Potential of fibroblasts to regulate the formation of three-dimensional vessel-like structures from endothelial cells in vitro

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Kunz-Schughart, Leoni A., Josef A. Schroeder, Marit Wondrak, Frank van Rey, Karla Lehle, Ferdinand Hofstaedter, and Denys N. Wheatley. Potential of fibroblasts to regulate the formation of three-dimensional vessel-like structures from endothelial cells in vitro. Am J Physiol Cell Physiol 290: C1385-C1398, 2006; doi:10.1152/ajpcell.00248.2005.—The development of vessel-like structures in vitro to mimic as well as to realize the possibility of tissue-engineered small vascular networks presents a major challenge to cell biologists and biotechnologists. We aimed to establish a three-dimensional (3-D) culture system with an endothelial network that does not require artificial substrates or ECM compounds. By using human skin fibroblasts and endothelial cells (ECs) from the human umbilical vein (HUGECS) in diverse spheroid coculture strategies, we verified that fibroblast support and modulate EC migration, viability, and network formation in a 3-D tissue-like stromal environment. In mixed spheroid cultures consisting of human ECs and fibroblasts, a complex 3-D network with EC tubular structures, lumen formation, pinocytotic activity, and tight junction complexes has been identified on the basis of immunohistochemical and transmission electron microscopic imaging. Tubular networks with extensions up to 400 μm were achieved. When EC suspensions were used, EC migration and network formation were critically affected by the status of the fibroblast. However, the absence of EC migration into the center of 14-day, but not 3-day, precultured fibroblast spheroids could not be attributed to loss of F viability. In parallel, it was also confirmed that migrated ECs that entered cluster-like formations became apoptotic; whereas the majority of those forming vessel-like structures remained viable for >8 days. Our protocols allow us to study the nature of tubule formation in a manner more closely related to the in vivo situation as well as to understand the basis for the integration of capillary networks in tissue grafts and develop methods of quantifying the amount of angiogenesis in spheroids using fibroblast and other cells isolated from the same patient, along with ECs.

endothelium; angiogenesis; human umbilical vein endothelial cell; multicellular spheroid; coculture; tubular structures

ANGIOGENESIS IS A COMPLEX morphogenetic process initiated primarily by sprouting of endothelial capillaries from existing blood vessels to form an endothelial plexus. After being remodeled, the vasculature matures by recruitment of perivascular cells and smooth muscle cells. Vascular maturation attenuates the rate of vascular sprouting and prevents vascular collapse and regression (18, 24), which is partly reflected by contact inhibition of endothelial cell (EC) proliferation in coculture systems with pericytes or smooth muscle cells (7, 12, 29, 47). In adults, the angiogenic process is essential during wound healing, tissue repair, and remodeling and for female reproductive cycles, among many normal and pathological conditions. Mechanistic studies designed to gain a better understanding of, and an ability to manipulate, the angiogenic process in health and disease have intensified during the past 30 years, having been stimulated by Folkman’s (2, 16, 19, 25, 27) initial hypotheses regarding the angiogenic switch during tumor cell growth and the development of the concept of antiangiogenic therapy. In the interim, a number of angiogenic and angiostatic mediators have been identified, and inhibition of angiogenesis is now a therapeutic strategy implemented for patients with rheumatoid arthritis, cancer, and hypertrophic scarring (4, 11, 17, 41, 50), whereas systemic sclerosis as a fibrotic disease with a reduced and insufficient vascular network instead requires the support of EC network formation (9, 30). The latter is also true for the remodeling and recovery of vascular structures that are reversibly or apparently irreversibly damaged during various disease states, such as arteriosclerosis or after organ failure.

With respect to the remodeling of vascular structures, much has already been learned from currently available model systems that mimic vessel formation under defined in vitro conditions and have been established to identify and assay molecules affecting angiogenesis. The most widely applied assay systems are combinations of Matrigel, collagen gels, or mixed ECM gels with ECs, which grow and differentiate to form loose network-like structures of cords in the presence of VEGF. However, gel-type EC culture systems have been developed on the basis of the assumption that the ECs form vessels in an appropriate matrix, grossly underestimating the intimate association and/or involvement of live fibroblasts. Because vessel sprouting and maturation are intricately tuned by diverse positive and negative regulators that are also expressed by adjacent cell types, coculture systems are clearly much more relevant, and although they are inevitably more complex, they nevertheless provide a better approximation of a three-dimensional (3-D) capillary-like network that would be found in vivo (46). This hypothesis is supported by the results of several in vitro studies, primarily in the field of cancer research. Janvier et al. (26), for example, showed that fibroblasts enhanced the formation of a capillary-like network in a sandwiched collagen gel culture system containing fibroblasts and PC-3 human prostate adenocarcinoma cells. In addition, Walter-Yohrling et al. (54) described that cocultures of an ovarian carcinoma cell line with myofibroblasts facilitated the

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invasion of ECs in a Matrigel-based 3-D culture system. The supporting effect of fibroblasts was also noted, however, in tumor cell-free applications. In 1994, Villaschi and Nicosia (53) documented microvessel stabilization and EC life span prolongation by fibroblasts in a collagen gel system and in the rat aorta assay. This was confirmed by Martin et al. (40), who showed that fibroblasts in a bioengineered but otherwise naturally occurring ECM enhanced angiogenesis and EC motility, both in the rat aortic ring and in a Matrigel tube formation assay.

