Vascular endothelial growth factors (VEGFs) are a highly conserved family of growth factors all angiogenic in vivo with mitogenic and chemotactic activity on endothelial cells. VEGFs are expressed in fibroblasts either in hypoxia or in response to growth factors. Here we report that, differently from the other members of the family, Vegf-D is induced by cell-cell contact. By in situ hybridization we demonstrated that noninteracting fibroblasts express low levels of Vegf-D mRNA, whereas contacting cells express high levels of Vegf-D transcripts. By immunostaining we observed that the surface protein cadherin-11 is localized at the opposite sites of interacting cell surfaces. Ca²⁺ deprivation from the culture medium determined the loss of cadherin-11 from the cell surfaces and down-regulation of Vegf-D mRNA. Moreover, a cadherin-11 antisense RNA construct inhibited Vegf-D expression in confluent BALB/c fibroblasts, whereas in NIH 3T3 cells, which express low levels of cadherin-11, Vegf-D induction could be obtained by overexpression of cadherin-11. This suggests that cell interaction mediated by cadherin-11 induces the expression of the angiogenic factor Vegf-D in fibroblasts.

The VEGF family is composed of several structurally and functionally related growth factors involved in vascular development. This family includes the vascular endothelial growth factor (VEGF), the placental growth factor, VEGF-B, VEGF-C, VEGF-D, and VEGF-E (1–11). All members of this family are angiogenic in vivo and able to stimulate proliferation of endothelial cells in vitro. Each member of the family recognizes and activates specific receptors on endothelial cells: VEGF recognizes VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1); placental growth factor and VEGF-B recognize VEGFR-1 (12–14); VEGF-C and VEGF-D recognize VEGFR-2 and VEGFR-3 (Flt-4) (7, 15, 16). This latter is almost exclusively expressed in lymphatic vessels, suggesting that these factors, beside playing a role in angiogenesis, are also involved in the formation of lymphatic vessels.

Due to the similarity of structure and promiscuity of receptor recognition, the specific role of each member of the family has not yet been identified. In differentiating tissues, specific receptors on endothelial cells.

succession and composition of the appropriated angiogenic factors for vessel formation. VEGF expression has been extensively studied. It responds to low levels of oxygen with induced transcription and increasing mRNA stability (17–19). Moreover, VEGF mRNA expression is up-regulated by epidermal growth factor, transforming growth factor-β, IL-6 in several cell types, and by IL-1β in smooth muscle cells (20–22). VEGF-C expression in cultured fibroblasts is induced by serum, phorbol 12-myristate 13-acetate, and several factors, including IL-1β and tumor necrosis factor α (23, 24). Vegf-D appeared to be differently regulated, because it was expressed in cells grown in low serum conditions (9).

Analyzing Vegf-D mRNA expression in mouse fibroblasts we observed that this growth factor, differently from the other members of the VEGF family, was induced by calcium-dependent cell-cell interactions. Cell-cell adhesion is mediated by cadherins, a large family of transmembrane calcium-dependent adhesive glycoproteins that form homotypic binding with their extracellular domain on adjacent cells (25–27). Although it is generally thought that cadherin expression results in a tight cell association, this is not a general principle and mesenchymal cells, which are loosely associated, express mesenchyme-specific cadherins like cadherin-11 (28–31).

The data presented in this report demonstrate that Vegf-D messenger is strongly induced by direct cell-cell contact. This induction can be inhibited by depletion of extracellular Ca²⁺ from the culture medium. Inhibition of cadherin-11 expression in contacting fibroblasts reduces Vegf-D mRNA induction, whereas cadherin-11 expression in fibroblasts, that do not express cadherin-11, restores Vegf-D induction. These results identify cadherin-11 as a surface molecule involved in Vegf-D regulation by cell-cell interaction.

**EXPERIMENTAL PROCEDURES**

**Cloning and Cell Culture**—The mouse cadherin-11 full-length cDNA was amplified from a mouse fibroblast cDNA library (9), using the primers 5’-GAGAGGATCCACCAAGATGCAGACTCTG-3’ and 5’-GAGACTCGAGTGAAGATACACTGTA-3’. The polymerase chain reaction product was cloned into the plasmid pcDNA3 (Invitrogen Corp.) in the sense (giving MO447) and antisense (giving MO334) orientation under the control of the cytomegalovirus promoter. The oligonucleotide sequences were obtained from the EBI Nucleotide Sequence Data Base under accession numbers D21253 (OB-cadherin) and D31963 (cadherin-11). All constructs were checked by automated sequencing.

