An Antisense Oligonucleotide to the Notch Ligand Jagged Enhances Fibroblast Growth Factor-induced Angiogenesis in Vitro*

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Ann B. Zimrin, Michael S. Pepper†, Grainne A. McMahon, Frank Nguyen, Roberto Montesano§, and Thomas Maciag§

From the Department of Molecular Biology, Holland Laboratory, American Red Cross, Rockville, Maryland 20855 and the Department of Morphology, University of Geneva Medical Center, 1, rue Michel-Servet, 1211 Geneva 4, Switzerland

Angiogenesis, or the formation of new blood vessels, plays a central role in a number of physiologic and pathologic conditions, including wound healing, diabetic retinopathy, and solid tumor growth, and endothelial cells can be induced to mimic this process in vitro. Using a modification of the differential display method (Zimrin, A. B., Villeponteau, B., and Maciag, T. (1995) Biochem. Biophys. Res. Commun. 213, 630–638), we isolated the human homolog of the Jagged ligand for the Notch receptor from human endothelial cells exposed to fibrin and demonstrate that the Jagged transcript, but not the Notch 1 or Notch 2 transcripts, are up-regulated by fibrin. Interestingly, the addition of an antisense Jagged oligomer to bovine microvascular endothelial cells grown on a collagen gel resulted in a marked increase in invasion and tube formation in the underlying gel in response to fibroblast growth factor. In contrast, no effect was observed on vascular endothelial growth factor-induced angiogenesis under identical conditions. These data suggest that Jagged-Notch signaling is able to regulate fibroblast growth factor-induced endothelial cell migration in vitro, an early event during angiogenesis in vivo.

Notch proteins are a family of closely related transmembrane receptors initially identified in developmental studies in Drosophila and Caenorhabditis elegans, which have been shown to be instrumental in cell fate decisions (1). Several Notch ligands have been identified in vertebrates and invertebrates, including Delta, Serrate, and Jagged (2). The Notch ligands are also transmembrane proteins and show a high degree of structural conservation. Studies with constitutively activated Notch proteins missing their extracellular domains have shown that activated Notch suppresses neurogenic and myogenic differentiation (3, 4). Studies of Notch signal transduction have suggested that a proteolytically processed intracellular fragment of Notch is translocated to the nucleus, where it interacts with the Suppressor of Hairless protein to activate transcription of a member of the Enhancer of Split protein complex (5, 6).

Angiogenesis is the formation of new blood vessels and involves the regulation of endothelial cell migration, growth, and differentiation by members of the fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) gene families (7). In order to gain insight into the molecular mechanisms responsible for the regulation of angiogenesis, we attempted to isolate and characterize human cDNAs induced during angiogenesis in vitro (8). We have identified the human homolog of Jagged as an angiogenesis-induced gene that is able to modify FGF-induced, but not VEGF-induced, angiogenesis in vitro. Since Jagged signaling is mediated by Notch receptor gene family members, these data implicate Notch signaling as a regulatory feature of the angiogenic process. Further evidence supporting a central role for the Notch receptor in cell differentiation is the identification of the mouse mammary tumor virus (MMTV) oncogene, int-3, as a truncated intracellular version of Notch (9), a Notch family member whose proto-oncogene expression has been shown by in situ hybridization to be endothelial cell specific in both fetal and adult tissues (9). Since the MMTV oncogene int-2 is the growth factor FGF-3 (10), a member of the angiogenic FGF gene family (11), our data also suggest that MMTV may ultimately target angiogenesis as a component of its tumor promoting activity.

