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Neuroangiogenesis: A Vascular Basis for Alzheimer's Disease and Cognitive Decline during Aging

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Abstract

Angiogenesis directs development of the brain's microcirculation during antenatal and postnatal development, but its role later in life is less well recognized. I content that during senescence a reduced cerebral capillary density (CCD) accounts in part for the vascular cognitive impairment (VCI) observed in many older persons and possibly for some forms of Alzheimer's disease (AD). I propose that neuroangiogenesis (NAG) is essential throughout adult life for maintaining the microcirculation of the cerebral cortex and elsewhere in the brain and that it commonly declines with old age. To support this hypothesis I have examined the neurological literature for relevant studies on CCD and NAG throughout the three stages of life and in persons with senile dementias. Finally, I discuss therapeutic approaches employing angiogenic factors for treating VCI and AD.

Key words: capillary density, angiogenesis, senile dementias, Alzheimer's disease, vascular cognitive impairment

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1. Introduction

Throughout the world senile dementias affect 5-7% of people over age 60. This number is expected to double in the next 20 years [1]. In the United States at present, Alzheimer's disease (AD) afflicts13% of persons age 65 or older and 43% of those 85 and older, or an estimated 5.4 million [2]. By 2030 the number of persons in the country with AD is expected to reach 7.7 million and by 2050 will approach 11 to 16 million -- "barring the development of medical breakthroughs to prevent or more effectively treat the disease" [2].

The pathogenesis of senile dementias is likely to be mutifactorial and involve several or many sequential cellular and biological interactions. However, most investigators have focused their attention mainly on one particular etiopathology. For example, I suggest that there is a common denominator accounting for the cognitive decline experienced by many aged people and the localized motor skills highly developed in young intensely trained musicians – namely, the microcirculation and the attendant level of angiogenesis in the brain. In a 2010 paper in the *American Scientist* I proposed that the remarkable finger/hand/foot dexterity displayed by concert pianists is due not only to augmented

synaptic connections in the primary motor cortex but also to an increased local capillary density -- initiated and maintained there by continuing angiogenesis [3].

The involvement of angiogenesis in cerebral capillary development during the early postnatal period has been established, but its function during maturity and old age is less well understood [4-6]. In this essay I contend that neuroangiogenesis (NAG) is also essential throughout adult life (albeit at a low level) for maintaining the microcirculation of the cerebral cortex and elsewhere in the brain and that it declines with aging, leading to a reduced cerebral capillary density (CCD) in the elderly – a condition fully reported and reviewed below. This may account for the memory loss and vascular cognitive impairment (VCI) observed in many older persons and for the common form of Alzheimer's disease (AD). VCI includes mild to severe cognitive decline, while the extreme state is called vascular dementia (VaD) [6].

This essay is outlined in some detail to enable thereader to find and focus on those areas of specific interest. It begins with a section covering background information on cerebral capillaries and angiogenesis. The following three sections summarize reports on CCD and NAG during the three stages of life (postnatal, maturity, senescence). Data in each are presented first from human subjects and then from experimental animals. Next are cited studies on CCD and NAG in Alzheimer's disease and other senile dementias. The hypothesis proposed here is then discussed along with related or rival ones. The final sections concern seven potential routes for administering angiogenic factors to the brain and the cautions to be observed when considering such treatment.

2. Background: CCD & NAG

Studies on cerebral microvascularity preceded by half a century the discovery of angiogenesis. In the early 1920s, Edward Horne Craigie (1894-1989), a biologist at the University of Toronto, reported on capillary densityin various parts of rat brains by counting certain structural features of the capillaries in thinly cut slices (mostly 2-6 μm thick), where only segments of vessels remain. The most widely used parameter has been the sum of the lengths of fragments visible in a fixed area of each brain slice over a fixed number of slices, yielding an L_v in μ/mm^3 [8-12]. To exclude arterioles, only vessel fragments with diameters generally smaller than 12μm were included in these measurements [13]. Other indices of capillary morphometry employed by different investigators have been the number of capillary sprouts, capillary volume of fragments per unit volume of brain tissue (V_v, per mm^3), capillary surface area/area of brain slice (S_v, per mm^2), or the sum of capillary diameters in a given area (D) [11,14].

More recent studies on brain sections have employed automated optical scanning systems to measure florescence staining by various antibodies to capillary endothelial markers: phosphatase, transferrin, laminin, CD105, CD31, etc. [15-18]. The endothelial cell content of mouse brain tissue homogenates have also been analyzed on SDS-Page gels by immunoblot staining of capillary endothelial markers [18,19]

I.C. (Isaac Chesar) Michaelson (1903-1983), a British ophthalmologist, studied capillary growth in the developing retina of man and cats and in 1948 was the first to postulate the existence of a vasculogenic factor [20]. In 1971 Judah Folkman (1933-2008) at Harvard Medical School reported isolating such a factor from various tumors but not from non-

malignant tissues. He proposed that solid tumor growth depends "on the continuous build-up of a new capillary network" by his tumor angiogenesis factor (TAF) [21]. Angiogenesis is also a physiological response in various non-tumor conditions – e.g., during embryonic and fetal development, the formation of ovarian follicles and transiently in the corpus luteum during ovulation and during the development of the placenta. Neovascularization is a vital part of wound healing and fracture repair but ceases when the tissue is fully recovered. In contrast, uncontrolled angiogenesis promotes progressive solid tumor growth, occurs also during chronic inflammation, and is associated with over 70 human disorders, which Folkman grouped together as "angiogenic diseases." Among them are diabetic retinopathy, rheumatoid arthritis, psoriasis, etc. [22, 23].