Mouse embryo fibroblasts were isolated from 14-day CD1 mouse embryos as described previously (32). Unless otherwise stated, mouse embryo, mouse 3T3-type, NIH 3T3, and BALB/c 3T3 fibroblasts were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Life Technologies, Inc.), 100 units/ml penicillin, and 100 µg/ml streptomycin at 37 °C in a humidified, 5% CO₂ atmosphere. Stable clones expressing mouse cadherin-11 were obtained from NIH 3T3 cells transfected with the cadherin-11 expression vector MO447 by standard CaPO₄ precipitation procedures (32). Transfectants were selected using 1 mg/ml G418 (Life Technologies, Inc.). Stable clones expressing mouse cadherin-11 in the antisense orientation were obtained

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The abbreviations used are: VEGF, vascular endothelial growth factor; IL, interleukin; PBS, phosphate-buffered saline; poly-HEME, poly-2-hydroxyethyl methacrylate.
from BALB/c 3T3 fibroblasts transfected with the plasmid MO334, and transfectants were selected using 0.4 mg/ml G418. The same empty vector was used to generate mock stable clones.

Cell-Cell Contact and Induction Experiments—Mouse fibroblasts were plated 14–16 h before day 0 on 10-cm tissue culture dishes at different densities (starting from about 20% confluence). At every 2 days. Low, medium, and high cell densities corresponded to cells plated from about 20% to about 70% confluence. The degree of cell confluence was monitored under an inverted microscope. The cell cycle was arrested by adding cyclosporin A (0.95 μg/ml), colchicine (0.11 μg/ml), or tunicamycin (0.5 μg/ml) in the culture medium of subconflu- ent fibroblasts for 24 h. Conditioned medium and high cell density culture medium of fibroblasts growing at high cell confluence, diluted 1:1 (v/v) with complete medium, and used to stimulate subconfluent fibroblasts for 33 h. The heparin wash of confluent fibroblasts was performed by using a solution of heparin (100 μg/ml, Sigma-Aldrich) in PBS. After taking off the medium, the heparin solution was left on confluent cells 2 h at room temperature, collected, centrifuged, and finally the bound antibodies were detected by enhanced chemilumi-

cence (Amersham Pharmacia Biotech).

Northern Blot Analysis—Total cellular RNA was extracted from cells by the guanidinium thiocyanate method (33). Total RNA (10 μg) was run on denaturing formaldehyde-agarose gel, transferred onto nylon membranes, and cross-linked by UV irradiation using a Stratalinker (Stratagene). Filters were hybridized with 32P-labeled probes, washed as described (9), and analyzed by using a PhosphorImager (Molecular Dynamics). Rat glyceraldehyde-3-phosphate dehydrogenase (g3pdh) was used as a control for RNA loading.

In Situ Hybridization—Digoxigenin-labeled Vegf-D sense and anti-
sense RNA probes were generated from a cDNA fragment correspond-
ing to the complete coding sequence of the mouse Vegf-D gene. Mouse fibroblasts were grown on microscopic slides at different densities (lane 1), washed as described (9), and analyzed by using a PhosphorImager (Molecular Dynamics). Rat glyceraldehyde-3-phosphate dehydrogenase (g3pdh) was used as a control for RNA loading.

RESULTS

Vegf-D mRNA Is Expressed in Confluent Cells—Unlike VEGF-C, whose expression is induced by several growth factors (23, 24), Vegf-D is not induced by cell treatment with platelet- derived growth factor, epidermal growth factor, fibroblast growth factor 4, basic fibroblast growth factor, or transforming growth factor β (data not shown). Moreover, we previously observed that in low serum conditions fibroblasts expressed high levels of Vegf-D transcripts (9).

