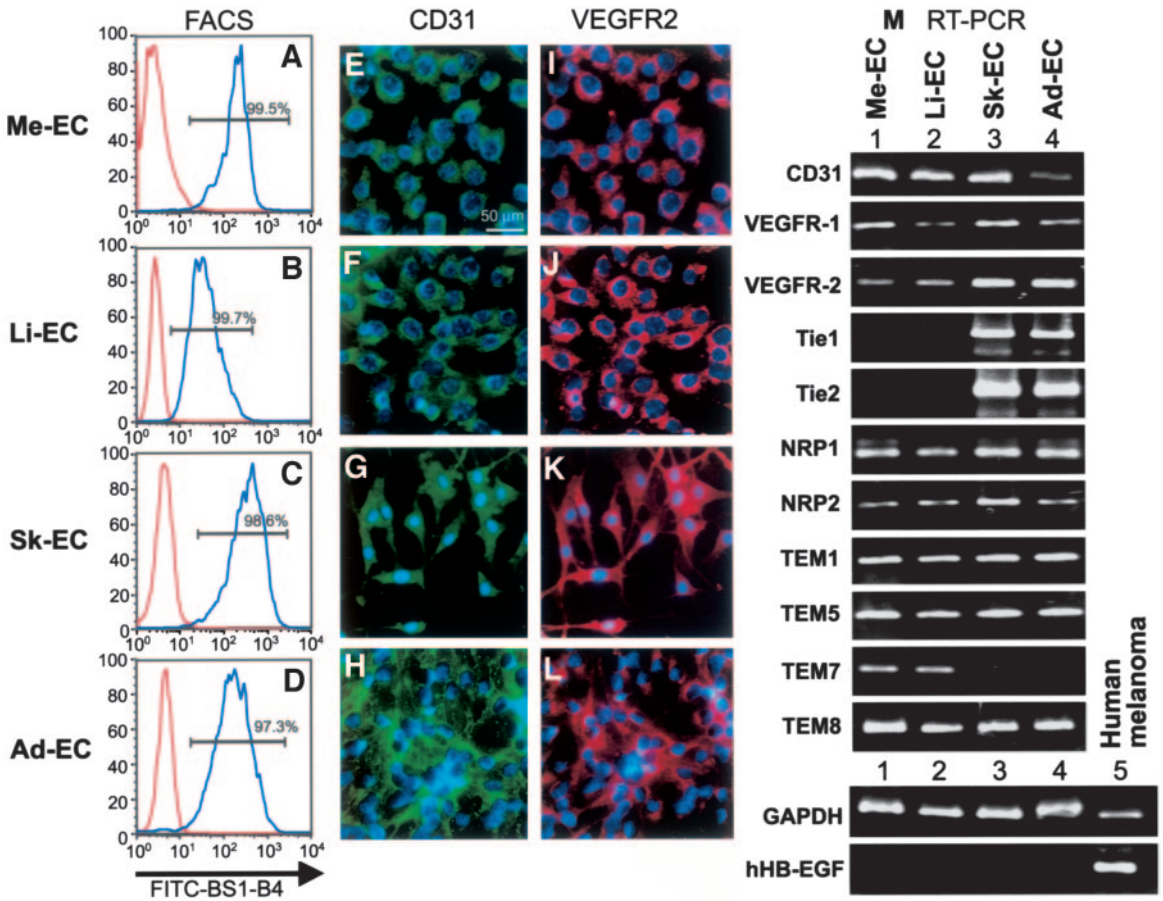

Fig. 1. Isolation and characterization of tumor and normal EC. *A–D,* FACS of binding to FITC-BS1-B4, showing purity of EC. *E–H,* immunostaining for CD31 (*green*); *I–L,* immunostaining for VEGFR-2 (*red*); *E–L,* nuclei (*blue*); *scale bar,* 50 μm. *M,* RT-PCR: The EC lines (*lanes 1–4*) were analyzed by RT-PCR for EC markers using mouse-specific primers. Human HB-EGF is expressed in human melanoma cells (*lane 5*) using human primers but not in mouse EC. (EC, endothelial cell; Me, melanoma; Li, liposarcoma; Sk, skin; Ad, adipose; FACS, flow cytometric analysis of cell surface protein)

