Recent data have demonstrated that vascular endothelial growth factor (VEGF) is expressed by subsets of neurons, coincident with angiogenesis within the developing cerebral cortex. Here we investigate the characteristics of VEGF expression by neurons and test the hypothesis that VEGF may serve both paracrine and autocrine functions in the developing central nervous system. To begin to address these questions, we assayed expression of VEGF and one of its potential receptors, Flk-1 (VEGFR-2), in the embryonic mouse forebrain and embryonic cortical neurons grown in vitro. Both VEGF and Flk-1 are present in subsets of post-mitotic neurons in vivo and in vitro. Moreover, VEGF levels are up-regulated in neuronal cultures subjected to hypoxia, consistent with our previous results in vivo. While the abundance of Flk-1 is unaffected by hypoxia, the receptor exhibits a higher level of tyrosine phosphorylation, as do downstream signaling kinases, including extracellular signal-regulated protein kinase, p90RSK and STAT3a, demonstrating activation of the VEGF pathway. These same signaling components also exhibited higher tyrosine phosphorylation levels in response to exogenous addition of rVEGFA165. This activation was diminished in the presence of specific inhibitors of Flk-1 function and agents that sequester VEGF, resulting in a dose-dependent increase in apoptosis in these neuronal cultures. Further, inhibition of MEK resulted in increased apoptosis, while inhibition of phosphatidylinositol 3-kinase had no appreciable affect. In addition to the novel function for VEGF that we describe in neuronal survival, neuronal VEGF also affected the organization and differentiation of brain endothelial cells in a three-dimensional culture paradigm, consistent with its more traditional role as a vascular agent. Thus, our in vitro data support a role for neuronal VEGF in both paracrine and autocrine signaling in the maintenance of neurons and endothelia in the central nervous system.

VEGF, a hypoxia-inducible endothelial cell mitogen, has been characterized as a potent vascular permeability factor and a critical factor in vasculo- and angiogenesis (1–3). VEGF is known to exert its effects via two high affinity receptors, feline sarcoma virus-like tyrosine kinase (Flt-1, VEGFR-1) and fetal liver kinase receptor (Flk-1, VEGFR-2) (4–9). Both receptors are critical for the proper differentiation and organization of endothelial cells into vascular beds. In the central nervous system, a cellular response to hypoxic exposure is increased VEGF production by glial cells that invest cerebral vessels (10–12), which results in increased angiogenesis and changes in vessel homeostasis.

More recently, in the peripheral nervous system VEGF has been shown to be a neurotropic factor, which stimulates axonal outgrowth, enhances cell survival, and increases Schwann cell proliferation in cultured superior cervical and dorsal root ganglia from adult mice. Co-expression of VEGF and Flk-1 in many neurons in these superior cervical ganglia cultures was also noted (13–15).

In addition, VEGF expression has been localized to subpopulations of neurons in the developing and mature central nervous system (14–18). Jin et al. demonstrated VEGF gene and protein expression in neurons of the cortex and hippocampus following a model for global cerebral ischemia in the adult rat brain, and the application of exogenous VEGF was shown to promote survival of rat cerebral neurons in culture and rescue HN33 hippocampal cells from death by serum withdrawal (18).

Furthermore, our previous work has demonstrated that VEGF expression by neurons of the developing cerebral cortex coincides both spatially and temporally with angiogenesis and that levels of VEGF in cortical neurons increase following hypoxic exposure (11). Thus, this previously unknown source of endogenous VEGF production in the brain presents a novel paradigm for examining VEGF function.

Interest in VEGF as a therapeutic molecule in a wide number of pathological conditions such as stroke and peripheral nerve damage led us to test the hypothesis that neuronal VEGF secretion may have specific signaling functions in the central nervous system (17, 19–21). Here we report that neuronal VEGF and its receptor Flk-1 are expressed by cortical neurons of E15 embryos in vitro and in vivo. Moreover, use of an in vitro culture model demonstrates that neuronal VEGF expression is hypoxia-inducible, with the resulting VEGF capable of supporting tube formation in a brain microvascular endothelial cell line. Co-localization of VEGF and its Flk-1 receptor, as well as changes in tyrosine phosphorylation of both Flk-1 and downstream signaling molecules, including mitogen-activated protein kinase, p90RSK, and STAT family members, suggests that VEGF mediates both autocrine and paracrine signaling functions in...
central nervous system neurons. Finally, inhibition of either VEGF function or Flk-1 activity results in increased cell apoptosis, indicating that endogenously produced VEGF acts as a neuronal survival factor.