Folkman's isolation of TAF (c. 10 kDa) in 1971 was followed by the discovery of other endothelial growth factors. Those listed below have been found in the brain. The first identified from non-tumor sources was the fibroblast growth factor (FGF, 18 kDa) [24]. The most thoroughly examined angiogenic agent has been vascular endothelial growth factor (VEGF, 46-48 kDa), which was originally called vascular permeability factor (VPF) [25, 26]. It exists in five isoforms with $VEGF_{165}$ being "the most abundant in the human brain" [27]. Other brain-localized factors include epidermal growth factor (EGF, 6 kDa), transforming growth factor-beta (TGF-β, 5.5 kDa), and platelet-derived growth factor B (PDGF-β, 46kDa) [28-31]. PDGF-β is secreted by brain endothelial cells; the cognate receptor, PDGF-R, is located on brain pericytes.

Another family of angiogenic factors includes angiopoietins which, like the family of VEGFs, are endothelial cell-specific cytokines whose cognate receptors are expressed only

on ECs [27, 32]. The various factors/cytokines associated with angiogenesis have been thoroughly reviewed [27, 29, 33].

3. CCD and NAG during Postnatal Development

CCD. The most rapid growth of the human brain occurs between ages 3-6 years in the frontal lobes and from 6-15 years in the temporo-parietal region [34]. Diemer compared the capillary density in the frontal cortex of newborn infants and adults and found the former to be 98.4 capillaries/mm^2 and the latter to be 290 cap./mm^2 [4]. Rats have been a more accessible model for exploring cerebral microcirculation postnatally [12, 14]. In 1925 E. H. Craigie pioneered such studies in male albino rats from their birth to 13 months of age (Day 364). Shortly before being sacrificed, each was injected with carmin gelatin to fill the cerebral vascular bed. Craigie examined the five laminae (Brodmann's layers) in each of five cortical areas for the total length of capillary fragments in a unit volume of brain tissue (L_v in μ/mm^3). Vascularity in all areas rose rapidly up through days 10 to 21 but only slowly thereafter. In three cortical regions (*praecentralis, occipitalis*, and *parietalis*) vascularity rose further to a maximum in 13-month old rats [8].

NAG during postnatal life. The following two reports ascribe control of early postnatal neovascularization in the brain to an angiogenic mitogen. 1) Breier *et al.* determined that vascular endothelial growth factor transcript levels (VEGF mRNA) in mice are "abundant in the ventricular neuroectoderm of embryonic and postnatal [6-day] brain when endothelial cells proliferate rapidly but [are] *reduced* in the adult when [as the authors state] endothelial cells proliferation has ceased" [5]. 2) Ment *et al.* concluded that "VEGF

may mediate reactive angiogenesis in the developing brain" based on observing that newborn rats subjected to sublethal hypoxia exhibited a 28% increase in vascular density in cortical areas and a 2.4-fold increase in VEGF mRNA [35].

4. CCD and NAG during Maturity (Adulthood)

CCD. After the brain's full development, the functional status of the cerebral microcirculation depends on the maintenance and repair of capillary endothelial cells. The turnover rates for these cells inhuman or animal brainshave not been reported, but such values for endothelial cells elsewhere in the body of various animals are available from studies which employed thymidine labeling of capillary endothelium and autoradiographs of tissue sections [36, 37]. For example, in 1967 Engerman and co-workers found that 0.01% of the capillary cells in the adult mouse retina were labeled after a single injection of tritiated thymidine and interpreted this as a turnover time of three or more years [38]. More recently, based on the "mean of 14 studies" by numerous investigators, Denekamp concluded that the "potential turnover time" of capillaries of "normal tissues" is 60 days (6x/yr) [39].

Again, none of the above values are for human cerebral capillaries. However, adopting Denekamp's mean value of 6x/yr as a possible turnover number in the human brain and assuming an average life span of 60 years after puberty, I calculate that cells in the cerebral capillary bed undergo 360 turnovers during the average adult's life. On the other hand, if one adopts here Engerman's 3-year estimate of capillary endothelial turnover, then

these cerebral capillaries would undergo replacement twenty times during an average adult lifetime.

NAG during maturity. Whatever the true value, the repair or replacement of the cerebral microcirculation throughout human adulthood must require the presence of endothelial growth factors in the brain and a persisting low level of angiogenesis. But the data for this are sparse. Two groups have used Northern blot analysis to measure transcript mRNA levels. Maxwell *et al.* examined the brains of patients with astrocytomas. In the adjacent normal brain tissue they found "a weak expression of TGF-αmRNA [transforming growth factor] and near background levels of EGF-R mRNAs [epidermal growth factor] [30]. Breier *et al*. studied adult mouse brains and reported that VEGF mRNA levels in the choroid plexus "appeared slightly *reduced*" compared with that in 6-day old post-natal brains [5].

Angiogenesis was inferred by Black *et al*. in studies measuring the capillary density in the visual cortex in rats of various ages raised for 50 days under two different conditions – either paired together in a standard cage containing only bedding (= control group) or housed with 4-5 other rats in a complex/enriched environment containing toys, changed and rearranged daily (= enriched group). Brain sections from the visual cortex (area 17) of both hemisphere of all rats were examined for capillary diameter and spacing (reflective of newcapillaries and angiogenesis). These values were higher in enriched groups of all ages than in similarly aged control rats. The authors also concluded that "2-year-old enriched animals ["old" rats] exhibit an impaired response to complex experience, in comparison to younger… counterparts," and that "angiogenesis, while it does occur, is substantially impaired in the middle-aged animals" [40].

The idea of a sustaining level of angiogenesishas been considered by others. In discussing angiogenesis inhibitors in human beings, Carmeliet concluded that there are "threshold levels of VEGF for the survival and maintenance of quiescent vessels in healthy organs" [23]. Based on his studies in rats, Bär wrote that the maintenance of capillary plexusesor its repair "needs a continuous action of an angiogenetic stimulus" [41].