(80%) that were counted (Table 1). These tumor endothelial cells had many missing chromosomes, markers of unknown origin, and double minute chromosomes in addition to aneuploidy. In contrast, skin endothelial cells (passage 15) and adipose endothelial cells (passage 18) were karyotypically normal with near diploid counts (Fig. 2*B, panels c* and *d*). For skin endothelial cells, 3 cells of 20 (15%) were noted to be precisely tetraploid and the rest were diploid, whereas all 20 of the adipose endothelial cells were diploid (Table 1). Low levels of aneuploidy are not uncommon in cultures of normal cells and need not be interpreted as reflective of genetic instability.

M-FISH (21-color FISH) was carried out to detect chromosomal rearrangements in tumor endothelial cells (passage 14; Fig. 3*A*). Two aneuploid melanoma endothelial cells are shown with heterogeneous genetic profiles including unbalanced translocations; t(12;17) (Fig. 3*A*), t(10;15) (Fig. 3*B*). Two liposarcoma endothelial cells are shown with heterogeneous aneuploid karyotypes with deletions and various marker chromosomes (Fig. 3, *C* and *D*). These heterogeneities suggest that the cytogenetic alterations seen in tumor endothelial cells are not clonal but arise independently in different endothelial cells.

Freshly Isolated, Noncultured Tumor Endothelial Cells Are Aneuploid. To exclude the possibility that abnormal tumor endothelial cell karyotypes arise as a result of growth in culture dishes, exposure to serum, and/or selection of abnormally growing cells, uncultured endothelial cells were examined by FISH using a mouse DNA bacterial artificial chromosome probe obtained from chromosome 17 to assess ploidy (Fig. 4, *A–D,* Table 1). Endothelial cells were isolated directly from tumors or normal tissue with >95% purity, and immunostained for CD31 (green) followed by FISH. Chromosome counts were analyzed in 100 interphase nuclei for each cell line. Nuclei with aneuploidy for chromosome 17 (red) were observed in melanoma endothelial cells (Fig. 4*A*) and liposarcoma endothelial cells (Fig. 4*B*). Quantitative analysis (Table 1) indicated that 34% and 16% of melanoma endothelial cells and liposarcoma endothelial cells were aneuploid, respectively. Of the 34 of 100 aneuploid melanoma endothelial cells, 15 had 3, 18 had 4, and 1 had 6 copies of chromosome 17, respectively. Of the 16 of 100 aneuploid liposarcoma endothelial cells, 12 had 3, and 4 had 4 copies of chromosome 17, respectively. These data are consistent with uncultured tumor endothelial cells being indeed aneuploid rather than being precisely tetraploid.

When cultured, FISH analysis showed that the frequency of aneuploidy increased to 85% and 87% for melanoma endothelial cells and liposarcoma endothelial cells, respectively, by passage 6 (Table 1). On the other hand, FISH analysis showed that uncultured skin endothelial cell (Fig. 4*C*) and adipose endothelial cells (Fig. 4*D*) were diploid with only 3% aneuploidy in skin endothelial cells and none in adipose endothelial cell (Table 1*B*, *left*). Even after 19 passages, FISH analysis showed that skin endothelial cells maintained their largely diploid state (Fig. 4*E*) with only 8% aneuploidy (Table 1*B*, *right*). Thus, tumor endothelial cells display impressive cytogenetic aberrations in contrast to normal endothelial cell.

