A new role for angiogenin in neurite growth and pathfinding: implications for amyotrophic lateral sclerosis

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Mutations in human angiogenin (hANG), an angiogenic member of the RNase A superfamily, have been recently reported in patients with amyotrophic lateral sclerosis (ALS), a progressive late-onset neurodegenerative disorder. However, very little is known about the expression and subcellular distribution of ANG in the nervous system or its role in differentiation. Here we report that mouse angiogenin-1 (mAng-1) is strongly expressed in the developing nervous system during mouse embryogenesis and neuroectodermal differentiation of pluripotent P19 embryonal carcinoma cells. mAng1 is strongly expressed in motor neurons (MNs) in the spinal cord and dorsal root ganglia as well as in post-mitotic MNs derived from P19 cells. We also show for the first time that ANG expression is in the growth cones and neurites. NCI 65828, an inhibitor of the ribonucleolytic activity of hANG, affected pathfinding by P19-derived neurons but not neuronal differentiation. Our findings clearly show that ANG plays an important role in neurite pathfinding and this has implications for ALS.

INTRODUCTION

The angiogenins are a family of angiogenic growth factors belonging to the ribonuclease A (RNase A) superfamily (1–3). It has been recently shown that patients with familial and sporadic forms of the progressive neurodegenerative disorder amyotrophic lateral sclerosis (ALS) have mutations in human angiogenin (hANG) (4,5), which is expressed in motor neurons (MNs) (5), suggesting that ANG may have an important function in the nervous system.

hANG has 33% sequence identity with bovine pancreatic RNase A, and the catalytic site residues of RNase A are conserved in hANG (6,7). However, the RNase activity of hANG is very weak (8). The amino acid residues critical for the RNase activity of hANG have been identified and confirmed by site-directed mutagenesis (8–11). Several other functional regions have been identified in hANG, including a nuclear localization sequence which is critical for its nuclear translocation, a putative receptor-binding region and a region important for immuno-modulation (12–17). Orthologues of hANG have now been identified in several species, and in the case of the human and murine ANGs, it has been reported that the weak ribonucleolytic activity is necessary for angiogenic activity (3,17).

hANG mediates neovascularization and is upregulated in pathological conditions such as cancers, diabetic retinopathy and rheumatoid arthritis (17). Expression of hANG is also induced by hypoxia (18–21). Structural studies combined with mutational analysis have provided considerable information on the mechanisms underlying its activity (22–26), but there has been no detailed study of the developmental expression of ANG, its role in neuronal differentiation or on the ‘in vivo’ function. Investigating these is essential to assess the role of ANG in the pathogenesis of ALS.

In our report here, we describe the expression of mouse ANG-1 [mAng-1, hANG’s murine orthologue with ~76% amino acid sequence identity; the ANG family in mice consists of six members and three pseudogenes (27,28)] in the nervous system during mouse embryonic development and during neural differentiation of the well-characterized pluripotent P19 EC cell model system. We chose this cell culture model of neuroectodermal differentiation for our investigations, as it would help us identify the stage(s) in differentiation and cell types expressing mAng-1. We find that...
mAng-1 is expressed in the nervous system, in MNs and in MNs derived from P19 EC cells. It localizes in growth cones and in neurite outgrowths. Using the P19 model of neuroectodermal differentiation, we also show that the inhibitor of ANG-NCI 65828 (29) inhibits neurite pathfinding. Taken together, our results indicate the important role played by ANG in neurite pathfinding and provide the basis for further investigating the role of ANG in pathfinding and in the pathophysiology of ALS.

RESULTS

mAng-1 expression during the development of the mouse nervous system

In order to understand the role that ANG plays in ALS, we first studied the expression and developmental distribution of mAng-1 in the nervous system during mouse embryogenesis. mAng-1 is widely expressed in the developing nervous system both in the brain and in the spinal cord, predominantly in the neurons. The expression of mAng-1 is downregulated in the nervous system as development proceeds but is still clearly detectable into adulthood.

mAng-1 is expressed strongly in the nervous system at 9.5 days pc with the highest expression in the presumptive fore brain, midbrain, hind brain and spinal cord (Fig. 1A). At 11.5 days pc, the high level of mAng-1 is maintained in the telencephalon, mesen and myelencephalon (Fig. 1B and C) as well as in the spinal cord, spinal ganglia and choroid plexus. Until mid-gestation, the expression of mAng-1 is stronger in the nervous system than in any other tissue.