5. CCD and NAG during Senescence (… in the absence of recognized dementia)

CCD. The effect of aging on cerebral capillary density has yielded conflicting reports [42]. Riddle *et al.* published a summary which covered three decades of investigations (1971-2001) concerning such age-related changes and which listed14 reports of decreases (6 human studies, 8 in rats) and seven with no decrease (3 human studies, 4 in rats) [43]. The following studies found decreased CCD in aged human subjects and rats.

Impairment of cerebral capillaries in older persons was observed by Stewart and colleagues. They obtained biopsy specimens from the neocortex and underlying white matter in patients ranging from 20 years to 80 years in age who underwent surgery for glial tumors or corticectomy for intractable epilepsy. Tissue was removed from the frontal, parieto-occipital, or temporal lobes in areas "as distant as possible from tumor or epileptogenic focus." The wall thickness of capillaries from these areas was measured under electron microscopy at 12 equally spaced points around the entire perimeter [44]. Gray matter capillaries of the older subjects had 21% thinner walls than the younger ones (0.97μm *vs.*1.22 μm) and 28% thinner walls in their white matter (1.01 μm*vs.*1.40 μm) [44]. The authors

interpreted the age-related thinning as being caused by "a net loss of endothelial cells, with resultant elongation of the remaining cells" [44].

A deceased CCD was also observed by Abernethy *et al.* in the paraventricular nuclei of 19 older human subjects, who ranged in age from 30 to 85 years and had no history of psychiatric treatment [15]. Somewhat related is an earlier study by Kuwabara and Cogan, who had noted a decline in the number of endothelial cells "in the capillaries of the peripheral retina in persons past middle life (50 years)" [45]. Studies comparing the brains of aged persons with those exhibiting senile dementia are presented in the next section.

As previously mentioned, in the 1920s Craigie studied CCD in male albino rats from their birth to 13 months of age. In three cortical regions vascularity rose further to a maximum in 13 month old rats, but in the two other regions (*regioinsularis* and *temporalis*) vascularity *declined* by that age [8]. In the *region insularis* it rose to a peak of 856μ at Month 5 but *fell* to 638μ by Month 13. And in *regio temporalis* a peak vascularity of 958μ was reached at Month 3 but *fell* to 855μ at Month 5 and was 850μ at Month 13 [8]. More recently, Buchweitz-Milton and Weiss recorded values for L_v (sum of capillary lengths in mm/mm^3) in the cerebral cortex of "young" rats (8-10 months) as 830 and of senescent rats (28-33 months) as 577 [11].

A decrease in "vascular elements" in the frontal and occipital neocortex of old rats was similarly observed by Klein and Michel, who measured various neocortical components (neurons, glial cells, vascular tissue) in young adult rats (6-8 months old) and aged ones (25-27 months old) -- both of whom were challenged to master a complex maze. Young adult rats succeeded after only 20 days. Some old rats could do so and were termed "old maze-

bright," while others could not and was called "old maze-dull." Combined counts of neocortical vascular elements in the young adult group was 448, that for the old maze-bright was 388 and for the old maze-dull was 314 [46].

NAG during senescence. While angiogenic growth factors have been identified in adult/maturehuman brains (see above), no data are available on levels in neurologically healthy aged persons. In a report cited earlier on capillaries in the visual cortex of rats housed under different environmental conditions (standard vs. enriched), Black *et al.* concluded that there is "a failure of angiogenesis in old rats" [40]. The following report by de la Torre contains observations which I have interpreted to indicate that angiogenesis *wanes* with age in rats. In experimental support of his vascular hypothesis of human dementia (to be discussed later), he developed two rat models of chronic brain hypoperfusion. These involved occluding for set periods both common carotid arteries (2-VO) or both carotids plus the left subclavian artery [= left vertebral a.] (3-VO) [47]. Neither 2-VO nor 3-VO elicited any sensory-motor deficits in the rats but did produce visuospatial memory impairment (VMI), as measured by the Morris water maze test. Three different age groups of rats were studied with the following results.

Young rats subject to 3-VO for 9 weeks recovered from VMI. Middle age rats could survive 3-VO just beyond 8 weeks but did not recover following 3-VO for 9 weeks and developed degeneration of the CA1 hippocampal sector and eventually atrophic necrosis of parietal and temporal cortex. Old rats (19-22 months) recovered following 2-VO for 1-2 weeks but could not survive 3-VO much beyond 8 weeks. These findings could be explained if angiogenesis in these rats *declines* with age. By this interpretation the above middle aged

and older rats lacked the level of angiogenesis which young rats possessed and thus could not survive the more prolonged (9 weeks) or more severe (3-VO) hypoperfusion which the younger ones did.

Until recently, the idea of a waning level of angiongenesis in aging human brains has received little detailed attention. But in 1992 Ferrara *et al.* had noted that VEGF mRNA is expressed in rat brains and suggested that the "presence of the growth factor may be required to maintain the differentiated state of those vessels, which otherwise might undergo involution" [48]. His conjecture made two decades ago raises the question of cerebral capillary density and neuroangiogenesis in patients with various senile dementias.

6. CCD and NAG in Senile Dementias – mainly AD

CCD. Bell and Ball measured microvascular densities of capillaries and arterioles in the hippocampus of 3 groups: normal young persons (mean age of 38 years), normal old (74 yrs.), and Alzheimer patients (78 yrs.) and recorded the overall mean values as 129, 108 and 107 mm/mm^3, respectively [49]. Fischer *et al.* examined four areas in normal vs. AD brains and found average vascular density indices (rounded off) as follows: basal forebrain: 87 *vs.* 43, hippocampus: 82 *vs.* 50, pre-frontal: 95 *vs.* 75, and motor/sensory: 94 *vs.* 83 [50]. And finally, Buée *et al*. reported the "percentage of the ratio capillary surface area/cortical … field area" for three groups – young controls: 26.32%, elderly controls: 18.95%, and AD patients: 16.50% [51]. This subject has been extensively reviewed elsewhere [52, 53].