Considering that many normal physiological processes that rely on angiogenesis also require activation and migration of fibroblasts, cocultivation of fibroblast and ECs is a logical approach to enhance the quality of vessel-like structures formed in vitro. Indeed, one of the most authentic in vitro angiogenesis test systems was presented 5 years ago and showed extraordinary in vitro tubule formation in mixed cultures of human diploid fibroblast and HUVECs in a monolayer coculture (8, 14). Most notably, no artificial or additional matrix proteins were required, and vessel-like structures with true luminal development occurred during a 14-day period in multilayered areas formed by the two cell types. This occurred despite the fact that no extant vessels were present and that there was no involvement of blood or fluid producing backup pressure as would be found during angiogenesis in vivo. The crucial role of fibroblasts was recognized, and the conditions allowed experimental analysis of the cellular interactions with comparative ease. We set out to evaluate the power of human skin fibroblast in vessel formation in a true 3-D culture system that extends three cell layers but also avoids artificial matrices. By choosing the spheroid model system with cell cycle-arrested fibroblasts (36, 37) that are cocultured with HUVECs, we have established an easy-to-handle culture technique that permits experimental analysis of the cellular interactions with comparative ease. We set out to evaluate the power of human skin fibroblast in vessel formation in a true 3-D culture system that extends three cell layers but also avoids artificial matrices. By choosing the spheroid model system with cell cycle-arrested fibroblasts (36, 37) that are cocultured with HUVECs, we have established an easy-to-handle culture technique that permits experimental analysis of the cellular interactions with comparative ease.

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**MATERIALS AND METHODS**

**Cell types and routine culturing.** For most coculture experiments, two cell types were applied, human umbilical vein ECs (HUVECs) (23) and normal diploid human fibroblasts originating from foreskin material after routine circumcision. Primary fibroblast culturing and characterization were performed as detailed earlier (36, 39). Both EC and fibroblast stock cultures were stored in liquid N2 in supplemented media or FCS, respectively, containing 10% DMSO and subsequently recultured as monolayers in complete endothelial growth medium (EGM)-2 medium (EC; Cambrex BioScience, Verviers, Belgium) or DMEM containing 0.2% phenol red (Biochrom, Berlin, Germany), 10% FCS, and 100 IU/ml penicillin + 100 μg/ml streptomycin (fibroblast; all from PAN-Biotech, Aidenbach, Germany). Cultures were incubated in a humidified atmosphere with 5% CO2 in air at 37°C. Cell transfer and preparation of single-cell suspensions were performed using mild enzymatic dissociation with a 0.05% trypsin-0.02% EDTA solution in PBS (PAN-Biotech). Cell counts and cell volumes were routinely recorded using a CASY1 cell analyzer (Schärfe System, Reutlingen, Germany) for culture quality assessment. Cells used for cocultivation had cumulative population doubling of <25 (passages 4 and 5) and were free of mycoplasmal contamination as verified using the MycoAlert mycoplasma detection kit (Cambrex Bioscience).

**Monolayer cocultures.** To pre-evaluate the capacity of EC and F to form vessel-like structures in coculture, dissociated single-cell suspensions were seeded as mixtures in 24-well plates according to previous investigations and according to the protocol provided with a commercially available culture system for angiogenesis (TCS Cell Works, Botolph Cladon, UK) (8, 14). EGM-2 medium with and without VEGF supplement (1–2 ng/ml according to the manufacturer’s instructions; Cambrex BioScience) was applied and refreshed every other day. The formation of tube-like structures in these cocultures was evaluated microscopically using EC-specific immunohistochemical staining after 8, 15, and 21 days in culture.

**Spheroid culture.** Spheroid mono- and cocultures were grown in liquid overlay using agarose-coated 96-well plates (50 μl of 1.5% agarose in DMEM/well; Sigma-Aldrich) and EGM-2 medium with or without VEGF supplement (10, 38). For long-term cultures up to 22 days, medium was renewed every 48 h to provide sufficient nutrient supply after an initial steady-state phase of 3–4 days. Spheroid volumes were routinely determined using a calibrated image-processing system consisting of an inverted microscope (Axiovert 200).

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equipped with a digital camera (Axiocam MRc) and software (KS3000) (all from Zeiss, Göttingen, Germany) with a protocol for reproducible morphometric analysis of spheroid data.

Different spheroid coculture strategies were used to demonstrate the impact of fibroblasts on the formation of a tubule-like network. For strategy 1, fibroblasts and EC were mixed in different concentrations (40:1, 4:1, or 2:1) and were seeded onto agarose-coated plates with a constant number of $1 \times 10^{4}$ fibroblast/well. Fibroblast-EC mosaic spheroids were fixed and processed for immunohistochemical staining after defined times in coculture up to day 21. In the other approach (strategy 2), fibroblast spheroid monocultures were prepared with an initial seeding concentration of $1 \times 10^{4}$ fibroblast/well. EC suspensions ($1 \times 10^{5}$) were added at days 3 and 14, respectively, after fibroblast inoculation. EC migration and tubule formation in these spheroid cocultures were monitored 2 and 8 days after addition of ECs.

**Immunohistochemistry.** Monolayer cultures were stained immunohistochemically according to an established protocol (8, 14). ECs were labeled using antibodies against human CD31 (platelet endothelial cell adhesion molecule 1) or CD146 (melanoma cell adhesion molecule) after fixation of the cultures with ice-cold 70% ethanol containing 15 mM glycine and blocked for nonspecific binding by incubation with 1% BSA in PBS. Anti-human CD31 antibodies were also applied to visualize ECs in spheroid cocultures using either immunohistochemical or immunofluorescence approaches. For both immunostaining techniques, spheroids and spheroid cocultures were fixed in 4% formalin, embedded in paraffin using a Hypercenter XP (Shandon, Frankfurt, Germany), and serially sectioned (5–6 μm). For immunohistochemistry, sections were processed according to routine protocols using the NexEs/HC module (Ventana Medical Systems, Tucson, AZ) to visualize the distribution of ECs and of the ECM molecules collagen types I and IV. Hematoxylin was used as a counterstain. All antibodies and secondary detection systems used for immunostaining are specified in Table 1. All unspecified chemicals were purchased from Sigma-Aldrich or Merck.