At 14.5d pc, the expression in the brain is maintained with strong expression in the ependymal layer, the cranial and facial ganglia and choroid plexus. Expression is also seen in the spinal ganglia. By 15.5 days of development, the level of mAng-1 is considerably lower in the brain, spinal chord and ganglia but stronger in the choroid plexus. This expression pattern continues in 16.5-day embryos and at 17.5 days, expression is retained in the spinal chord and spinal ganglia. In 14.5 and 16.5 days pc embryos stained for mAng-1, peripherin (marker for CNS MNs) and Islet1 (marker for post-mitotic MNs), there is co-expression of mAng-1 with Islet1 in the dorsal root ganglia (Fig. 1D–I) and co-localization with peripherin (Fig. 1J–L), indicating that mAng-1 is expressed in MNs. mAng-1 is also co-expressed with Islet1 in the spinal chord (Fig. 1M–O).

Dynamic distribution of mAng-1 in retinoic acid-induced neuronal differentiation of P19 EC cells and sub-cellular distribution of mAng-1

From the developmental expression pattern, it appears that mAng-1 may be involved in one, some or all of the following aspects of neuroectodermal development: neural precursor formation, neuronal differentiation, neurite outgrowth and pathfinding and/or survival. In order to identify the stage at which mAng-1 may be important in neural differentiation, we used the well-characterized P19 EC cell differentiation model (30,31).

P19 EC cells can be induced to differentiate into neural cell types by either one of two methods. One is through embryoid body formation in the presence of retinoic acid (RA). The embryoid bodies are then trypsinized and plated to form adherent cultures with or without a PA6 feeder layer. This gives rise to mostly neuroectodermal derivatives, but cells of other tissue types also arise. The second is a more directed and shorter approach and involves direct plating of the EC cells on a PA6 stromal layer. PA6 is able to induce neural differentiation from embryonic stem (ES) cells and this activity has been referred to as stromal cell-derived inducing activity. In such co-cultures, over 90% of the ES cells are converted to neural cells and a large proportion of these differentiate into dopaminergic neurons (32). ES cells can also be differentiated to MNs by co-culturing with PA6 or in media containing Shh in the presence of RA (33). We have used a modification of the method reported by Kawasaki et al. for directing differentiation of P19 EC cells to MNs by plating P19 cells directly (without the embryoid body step) on the PA6 stromal line under serum-free conditions (34) in media containing knockout serum replacement (KOSR) and RA (35). In a few initial experiments, we also co-cultured P19 EB derived cells with PA6 in the presence of RA. We then analysed the differentiating P19 cells for expression of mAng-1, nestin, GAP-43, 165 kDa neurofilament (NF), Islet1, βIII-tubulin, peripherin and GFAP.

The PA6 stromal cell line stained weakly for mAng-1 and the expression was nuclear. Undifferentiated P19 cells had barely detectable mAng-1 expression (data not shown). The signals in the differentiating P19 cells were strongest in the cytoplasm of the neurospheres, developing neurons and in the extending neurites in the first 48 h. Nuclear expression was undetectable. Nestin is an intermediate filament protein expressed by progenitor (radial glial) cells in the developing rat CNS, also in Schwann cells and their putative precursors in PNS, and is a marker for neural precursor cells (36). A substantial number of nestin-positive cells were present at 48 h and mAng-1 was co-expressed very strongly with nestin in the early stage of neuroectodermal differentiation in the neuronal precursor cells but did not co-localize with nestin (Fig. 2Aa–c). The mAng-1 signals were moderate from 72 h onwards, indicating that the expression of mAng-1 was downregulated to a degree but was still clearly detectable in neurons. mAng-1 expression co-localized with βIII-tubulin [embryonic NF (37)] in the cytoplasm and neurites in the early stage of neuronal differentiation (Fig. 2Ad–f). Staining for NF [165 kDa (38)] revealed that, at 96 h, the neurons had developed very long neurite outgrowths and built up very large networks. mAng-1 co-localized with NF in most of the neurons and was present in the cell bodies. However, there were some neurons that stained weakly or were completely negative for mAng-1 (Fig. 2Ah and i). This suggests that only a subset of neurons derived from differentiating P19 cells maintained expression of mAng-1.