Many studies measuring capillary densities in AD brain *have* also described" physical abnormalities in the capillaries [50, 51, 54]. I have encountered no reports stating that such

abnormalities may precede a decline in capillary density, but this seems a plausible sequence in the progression of AD. Ravens summarized the histological findings of vascular changes in the human senile brain beginning withthe study by Sir John Batty Tuke (1835-1913) in 1873 and continuing to 1976 with the several papers by Fang and colleagues [55]. In 1992 Kalaria and Kroon described capillaries in Alzheimer's disease as exhibiting "looping, kinking, and extensive tortuosity" [56]. In 1995 Kalaria and Hedera reported that over 90% of AD brains exhibit microvascular aberrations in neocortical tissue sections stained with antibodies to endothelial cell markers and basement membrane. The abnormalities included "capillaries with collapsed or degenerated endothelium" [57]. Grammas *et al.* contend that "perturbations of the brain microvascular endothelium" may cause release of neurotoxic proteins in Alzheimer's disease [58].

NAG in AD. Below are five reports on the presence of endothelial growth factors or their mRNAs in Alzheimer patients. Several papers suggest that the factors' presence here represents an ineffective response. 1) In 1998 Kalaria *et al.* examined the brains of 12 AD subjects and found prominent VEGF reactivity in 40% of the cells resembling astrocytes. The authors reasoned that this increased VEGF reactivity, which was "often localized around vessels," might "compensate for insufficient vascularity and reduced cerebral perfusion in AD" [5].

2) In 2004 *Yang et al.* reported that "VEGF is co-localized with Aβ plaques in the brains of patients with AD … most likely results in deficiency of available VEGF … [and] may contribute to neurodegeneration and vascular dysfunction in the progression of AD" [60].

3) In genome-wide expression profile studies, Pogue and Lukiw found in AD brains that the majority of specific mRNAs were down-regulated but that nine genes in a family having the potential to promote angiogenesis were up-regulated, with VEGF being the least elevated. The authors concluded that any advance in angiogenic signaling may be the result of dysfunctional cerebral vasculature and may be "both a consequence and a contributing factor to etiopathology" of AD [61].

4) Growth factors other than VEGF have been studied in demented and aged human brains. Increased levels of basic fibroblast growth factor were found in association with the neuritic plaques and neurofibrillary tangles in AD brains. The sequestered bFGF may represent a decreased "bioavailability to surrounding cells" [62].

5) Similar studies by Styren *et al.* showed increased levels of epidermal growth factor receptor on brain vascular endothelial cells of aged and demented persons (many with AD) as compared with neurologically normal subjects. EGFR was interpreted here as a "proliferative signal" elicited by prior damage to the CNS or brain vasculature [63].

However, in a different neurological disease Lambrechts *et al.* reported that VEGF plasma levels are 50% lower in persons with spontaneous amyotrophic lateral sclerosis (ALS). They found that the intraperitoneal treatment of an ALS mouse model with VEGF "could prevent motorneuron death in ischemic conditions" [64].

7. Two Rival Ideas about the Etiopathology of AD: Aβ *vs.* vascular

The presence of amyloid plaques and neurofibrillary tangles in the brain led Hardy & Higgins in 1992 to propose the amyloid cascade theory of Alzheimer's disease [65]. Two

troubling reservations about this theory are that 1) "some humans without symptoms of AD have many cortical Aβ deposits" and 2) "the number of amyloid deposits in the brain does not correlate well with the degree of cognitive impairment" experienced by patients [66]. However, in the face of theseobservations and as late as 2002, Hardy and Selkoe wrote that "an alternative hypothesis explaining the cause and early pathogenesis of AD that has as much experimental support as the Aβ hypothesis has not emerged" [66].

Meanwhile, during the past two decades the pathogenesis of AD has been aggressively pursued along many new avenues of research. Grammas noted that "the clinical entity AD has, by definition, been categorized as a 'non-vascular' dementia." This designation explains in part why "the role of neuro-vascular interactions in the evolution of neuronal injury in AD brain [had been] underappreciated" for so long [67].

The cerebral microcirculation attracted the attention of many students of AD, notably Jack C. de la Torre. In 1993 he and Mussivand had reported on the "extensive angioarchitectural distortions of cerebral capillaries in Alzheimer's brains" and proposed that "during ageing, brain capillaries ... may undergo progressive degeneration caused by amyloid deposits ... or genetic predisposition" [68]. In 1994 de la Torre ascribed a lowered cerebral blood flow in AD brain to various conditions, including "twisted, looped, and kinked" capillaries [69]. Over the next decade he defined risk factors responsible for the vascular disorder in AD persons – e.g., atherosclerosis, carotid artery pathology, diabetes, etc. [47, 70]. In 2010 he distinguished between *cerebral hypoperfusion* (a slow pathologic process involving many months or years) and *cerebral ischemia* (a more rapid progress involving only hours or days) [71]. Causes of the latter might be carotid artery stenosis,

strokes, etc. In support of his general vascular theory of AD he cited the related studies of ten different laboratory groups [71].

Many others have pursued this vascular thesis [51, 52, 56, 58, 72-77]. Notable among them was Zlokovic, who in 2005 advanced a "neurovascular hypothesis of AD," which maintains that the synaptic and neuronal dysfunction seen in AD is due to aberrant angiogenesis, faulty clearance of Aβ across the BBB, and senescence of the cerebral vascular system [78].

8. Other Etiological Considerations Concerning AD -- Genetic, etc.

According to Wu *et al.*, the above changes in AD listed by Zlokovic may reflect the low expression of the homeobox gene MEOX2 (mesenchyme homeobox, also known as *GAX*, growth arrest-specific homeobox) [79]. Genome-wide association studies show that three genes account for 10% or so of cases termed the early onset, or familial form of the disease [80]. But late onset AD and other forms of senile dementias may involve still other genes.