**Immunofluorescence.** The fluorescein-FragEL DNA fragmentation detection kit (Calbiochem/Merck, Darmstadt, Germany) was applied to label DNA strand breaks in cells undergoing apoptosis in sections of paraffin-embedded spheroid mono- and cocultures. In brief, sections of 8-, 15-, and 21-day-old spheroid monocultures or cocultures grown according to strategy 2 were deparaffinized, rinsed in Tris-buffered saline (TBS; 20 mM Tris, pH 7.6, and 140 mM NaCl), and permeabilized by being incubated with proteinase K solution (2 μg/ml in 10 mM Tris, pH 8.0, 100 μl/specimen) for 20 min at room temperature using a coverslip procedure. After being washed with TBS, terminal deoxynucleotidyl transferase (TdT) equilibration buffer was added according to the manufacturer’s instructions for a 20-min incubation interval at room temperature, followed by 1.5-h incubation with the TdT enzyme diluted in Fluorescein-FragEL TdT-labeling reaction mixture (100 μl/sample). Immunofluorescence-labeled sections were mounted using a specific mounting medium containing 4,6-diamidino-2-phenylindole (Vector Laboratories) to show all nuclei. Positive controls for paraffin-embedded materials were generated by treatment of fibroblast spheroid sections with 1 μg/ml DNase (HyPure RNA tissue kit; Roche Diagnostics, Mannheim, Germany) in TBS containing 1 mM MgSO$_4$ for 20 min at room temperature. In addition, a section of a large (>900-μm diameter) colonic tumor spheroid with central apoptotic cell death and secondary necrosis was stained as an independent positive control.

Because proteinase K treatment interfered with the labeling of CD31 antigen using commercially available anti-CD31 antibodies, we visualized ECs in parallel sections of cocultures using an established immunofluorescence protocol. Primary and secondary antibodies and incubation details are provided in Table 1. Chemicals from unspecified sources were purchased from Sigma-Aldrich or Merck.

**Flow cytometry.** Spheroid mono- and cocultures were also dissociated to determine the number of cells per spheroid using the CASY1 cell analyzer and to perform advanced flow cytometric analysis. In general, a defined number of spheroids (30–48) was collected for each measurement, and single-cell suspensions were prepared by applying enzymatic treatment for 5 min at 37°C using 0.05% trypsin-0.02% EDTA solution in PBS (PAN-Biotech) supplemented with 10 μg/ml collagenase type 1A (Sigma-Aldrich). For analysis of F-to-EC distribution, cell suspensions were stained with FITC-conjugated anti-human CD31 (clone MEM-05; Immunotools, Friesoythe, Germany). Propidium iodide (PI, 2 μg/ml; Sigma-Aldrich) was applied

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Fig. 1. Human skin fibroblasts (F) and human umbilical vein endothelial cells (HUVECs) in monolayer coculture form an endothelial cell (EC) network and show sprouting. Mixed cultures of F and EC were stained immunohistochemically with a mouse anti-human CD146 MAb to visualize the developing EC network as a function of time in monolayer cultures grown in supplemented endothelial growth medium (EGM)-2 with VEGF and bFGF. APAAP staining technique, Fast Red as chromogen. d7, day 7; d15, day 15; d21, day 21.
ad preassessed in a 2-D coculture angiogenesis system and analyzed using flow cytometry. Using a FITC-conjugated ELISA, VEGF concentration in supernatants of spheroid mono- and cocultures was measured after defined times using a commercially available ELISA kit (R&D Systems, Wiesbaden, Germany). Conditioned media of spheroid cultures grown in EGM-2 medium without VEGF supplement were collected at days 3, 5, 13, and 15 in culture. The 2-day time intervals were chosen to allow for calculation of 48-h cellular release rates, taking into account the respective cell numbers per spheroid at days 5 and 15, respectively. According to the feeding strategy with only 50% of the medium replaced at day 3 in culture and every 48 h thereafter, VEGF levels at day 3 or 13 had to be subtracted from the 5- and 15-day measurements. Unconditioned, fresh EGM-2 media with and without VEGF supplementation served as controls.

3-D reconstruction. To demonstrate the EC network in a representative mixed fibroblast-EC spheroid coculture, an entire series of 5- to 6-μm sections of one spheroid stained immunohistochemically for CD31 was imaged (45 individual sections). The resulting tagged image file format, or TIFF, files were processed and reassembled for 3-D reconstruction using Amira 3.1 software (TGS; Mercury Computer Systems, San Diego, CA). The procedure included image alignment, filtering, and conversion of color information using different algorithms (median and Gaussian) to extract CD31-stained areas (diaminobenzidine-positive, brown), as well as the production of gray-scale binary images. We then generated isosurfaces for 10–12 adjacent or for all sections using a voxel of 5 to dilate data sets into Z-direction and produced overlays.