MATERIALS AND METHODS

Immunohistochemistry—Sections of embryonic day 15 (E15) mouse brains or cortical neurons derived from them and grown in vitro (11) were fixed and subjected to immunohistochemistry. The antibodies used were polyclonal anti-VEGF (Neomarkers Inc., 1:2500), anti-Flk-1 (Santa Cruz Biotechnology Inc., 1:250), monoclonal anti-neuron-specific class III β-tubulin (Tuj-1) (Berkeley Antibody Co., Richmond, CA, 1:500), anti-Erk-2 (Santa Cruz Biotechnology Inc., 1:1000), anti-phospho-Erk (Santa Cruz Biotechnology Inc., 1:1000), anti-STAT1, anti-STAT3a, and anti-p-STAT3a (Chemicon, International, Inc., Temecula, CA; 1:1000), anti-PI 3-kinase (Upstate Biotechnology, Lake Placid, NY; 1:1000), anti-p90RSK and anti-p90RSK PY (Cell Signaling Technology, Inc., New England Biolabs, Beverly, MA, 1:1000), and anti Cleaved Caspase 3 (D175) (Cell Signaling, Beverly, MA, 1:500). Wortmannin, LY294002, and PD98059 were purchased from Sigma. TUNEL labeling was performed as recommended by the manufacturer (Roche Diagnostics, Indianapolis, IN).

Primary Cortical Neuronal Cultures—The dorsal telencephalon was dissected from E15 mice (Charles River Laboratories), as previously described (22). Cells were plated on either glass coverslips or plastic Petri dishes coated with poly-l-ornithine and laminin. Cells were incubated for 6 days in normal atmosphere with 5% CO2 or in hypoxic conditions consisting of a mixture of 5% CO2, 10% O2, 85% N2 (BOC Gases, North Haven, CT). In some experiments cultures were incubated with 5% CO2, 10% O2, 85% N2 and 100 nM 6,7-dimethoxyquinazoline (CB676475) (24), a potent and selective inhibitor of PI 3-K, (Sigma). We performed immunohistochemistry—Sections of embryonic day 15 (E15) mouse brains or cortical neurons derived from them and grown in vitro (11) were fixed and subjected to immunohistochemistry. The antibodies used were polyclonal anti-VEGF (Neomarkers Inc., 1:2500), anti-Flk-1 (Santa Cruz Biotechnology Inc., 1:250), monoclonal anti-neuron-specific class III β-tubulin (Tuj-1) (Berkeley Antibody Co., Richmond, CA, 1:500), anti-Erk-2 (Santa Cruz Biotechnology Inc., 1:1000), anti-phospho-Erk (Santa Cruz Biotechnology Inc., 1:1000), anti-STAT1, anti-STAT3a, and anti-p-STAT3a (Chemicon, International, Inc., Temecula, CA; 1:1000), anti-PI 3-kinase (Upstate Biotechnology, Lake Placid, NY; 1:1000), anti-p90RSK and anti-p90RSK PY (Cell Signaling Technology, Inc., New England Biolabs, Beverly, MA, 1:1000), and anti Cleaved Caspase 3 (D175) (Cell Signaling, Beverly, MA, 1:500). Wortmannin, LY294002, and PD98059 were purchased from Sigma. TUNEL labeling was performed as recommended by the manufacturer (Roche Diagnostics, Indianapolis, IN).

Western Blotting—Western blotting was carried out on lysates of E15 neurons and E15-conditioned media as previously described. (11) Anti-sera directed against VEGF, Flk-1, PI 3-kinase, Akt, pAkt, Erk-2, pErk, p90RSK, PY-p90RSK, STAT3, pSTAT3, and STAT1 were used at 1:500 dilution sera directed against VEGF, Flk-1, PI 3-kinase, Akt, pAkt, Erk-2, pErk, LY24002, inhibitors of PI 3-K, (Sigma).