EXPERIMENTAL PROCEDURES

Total RNA was obtained using standard protocols. The differential display was performed as described previously (8), using primers with the sequences 5'-GAGACCAGTGAAGATACTT-3' and 5'-GGACAGCTCGG-3'. The clone isolated was used to screen a CDNA library made in the ZAP Express vector (Stratagene) using RNA isolated from human umbilical vein endothelial cells plated on fibrin in the presence of crude FGF-1 for 1, 3, and 5 h. The overlapping CDNA clones obtained were sequenced using an ABI DNA synthesizer and assembled with the DNASTAR program. Analysis by reverse transcription and polymerase chain reaction (RT-PCR) amplification was performed as described (8) using the following primers: Jagged sense, 5'-CGCAGCTGAGGATAAACATC-3'; Jagged antisense, 5'-TTTGGATCTGGTTCAGCT-3'; Notch 1 sense, 5'-TTTCAATGGACGCGCAGTGA-3'; Notch 1 antisense, 5'-CAAGGATGATGTCGCT-3'; Notch 2 sense, 5'-GACAGCTCGG-3'; Notch 2 antisense, 5'-TGATGAGTCCTCCCATAGTC-3'; Notch 3 sense, 5'-TGTGTGTCAGG-3'; Notch 3 antisense, 5'-TACTAGAGCGGCTAGGATC-3'; Notch 4 sense, 5'-AACCACTGAGATC-3'; Notch 4 antisense, 5'-CCATGAGCATTCTAC-3'; Notch 5 sense, 5'-TGGATCCTGACGCT-3'; Notch 5 antisense, 5'-GAGGATTACCA-3'; Notch 6 sense, 5'-TTTCAATGGACGCGCAGTGA-3'; Notch 6 antisense, 5'-GACAGCTCGG-3'; Notch 7 sense, 5'-CAAGGATGATGTCGCT-3'; Notch 7 antisense, 5'-GACAGCTCGG-3'. Adrenal cortex-derived bovine microvascular endothelial (BME) cells were provided by Drs. M. B. Furie and S. C. Silverstein (Columbia University, New York) and were grown in α-modified minimal essential medium (Life Technologies, Inc. AG, Basel, Switzerland), supplemented with 15% heat-inactivated donor calf serum (DCLS, Life Technologies, Inc.), penicillin (110 units/ml), streptomycin (110 μg/ml), and streptomycin (110 μg/ml). BME cells were subcultured at a 1:4 split ratio in 1.5% gelatin-coated tissue culture dishes or flasks (Falcon Labware, Becton Dickinson Company, Lincoln Park, NJ). The in vitro angiogenesis assay was performed as described (12) in 16-mm tissue culture wells (Nunc, A/S Nunc, Roskilde, Denmark). BME cells were seeded at 5.0 × 10^4 cells/well in 50 μl of α-modified minimal essential medium, 5% DCLS.

1 The abbreviations used are: FGF, fibroblast growth factor; BME, bovine microvascular endothelial; MMTV, mouse mammary tumor virus; RT-PCR, reverse-transcribed polymerase chain reaction; VEGF, vascular endothelial growth factor; DCLS, donor calf serum.

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§To whom correspondence should be addressed. Tel.: 301-738-0653; Fax: 301-738-0645.

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Upon reaching confluence (3 days), DCS was further reduced to 2%, and cells were incubated with recombinant human FGF-2 (provided by Dr. P. Sarniments, Farmitalia Carlo Erba, Milan, Italy), recombinant human VEGF (165-amino acid homodimeric species, PeproTech Inc., Rocky Hill, NJ), and oligonucleotides (Integrated DNA Technologies, Coralville, IA). Oligonucleotides were added to the cells 2 h before cytokines on the first day of treatment. Medium and cytokines were renewed after 2 days, and oligonucleotides were added either every day or every other day during the 4-day assay period. Cultures were fixed in situ after a further 2 days with 2.5% glutaraldehyde in 100 mM sodium cacodylate buffer (pH 7.4), and photographed. For quantitation, randomly selected fields measuring 1.0 × 1.4 mm were photographed in each well at a single level beneath the surface monolayer by phase contrast microscopy, using a Nikon Diaphot TMD inverted photomicroscope. In each experiment, invasion was quantified from at least three three photographic fields by determining the total additive length of all cellular structures that had penetrated beneath the surface monolayer either as apparently single cells or in the form of cell cords or tubes (13). Results are shown as mean additive sprout length ± S.E. (in micrometers) for at least three experiments per condition. Mean values were compared using Student’s unpaired t test, and a significant p value was taken as <0.05.

RESULTS AND DISCUSSION

In an attempt to identify the molecular events involved in the regulation of angiogenesis (12, 14), we used a modified differential display procedure (8) to isolate transcripts that were differentially expressed in human umbilical vein endothelial cells plated on fibrin in the presence of FGF-1 over the course of 24 h. One of the cDNAs that was amplified at 2 h was found to be highly homologous to the rat Jagged transcript (15), suggesting that we had isolated the human Jagged homolog. The predicted protein sequence includes a signal peptide, a DSL domain shared by the Notch ligands Delta, Serrate, LAG-2 and APX-1, 16 tandem epidermal growth factor-like repeats, a cysteine-rich region, a transmembrane domain, and a 125-base pair cytoplasmic tail (Fig. 1a). The 5’ end of our 5444-base pair sequence corresponds to position 425 of the rat sequence, at the 14th codon of the predicted 21-residue signal peptide (15). Two additional Jagged clones were also obtained containing identical deletions, 88 and 1307 base pairs in length. The first deletion is located in the middle of the cysteine-rich region and predicts a frameshift in the translation product, resulting in the insertion of 15 novel amino acid residues followed by a premature termination of the protein, effectively deleting the transmembrane and cytosolic domains from the Jagged translation product (Fig. 1a).