Transmission electron microscopy. Selected spheroid cocultures were fixed in 0.1 M cacodylate-buffered Karnovsky solution (2.5% glutaraldehyde and 1% paraformaldehyde overnight at room temperature) and postfixed in 1% osmium tetroxide (2 h), pH 7.3, dehydrated in graded ethanol, and embedded in EmBed-812 epoxy resin (all reagents were obtained from Service, Munich, Germany; automated LYNX tissue processor, Leica/Germany). After 48-h heat polymerization at 60°C, semithin (0.8 μm) sections were cut, stained with toluidine blue/fuchs in, and after light microscopic spheroid selection, the epon block was trimmed for ultrathin sectioning. Ultrathin (80 nm) sections were prepared with a diamond knife on a Reichert Ultracut-S ultramicrotome and double contrasted with aqueous 2% uranyl acetate and lead citrate solutions for 10 min each. The sections were examined using a LEO912AB electron microscope operating at 80 kV.

RESULTS

EC network in 2-D fibroblast endothelial mixed cultures. Primary EC cultures differ individually in their capacity to form an EC network in vitro angiogenesis systems. The capacity of HUVECs to be applied in 3-D coculture was therefore preassessed in a 2-D coculture angiogenesis system in parallel, which allowed us to assess selected human foreskin fibroblast cultures as a supportive stromal cell type. Figure 1 documents the development of a planar EC network in this two-cell-type system as a function of incubation time. Between days 15 and 21, clear EC sprouting activity was observed. According to previous studies in which this coculture model was used, a few EC sprouts were obtained even in medium without added VEGF, but sprouting became more pronounced with increasing supplementation up to 10 ng/ml VEGF. EC sprouting and network formation was also affected by the addition of other physiologically relevant paracrine modulators, including the pluri peptide growth factors TGF-β1 and PDGF (data not shown).

EC network in 3-D fibroblast endothelial mixed cultures. Preassessed EC and fibroblast cells were cultured in a 3-D format using diverse spheroid coculture strategies in liquid overlay. The most convenient approach to the coculture of different cell types as spheroids is the inoculation of mixed single-cell suspensions (with defined cell ratios) on agarose-coated 96-well plates (strategy 1). Indeed, although this protocol has shown little previous success in producing tumor-endothelial mixed spheroids with the intent of mimicking particular aspects of tumor angiogenesis as discussed later, seeding of suspensions of fibroblasts and EC with different ratios did result in the formation of mixed spheroid cultures as visualized immunohistochemically in 5- to 6-μm sections (Figs. 2 and 3). In these mixed spheroid cultures, a capillary-like network of ECs developed during an 8-day period and remained histomorphologically intact for >7 days. (The term capillary-like was used during our study until we had verified that a true vessel structure had indeed formed, although not throughout the whole network.) The establishment of strands (cf. Matrigel vessel formation) of ECs was accompanied by morphological alterations in fibroblasts in the central spheroid region expressing a rather rounded, epithelium-like cell shape (Fig. 2A).

EC clustering within spheroids was more evident in cultures derived from cell mixtures with increasing proportion of ECs, which ranged from 40 to 1 to 2 to 1. However, the size of mixed spheroids was unaffected by an increased amount of EC seeded per spheroid when constant numbers of fibroblasts were applied, e.g., in one experimental series of three cultures (A–C), the spheroid diameter at day 3 in culture averaged 453 ± 9 μm (1:40), 434 ± 9 μm (1:4), and 445 ± 13 μm (1:2), respectively, indicating that either the majority of ECs were located in the extracellular space of the 3-D fibroblast structure not contributing to an overall increase in spheroid size or the number of surviving ECs per spheroid was relatively constant. To answer this question, spheroids inoculated with fibroblast-EC ratios of 40 to 1 and 4 to 1 were collected, dissociated, and analyzed using flow cytometry. Using a FITC-conjugated...
anti-human CD31 MAb and differences in the autofluorescence signal, we were able to distinguish fibroblasts and ECs in FL-1 vs. FL-2 dot blot diagrams as shown in Fig. 2B. Membrane-intact cells were identified by PI exclusion. The comparison of 4-to-1 and 40-to-1 fibroblast-to-EC seeding ratios revealed that the 40-to-1 approach results in a significantly ($P < 0.05$) lower number of cells per coculture both at early (day 3) and late (day 15) culture phases. As expected, this phenomenon is due primarily to the reduced number of ECs ($P < 0.001$). However, our data also clearly show that the difference in the number of ECs per coculture neither at day 3 nor at day 15 differ by a factor of 10 as expected on the basis of the seeding protocols (4 to 1 vs. 40 to 1). Indeed, in the 4-to-1 approach, $-65$ to $-70\%$ of the seeded ECs were lost during the 3-day initiation and/or aggregation interval, whereas the EC fraction in the 40-to-1 series not only survived but increased. This increase was consistent but highly variable, ranging between 10 and 70\% ($n = 6$). However, as a result of EC increase in one setting and loss in the other, the number of membrane intact ECs at day 3 in coculture differed by a factor of only 2–2.5 between the 4-to-1 and 40-to-1 seeding experiments (Fig. 2B).

Impact of exogenous VEGF on EC network in 3-D mixed cultures. Superfluous VEGF medium supplementation was not required for the formation of the EC network in the fibroblast endothelial mixed spheroid culture system. This was indicated in immunohistochemically stained sections of spheroid cocultures during a period of 21 days (Fig. 3A) and confirmed using flow cytometric analysis (Fig. 3B). We therefore hypothesized that routine addition of 2% FCS plus fibroblast-derived paracrine factors provide a sufficient level of VEGF potentially required for EC network formation. Accordingly, VEGF concentrations were determined in supernatants of fibroblast mono- and fibroblast + EC cocultured spheroids at defined time intervals and cellular VEGF release rates (free VEGF/cell) were calculated on the basis of cell number per spheroid. The data in Fig. 3C show that the VEGF level in the supernatant of fibroblast spheroid cultures at day 3 was one-tenth that in VEGF-supplemented medium (~100 pg/ml vs. 1,000 pg/ml). Unsupplemented medium controls contained only ~10 pg/ml. Thus cellular VEGF release was $2.0 \pm 0.6$ fg/cell for the 72-h initiation interval. VEGF levels then continuously decreased throughout further cultivation as cellular release rates rapidly dropped to zero. VEGF levels in the fibroblast + EC cocultures already were significantly lower at day 3 in