To test whether Vegf-D mRNA induction may require high cell density, RNA was collected at various time points from fibroblasts plated at different densities. At day 0, when cells were plated at low (20% confluence) or medium (40% confluence) density, the expression of Vegf-D was barely detectable, whereas some Vegf-D expression could be detected in cells plated at the highest density (70% confluence) (Fig. 1A, lanes 1–3). Two days later, after the cells reached a higher conflu-

cence, we observed a correspondent induction of Vegf-D tran-

scripts. In particular, Vegf-D expression was increased in the cells originally plated at medium and high density (lanes 5 and 6). In fact, after 2 days, these cells reached about 90 and 98% confluence, respectively, with elevated cell-cell interactions. At day 2, cells that were originally plated at low density (lane 4) were at about 30% confluence and showed still low Vegf-D expression. Quantitative analysis revealed that cells plated at higher density reached the highest expression of Vegf-D between days 4 and 6 from plating, cells plated at medium density at day 6, and cells plated at low density at about day 8 (Fig. 1B). Thus, the levels of Vegf-D transcripts and cell density are directly correlated.

Extracellular Ca2+ Is Required for Vegf-D Expression—Next we examined whether cell cycle arrest or soluble autocrine growth factor(s) accumulating in the culture medium, or on the surface of cells growing at high cell density, could be responsible for Vegf-D induction. The treatment of subconfluent cells with the cell cycle inhibitors cyclosporin A, colchicine, or tunicamycin did not lead to Vegf-D induction (Fig. 2A). Neither the treatment of subconfluent cells with conditioned medium nor with a heparin wash from confluent cells could induce Vegf-D expression, suggesting that autocrine-soluble factors were not involved in Vegf-D induction (Fig. 2B, lanes 2 and 4). To test whether cell-cell interaction and/or cell-cell contacts would play a role in Vegf-D induction, we analyzed Vegf-D mRNA levels in cells growing either in the presence of EGTA or in plates precoated with poly-HEMA. In the presence of EGTA, cells grew at a normal rate, acquired a round shape, and lost interactions with each other and with the culture plate. Deprivation of Ca2+ from the culture medium
strongly inhibited Vegf-D mRNA accumulation (Fig. 2C, lane 1). On the contrary, culture plates precoated with poly-HEME, which inhibited cell adhesion to the plate, did not affect Vegf-D induction (Fig. 2C, lane 2), suggesting that cell-matrix interactions are not involved in Vegf-D expression. Because Ca\textsuperscript{2+} depletion from the culture medium might affect calcium influx into the cells, we tested the expression of Vegf-D mRNA in cells treated with different Ca\textsuperscript{2+} channel blockers. The treatment of confluent cells with the calcium channel antagonists nifedipine, verapamil, amiloride, diltiazem, or ω-conotoxin GVIA did not inhibit Vegf-D mRNA expression, and the treatment of sparse cells with the calcium channel agonist BAY K-8644 did not induce Vegf-D expression (Fig. 2D). Therefore, this excluded that calcium influx plays a role in the Vegf-D up-regulation.

Vegf-D mRNA Is Strongly Induced by Direct Cell-Cell Interaction—To directly observe Vegf-D mRNA expression in contacting cells we performed in situ experiments with cultured fibroblasts plated at various degrees of confluence. Hybridization with Vegf-D antisense probe showed that single cells expressed very poor levels of Vegf-D mRNA, whereas contacting fibroblasts did express high levels of Vegf-D messenger. This could be observed even at the level of two interacting cells (Fig. 3, A and B).

Taken together, the above experiments demonstrated that both cell-cell interaction and extracellular calcium ions are required for Vegf-D up-regulation in mouse fibroblasts. However, Vegf-D mRNA is not induced by autocrine growth factor(s). Subconfluent cells not treated with conditioned medium (untreated); subconfluent cells treated with conditioned medium (treated); fibroblasts at confluence (c. m.); subconfluent cells treated with a solution of heparin (heparin); subconfluent cells treated with a heparin wash of fibroblasts at confluence (he-extr.); fibroblasts at confluence (control). C, Northern blot analysis of RNA extracted from fibroblasts grown for 24 h in the presence of 2.2 mM EGTA or for 48 h in culture plates precoated with poly-HEME (poly-H), which inhibits cell adhesion to the culture plate. Deprivation of Ca\textsuperscript{2+} from the culture medium inhibited Vegf-D mRNA accumulation. Total RNA from untreated confluent cells (control). D, Northern blot analysis of RNA extracted from fibroblasts grown in the presence of calcium channel modulators. Fibroblasts were grown either at high cell density for 18 h with calcium channel antagonists (diltiazem, amiloride, nifedipine, verapamil, ω-conotoxin GVIA) or at low cell density for 24 h with a calcium channel agonist (BAY K-8644). Vegf-D expression was not induced by calcium flux through calcium channels. Control of untreated fibroblasts confluent (lane 1) and subconfluent (lane 7).