We investigated if the subset of neurons expressing mAng-1 were MNs by staining for mAng-1, Islet1 and peripherin. Islet1, a transcription factor of the LIM HD family, is the first molecular indicator of MN differentiation (39–41). Islet1-positive cells appeared at 96 h, increased by 120 h (18%) followed by a reduction in Islet1-positive cell at 144 h (6%).
Figure 1. mAng-1 is developmentally regulated in the CNS during mouse embryogenesis. (A) mAng-1 in 9.5-day pc mouse embryo; (B) and (C) mAng-1 in 11.5-day pc mouse embryo; (D–F) co-expression of mAng-1 and Islet1 in DRG of 14.5-day pc mouse embryo; (G–I) co-expression of mAng-1 and Islet1 in DRGs of 16.5-day pc mouse embryo; (J–L) co-expression of mAng-1 and peripherin in DRG of 16.5-day mouse embryo; (M–O) co-expression of mAng-1 and Islet1 in sc of 14.5-day mouse embryo. (A–C) sagittal sections; (D–O) transverse sections. fb, forebrain; mb, midbrain; hb, hindbrain; sc, spinal cord; spg, spinal ganglia.
Figure 2. mAng-1 is expressed in neural precursor cells and in neurons during P19 neural differentiation. (A): (a–c) ANG and nestin at 48 h; (d–f) ANG and tubulin at 72 h; (g–i) ANG and NF at 96 h. (B): (a–c) ANG and Islet1 at 120 h; (d–f) ANG and peripherin at 120 h; (g–i) ANG and GFAP expression at 144 h. Bar: 50 μm.
The Islet1-positive neurons co-expressed mAng-1 in the cell bodies and neurites (Fig. 2Ba–c). A proportion of other neurons were also mAng-1 positive. Peripherin (marker for CNS MNs)-positive neurons showed co-localization of mAng-1 at 120 h of differentiation (Fig. 2Bd–f). No positive staining for GFAP (42) was observed until 120 h but at 144 h a very small proportion of cells that were GFAP-positive were detectable which were negative for mAng-1. This suggests that mAng-1 is not expressed in astrocytes (Fig. 2Bg–i).

mAng-1 expression was found to assemble particularly in the tips of the processes within the developing immature neurons and co-localized with the neural-specific growth-associated protein-43 (GAP-43), which is a major protein of axonal growth cones (43) (Fig. 3A–F).

In the P19 EC cell culture model of neuroectodermal differentiation, mAng-1 is not expressed in the undifferentiated pluripotent stem cells. With the commitment to neuroectoderm and the generation of neural precursor cells that are nestin positive, mAng-1 expression is very strong. During differentiation of the P19 cells to neurons, mAng-1 is seen to co-localize with GAP-43 (Fig. 3) and later in the neurites with NFs (Fig. 2A and B). We also demonstrate the subcellular localization of mAng-1 during the differentiation of P19 into neurons; mAng-1 is initially strongly expressed in the cytoplasm of the precursor cells in the neurospheres, it then accumulates in the neurites and cell bodies of the neurons but not in the nucleus. It is not expressed by astrocytes as seen by the lack of mAng-1 in the GFAP-positive cells.

Effect of the ANG inhibitor NCI 65828 on neurons differentiating from P19 EC cells

NCI 65828, an azo-naphthalene sulphonate compound identified by high throughput screening is an inhibitor of hANG (29). It binds hANG ($K_i = 81 \mu M$) and is able to inhibit its ribonucleolytic and angiogenic activity, thereby suppressing hANG-induced neovascularization and tumour growth (29). This antagonist of hANG provided us with a handle to perturb the function of ANG during neuronal differentiation in our P19 EC cell neural cell differentiation model system.

We first investigated if mAng-1 was necessary for the formation of neural precursor cells by staining differentiating P19 cells incubated with and without the hANG inhibitor NCI 65828, for the expression of nestin. Control cultures showed a clear cytoplasmic network of intermediate filaments staining positive for nestin at 24 and 48 h of differentiation (Fig. 4A and C). Differentiating P19 cells exposed to the hANG inhibitor showed expression of the neural precursor marker nestin by 24 h (Fig. 4B). However, the neural precursor cells clumped together and were not well spread which was prominent at 48 h (Fig. 4D). Nestin level was downregulated by 72 h and the appearance of NF-positive cells was considerably lower by 96 h of differentiation. This dynamic reciprocal pattern of expression of nestin and NF by inhibitor-treated cells was similar to that seen in the control cells.