To investigate the role of Jagged and Notch in endothelial cell behavior, we used RT-PCR analysis to evaluate the steady-state mRNA levels of Jagged and two related Notch proteins, human TAN-1, and human Notch group protein (Notch 2), in human endothelial cells on fibrin (Fig. 1b). We found that although the Jagged transcript was up-regulated in populations of human endothelial cells exposed to fibrin at the 3-h time point, the steady-state levels of the two Notch transcripts were not changed over the course of 24 h. These results were interesting because they suggest that the human endothelial cell population is able to express both the ligand, Jagged, and its receptor, Notch. Thus, the human endothelial cell may be able to complete an autocrine/juxtacrine signal using fibrin to activate the Notch signal transduction pathway by the regulation of the Jagged gene. Although we suggest the term "auto-

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2 A human Jagged cDNA clone containing the presumed translation start site has been isolated and sequenced by Drs. S. Gray and S. Artavanis-Tsakonis (Yale University) and will appear in the GenBank™ data base with the accession number U61276. Our cDNA clone, which has minor sequence differences and a longer 3’-untranslated region, has been submitted and has accession number U77720. The sequence of the longest Jagged clone containing the two deletions has also been submitted and has accession number U77914.

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FIG. 1. The human jagged protein and its expression in human umbilical vein endothelial cells. a, total RNA was isolated from human endothelial cells plated on fibrin in the presence of crude FGF-1 at 0, 2, 5, and 24 h and subjected to the differential mRNA display. A cDNA species that was increased at 2 h was cloned and used to screen a cDNA library synthesized using RNA from human umbilical vein endothelial cells plated on fibrin gels. Eight overlapping clones were isolated and sequenced, resulting in a consensus sequence 5444 base pairs in length. Two further clones were isolated containing two identical deletions each. A search of the GenBank™ data base revealed homology with the rat Jagged mRNA. The features of the deduced Jagged protein are described in the text. The cross-hatched area represents the predicted translation product. b, RNA was obtained at 0, 1, 3, 5, 8, and 24 h from human endothelial cells seeded on fibrin in the presence of crude FGF-1 and subjected to RT-PCR analysis to analyze the steady-state levels of the transcripts for Jagged, Notch 1, Notch 2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).
A quantitative evaluation of the ability of the antisense Jagged oligomer to potentiate the induction of angiogenesis revealed that the potentiating effect was more prominent if the antisense Jagged oligomer was added every day rather than every other day during the 4-day assay period. When added every day, the antisense Jagged oligomer potentiated FGF-2-induced invasion in a concentration-dependent manner with a maximal effect at 10 μM oligomer (Fig. 3). FGF-induced capillary endothelial cell invasion was not significantly affected by three control Jagged (16-mer) oligonucleotides (a sense Jagged, a 3'-antisense Jagged (AS) and a mutated 5'-antisense Jagged (MUT) oligomer) added at 2 μM every day during the assay period.

An analysis of the structure of the Notch protein provides some insight into the possible involvement of the Jagged-Notch signaling pathway in the process of angiogenesis in vitro. The predicted protein sequence of the Notch receptor contains a cytoplasmic region that has six tandem ankyrin repeats (19), a motif found in many functionally diverse proteins (20), including members of the rel/NF-κB family (21), and thought to be responsible for protein-protein interactions. Notch has been shown to interact with a novel ubiquitously distributed cytoplasmic protein deltex through its ankyrin repeats, a domain shown by deletion analysis to be necessary for activity (22). Another probable component of the pathway is the protein Suppressor of Hairless, which when coexpressed with Notch in Drosophila cells, is sequestered in the cytosol, but is translocated to the nucleus when Drosophila Notch binds to its ligand Delta (23). Although the involvement of these signaling proteins in the regulation of endothelial cell migration is not known, the endothelial cell specificity of the Notch 4 transcript in vivo (9) argues that downstream effectors of Notch gene family members are likely to be involved. Because the sensitivity of the capillary endothelial cell to the antisense Jagged oligomer is specific for FGF receptor signaling, it is likely that Jagged-induced Notch signaling represents a process that is able to discriminate between the FGF receptor and VEGF receptor signal transduction pathways. Interestingly, EM5, an intracellular protein that binds to the C. elegans Notch protein, LIN-12, contains a Src homology domain (24) which may enable the Notch and FGF receptor signaling pathways to interact in the endothelial cell. RT-PCR analysis of the human EMB-5 homolog has demonstrated the presence of the EMB-5 transcript in human endothelial cells (data not shown). Furthermore, since Jagged-mediated signaling is involved in the attenuation of the signaling pathways responsible for the terminal differentiation of myogenic cells (15), it is also possible that Jagged-induced signaling mediated by one or more of the Notch gene family members may play an important role in the maintenance of endothelial cell organization in vitro. These observations may also be particularly applicable to breast tumor angiogenesis in that (i) the MMTV int-3 oncogene encodes the intracellular signaling domain of the endothelial cell-specific Notch 4 proto-oncogene (9), (ii) the MMTV int-2 oncogene encodes a secreted member of the FGF gene family, FGF-3 (10), (iii) the MMTV int-1 oncogene encodes wnt-1, a ligand for the Drosophila frizzled gene that signals through the disheveled gene product, a recently characterized Notch-binding protein (25), and (iv) angiogenesis plays an significant role in the pathogenesis of invasive breast cancer (26).

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**Regulation of Angiogenesis by Jagged**