Fig. 3. Addition of VEGF does not critically affect the formation of the EC network in mixed spheroid cultures. A: representative images of 5- to 6-μm median sections of paraffin-embedded spheroid cocultures established from F-to-EC mixtures (1 to 4) cultured over a period of 3, 14, or 21 days, respectively, in medium supplemented with or without VEGF (~1 ng/ml). EC labeling was performed as described in Fig. 2A. B: analysis of cell suspensions derived from dissociated F + EC cocultures grown in medium supplemented with or without VEGF. Flow cytometry was performed as described in Fig. 2B. Mean cell counts per spheroid coculture (±SD) of 3 independent experiments are shown. Number of cells (F + ECs) decreased only at later growth phases (day 15). Number of membrane-intact ECs did not differ between the two culture conditions. C: VEGF content in supernatants of F monocultures and F + EC cocultures was determined by ELISA and is shown with respect to VEGF-supplemented and -nonsupplemented medium controls. According to these data, which show a rapid drop in VEGF content as a function of time, F released VEGF only at the onset of spheroid culturing. VEGF level is significantly lower in mixed spheroids compared with F monocultures up to day 13 in culture.
culture with a free VEGF concentration of 0.7 ± 0.1 fg/cell, indicating either reduced cellular release rates during the 72-h aggregation phase because of reciprocal interactions between fibroblast and EC or some VEGF consumption required for EC survival and network formation at early culture phases.

Similarly, we examined mixed spheroids cultured in EGM-2 medium without external addition of both VEGF and bFGF using flow cytometric approaches. Under these conditions, the total number of membrane intact cells was reduced by ~10%. However, this was primarily due to a reduction of fibroblasts in these mixed cultures, at least during early culture phases (day 3). At days 8 and 15, the number of ECs was slightly reduced compared with bFGF-supplemented controls (data not shown). However, the difference in the number of ECs was not as pronounced as expected in a bFGF-deficient environment. As for VEGF, this phenomenon is under further investigation, including bFGF release in fibroblast spheroids.

**Tubule-like structures in fibroblast endothelial mixed cultures.** Serial sections through entire mixed spheroids seeded at a 4-to-1 ratio were stained for CD31 to confirm that the EC arrangement was not planar but was a truly 3-D interconnected cellular network (Fig. 4A). Reconstruction of the filtered immunohistochemical color information revealed a complex, capillary-like 3-D structure (Fig. 4B). To further show whether tubular structures with lumina formed by ECs were present in the in vitro system reflecting an in vivo-like capillary EC network, 14-day mixed spheroids were examined using transmission electron microscopy. Figure 5 highlights the tubular arrangements of ECs with lumen formation (cf. structures described in Ref. 8). Tubule walls were no thicker than a single EC layer. Points of tightly apposed cell membranes were frequent. EC tight junctions and junctional complexes (Fig. 5, A and B), as well as luminal pinocytotic activity, were clearly visible (Fig. 5B). In some luminal structures, fingerlike EC protrusions characteristic of EC over laps and adherence could be distinguished. Cell debris and degradation material were found frequently in the lumina (Fig. 5C). The EC tubular structures were covered by a segmental basement membrane confining the ECs from surrounding fibroblasts (Fig. 5A). Rod-shaped microtubulated bodies (Weibel-Palade bodies), specific organelles present in ECs of arteries, veins, and capillaries in different quantities that contain von Willebrand factor, could not be identified clearly, although some cytoplasmic inclusions suggestive of such configurations were observed. Observations of myelin in EC cytoplasm were frequent. In some cases, zones of amorphous breakdown material within the cytoplasm of EC, as well as single ECs undergoing destruction, occurred, with both processes appearing to participate in lumen formation.

**EC migration in 3-D fibroblast cultures.** An alternative strategy for potential coculturing of heterologous cell types in a 3-D format is the preestablishment of spheroids of one cell type that are then exposed to single-cell suspensions of the other cell type (strategy 2). This protocol allows one to study adherence and migration of the second cell type but also requires these processes to achieve heterologous spheroid cocultures consisting of both cell types. In the present study, we prepared fibroblast spheroids that were cocultured with EC suspensions. Most important, we have shown that ECs definitely infiltrated fibroblast spheroids (Fig. 6). Frequently, clusters of EC were found on one side within the fibroblast spheroid, indicating that infiltration occurred primarily on one spheroid side, supposedly the side located toward the agarose, where cell suspensions concentrate under steady-state conditions.

Migration of ECs was critically affected by the time of fibroblast spheroid preculturing. Although EC finally migrated throughout the entire fibroblast spheroid when cocultured after an fibroblast initiation interval of only 3 days, 14-day precultured fibroblast spheroids became infiltrated by ECs only in the peripheral zone (Fig. 6). Those ECs with short-term preculturing established an EC network almost comparable to that in mixed cultures after a 14-day incubation period (Fig. 7). However, its establishment was clearly delayed compared with spheroids of different coculture strategies at days 2 and 8, respectively (Fig. 6 vs. Fig. 2).