RESULTS

VEGF and Flk-1 Are Expressed by E15 Mouse Cortical Neurons in Vivo and in Vitro—We performed immunohistochemistry to localize VEGF and Flk-1 in E15 forebrain sections using a post mitotic neuronal marker (Tuj-1) to identify neuronal cells. We found that VEGF was expressed by neurons throughout the wall of the developing cerebral cortex (Fig. 1a). Flk-1 was also expressed by neurons of the E15 forebrain and widely distributed and at low levels (Fig. 1b). Additionally as expected, both VEGF and Flk-1 were present within vascular structures superficial to the cortical plate (Fig. 1, a and b).

To determine whether cells in vitro expressed VEGF and Flk-1, we prepared primary cultures of E15 cortical neurons and performed immunohistochemistry. Staining revealed that ~30% of Tuj-1-positive cells in culture expressed VEGF (Fig. 1c, e, and f); about 25% of Tuj-1-positive cells expressed Flk-1 (Fig. 1f). Furthermore, ~90% of cells that expressed VEGF also expressed Flk-1 (Fig. 1g). To determine whether expression changed when cells were grown in vitro, we assayed levels of VEGF and Flk-1 in freshly dissociated but unplated neurons, as well as cells grown in culture. Both populations of cells expressed VEGF (Fig. 1e, lanes 1 and 2) and Flk-1 (Fig. 1h, lanes 1 and 2), and furthermore, the VEGF produced by these neurons was secreted into the media (Fig. 1e, lane 3). Similarly, E15 cortical neurons express Flk-1 as dissociated cells or cells grown in vitro (Fig. 1h, lanes 1 and 2). These results demonstrated that neurons in culture mimic neurons in their ability to synthesize and secrete VEGF. In addition, they have a receptor with which they could respond to this factor.

Flk-1 and Its Downstream Targets Are Phosphorylated in Cortical Neurons—Next, we examined the activation state of Flk-1 in cortical neuronal cultures and found that the Flk-1 was tyrosine-phosphorylated, indicating that a portion of the
Levels of total ERK, p90RSK, Akt, and STAT3a protein did not vary greatly among cells grown under normoxic, hypoxic, and normoxic conditions. However, comparable with results with hypoxic conditions, levels of tyrosine-phosphorylated ERK, p90RSK, and STAT3a were significantly increased following sustained culture in hypoxic (Hx) conditions. Following daily administration of 10 ng/ml rVEGF-A165 for 6 days, we observed that levels of total ERK, p90RSK, Akt, and STAT3a protein did not vary greatly among cells grown under normoxic, hypoxic, and normoxic + rVEGF-A165 conditions. However, comparable with results with hypoxic cultures, levels of tyrosine-phosphorylated ERK, p90RSK, and STAT3a were significantly increased following addition of rVEGF-A165, indicating activation of a VEGF-inducible signaling pathway (Fig. 2f, fold change 2.0 ± 0.16, 4.2 ± 0.9, and 2.0 ± 0.41, respectively). Notably, as in hypoxic cultures, Akt phosphorylation levels and STAT1 and PI 3-kinase expression levels were not significantly changed by VEGF treatment.

**Inhibition of VEGF Signaling Induces Apoptosis in E15 Neuronal Cultures**—To examine the effects of blocking VEGF/Flk-1 signaling in cortical neurons two approaches were taken. First, we inhibited VEGF receptor tyrosine kinase activity using SU1498, a potent and selective Flk-1 tyrosine kinase inhibitor (23), or added CB676475, a broad range VEGF receptor tyrosine kinase inhibitor (24). SU1498 was found to elicit increased apoptosis (as determined by TUNEL labeling) in a dose-dependent manner when added to E15 neuronal cultures (Fig. 3a). CB676475 exhibited similar properties (Fig. 3b, upper two panels).