Transcriptional profiling of brain endothelial cells from the frontal cortex of AD patients revealed that MEOX2 is one of 34 genes (out of 12,600 genes examined by Wu *et al.*) significantly altered compared with control age-matched autopsy samples [79]. MEOX2 is a regulator of vascular differentiation, while the other 33 genes, which are variously up- or down-regulated in AD, encode transcriptional factors controlling diverse cellular events (cell differentiation, metabolism, apoptosis, etc.). Wu reported that in AD cortical tissue the

capillary tubes were 65% fewer and the total capillary length was only 60% of controls. Deletion of *Meox2* in mice resulted in reductions in brain capillary density.

Wu *et al.* transduced cultures of human brain endothelial cells with an adenovirus containing an oligonucleotide construct for Meox2 gene and found that the cells expressed "40% the level of GAX homeoprotein, formed 60% fewer capillary tubes, and had substantially lower survival rates after VEGF stimulation compared to controls" [79]. The authors suggested that in AD the low expression of MEOX2 activates a proapoptotic pathway, but the story is complicated by the presence of both apoptotic antagonists and apoptotic accelerators [80, 81].

Genetics represents only one of the many promising avenues for basic research on AD. Other aspects of the brain's microvascular system are the associated non-neuronal cells – i.e., mural cells (smooth muscle cells & pericytes ensheathing the ECs) and adjacent glial cells (astrocytes, microglia, & oligodendrocytes). Some of them are sources or targets of the various endothelial growth factors. All are subjects of intense research [83, 84]. For example, Winkler *et al.* summarized the many reports about pericyte deficiency leading to BBB breakdown and brain hypoperfusion [85]. But the above topics and many others (hypoxia-inducible factors, BBB, Notch signaling pathways, Il-1β and other immunological factors influencing inflammation, etc.) are not discussed further here in order not to stray from this essay's main theme -- CCD and NAG.

9. An Alternate Focus for AD & Senile Dementias: Neuroangiogenesis

The hypothesis offered at the beginning of this essay proposes that during the life time of people, rats, mice, etc. the endothelial cells of cerebral capillaries, like most others cells in the body, deteriorate requiring repair by angiogenic factors. As noted before, in the early 1990s Ferrara had speculated that the presence of VEGF in the adult kidney or *brain* "may be required in order to maintain the differentiated state of those vessels, which otherwise might undergo involution" [26] The same idea was later expressed in a paper on retinopathy of prematurity by Alon, who wrote that "VEGF may act as a vascular survival factor" [86].

More precisely, I propose that after postnatal development, various growth factors (FGF, VEGF, EGF, TGF-α, angiopoietins, and maybe others yet to be identified) persist throughout adult life in the brain at low but progressively waning levels. Some of these factors help maintain cerebral capillaries then and perhaps even generate additional ones, as in the primary motor cortex of heavily engaged concert pianists. Following the general pattern of many hormones, such factors/cytokines in the brain commonly decline (I contend) during senescence, leading to a waning CCD which in time may account for a reduced cognition in aged persons and the emergence of AD.

The hypothesis does not exclude a role for amyloid plaques and neurofibrillary tangles contributing to capillary disturbance and neuronal loss. Indeed, Paris *et al.* found that Aβ inhibited capillary formation in three in vitro systems: 1) human brain endothelial cells (HBEC) plated on Matrigel, 2) CAM assay, and 3) bFGF-induced blood vessel formation in the corneal micropocket [87]. Also intra-tumor injection of Aβ inhibited vascularization of several different human tumor xenografts in nude mice [87].

10. Challenges to the NAG Hypothesis of AD

Two papers represent challenges to the NAG hypothesis, for one concludes that neuroangiogenesis is increased in AD and the other contendsthat treatment of AD should involve suppression of neuroangiogenesis.

Paper #1. Biron *et al*. noted that the well-recognized increased permeability of the BBB in AD has usually been blamed on cerebral hypoxia and neuroinflammation, but the authors suggested that instead the leakage may be due to neoanagiogenesis and hypervascularization induced by amyloidogenesis [18]. This conjecture was based on their human and mouse studies in which various markers of microvascular density (MVD) and tight junctions (TJ) were targeted/labelled with primary antibodies, which were then revealed by secondary antibody staining and measured by confocal microscopy [17]. For example, MVD "was defined as a ratio of the TFA (total fluorescence area in μm^2 in hotspots) to total area of an imaged field and was used as a surrogate measure of angiogenesis" [18]. Some of the results of this very detailed paper are abstracted below.

Biron *et al.* assessed microvessel density in human brains with AD and those with no disease (ND) using sections stained with a primary antibody to laminin. This marker for basement membrane was used here as a measure of MVD [88]. In AD brains compared with ND brains, the MVD appeared 69% higher in the cerebral cortex sections and 60% higher in the hippocampal sections – reflecting, according to these investigators, hypervascularity in the former. (I derived the above percentages from data given in the text.) These findings contrast with the reduced CD in AD brains reported in most previous studies and may reflect

different assays employed – i.e., confocal fluoroscopy used here versus the more tedious visual counting of capillary segments in brain sections, as described earlier in this essay.

Biron *et al*. also used transgenic AD model mice (TgAPPsw line 2576), which overexpress the human amyloid precursor protein. He examined them for markers of vascular density (CD 105), apoptosis (caspase-3), and tight junctions (occludin, ZO-1). Compared with normal littermates, the brains of aged Tg 2576 mice showed an enhanced MVD. From the data given in the text, I calculate an 80% increase in the neocortex and a 60% increase in the hippocampus. There were "significant disrupted tight junctions" but no evidence of vascular apoptosis [18]. The authors inferred that the BBB leakage was due to the increased microvascularity/angiogenesis.