We first hypothesized that the lack of EC migration into the central area of old fibroblast spheroids might have resulted from the loss of fibroblast viability. Because there was no indication of loss of fibroblast membrane integrity and necrotic cell death in fibroblast spheroids, we visualized the local distribution of apoptotic cells in sections using a fluorescence-based DNA fragment end-labeling technique. It was clear that central fibroblast areas did not obviously become apoptotic or necrotic during a period of up to 21 days in culture (Fig. 7A), fibroblast spheroid dissociation combined with cell count and flow cytometric analysis confirmed this observation. The number of membrane-intact fibroblast was ~75–85% and did not change dramatically as a function of time in culture, and we did not observe high proportions of apoptotic (AnnexinV+) cells. In spheroid cocultures, most of the migrated ECs were viable (where they had formed a vessel-like network within the fibroblast spheroid). Those that remained in clusters within the fibroblast spheroids were usually apoptotic (Fig. 7B). Dissociation and flow cytometric analysis of 14-day-old fibroblast spheroids cocultured with EC suspensions during a period of 3–8 days revealed the following. 1) Only a small proportion of the 10,000 ECs added to fibroblast spheroids had attached and migrated (~5–10%). 2) At day 3, we found 39.1 ± 1.5% (n = 3) of the attached ECs to be AnnexinV positive (data not shown). Accordingly, the number of membrane-intact ECs decreased from days 3 to 8 because of some apoptosis. 3) The proportion of AnnexinV-positive ECs was significantly lower at day 8 (12.8 ± 0.7%) (Fig. 7C) than on day 3, which confirmed our observation in paraffin-embedded sections (Fig. 7B), implying that cells forming the EC network within fibroblast spheroids indeed show reduced apoptosis.

We also questioned whether ECM components accumulated in the fibroblast-spheroid center. We previously studied the distribution of collagen types I and IV and did not find systematic and massive alterations in these ECM components. Taking into account a collagen type I-positive tumor tissue control, it is noteworthy that this same collagen was below the level of detection in paraffin-embedded fibroblast spheroids. Collagen type IV in cocultures was found primarily in the EC areas.

**DISCUSSION**

*Spheroids as experimental tools.* The idea of using the spheroid model as a basis for the study of angiogenesis, among other approaches, may seem obvious, considering that it has...
Fig. 4. EC in mixed spheroid cultures form a complex 3-D network. A: series of 45 consecutive, aligned, immunohistochemically stained 5- to 6-μm sections of one paraffin-embedded spheroid coculture seeded at a ratio of 40 to 1 and cultured over a 14-day period in VEGF-supplemented EGM-2. EC labeling was performed according to methods described in Fig. 2. Color information was filtered and converted to create binary images. B: isosurface calculation from original images (left lower panel) and overlay production from binary images shown in A of each of 11 adjacent sections (top left) or of all 45 sections (top right). Combination of isosurface and overlay productions (bottom right).
been used in biomedical research since the early 20th century to study the mechanisms of organogenesis and the expression of malignancy, as well as to mimic tissues in vivo (34, 37, 42, 43). However, attention was mostly focused on isolated cell types of epithelial and tumor origin. This (simple) spheroid system clearly is of intermediate complexity with regard to pathophysiological micromilieu and histomorphology, but it does not reflect organ- and/or tissue-specific cellular heterogeneity. Heterologous intercellular interactions began to be appreciated by cell culturists decades later, and investigators have made sporadic attempts to reestablish spheroid cultures during the past 15 years using diverse stromal cell populations relevant in tumor tissue, such as immune cells and fibroblasts (13, 33, 37, 38). The application of EC suspensions with tumor cell spheroids has not been successful, however, supposedly because ECs do not survive in this approach. This clearly was one of the rationales for Korff and Augustin (31) to establish a tumor cell-free EC spheroid model, even though such 3-D EC

Fig. 5. EC network in mixed spheroid cultures shows tubule-like structures. A: transmission electron photomicrograph of a selected area of a 14-day-old spheroid coculture (4-to-1 F-to-EC ratio) displaying lumen formation by tubular arranged ECs. Note the numerous tight junctions laterally connecting adjacent cells (small open arrows) and segmental basement membrane formations (thick open arrows) at the adluminal cell pole, which delineate the tubular structure to the surrounding region (cf. Ref. 12). B: higher magnification of area depicted in A showing multiple pinocytotic vesicles (solid arrowheads) in the luminal cell surface as well as tight junctions (small open arrows) of the lateral junctional complex sealing the luminal space (asterisk). In one of the cells, cytoplasmic inclusion suggestive of a Weibel-Palade body is shown (long open arrow); however, due to the oblique plane of the section, it could not clearly be distinguished from myelin figures observed in many cells. C: luminal aspect of another tubular structure showing fingerlike cell protrusions (thick solid arrows) in the top part of the image and cell debris (solid cross) in the bottom part. Bar, 1 μm.

Fig. 6. ECs migrate into F spheroids. EC migration and network formation in 3-D coculture are critically affected by F-derived factors. Representative images of 5- to 6-μm sections of 3-day (top) and 14-day-old F spheroids (bottom) cocultured with EC suspensions (1 × 10⁶ ECs/F spheroid) for an additional period of 2 to 3 or 8 days, respectively. Immunohistological processing of paraffin-embedded material and EC labeling (CD31) was performed as described in Fig. 2. EC migration is incomplete in long-term precultured F spheroids. Histomorphological observations imply that F differentiation or cell death phenomena throughout culturing confine EC migration.
clusters were expected to be poor mimics of the in vivo situation. Indeed, interesting differentiation phenomena were observed at the periphery of these EC spheroids, and apoptosis in the spheroid center was manipulable. This model became more advanced with the use of two different approaches. In the first, Vernon and Sage (52) embedded EC spheroids initiated with a hanging drop technique in type I collagen to analyze radial EC migration and morphology. This model, radial invasion of matrix by aggregated cells, has been expanded using a confrontation culture approach with tumor cell spheroids (20, 21). In the second model, ECs and stromal cells, namely, smooth muscle cells, were co-inoculated in nonadherent, round-bottomed, 96-well plates to form EC-smooth muscle cell spheroid cocultures (32). To some extent, this system reflects an inside-out presentation of a vessel with a central region of smooth muscle cells surrounded by one layer of ECs oriented toward the nutrient supply and culture medium.