The second approach taken was to sequester endogenously expressed VEGF with either a neutralizing VEGF antibody or a recombinant soluble chimeric Flt-1 molecule. In both instances, cultures containing either of these agents exhibited increased apoptosis as evaluated by TUNEL labeling (Fig. 3b, lower three panels).
VEGF was sequestered by adding 1.0 μM and fourth panel b cultures daily. As illustrated in -
nant soluble Flt-1 (mFlt(1

As illustrated in Fig. 4, only occasional apoptotic cells were iden-

ted in Fig. 4A) and normoxic cultures incubated with 20 μM of the PI 3-kinase inhibitors LY (Fig. 4B) or Wortmannin (data not shown). In contrast, normoxic cultures incubated with 20 μM of the MEK inhibitor PD98059 exhibited markedly increased apoptosis as evidenced by the increase in caspase 3 staining (Fig. 4C).

Neuronal VEGF Supports the Organization of Endothelial Cells into Tubes—To determine whether neuronal VEGF is capable of promoting endothelial cell organization and differentiation, we assessed the effects of media conditioned by cortical cultures on brain microvascular endothelial cells cultured in three-dimensional collagen gels.2 We found that neuronal-conditioned media supports rearrangement and differentiation of rat brain endothelial (RBE4) cells, resulting in the formation of tube-like structures (Fig. 5). The ability to affect tube formation correlated with levels of VEGF in the media, as no tubes form in cultures treated with unconditioned media (Fig. 5, panel 1). Modest tube formation is observed in cultures supplemented with media from normoxic neuronal cultures (Fig. 5, panel 2), and robust tube formation occurs in cultures that were supplemented with media from hypoxic neuronal cultures (Fig. 5, panel 3). Thus, although produced by cortical neurons in culture, neuronal VEGF has the ability to influence endothelial function and subsequent angiogenesis.

Inhibition of PI 3-Kinase and MEK Signaling Induces Differential Apoptosis in E15 Neuronal Cultures—To further examine the specific signaling pathways downstream of the VEGF-induced inhibition of apoptosis a pharmacological approach was taken. Namely, chemical inhibitors of either MEK (PD98059, 20 μM) and PI 3-kinase (Wortmannin and LY24002, 20 μM) were added daily from day one to day six of culture of the E15 neurons. As illustrated in Fig. 4, only occasional apoptotic cells were identified by caspase 3 staining of normoxic cultures (Fig. 4A) and normoxic cultures incubated with either 20 μM of the PI 3-kinase inhibitors LY (Fig. 4B) or Wortmannin (data not shown). In contrast, normoxic cultures incubated with 20 μM of the MEK inhibitor PD98059 exhibited markedly increased apoptosis as evidenced by the increase in caspase 3 staining (Fig. 4C).

DISCUSSION

We show here that embryonic cortical neurons, in vivo and in vitro, express VEGF and one of its receptors, Flk-1 (Flt-1 was not detected in these cultures). Moreover, Flk-1 is activated in cultured neurons and appears to initiate signaling via a cascade that likely includes PI-3 kinase, Akt, Shc, Nck, and the kinases ERK, p90RSK, and STAT3a. Under hypoxic conditions, cortical neurons express elevated levels of VEGF and Flk-1. The MEK inhibitor PD98059 (20 μM) induces markedly increased apoptosis in cultured E15 neurons (panel C) compared with cultured E15 neurons in control normoxic conditions (panel A) and E15 neurons cultured in the presence of the PI 3-kinase inhibitor LY294002 (panel B). Tuj-1 labeling (green fluorescence) was utilized to identify neurons, while red fluorescence was used to mark apoptotic cells. The orange color denotes apoptotic neurons. Scale bar = 100 microns.

2 Chow, J., Ogunshola, O. O., Fan, S. Y., Li, Y., Ment, L. R., and Madri, J. A. (2001) Brain Res. Dev. Brain Res. 130, 123–132.
way is responsible, in part, for mediating neuronal survival in this culture model. Interestingly, the PI 3-kinase-Akt pathway does not appear to be involved in the modulation of neuronal apoptosis in our in vitro model (Figs. 2, 4, and 6B). Finally, media conditioned by both normoxic and hypoxic cortical neurons promoted in vitro angiogenesis, with levels of VEGF correlating with the extent of tube formation. Our previous findings that neurons in the brain express VEGF are extended by these studies (11). Indeed, the maintenance of expression of VEGF by neurons in culture demonstrates that this expression is intrinsic to these neurons, since it exists in the absence of proper cellular contact and connectivity. Furthermore, the observation that VEGF and Flk-1 are co-localized supports the notion that neuronal VEGF in the brain can act as an autocrine as well as a paracrine factor for neurons (Fig. 6, A and B).