FISH of Sections. FISH carried out on frozen sections showed that normal skin endothelial cells were all diploid (Fig. 5*A*). In contrast, melanoma endothelial cells (Fig. 5, *B* and *C*) and liposarcoma endo-

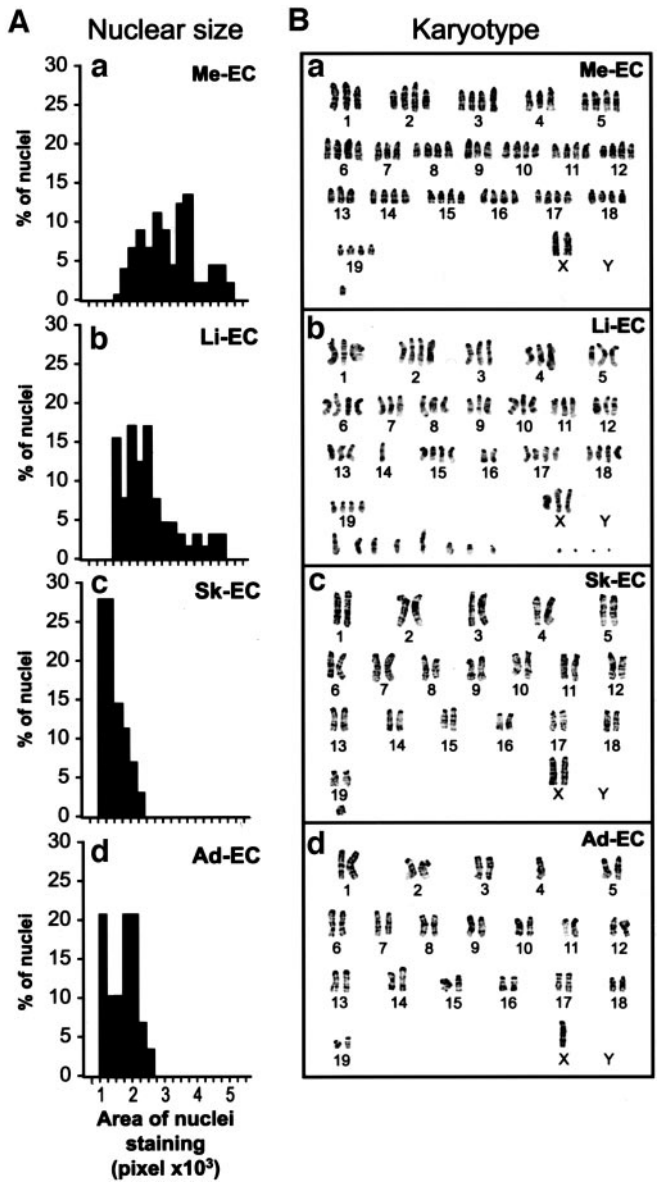

Fig. 2. Tumor ECs have large heterogeneous nuclei and abnormal karyotypes. *A*, the histogram shows nuclear size distribution using the IP Lab software. Average nuclear size (pixel $\times 10^3$): Me-EC (*a*): 3.36 ± 1.06 SD; Li-EC (*b*): 2.58 ± 0.79 SD; Sk-EC (*c*): 1.51 ± 0.37 SD; Ad-EC (*d*): 1.66 ± 0.49 SD. *B*, karyotypes of EC. Four to five karyotypes in each cell line were analyzed, and one of each is shown. Tumor ECs (*a* and *b*) had complex abnormal karyotypes and were aneuploid. *a*, Me-EC: 75,XX,-X,-X,-1,-4,-7,-9,-13, one marker chromosome. *b*, Li-EC: 71,XXX,-X,-1,-3,-4,-5,-7,-8,-9,-10,-11,-12,-13,-14,-14,-14,-16,-16, 8 marker chromosomes and 4 double minutes. In contrast, normal ECs were essentially diploid (*c* and *d*). *c*, Sk-EC: 41,XX, one marker chromosome. *d*, Ad-EC: 38,X,-Y,-4. (EC, endothelial cell; Me, melanoma; Li, liposarcoma; Sk, skin; Ad, adipose)