Staining for NF revealed that the neurons clumped together in inhibitor-treated cultures and the neurites did not form extended networks. The inhibitor did not affect the generation
of neural precursors from P19 EC cells or their differentiation to neurons. However, pathfinding by the neurites was severely compromised.

To confirm that ANG is indeed important for extension of neurites and pathfinding, we developed a method to induce P19 EC cells cultured on Cytodex-3 beads to undergo neuronal differentiation when co-cultured on a PA6 feeder layer in the presence of RA. Neuronal differentiation in this model culture system was confirmed by the expression of neuronal markers (data not shown; Crabtree and Subramanian, manuscript in preparation). The beads (average diameter of 150 μm) provide a focal point and allowed us to monitor both the differentiation and migration of the neurite outgrowths. It also allowed us to measure the length and number of outgrowths. We then used this system to study the effects of NCI 65828 on neurite outgrowth and migration.

In P19 bead cultures induced to differentiate in the absence of NCI 65828, cells staining positive for the neuronal marker NF first appeared at 48 h of culture with a few neurite outgrowths clearly extending out from the beads. By 72 h, the number of neurites and the distance they extended increased and was maximal at 96 h. The strongest expression of NF was seen at 96 h and an extensive network of neurite outgrowths radiated out from the beads (Fig. 4E, G and I). NF-positive cells first appeared on the inhibitor-treated beads at 48 h. This increased by 96 h and the majority of the cells on the beads stained for NF. However, the neurons that formed did not extend out and away from the beads and covered the beads as a mesh of NF-positive cells. The extensive network of neurite outgrowths seen by NF staining in the control was absent in the inhibitor-treated beads, indicating that neurite pathfinding was affected in the presence of the ANG inhibitor (Fig. 4F, H and J).

**DISCUSSION**

Our significant finding from this study is that ANG is critical for neurite pathfinding and is the first to link the inhibition of the activity of ANG to neurite pathfinding. This has implications for ALS.

Recent reports indicate that angiogenic growth factors, such as VEGF which cause sprouting, branching and growth of blood vessels and capillaries, also have a neurotrophic function (44,45). Ligand–receptor signalling systems involved in neuronal guidance are also being identified as having a function in vessel guidance and patterning (46). Our observation that high levels of ANG are present in early stages of neuronal differentiation and localized in growth cones and eventually in the neurites suggests that ANG, like VEGF, has a functional role in the nervous system. The ability of the small molecule ANG inhibitor NCI 65828, in inhibiting the pathfinding by neurite outgrowths, confirms that this is indeed the case. This is yet another instance where an angiogenic factor has a pleiotropic role.

The ability of hANG to organize endothelial cells into tube-like structures, induce secondary messengers and support endothelial cell migration during neovascularization is well established (47–51). ANG may be also be acting through similar pathways in neurite pathfinding. Endogenous hANG has been implicated in cell proliferation induced by other angiogenic proteins such as VEGF. Downregulation of ANG expression by RNAi decreases VEGF-induced rRNA transcription and inhibition of nuclear translocation of ANG rescinds the angiogenic activity of VEGF (52). Whether ANG and VEGF act through similar pathways in neurite pathfinding and survival needs to be established.

In summary, we have shown conclusively that ANG is expressed in the developing and adult nervous system and in P19-derived MNs. Expression of ANG is crucial to neurite pathfinding. However, several questions still remain to be answered. Among them, the molecular mechanism by which ANG mediates neurite pathfinding and how this is affected
by the autosomal dominant mutant hANGs. It is also not clear if the ribonucleolytic activity of ANG is essential for its function in the nervous system. Gene disruption of mAng-1 and knock-in of the ANG ALS mutations combined with biochemical studies should provide some of the answers. However, the knockout approaches may not prove to be easy since the mAng locus has undergone duplication to give rise to six members and three pseudogenes (28,29) unlike in the humans which have a single ANG gene.