In addition to its more widely recognized role in angiogenesis, VEGF is involved in regulation of the early developmental program of retinal neurogenesis (31). In the peripheral nervous system, the neurotrophic and mitogenic effects of exogenous VEGF result in stimulation of axon outgrowth through the Flk-1 receptor as well as increased survival of peripheral neurons in vitro, suggesting that further functions may exist (14, 15). In the central nervous system, exogenous VEGF reduced neuronal cell death in an in vitro model of cerebral ischemia (18, 30, 32). In this study we attribute a role to the endogenous production of VEGF by neuronal cells and suggest that not only VEGF from exogenous sources (such as astrocytes and endothelial cells) is important for neuronal survival. Our findings add to the complexity of biological roles that have been attributed to VEGF.

We show that increased cell death results from a blockade of endogenous VEGF signaling indicating the importance of both VEGF and the Flk-1 receptor in neuronal survival. Our studies...
illustrate that complex signaling cascades are susceptible to elevation of endogenous VEGF levels in cortical neurons exposed to hypoxic conditions in vitro. Interestingly, contrary to findings by Jin et al., which demonstrate the importance of PI 3-kinase/Akt signal transduction system in VEGF-mediated neuroprotection in HN33 cells (30, 32), we find that the MEK-ERK pathway is crucial to the survival of cortical neurons in vitro. This discrepancy could be due to differences in experimental design as well as differences in the cell populations tested. Potentially acting through both autocrine and paracrine Fk-1 activation, ERK, p90RSK, and STAT 3a phosphorylation levels are up-regulated, either in abundance or in activity in cortical neurons under hypoxic conditions or after exposure to rVEGF. These findings, along with the observation of persistent stable Akt phosphorylation, highlight the fact that VEGF can modulate a number of downstream pathways some of which have not yet been identified. Furthermore our data suggests that neurons themselves provide a source of VEGF to aid their survival under adverse conditions, a response that will presumably depend on the severity of the insult. Perhaps this initial release of VEGF into the local environment may prolong survival of cells that are not irreversibly damaged until angiogenesis is initiated. Additional functional and novel responses of neurons to changes in local VEGF expression and their impact are still to be identified.

We show that neuron-conditioned media supports differentiation and tube formation in a three-dimensional model of angiogenesis. Although we cannot exclude the presence of other pro-angiogenic factors in the conditioned media, our study suggests that neuronal VEGF is bioactive, can signal to surrounding endothelial cells, and can potentially produce a gradient to direct angiogenesis during development and in response to injury and trauma. Furthermore, hypoxia-conditioned media augments tube formation, consistent with our previous finding that neuronal VEGF can produce a gradient to direct angiogenesis in the developing cortex.

Since cortical neurons are diverse and because we know that this diversity is at least in part recapitulated in vitro, it will be important in the future to understand the effects of VEGF stimulation on distinct cortical neuron subtypes. It is also tempting to speculate that some of these may be neuronal-specific form of VEGF. Interestingly, not all cortical neurons express VEGF and/or flk-1 receptors, which may be a reason why some cells are more susceptible to injury and insult. In addition, the issue of whether modulating levels of Flk-1 signaling under hypoxic conditions will protect cells from the apoptosis they are destined to undergo is an interesting and pertinent question, as it may have profound implications for the treatment of cortical injury. Finally, our results suggest that there appears to be a coordinated process in which neurogenesis and angiogenesis share common molecular triggers. In the future, we will examine the roles that VEGF plays in neuronal maturation and assess the potential roles of VEGF in modulating neuronal plasticity within the developing brain and the coordination that exists between neurons and the blood vessels that supply them.

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