thelial cells (Fig. 5*D*) were sometimes aneuploid. There were no FISH signals in the human tumor cells, because the probe used is mouse specific, ensuring that any aneuploid cell stained with CD31 was a mouse endothelial cell. These results are consistent with the FISH analysis of purified endothelial cells before culture (Fig. 4). However, it should be noted that these are representative results and that carrying out FISH on sections is technically difficult. Only a minority of the cells in a tumor are endothelial cells, and of these only a small percentage of endothelial cells are aneuploid. In addition, whole endothelial cell nuclei were not always detectable, because the diameter of tumor endothelial cell nuclei often exceeded the 10-μm section thickness. We have concluded that FISH analysis of isolated endothelial cells appears to be the most reliable, reproducible, and quantifiable method for showing that tumor endothelial cells are aneuploid, because there are few if any contaminating nonendothelial cells.

Tumor Endothelial Cells Have Abnormal Centrosomes. Abnormal centrosomes, characterized by excess number (≥3 per cell), enlarged size, or aggregation, are highly correlated with aneuploidy and chromosomal instability of tumors (21, 22). Uncultured endothelial cells were immunostained with antipericentrin antibody (Fig. 4, *F–I*). Uncultured normal endothelial cells had only one centrosome per nucleus (Fig. 4, *H* and *I*), and this centrosome number was maintained in skin endothelial cells even after 19 passages (Fig. 4*J*) showing that culture conditions did not create centrosome abnormalities. On the other hand, uncultured melanoma endothelial cells and liposarcoma endothelial cells had multiple centrosomes, as many as 5 per nucleus (Fig. 4, *F* and *G*). Centrosome abnormalities occurred in 32% and 26% of melanoma endothelial cells and liposarcoma endothelial cells, respectively, compared with 4% and 1% for skin endothelial cells and adipose endothelial cells, respectively (Table 1). The 1% to 4% abnormalities are within the range observed by others in normal cells (23). The number of centrosome abnormalities in uncultured cells (Table 1) correlated very well with the level of aneuploidy in uncultured cells (Table 1). Taken together, several different measurements indicate that these tumor endothelial cells are genetically abnormal.

DISCUSSION

We have provided evidence that tumor endothelial cells are cytogenetically abnormal contrary to the common presumption that these endothelial cells are normal diploid cells. Mouse endothelial cells were isolated from human melanoma and liposarcoma tumor xenografts and compared with normal mouse endothelial cell counterparts. Tumor endothelial cells grew faster, had a lower serum-requirement, and were more responsive to growth factors such as FGF-2 and EGF. In addition, tumor endothelial cells had relatively larger nuclei and a more heterogeneous nuclear size portending differences in DNA content. Most strikingly, both melanoma endothelial cells and liposarcoma endothelial cells were aneuploid. Uncultured CD31-positive tumor endothelial cells showed 16% (liposarcoma endothelial cells) to 34% (melanoma endothelial cells) aneuploidy by FISH. Tumor endothelial cell aneuploidy could also be detected *in situ* in frozen sections. When cultured, tumor endothelial cell aneuploidy of both increased to ~85%. Furthermore, these tumor endothelial cells were characterized by structural aberrations such as nonreciprocal translocations, missing chromosomes, marker chromosomes, and double minutes. Individual tumor endothelial cells had different cytogenetic profiles indicating that tumor endothelial cells are heterogeneous and not clonal. In

Table 1 *Percentage of aneuploid cells and centrosome abnormalities*

	A. Karyotype (p14–p18) * aneuploid cells (%)	B. FISH † aneuploid cells (%)		C. Centrosome ‡ abnormal centrosomes (%)
		Uncultured	Cultured	Uncultured
Me-Ec	75 (p14)	34 §	85 (p6)	32
Li-EC	80 (p14)	16 ¶	87 (p6)	26
Sk-EC	15 (p15)	3 ‖	8 (p19)	4
Ad-EC	0 (p18)	0	ND **	1

Abbreviations: EC, endothelial cell; ME, melanoma; Li, liposarcoma; Sk, skin; Ad, adipose; p, passage number.

