Commentary

Alzheimer's Disease in Man and Transgenic Mice *Females at Higher Risk*

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The major risk factors for Alzheimer's disease (AD) are aging, Down's syndrome, genetics, and a positive family history. Most, but not all, studies also find that aging women have a higher risk of developing AD than men.¹⁻⁴ Female apolipoprotein E4 (ApoE4) carriers are also at higher risk than males.⁵⁻⁸ Together with the greater life expectancy of women in developed societies, this means that the majority of patients who live and die with AD are women. The etiology of the higher risk of AD in females is unknown. Perhaps because cognitive functions tend to be more distributed and bilateral in females compared to males, the neuronal disconnections associated with AD may affect women more profoundly. A role for estrogen, by influencing brain development (pre- or postmenarche) or senescence (postmenopause), is a leading hypothesis.

In agreement with this notion, some studies suggest a protective effect of estrogen replacement therapy in the risk of developing AD. For example, the risk of developing AD among self-reported estrogen users is about one-third less than among non-users (odds ratio 0.65), and the risk decreases with increased duration and dosage of conjugated equine estrogen, the major form of estrogen replacement in clinical use.^{9,10} Women who begin menstruation at later, rather than earlier, ages have a higher risk of developing AD.⁹ A protective effect of estrogen is also found in a cohort of elderly women in Manhattan. In this study, estrogen use decreases the risk of developing AD by about half (odds ratios 0.40 and 0.50 after adjustment for education) and is independent of ApoE genotype.¹¹ Other studies, however, find no protective benefit of estrogen replacement therapy^{12,13} and suggest that artifactual variables, such as higher socioeconomic status, more education, or better access to medical care among women who are prescribed estrogen, mediate this difference. A subsequent meta-analysis of ten studies, including eight case control and two prospective studies, found a risk reduction of 29% in postmenopausal women taking estrogen replacement therapy compared

to untreated women.¹⁴ Two more recent case-control studies also demonstrate a risk reduction of 58 to 72%.^{15,16} Finally, a recent review of 15 case-control studies published since 1990 suggests that estrogen replacement decreases the risk of developing AD by half.¹⁷

Given this epidemiological evidence, as well as positive results from brief, small, open trials of estrogen replacement in AD,¹⁴ three large double-blind placebo-controlled clinical trials of patients with probable AD have now been published—all three with disappointing results.¹⁸⁻²⁰ With this information, estrogen replacement therapy cannot currently be recommended as a treatment for AD. Furthermore, the risk of developing AD should not factor into the difficult clinical and personal decision to use estrogen replacement, balancing its beneficial effects on osteoporosis, cardiovascular disease, and other conditions with its carcinogenic potential. The debate now centers on whether the gender effect of AD risk is in fact mediated by postmenopausal estrogen decline and, if so, whether treatment of patients diagnosed with probable AD is too little, too late. Perhaps estrogen must be given in preclinical stages of AD, such as in higher-risk subjects with isolated memory impairment or minimal cognitive impairment, as a preventive therapy. Testing of this hypothesis is underway in 5- and 10-year AD prevention trials, the results of which will be available in 2003 and 2008. Furthermore, the correct estrogen preparation, dosage, and duration, with or without progestins, route of administration (transcutaneous *versus* oral), and use of drug holidays may all influence the outcome of clinical trials.

Transgenic Mouse Models of AD Develop a Partial AD-Like Phenotype with Aging

Transgenic (tg) mouse models have proven to be useful tools in testing hypotheses of AD pathogenesis as well as