mophilic calcium-dependent cell-cell interactions are mediated by cadherins (25). We therefore tested the hypothesis that direct interaction between contacting fibroblasts, mediated by cadherins, could be responsible for Vegf-D up-regulation.

First we examined by Northern blot analysis the expression of cadherins in sparse and confluent fibroblasts with different cadherin probes. We observed that, in fibroblasts, grown at different degrees of confluence, cadherin-11, a mesenchymal-specific cadherin, was strongly induced by cell-cell contact (Fig. 4). Importantly, cadherin-11 mRNA induction was preceding Vegf-D mRNA of 8–12 h, suggesting that cadherin-11 could be involved in Vegf-D regulation.

Immunostaining of contacting fibroblasts using anti-cadherin-11 antibodies revealed a positive staining at the cell surfaces (Fig. 5). In sparse cells cadherin-11 was localized at intercellular contacts, whereas it was mostly absent from surfaces free of cell contact (Fig. 5A). At confluence cadherin-11 staining was observed at the level of the whole cell membrane (Fig. 5B). To examine whether the localization of cadherin in fibroblasts depends on Ca\textsuperscript{2+}, contacting cells were treated with EGTA. As expected, within the first hour cadherin-11 signal disappeared from the cell surface membrane, mostly cytoplasmic (Fig. 5C). Addition of Ca\textsuperscript{2+} to the media restored cell-cell contacts, with reappearance of cadherin-11 at the intercellular contacts and induction of Vegf-D mRNA in the cells (not shown).

Cadherin-11 Is Required for Vegf-D Induction in Interacting Cells—In our study, mouse 3T3 type fibroblasts, derived from mice strain 129/SvJ × C57BL/6J (129-B6) (35), were used for the experiments.
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and the RNA was collected. A culture medium was changed, the degree of confluency was monitored,

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Vegf-D

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, mouse fibroblasts, grown on microscopic slides at different degrees of

comparative analysis of gene expression, because Vegf-D was

strongly expressed in these cells. We tested whether other fibroblasts showed the same Vegf-D mRNA regulation. Primary embryo fibroblasts obtained from CD1 mice and BALB/c 3T3 fibroblasts revealed a Vegf-D mRNA strong induction that correlated with cadherin-11 high expression in confluent cells. Instead, NIH 3T3 fibroblasts expressed barely detectable levels of both cadherin-11 and Vegf-D mRNAs (Fig. 6). Thus, extending the correlation between Vegf-D mRNA induction and cadherin 11 expression in the same cells.

To directly evidence that cadherin-11 is required for Vegf-D expression, we generated, from BALB/c 3T3 cells, stable cell

lines overexpressing cadherin-11 in the antisense orientation. Analysis of several stable clones revealed a variable level of cadherin-11 measured by Western blot of cell lysates. Two clones expressing low levels of cadherin-11 were analyzed for Vegf-D expression (Fig. 7A). By Northern blot analysis of the cadherin-11 antisense clones using Vegf-D probe, we observed that inhibition of cadherin-11 resulted in a strong reduction of Vegf-D expression (Fig. 7, compare A and B).

The converse experiment was performed in NIH 3T3 fibroblasts, because these cells expressed low levels of cadherin-11. From NIH 3T3 we generated stable clones expressing, under the control of a constitutive promoter, cadherin-11 and analyzed Vegf-D mRNA expression levels in contacting cells. Two clones expressing higher levels of cadherin-11 were chosen for Vegf-D expression analysis (Fig. 7C). In these cells the ectopic expression of cadherin-11 induced a significant increase of Vegf-D transcripts (Fig. 7, compare C and D).