* Karyotype of cultured EC. Percent of aneuploid cells; 20 cells are scored.

† FISH analysis of uncultured (left) and cultured (right) EC. Percent of aneuploid cells; 100 cells were scored.

§ Me-EC: 15% had 3, 18% had 4, and 1% had 6 copies of chromosome 17.

¶ Li-EC: 12% had 3 and 4% had 4 copies of chromosome 17.

‖ Sk-EC: 3% had 3 copies of chromosome 17.

** No data available for cultured Ad-EC, but karyotype at p18 shows 0% aneuploidy.

‡ Percent of cells with abnormal centrosomes; 100 uncultured cells were scored.

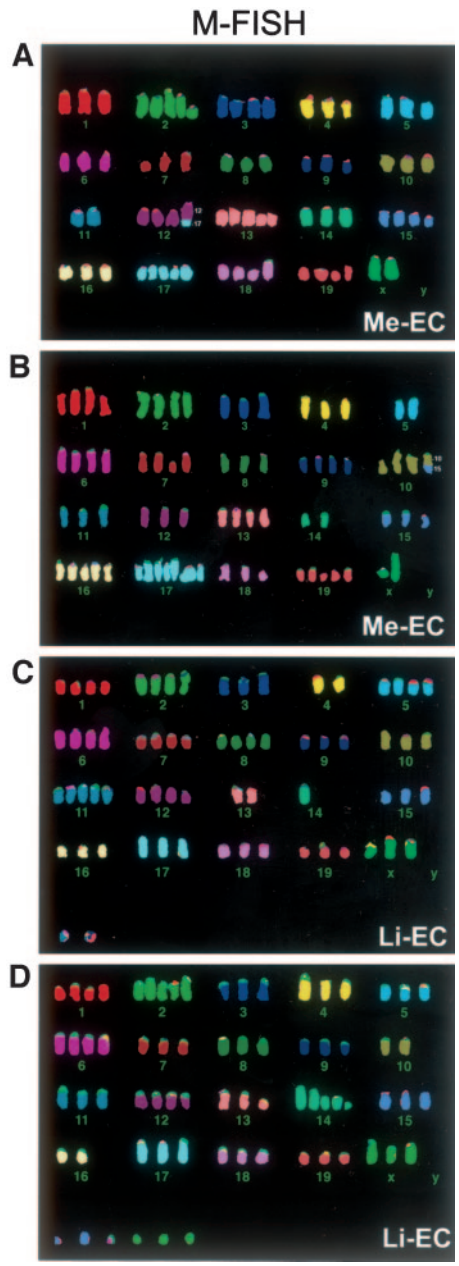

Fig. 3. M-FISH of Me-EC and Li-EC using SKY paint probe mixtures. Two representative Me-EC and Li-EC cells are shown illustrating heterogeneous aneuploid karyotypes with different chromosomal translocations: (*A*) t(12;17), (*B*) t(10;15). Two representative Li-EC (*C* and *D*) cells are shown also illustrating heterogeneous aneuploid karyotypes and various marker chromosomes. (EC, endothelial cell; Me, melanoma; Li, liposarcoma)

contrast, uncultured normal endothelial cells were diploid and remained so, when cultured, for at least 19 passages. These results suggest that tumor endothelial cells, unlike normal endothelial cells, are inherently genetically unstable, and the degree of tumor endothelial cell aneuploidy was exacerbated in culture.