**MATERIALS AND METHODS**

**Immunohistochemistry of mouse embryos**

Embryos were obtained from matings of male and female (C57BL) mice. The morning of the vaginal plug was designated as day 0.5 pc. Embryos from days 8.5 to 16 pc and adult brains were fixed in 4% paraformaldehyde (Sigma) in PBS (Oxoid) overnight at 4°C, dehydrated and processed for histology. Sections were cut (5 μm) and mounted on subbed slides and processed for immunostaining after dewashing and rehydration. Sagittal and transverse sections were stained after antigen retrieval using the Vector antigen retrieval kit following manufacturer’s instructions. Primary antibodies used were rabbit anti-mouse angiogenin (1:400 from Karen Olson), mouse anti-Islet1+2 (1:5, 40.2D6, DSHB), mouse anti-NF (1:10, 2H3, DSHB) and rabbit anti-peripherin (1:200, Chemicon). Secondary antibodies used were goat anti-mouse Alexa 488, goat anti-rabbit Alexa 488, goat anti-rabbit Alexa 546 and donkey anti-mouse Texas red (GE Healthcare).

**Cell culture**

Mouse embryonal carcinoma cells P19 (34,53) and PA6 cells (54) were cultured in Alpha minimum essential medium (alpha-MEM) supplemented with 10% FCS and 1% NEAA at 37°C in 5% CO₂.

**Induction of differentiation by direct plating on PA6 feeders**

P19 EC cells were induced to differentiate by RA treatment (5 × 10⁻⁷ M) and co-culture with PA6. PA6 cells were plated on to gelatinized cover slips in 12-well plates at a density of 2 × 10⁴ cells/ml, 2 days prior to seeding with P19 EC cells and allowed to form a confluent monolayer. P19 cells (5 × 10³ cells/ml) were plated on the PA6 feeder in alpha-MEM medium containing 0.5% (v/v) KOSR, 1% (v/v) NEAAs and 5 × 10⁻⁷ M RA. Cell morphology was monitored daily using a Nikon inverted microscope and photographed on Kodak 64T film. Cells were fixed in 4% buffered PFA at 24 h intervals over a period of 120 h and immunostained for markers of neuroectodermal differentiation.

**Treatment of P19 cells with NCI 652828**

P19 cells were grown on Cytodex 3 bead (GE Healthcare) O/N. The cell-coated beads were plated on a confluent monolayer of PA6 cells on gelatinized cover slips and treated with RA (5 × 10⁻⁷ M) to induce neuronal differentiation (Crabtree and Subramanian, manuscript in preparation). Twenty-four hours after plating, the media was replaced with media containing 0.5% KOSR, 5 × 10⁻⁷ M RA and ANG inhibitor NCI 652828 at a concentration of 80 μM. Controls were differentiated as normal. Cells were fixed in 4% PFA in PBS at 24 h intervals over a period of 120 h.

**Indirect immunofluorescence of P19 and differentiated neuronal lineages**

Cells cultured on gelatinized cover slips were fixed in 4% PFA in PBS and immunostained. Non-specific binding was blocked with 0.5% BSA in PBS containing 1% Triton X-100 for 1 h at room temperature. The primary antibodies used for staining were rabbit anti-mouse angiogenin 1:400 (from Karen Olson), mouse anti-Isl1+2 (1:5, 40.2D6, DSHB), mouse anti-NF (1:10, 2H3, DSHB), mouse anti-GFAP (1:100, Chemicon), mouse anti-GAP-43 (1:300, Sigma), mouse anti-nestin (1:100, Rat 401, DSHB), anti-βIII-tubulin (TU20 1:400, Chemicon) and rabbit anti-peripherin (Chemicon). Primary antibodies diluted in block solution were allowed to bind overnight at 4°C with shaking, washed with 0.1% Triton X-100 in PBS (3 × 10 min). Binding was detected by incubating with a suitable secondary antibody [goat anti-mouse Alexa 488 (1:1000 Molecular Probes), goat anti-mouse Alex 546 or goat anti-rabbit Alexa 488 (1:1000 Molecular Probes), donkey anti-rabbit Alexa 546 (1:500)]. Cells were washed with wash buffer as earlier and stained with DAPI (0.1 μg/ml) in PBS for 5 min at room temperature. Cells were mounted in Mowiol. Staining was observed and photographed using a Leica DMRB.

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**Conflict of Interest statement.** None declared.

**REFERENCES**