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testing novel therapeutic strategies. Tg human amyloid precursor protein (hAPP) mice recapitulate some, but not all, features of human AD, and may therefore be best described as developing a partial AD-like phenotype with aging. For unclear reasons, the distribution of amyloid pathology in tg hAPP mouse brain is remarkably similar to the human disease. One of the more widely studied hAPP tg mouse lines, Tg2576 mice, developed by Hsiao et al,^{21,22} expresses the familial AD gene hAPP swe (Swedish mutation; APP_{K670N/M671L} in the APP770 numbering system) in a C57B6/SJL genetic background. The neuron-specific prion protein promoter drives expression of the transgene. With aging, Tg2576 mice exhibit a phenotype that includes learning and memory deficits, an abnormal pattern of glucose metabolism in brain, and pathological changes including amyloid plaque deposition, elevated A β 40 and A β 42 levels, neuritic changes, phosphorylated tau epitopes, α -synuclein-positive dystrophic neurites, gliosis, and inflammatory responses; however, aging mice develop neither neurofibrillary tangles nor significant neuronal loss.^{21–33} Cholinergic abnormalities in the immediate vicinity of amyloid plaques are apparent in immunostained brain sections from older hAPP tg³⁴ and hPresenilin-1 (mutant)/hAPP double tg mice,³⁵ but more macroscopic studies of cholinergic function have not been reported.

Amyloid plaque deposition in aging hAPP tg mice may be modulated pharmacologically, immunologically, and genetically. For example, amyloid pathology is accelerated in hPresenilin-1 (mutant)/hAPP double tg mice,²⁵ and absent in murine ApoE null (–/–)/hAPP tg mice.³⁶ In the latter mice, hApoE4 transgene expression promotes more fibrillar amyloid deposition than hApoE3.³⁷ Human transforming growth factor β 1/hAPP double tg mice develop increased A β deposition within plaques, with a greater proportion of meningeal and vascular deposition, reflecting a role of inflammation in amyloidogenesis.³⁸

Amyloid plaque deposition and behavioral decline in hAPP tg mice is prevented, and perhaps partially reversed, by immunization with intraperitoneal A β 42^{39–41}; mucosal (nasal) administration of A β 40 may be somewhat less effective.⁴² Protection from amyloid deposition may be transferred passively by immunoglobulin, suggesting activation of an immunoglobulin and phagocytic microglial clearance mechanism.⁴³ The response to immunization (as measured by serum titer to A β) may correlate with lower amyloid burden in brain. Immunization does not prevent all amyloid deposition, perhaps due to poor penetration of immunoglobulins across the blood-brain barrier. Taken to an extreme, one may speculate that the risk of developing sporadic AD with aging may correlate with innate immune, inflammatory, or cellular responses to amyloid deposition in brain.

Finally, amyloid pathology in hAPP tg mice may be prevented by pharmacological treatment with the phosphatidylinositol kinase inhibitor wortmannin, which inhibits A β production *in vitro*,⁴⁴ the Cu²⁺/Zn²⁺-chelator/antibiotic clioquinol, which blocks amyloid fibril formation *in vitro*,⁴⁵ or the nonsteroidal anti-inflammatory drug ibuprofen.⁴⁶ The efficacy of this wide variety of pharmacological treatments in preventing amyloid deposition in tg AD

mice reveals multiple alternative and competing therapeutic strategies, and suggests that we may ultimately employ a clinical cocktail for AD prevention in humans. Novel therapeutics targeting the recently identified γ -secretase complex and β -secretases that generate A β 40 and A β 42 from APP are also being vigorously pursued.⁴⁷

Taken together, these studies reveal mechanisms of amyloidogenesis and new therapeutic targets based on the amyloid hypothesis of AD. None of these studies examined gender as a variable. Furthermore, the obvious studies of estrogen treatment trials in tg hAPP mice have not yet been reported. Ovariectomy increases A β levels in guinea pig brain (1.5-fold for A β 40 and 1.8-fold for A β 42), and this effect is partly reversed with either low-dose or high-dose 17 β -estradiol treatment.⁴⁸ This study leads directly to a hypothesized acceleration of pathology in ovariectomized hAPP tg mice and a beneficial effect of estrogen replacement in the prevention of amyloidogenesis in aging hAPP tg mouse brain.