Centrosomes play an important role in ensuring the proper segregation of chromosomes and, thus, maintaining the establishment of cell polarity and genetic stability (18, 21–24). Loss of polarity and chromosome missegregation (aneuploidy) in aggressive human malignant tumors result from defects in centrosome function. Both invasive and preinvasive carcinoma have abnormal centrosomes (18, 22, 24, 25). These defects occur concurrently with chromosome instability. In our case, 26% to 32% of uncultured tumor endothelial cells had centrosome abnormalities. These results correlate very well with the percentage of aneuploid uncultured tumor endothelial cells, reinforcing the premise that aneuploidy and centrosome abnormalities are linked (22). There are several pathways that can lead to aneuploidy

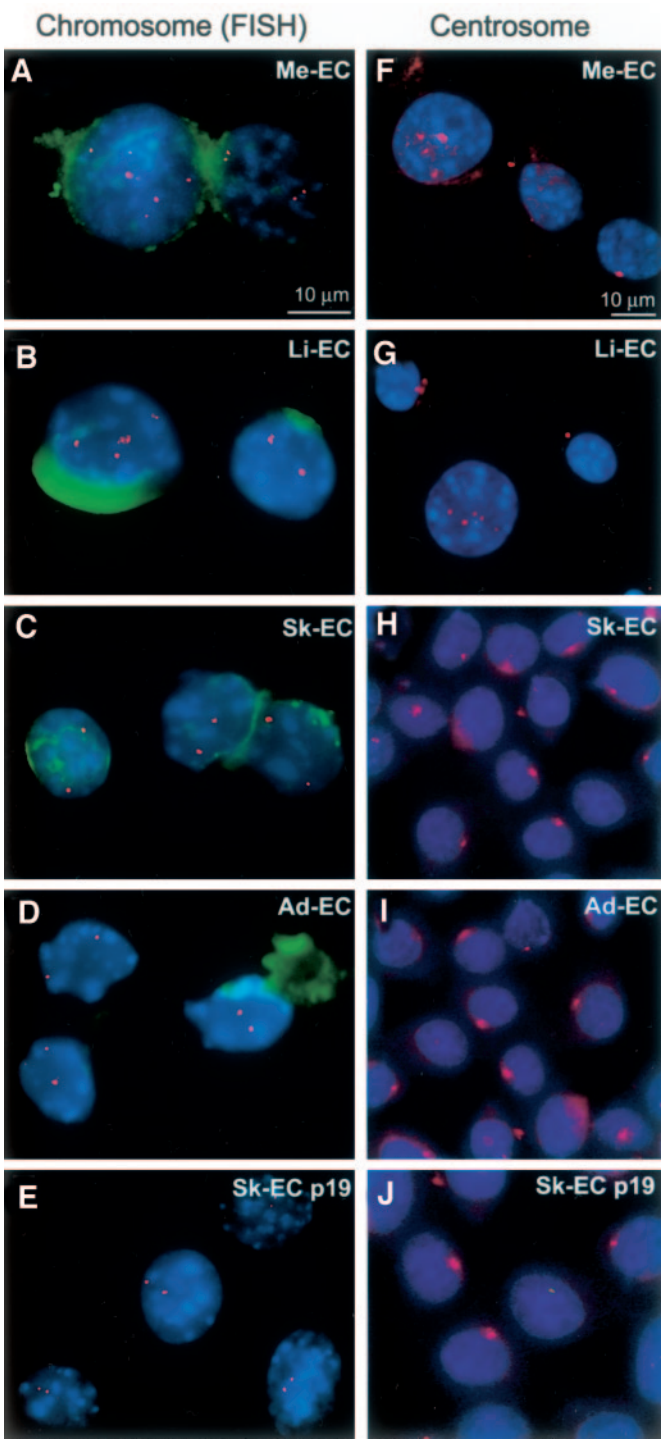

Fig. 4. Uncultured tumor ECs are aneuploid and have abnormal centrosomes. *A–E,* FISH analysis with a mouse chromosome 17 locus-specific probe. Three or more signals (*red*) were detected in uncultured tumor ECs (*A* and *B*), whereas uncultured normal ECs (*C* and *D*) and cultured Sk-EC (p19; *E*) had two signals. CD31 (*green*), 4′, 6-diamidino-2-phenylindole (*blue*). *Scale bar,* 10 μm. *F–J,* immunostaining of centrosomes with pericentrin antibody. Three or more centrosomes (*red*) were detected in uncultured tumor ECs (*F* and *G*), indicating centrosome abnormality, whereas uncultured normal ECs (*H* and *I*) and cultured Sk-EC (p19; *J*) showed only one centrosome. 4′, 6-diamidino-2-phenylindole (*blue*); *scale bar,* 10 μm. (EC, endothelial cell; Me, melanoma; Li, liposarcoma; Sk, skin; Ad, adipose)