The above findings of an increased MVD in the transgenic AD mice contrast with the decreased vascular densities in Tg2576 AD mice reported by Paris *et al*. [89]. Using an automated optical fractionator/scanner, they examined the cortical and hippocampal sections of 3-, 9-, & 17-month old TgAPPsw and control littermates after immunostaining with PECAM-1 (platelet endothelial cell adhesion molecule = CD31). Compared with the controls, the TgAPPsw mice of all ages showed reductions in vascular density in both brain areas – 33% in the cortex and 32% in the hippocampus [my calculations derived from Fig. 4]. These data led Paris *et al.* to suggest "that the overexpression of APPsw in the vasculature may oppose angiogenesis" and to conclude that Aβ peptides have "profound anti-angiogenic effects in vitro and in vivo" [89].

In discussing the above discrepancy, Biron *et al.* wrote that "PECAM [a.k.a., CD31] is not favoured as an angiogenic marker as compared to CD105," since PECAM is present

elsewhere than on the panendothelium, such as stromal or inflammatory cells [18, 90]. But if CD31 is less specific/more widespread than CD105, then the lowered CD reported by Paris et al. seems to me all the more impressive. Finally, early students of CCD worried about tissue shrinkage producing erroneously high counts [49]. The same concern was recently raised in studies on microglial clusters in AD [88]. The discrepancy between these two reports remains unresolved.

In any case, I suggest that the BBB leakage noted in Biron's study and similar ones may represent abnormal neoangiogenesis, reflecting an imbalance of vascular growth factors – e.g., perhaps a predominance of VEGF (a.k.a., VPF, vascular permeability factor) over angiopoietin.

Paper #2. Vagnucci and Li also linked Alzheimer's disease with angiogenesis in a way quite different from that proposed in this essay. They stated that "the brain endothelium secretes the precursor substrate for β-amyloid plaque and a neurotoxic peptide that selectively kills cortical neurons." Since long term use of various non-steroidal anti-inflammatory drugs "seems to prevent Alzheimer's disease," they concluded that "this benefit is largely due to these drugs' ability to inhibit angiogenesis." They suggested that "antiangiogenic drugs targeting the abnormal [sic] endothelial cell might be able to prevent and treat this disease" [91]. The authors used the phrase "abnormal endothelial cells" without explaining it further. The 12 drugs listed in their Table 2 have effects other than anti-angiogenesis which may be altering the clinical course of AD. Any "abnormality" in this system may have been due to an imbalance of angiogenic growth factors elicited, as I postulated above.

11. An Approach for Treating Cerebral Microvascular Hypoperfusion

Folkman's research on angiogenesis included efforts to develop anti-angiogenesis agents (e.g., Avastin, a monoclonal antibody) which investigators have used to suppress the vascularization of solid tumors and to treat various angiogenic diseases [27, 91]. The reverse side of the angiogenic coin concerns promoting capillary formation [92, 93]. Folkman wondered whether "purified angiogenesis factors [could] be administered in vivo, either locally or systemically, to accelerate the healing of wounds and fractures, or to increase neovascularization in the ischemic or infarcted heart" [22]. Others have pursued this idea in people, dogs, and rabbits [94-96]. In 1992 Wang examined cerebral blood vessel development in postnatal mice with videomicroscopy and was led to propose studies involving "localized application of angiogenic factors in the brain" [12]. The following seven reports (1-7) describe growth factors being administered directly into the brain of experimental animals. Some of these authors had in mind using a similar approach in the future for treating people with ischemic brain conditions, such as strokes. An eighth report (8) concerns exogenous VEGF which "ameliorated the cognitive impairment" in transgenic AD mice.

1. In 1990 Puumala *et al.* infused a high dose of basic fibroblast growth factor (HBGF-2, or heparin binding growth factor) into the left lateral ventricle of the brains of Wistar rats six times over 26 days. Four days after the last infusion, the cerebral vessels of the experimental and control animals were injected with carbon black and their brains removed for sectioning. Eight areas of each brain were examined. Only the section taken from the

left perilateral ventricular cortex showed a significant increase in capillary count compared to the same section in the sham operated animal. Here the capillary density in the parenchyma adjacent to the left lateral ventricle was 315/mm^2 compared to 261/mm^2 in the same area in the control animal [97].

2. Using VEGF, Rosenstein performed similar experiments on young adult Wistar rats whose brains were cannulated to a 3 mm depth at a site posterior to the coronal suture and lateral to the sagittal suture [98]. During 7 days an osmotic minipump delivered 0.05µg/ml recombinant VEGF at 1 µl/hr and in the process produced a cavity. In other rats a comparable cavity was produced via a control infusion. In the VEGF-treated rats the infusion site was filled with "remarkably vascular tissue" containing vessels which were "tortuous and dilated and lacked uniform spacing" [98].

3-5. Recombinant $VEGF_{165}$ was also used by three other laboratory groups in rats subjected experimentally to middle cerebral artery (MCA) occlusion for 90 minutes on Day 0. Hayashi employed a ligature occlusion technique (Day 0), applied Gelfoam impregnated with 9 ng of $VEGF_{165}$ to the surface of the cerebral cortex through a burr hole on Day 1, and sacrificed the animals 24 hrs later (Day 2) [99]. Zang applied a fibrin clot, injected $rhVEGF_{165}$ (1 mg/kg) intravenously via a pump 48 hours later (Day 3), and removed the brains on Day 9 [100]. Sun occluded the MCA with a suture, administered $VEGF_{165}$(10 µg/ml) via an osmotic minipump into the left lateral ventricle (at 1 µl/hr x 3 days), and sacrificed the animals at various times thereafter [101]. The infarct volume was measured in allbrain sectionsand found to begreatly reduced in the treated animals compared with control animals. The results of these three occlusion studies are summarized below.

Hayashi reported that the mean infarct volume of the sham-treated rats was 33% of their ipsilateral hemisphere, while that of the six VEGF-treated rats was only 18%. The interval between treatment and sacrifice was too short for angiogenesis to occur in the penumbra of the cortex and suggests a protective action on the capillaries of the factor absorbed from the Gelfoam. Zang's 9-day interval would have allowed time for the formation newly grown vessels. Sun concluded that VEGF reduced the infarct size in his rats and enhanced cerebral angiogenesis and neurogenesis [101].