DISCUSSION

In multicellular organisms, intercellular interactions and inductive signals play a major role in cell fate during development. Cell-cell adhesion, dictated by homophilic surface molecules like cadherins, determine cell patterning establishing the tissue architecture; however, secreted growth factors, which
act at a few cell diameters, modify the expression pattern of neighboring target cells. Here we demonstrate that in cultured fibroblasts direct cell-cell interaction, mediated by the mesenchyme-specific cadherin-11, triggers Vegf-D mRNA induction, suggesting cross-talk occurs between cell adhesion and growth factor signaling.

Several lines of evidence support the model that Vegf-D expression is regulated by direct cell-cell interactions via cadherin-11. First, Vegf-D is not induced in subconfluent cells under diverse culture conditions, whereas its expression is dramatically increased in cells that reach confluence. Second, the addition of conditioned media from cells highly expressing Vegf-D does not induce its expression in cells grown at low density, excluding the possibility that autocrine-diffusible factors are instrumental in this activation. Third, depletion of extracellular calcium ions, but not inhibition of cells to signal through calcium flux, blocks Vegf-D expression, demonstrating that direct calcium-dependent cell-cell interactions are required. Fourth, Vegf-D expression directly correlates with cadherin-11 localization on the cell-interacting surfaces. Fifth, down-modulation or overexpression of cadherin-11 in fibroblasts affects Vegf-D expression in a negative and positive manner, respectively. Thus, the experiments described in this report provide evidence that cadherin-11 mediates a cell interaction signaling that leads to the regulation of Vegf-D in contacting fibroblasts.

Cadherins play an important role in cell recognition and sorting during development (36, 37; and references therein). Their function has been perceived to link and stabilize connections between cells through interaction with the cytoskeleton. However, cytoplasmic domains of cadherins are highly diversified, and examples of cadherins have been found associated with signal transduction molecules and/or able to induce intracellular messengers, suggesting that cadherins mediate signal transduction pathways upon ligand binding (for review see Ref. 38). During development Vegf-D mRNA expression appears to be restricted to cadherin-11-positive mesenchymal cells. In the developing mouse embryo cadherin-11 is expressed in migratory cells derived from neural crest cells and in cells involved in mesenchymal condensation (30, 31). Vegf-D expression shows a striking overlap with cadherin-11 (30, 31, 39).

VEGFs are a family of angiogenic factors that are known to induce vessels sprouting in vitro and activation of endothelial specific receptors in vitro which are in part overlapping (7, 12–16). Although it is difficult to assign a specific role to each member of the family, it is likely that a coordinated regulation of each factor is required for the correct three-dimensional formation of new vessels. Therefore, the pattern of expression of each factor may contribute to explain its role in the angiogenic process. Interestingly, VEGF is strongly induced by hypoxia whereas VEGF-B, VEGF-C, and VEGF-D are not (17, 19, 23, 40). In lung fibroblasts VEGF-C is up-regulated by growth factors (23, 24). By contrast, in mouse fibroblasts Vegf-D is not induced by growth factor treatment,2 whereas it is up-regulated by cadherin-dependent cell-cell interaction. Vegf-D regulation by cell-cell contact is consistent with a model in which aggregating fibroblasts switch on the expression of a factor that is chemotactic to endothelial cells and thus could recruit them to the forming tissue. The cell-cell contact mediated by cadherin-11 in vitro may mimic, in part, the angiogenic process and suggests that Vegf-D expression might be involved in the initial steps of the angiogenic process.

The neovascularization and/or formation of lymphatic vessels in solid tumors is induced by tumor cells that express inductive angiogenic factors, which promote vessels sprouting with the recruitment of endothelial cells to the growing tumor mass (10, 41, 42). However, tumor vasculature is highly disorganized with vessels being dilated, tortuous, and fenestrated and having excessive branching and uneven diameters (43).

2 M. Orlandini and S. Oliviero, unpublished observations.
This could be the result of the imbalance of angiogenic factors. The fine regulation of VEGF-D by cadherin signaling is probably compromised in tumor cells especially considering that cadherin expression is altered during the malignant progression of tumors (44–49). In this respect it is interesting that the expression of VEGF-D has been found to correlate inversely with tumor size and node positivity in lung adenocarcinoma (50).

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