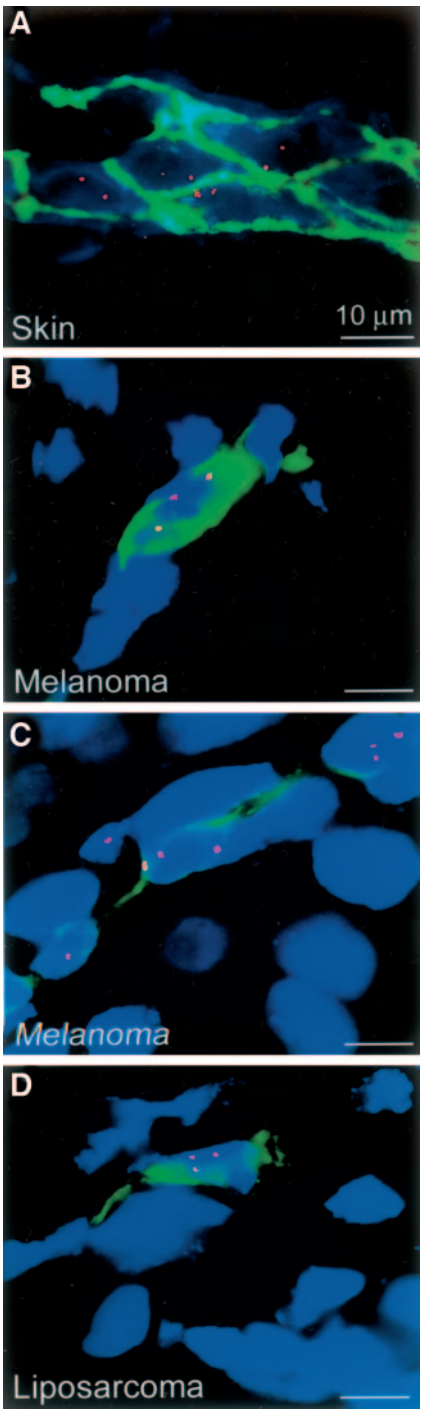

Fig. 5. Analysis of ploidy by FISH and CD31 costaining *in vivo*. Frozen sections of normal melanoma and liposarcoma tumors grown from subcutaneous injections in nude mice and sections of skin from nude mice were prepared, immunostained with CD31 (*green*) followed by FISH (*red*) using a spectrum red-conjugated mouse chromosome 17 locus-specific probe (*red*). Nuclei are stained with 4′, 6-diamidino-2-phenylindole (*blue*). Shown are representative CD31-positive ECs. *A,* normal skin EC has two signals. *B* and *C,* examples of melanoma ECs with three signals. *D,* liposarcoma ECs with three signals. *Scale bar,* 10 μm.

and supernumerary centrosomes. For example, if a cell does not complete mitosis or cell division, it will progress to a G_1-like state and be tetraploid with 2 centrosomes (26). However, in our tumor endothelial cells, we detected 3 to 5 centrosomes per cell, and these cells grew well, indicating that tumor endothelial cells escaped tetraploid cell-cycle arrest and maintained aneuploidy.