6. Similar studies by Kanya and by Wang in mice involved MCA occlusion and treatment within transventricular doses of VEGF. Both showed improved motor and memory functions compared with untreated mice [102, 103].

7. Thau-Zuckman *et al.* subjected mice to traumatic brain/closed head injury and then infused VEGF into the lateral ventricles. In comparison with untreated mice, they found increased numbers of "proliferating cells in the subventricular zone and in the perilesion cortex," indicating augmented angiogenesis at the site of injury and reduced lesion volumes there [104].

8. Finally, Wang *et al.* used the transgenic AD mouse model designated PDGF-hAPP^{V7171} (platelet-derived growth factor). These mice were given intraperitoneal injections of VEGF x3 days. Based on Morris water maze testing, they exhibited improved learning and memory. Hippocampal areas showed "new blood vessel formation" by the 7th and 14th days after treatment but fell to the control level by the 28th day [105].

In summary, numerous investigators have shown in brain-injured animals that VEGF administered into the ventricle contiguous to the injury reduces the ensuing cerebral deficit

for a time. VEGF injections in transgenic AD mice induced a transient angiogenesis and increased microvasculature. In her 2011 review on AD, Grammas discussed many of the above reports and topics, speculated about an "unexplored connections between angiogenesis and AD," and suggested that "new therapeutic approaches are desperately needed" [67]. And also in 2011 Carmeliet and Jain wrote, "The revascularization of ischaemic tissues would benefit millions, but therapeutic angiogenesis remains an unmet medical need" [33].

12. Seven Potential Delivery Systems for Treating Senile Dementias

The experimental brain injuries employed in experiments described above (carotid artery occlusion and local brain trauma) were acute cerebral events and contrast with the slow development of senile dementias in people. Nevertheless, these animal studies suggest that providing angiogenic factors chronically to the human brain might retard or ameliorate cerebral microvasculopathies associated with aging – i.e., VCI and AD.

Recent investigators have cautioned against the use of a single endothelial growth factor in therapeutic efforts to correct various vasculopathies, such as strokes, etc. As early as 2000 Yancopoulos *et al.* wrote that many members of the VEGF, angiopoietin, and ephrin families interact in a complementary and coordinated manner to form functional vessels without leaks, etc. They noted that the random delivery of "a single growth factor to reconstitute a vascular bed is now viewed as "somewhat naive and even misguided" [106]. In 2002 two papers reported on the synergism of VEGF and angiopoietin-1 (Ang1) in angiogenesis and warned that using VEGF alone risks the growth of leaky vessels [107, 108].

Manoonkitiwongsa *et al.* advised against "VEGF monotherapy" for treating stroke victims for fear of induced angiogenesis with BBB leakage [109]. And recently Thau-Zuchman emphasized that in treating traumatic brain injuries VEGF alone "could be a double edged sword" by increasing vascular permeability and exacerbating cerebral edema [104].

Seven potential modes of administering angiogenic factors to the brain are discussed below and involve broadly speaking the blood circulation, the CSF, the subarachnoid space, or the olfactory/trigeminal nerves. An important corollary of the NAG hypothesis is that more than one factor may be involved in producing functional capillaries. Thus in discussing the therapeutic approaches below, I will generally refer to "factor or factors," factor/s, or VEGF *et al.*

#1. VEGF along with a complementary factor/s could be given intravenously over many months via a programmable minipump placed subcutaneously in the body [110-112]. But such an IV treatment has the potential to stimulate angiogenesis throughout the body and not be limited to the brain, where it is sought. The desired localization might be achieved by chemically linking the factor/factors to an antibody specific for some antigen on capillary endothelial cells. There is a high concentration of transferrin receptors on the luminal surface of capillaries "throughout the entire cerebrovascular bed" [16]. A mouse monoclonal antibody (OX-26) is specific for these receptors in rats.

The potential success of this approach using VEGF *et al.* coupled to *OX-26* is suggested by the following two studies. When methotrexate was conjugated to OX-26 and the complex was injected intravenously, the drug was released across the blood brain barrier into the rat brain parenchyma [113]. Similarly, when the complex of nerve growth

factor and OX-26 was injected IV, NGF was observed within the rat brain capillaries and brain parenchyma, while it was not found there when injected alone [114].

#2. An alternative route via the cerebral spinal fluid has some precedent in studies on Parkinsonian patients in whom nerve growth factor (NGF) has been infused over a 12-month period into the lateral ventricle with the expectation that it would spread from the CSF into the adjacent nigrostriatal dopamine system [115]. Kordower stated that "NGF will diffuse readily from the ventricular space into brain parenchyma" of the basal forebrain [116]. In the case of VCI and AD patients, introducing VEGF *et al.* via a ventricle would allow the factor also to reach the surface of the cerebral cortex chronically, since the CSF circulates through the subarachnoid spaces over the pia mater covering the cerebral hemispheres.

#3. A more direct route would entail injecting the factor/s into the subarachnoid space over the cerebral hemispheres through catheters (1 mm outer diameters) containing multiple draining holes -- like those used in intraputamenal infusion studies [117].

#4. Another focused approach might involve the angiogenic factor/s coupled to magnetic nanoparticles (MNP, 2-10 nm). Here the complex/complexes introduced into the body via the blood or CSF would be directed to the cerebral capillaries or cerebral pia mater and held there by a magnetic field over the skullcap. "A simple external magnetic field is all that is necessary to target a drug [coating a nanoparticle] to a specific site inside the body" [118]. Such particles can "be retained at the site by application of a magnetic field gradient" [119]. For example, a study on stent angioplasty employed an anti-restenotic agent (paclitaxel) loaded onto magnetic nanoparticles and subjected to a uniform induced magnetic field at the stent site. The authors reported a 4- to 10-fold higher concentration

there of drug-loaded particles than of the agent injected alone [120]. Such a delivery system consists of three parts: a magnetic core (e.g., magnetite, Fe_3O_4), a surfacecoating (e.g., polyethylene glycol, etc.), and a functional outer coating (e.g., a drug or factor) [119].