We used fibroblast but not smooth muscle cells as a stromal cell type interacting with ECs and found an entirely different pattern. An EC surface layer was rarely observed; instead, fibroblast and EC mixed to form a capillary-like EC network within the spheroid. By using EC suspensions, we confirmed that ECs not only attach to fibroblast and fibroblast-derived ECM but also migrate into fibroblast spheroids. This obviously is not the case with smooth muscle cells. Accordingly, it would be most relevant in advanced vessel engineering to create human three-cell-type (spheroïd) culture systems consisting of ECs and both adjacent cell types relevant for vessel assembly and maturation.

**Fibroblast-EC spheroid cocultures.** We have successfully established the first cell system that does not require any artificial matrix material or animal cell systems and is capable of forming a 3-D network of EC tubular structures with extensions of up to 400 μm, applying human cell types on the basis of a similar strategy used by Bishop et al. (8). It has become evident that under these culture conditions, fibroblast could provide all factors, including VEGF, required for the survival, differentiation, and network formation of ECs in a 3-D environment but also that these processes critically depend on an optimal balance of heterologous interactions. For example, high proportions of ECs lead to cluster formation accompanied or followed by apoptotic EC death. This massive cell death, which is not found in areas of EC tubular structures in the spheroid cocultures, clearly does not reflect the process of EC apoptosis found in the middle of a cord interpreted to contribute to lumen formation and maturation of patent small blood vessels (15, 51). Both culture strategies applied in the present study imply an optimum fibroblast-to-EC ratio for EC attachment, survival, and network formation that is in the range of 10 to 1. Several observations support this interpretation. 1) In mixed cultures with high numbers of co-seeded ECs, the majority of ECs are lost during the 3-day initiation phase, with cultures at day 3 containing ~10% EC. This ratio stayed constant throughout further cultivation; that is, the number of ECs decreased in parallel with a loss of membrane-intact fibroblast. 2) In cultures with a 20-to-1 fibroblast-to-EC ratio at day 3, the number of ECs was stable throughout cultivation (day 15 vs. day 3) despite the decrease in viable fibroblast. Accordingly, the F-to-EC ratio at day 15 in culture declined to 10 to 1. 3) In fibroblast spheroid-EC suspension cocultures (strategy 2), the number of ECs added was extremely high (10,000), but only 5–10% of these cells attached and migrated into 14-day-old fibroblast spheroids. Attached and migrated ECs accounted for a 10-to-1 fibroblast-to-EC ratio after 3 days in coculture.

EC proliferation in our spheroid coculture model was not analyzed in detail. However, the lack of spheroid growth in volume indicates cell quiescence of both EC and fibroblast populations, much as the situation in fibroblast and EC spheroid monocultures (32, 36). Indeed, spheroid volume decreased at later growth phases as indicated in the median sections shown in Fig. 2A. Flow cytometric analyses of dissociated spheroid cocultures revealed this impression, showing that the overall number of membrane-intact cells is reduced at later culture phases independently of VEGF supplementation (day 15 vs. days 3 and 8). However, it was also shown that the number of membrane-intact ECs only decreased along with fibroblast; that is, there was clearly no reduction (but rather an opposite tendency) in the number of membrane-intact ECs from days 3 through 8 in culture. In the present study, the proportion of apoptotic ECs according to AnnexinV labeling also was minimal. The phenomenon of F loss at later coculture stages is under further investigation, as is the observation that the addition of EC to fibroblast using both culture strategies in general is accompanied by a deprivation of membrane-intact fibroblast (see, e.g., Fig. 7A vs. Figs. 2B, 3B, and 7C).

Although immature neovasculature is known to be leaky and fragile and depends on a continuous supply of VEGF for survival (6, 45), vascular maturation is associated with arrest of angiogenesis through contact inhibition of EC proliferation in coculture systems with pericytes or smooth muscle cells (5, 7, 29, 47). Accordingly, we hypothesize that our system mimics a relatively mature state of the tubular network that might also be found in vivo, although this hypothesis must rely on further detailed work to fully characterize the system. Our data clearly show that the formation of the EC network does not require the constant addition of exogenous VEGF. Fibroblast were shown to produce some VEGF at least at the onset of coculturing.
However, the level of VEGF was quite small and also rapidly decreased as a function of time in culture. Thus we conclude that either VEGF released at the onset of coculturing is operating and sufficient for EC survival or ECs become autonomous and thus require less VEGF as they become organized.

In contrast to tumor spheroid-based experiments (data not shown), ECs in suspension were capable of adhering to and migrating into the ECM-rich fibroblast spheroids. In the present study, interestingly, EC migration was restricted to peripheral areas when the period of fibroblast spheroid pre-culturing was increased. This lack of EC migration cannot be due to loss of fibroblast viability in the spheroid center. An impact of the early drop in VEGF production by fibroblast in 3-D culture is under discussion. However, we emphasize that EC migration into old fibroblast spheroids was limited in the presence of VEGF-supplemented media. Our morphological observations imply that the underlying mechanism may be causally related to fibroblast differentiation phenomena that could be identified in future studies, because they may become particularly useful for the selective manipulation of EC migration and network formation. In the present study, nutrient and oxygen gradients from the spheroid periphery to the center may play a role, although previously published data imply that central hypoxia with Po2 values <2% (~14 mmHg) is not expected in fibroblast spheroids with an ~400-μm diameter (35).