In general, the mechanisms that contribute to chromosomal abnormalities are not clear. In the case of tumor endothelial cells, it is plausible that normal host endothelial cells upon invading the tumor acquire their cytogenetic abnormalities from the tumor microenvironment. One possibility is that mouse endothelial cells fuse with human tumor cells (26) or take up human tumor oncogenes such as *H-ras* by phagocytosis of apoptotic bodies (27). Another possibility is that the tumor cells transdifferentiate into endothelial cells as has been reported for leukemia (28). In this tumor, leukemia endothelial cells were genotypically clonal cells derived from leukemia tumor cells in bone marrow. A recent publication reports that a median of 37% of endothelial cells in B-cell lymphomas harbor lymphoma-specific chromosomal translocations, and that the lymphoma cells and endothelial cells share chromosomal aberrations (29). It was suggested that there is a genetic relationship between the lymphoma cells and the endothelial cells, contradicting the assumption that endothelial cells are normally diploid cells that do not acquire mutations. It was noted in this study that for a reliable interpretation of the FISH hybridization signals the analysis of single cell suspensions was preferred to that of sections. This statement is in accordance with our own preference that FISH analysis of freshly isolated endothelial cells is much more reliable than FISH analysis of sections *in situ*.

One advantage of our using human tumor xenografts in a mouse is that the mouse and human karyotypes are readily distinguishable. Karyotype analysis indicated that there was no evidence for human genetic material in mouse tumor endothelial cells. Furthermore, because the DNA probe used in M-FISH was specific for mouse DNA, the hybridization would have been compromised if human DNA had been incorporated into the mouse genome to any significant degree. It has been suggested that human hematopoietic tumor cells can differentiate into human tumor endothelial cells (28, 29). However, because we are examining mouse endothelial cells, it would not be likely that the aneuploidy we detect in tumor endothelial cells is due to transdifferentiation of human tumor cells into endothelial cells. Thus, some other mechanisms must be involved in producing cytogenetically abnormal tumor endothelial cells in the relatively short period of time of xenograft growth. These could involve factors in the tumor microenvironment produced by tumor cells or stromal cells such as cytokines. These possibilities are under investigation.

Our tumor endothelial cells appear to have several altered functions, for example, alterations in growth factor receptor expression profiles and the response of the tumor endothelial cells to growth factors. Preliminary experiments indicate that our tumor endothelial cell up-regulate EGF receptor (EGFR). EGFR is not expressed in normal endothelial cells, and these cells do not respond to EGF. In contrast, tumor endothelial cells express EGFR protein, and EGF induces tyrosine phosphorylation of EGFR, phosphorylation of Erk 1/2, and mitogenesis in these cells. These results are consistent with immunohistochemical data *in vivo* showing that in human xenografts, tumor-associated endothelial cells express EGFR, which is activated, specifically in tumors expressing transforming growth factor α (30). These results are significant, because there is the possibility that EGFR tyrosine kinase inhibitors and anti-EGFR antibodies could target the tumor endothelium and provide antiangiogenesis therapy in addition to direct tumor cell therapy (31). In addition, preliminary results indicate that the tumor endothelial cells are highly responsive to FGF-2, suggesting a change in the FGF receptor profile. The dysregulation of receptor genes may be caused by chromosome abnormalities; however, this is not yet clear.

There are also some therapeutic implications in our results. For example, the existence of abnormal endothelial cells in tumors has implications for antiangiogenesis therapy, because chromosome instability might provide a mechanism to alter the endothelial cells in such a way that they become resistant to drugs. Some antiangiogenic

drugs have been shown to lose their effect over time (32), and we speculate that this may be due to tumor blood vessel endothelial cells becoming increasingly unstable, heterogeneous, and resistant. In support of this, it has been shown previously that tumor endothelial cells in culture are more resistant to vincristine chemotherapy than are normal endothelial cells (12). Our preliminary results indicate that tumor endothelial cells are more resistant to etoposide, a topoisomerase inhibitor, than are normal endothelial cells. On the practical side, tumor endothelial cells, with their altered properties emanating from being in a tumor microenvironment, might be better suited for screening antiangiogenesis drugs than are normal endothelial cells.

In conclusion, we have demonstrated that endothelial cells associated with tumors are cytogenetically abnormal. The instability of tumor endothelial cells raises questions about the long-term efficacy of antiangiogenesis therapy. Additional investigations will attempt to elucidate the mechanisms involved in promoting these chromosomal aberrations.

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