5. A general approach for introducing an angiogenic factor or factors overlying the cerebral parenchyma is suggested by Folkman's work with sustained-release polymers implanted in rabbit corneas and other sites. In his studies, nanogram (10^{-9}g.) quantities of tumor angiogenesis factor (TAF) were released and became therapeutically available over weeks to months [121, 122]. The therapeutic level of an angiogenic factor/s needed in the brain is a key concern. Rosenstein reported that cultures of fetal, neonatal, and adult rat neocortical explants treated with human recombinant VEGF showed "a dose-dependent increase in the ratio of vascular area/total explants area" over the range of 1-25ng [98]. Kurachi found that neovascularization on the chick chorioallantoic membrane occurs at femtomolar levels (10^{-15}g.) of angiogenin [123]. Parenthetically, Murray *et al.* noted that incorporating albumen in the polymeric delivery system allowed release of picogram (10^{-12} g.) quantity/day of epidermal growth factor (EGF) [124].

The use of sustained-released systems has been described in recent publications from the Department of Neurosurgery at Johns Hopkins University School of Medicine for the delivery of anticancer agents directly to sites of excised gliomas. Here the drugs were incorporated in biodegradable polyanhydride wafers (Gliadel wafers) placed in the excised site [125, 126]. Various studies in rats have employed four different intracranial resorbable, slow sustained release systems -- surgical foam, a thermal gel depot, a microcapsule, or biodegradable polymer beads [126-130]. If such a slow release device containing an

angiogenic factor/s were placed on the pia mater covering the cerebral cortex of persons with incipient VCI or AD, the factor/s might be absorbed into the parenchyma and help maintain existing capillaries or stimulate local capillary production over the long term. A *locally applied* device or system would prove more effective in the case of a factor having a short half life in the blood when administered systemically.

In light of the brain-penetrating therapies being tested in Parkinson's disease (e.g., intraventricular and intraputamenal infusions over many months), it is not implausible to consider two less invasive approaches for the chronic administration of an angiogenic growth factor merely to the brain surface – i.e., perfusion through multiple small holes in the skull or via trephination. The former route might involve VEGF bound to biodegradable, slow-release polymer beads, which are introduced via multiple microholes through the skullcap into the subdural space over the cerebral cortex [130].

#6. The latter route (trephination) might employ VEGF *et al.* in biodegradable polyanhydride wafers, which are distributed over the pia mater of the cerebral cortex through burr holes. Trephining has been a simple, successful operation performed since the Neolithic Age in Europe. Long before modern anesthesia and knowledge of asepsis, persons underwent trephination multiple times and recovered, as judged by the smooth, healed edges of the bony hole in their skulls. One ancient Inca patient in Cuzco, Peru survived seven successive operations with seven healed burr holes in his skull [131]. Today the operation can be performed in an outpatient setting in 20 minutes or so and requires only a local anesthetic to deaden pain in the epicranium.

#7. Another potential route involves the nasal cavity, where macromolecules may be absorbed across the olfactory membrane (olfactory receptor cells) and reach the brain parenchyma. For example, when gelfoam was soaked in a wheat germ agglutinin-horseradish peroxidase solution (WGA-HRP) and inserted into the nose of rats, neurons labeled with WGA-HRP were found "in the midbrain and pons and throughout the entire expanse of the olfactory cortex to the caudal pole of the cerebral hemisphere" [132]. Similar studies were reviewed by Illum [133].

Recombinant human nerve growth factor (rh NGF, M_w 37 kDa) given by nose drops to anesthetized rats was measured in the brain by enzyme-linked immunosorbent assay and found to reach concentrations within an hour of 3400 pM in the olfactory bulb and 660-1300 pM in four coronal sections of the cerebral hemisphere taken from anterior to posterior [134]. The authors of this 1989 paper suggested using this route for the long term treatment of Alzheimer's disease with nerve growth factor (NGF) [134].

Intranasal insulin is being tested in a 4-month long clinical trial in "adults with amnestic mild cognitive impairment or Alzheimer disease" [135]. Daily doses of an insulin aerosol are inhaled deeply via a drug delivery device. A variation of this study could examine the effects of long term administration of angiogenic factors intranasally to persons with VCI and AD. Agents encapsulated in cationic liposomes or onto nanoparticles and administered nasally show an increased absorption [136, 137].

A small fraction of an agent administered nasally is absorbed by the ophthalmic branch of the trigeminal nerves and reaches the posterior part of the brain [138]. When insulin-like growth factor-1 was administered to rats intranasally, the factor appeared within

30 minutes in the rostal brain areas near the olfactory bulb and in "caudal areas near where the trigeminal nerve enters the brainstem" [139].

13. Conclusion

The hypothesis advanced here attributes the vascular cognitive impairment (VCI) of old age and most cases of Alzheimer's disease (AD) to a reduced cerebral capillary density (CCD) and an underlying declining (or aberrant) neuroangiogenesis (NAG). Various mechanism not discussed in detail here determine the level of endogenous angiogenic factors in the brain throughout life. I suggest that treatment with comparable exogenous factors may correct the deficiency or imbalance presumed to be responsible for VCI and AD. The NAG hypothesis, if valid, has practical implication for preventing or reducing the incidence of these conditions and possibly ameliorating the existing states. Seven therapeutic approaches have been discussed for introducing angiogenic factors into the brain. Effective treatment will likely involve a careful balance of several exogenous factors in order to preserve or revive a healthy cerebral microcirculation. In future clinical studies on VCI and AD, investigators will be challenged to determine the most effective and safest combination of angiogenic factors.

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