Reconstruction of tubular structures. Many tests for angiogenesis are based on the measurement of the ability of flat ECs to turn into tubular structures. Assays mimicking vasculogenesis and/or angiogenesis (or simply tubulogenesis) are gel-based, often fibrin or collagen clots, but most commonly Matrigel, a matrix-rich product prepared from Engelbreth-Holm-Swarm tumor cells. More recently, an advanced biomatrix gel-type system consisting of complex mixtures of ECM components, e.g., collagen types I and IV, laminin, fibronectin, heparan sulfate proteoglycans, and other commercially available compounds have been developed that supposedly better represent the matrix architecture of stromal and basement membrane compartments to be applied for tumorigenesis and angiogenesis studies. Also, more complex systems, such as the organotypic (aortic ring), the chick aortic arch, and Matrigel sponge assays, were developed to analyze vascular responses to paracrine factors and angiogenic or proangiogenic substances in vitro. Several reviews covering different aspects of the multifaceted angiogenic process reflected in these in vitro assay systems can be found in the literature (3, 20, 48). Despite some general criticisms of the relevance of these in vitro vs. in vivo test systems, most investigators recognize that currently available in vitro assays, although useful in complex mechanistic studies, usually cannot be used in tissue engineering of blood vessels. One limitation is that a truly 3-D EC network and vessel maturation are seldom achieved, although the formation in a quasi-3-D monolayer system has its merits (8). One also must constantly keep in mind that in vitro systems do not have back-pressure of blood within the opening vessels. Another problem is the reliance of several models on nonhuman material or cells, which generally bars their reimplantation into humans unless immunosuppressed.

A startlingly ambitious study to create long-lasting blood vessels was presented recently by Koike et al. (28), who showed that 10T1/2 mesenchymal precursor cells seeded together with HUVECs in a 3-D fibronectin type I collagen gel critically supported the formation and stabilization of perfused vessels after implantation of defined pieces of the solidified gel construct into the cranial window in severe combined immunodeficiency mice. Although this study, again, is one that combines human ECs with a mouse (mesenchymal) cell type and matrix, it is in strong accord with our hypotheses and findings. Exactly how integration of existing vessels with the endothelial network in spheroids (or tissue grafts) occurs remains an intriguing yet highly complex behavioral process of ECs. However, our model is a nonadherent system that can be manipulated easily to be transferred into animal models to study this process.

Conclusions and perspectives. Today’s cell biologists and tissue engineers face two particular components of complex systems, namely, three-dimensionality and heterogeneity (1). Both of these issues were integral to our study in verifying that human fibroblast are essential supporters of human EC survival and migration, as well as important modulators (contributors) of expanded capillary-like networks in vitro. This extends the key role played by fibroblast in other aspects of wound healing (44). The particular focus of our study was to develop a system for future morphogenetic studies that would be useful in gaining deeper insight into the complex process of microvascular reconstruction both for studying tumor angiogenesis and for future-oriented, patient-specific, tissue-engineering purposes in regenerative medicine.

To test different culture protocols, we applied HUVECs as the most widely used EC population of human origin in vitro. However, HUVECs are embryonic and may differ from organ-specific adult ECs (20). It is our firm intent in future experiments to apply fibroblast and ECs from the same person when possible for tissue-engineering purposes. It will also be interesting to evaluate the capacity of adult macro- and microvascular ECs to form such a 3-D network in the spheroid system. On the basis of previous observations using the planar coculture model with human dermal microvascular ECs (8, 14), we hypothesize that different EC populations can be applied successfully under the culture conditions described. The same is true for fibroblast; adult fibroblast can be a highly heterogeneous multifunctional cell population despite their similar morphology (22, 49). However, fibroblast of different origins (skin, breast, and from normal and tumor stroma) were used in previous monolayer coculture systems, and the formation of tubular structures was in principle induced by all fibroblast types. Also, much of our work relies on the addition of FCS, which needs to be substituted so that homologous, and consequently autologous, sera can be used as medium supplements. A prospective experimental setup shall not only obviate the need for any animal-derived material but also improve the quality of the formed vessel-like structures by a defined increase in complexity, e.g., by addition of smooth muscle cells. Finally, it is most relevant to apply fibroblast + EC coculture strategies in nonspheroid alternative culture systems such as microstructures or biodegradable polymers that are designed to produce particular 3-D shapes for tissue-engineering purposes.

Two additional, specific developments are proposed, the first being to study the interaction of tumor cells with ECs, knowing that fibroblast must also play a part in this process. More than this, however, the second phase follows the abilities of different tumor types to instruct the fibroblasts and thereby control...
the levels of tumor angiogenesis that they indirectly induce. The spheroid coculture model that we devised may become useful for assaying pro- and antiangiogenic substances in a 3-D tissue context, but it has not been designed specifically for this purpose. Indeed, the system is related more to the study of the angiogenic process itself as a developmental process throughout and after tubule network development as a result of heterologous intercellular interactions. Thus it may be particularly useful to study and identify (cellular) modulators systematically in the process of vascular maturation rather than during the early onset of angiogenesis.

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DISCLOSURES

We claim no conflict of interest (e.g., consultancies, stock ownership, equity interests, patent-licensing arrangements, lack of access to data, lack of control of the decision to publish).

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