Female hAPP Tg Mice Develop More Amyloid Plaque in Brain

In support of these hypotheses, Callahan et al⁴⁹ report in this issue of *The American Journal of Pathology* that in aging Tg2576 mice, females exhibit a much greater amyloid plaque burden in brain than males. Given the current clinical quandary regarding the results of estrogen replacement in women with AD, this study could not have been more timely. Amyloid load was measured by a quantitative areal analysis of stained brain sections. In 91 mice studied at 15 months of age, the area occupied by plaques in female Tg2576 mice was nearly three times that in males. A slight trend was apparent but insignificant in female mice at ages 8 and 12 months. Amyloid plaque deposition begins in earnest between 12 and 15 months of age, and thus coincides with the age of mouse anestrus. This is the first report of a gender effect on amyloid deposition in hAPP tg mice, and reveals that tg hAPP mice recapitulate yet another feature of the human disease: the gender effect. There are no data regarding a gender difference in amyloid load in other hAPP tg mouse lines. Neither is there information regarding a gender effect in hTau tg mice that develop neurofibrillary tangles. Again the question of mechanism arises, and postmenopausal estrogen loss is hypothesized as a leading candidate. Several mechanisms may be proposed; they are not mutually exclusive. The first category of possible mechanisms is more direct and based on known pleiotropic effects of estrogen on neurons and brain:

- 1) Estrogen down-regulates A β generation from APP metabolism in cultured cells.^{50,51} Thus, the decline of estrogen with menopause may result in increased A β generation. Data to test this hypothesis with tg hAPP mice are lacking, however. As mentioned, there are no published studies of estrogen deprivation or treatment in tg hAPP mice.

- 2) Estrogen has trophic effects on neurons, including cholinergic neurons, and may protect them from morbid-

ity and mortality.^{52–55} However, since Tg2576 mice do not demonstrate significant neuronal loss, this mechanism may be more applicable to the human disease than to hAPP tg mice.

3) Estrogen is an antioxidant, and thus may decrease neuronal damage caused by oxidative stress.^{56,57} Two other antioxidants, α -tocopherol (vitamin E) and selegiline, have clinical benefits in patients with probable AD.⁵⁸ In this study, clinical benefits were defined primarily by functional outcome measures; however, no benefit of treatment on cognitive (secondary) outcomes was found.

The second category of possible mechanisms contains those which may indirectly affect another risk factor of AD:

4) ApoE appears to play a role in A β deposition and/or clearance from brain. Since estrogen up-regulates ApoE expression,^{59,60} the gender effect may be mediated by a postmenopausal decline in circulating ApoE levels.^{61,62}

5) Estrogen regulates cholesterol levels, and elevated cholesterol promotes A β generation in cell culture and animal models.⁶³ This effect of estrogen on cholesterol levels may be independent of ApoE-mediated clearance mechanisms.

6) Although not a known risk factor for AD, the immune, inflammatory, and glial response to amyloid in tg hAPP mouse brain modulates AD pathology. Likewise, estrogen levels may influence the immune and inflammatory response to amyloid deposition in brain.⁶⁴

Clearly, multiple mechanisms of estrogen may alter (i) A β generation from APP, (ii) the deposition and clearance of A β in brain, or (iii) cellular responses to amyloid. Although these effects suggest roles for estrogen, they may not explain the gender difference seen in aging Tg2576 mice, nor the increased risk of AD in women.

The Gender Effect on Soluble A β Levels in hAPP Tg Mouse Brain Is Less Pronounced

Callahan et al⁴⁹ also measure A β 40 and A β 42 levels from soluble and insoluble fractions of tg mouse brain tissue by enzyme-linked immunosorbent assay. In contrast to the marked gender effect on plaque burden in brain, the difference in soluble A β 40 levels is subtle. Soluble A β 40 levels are statistically significantly higher in 15-month-old female mice compared to males, but not markedly higher. As may be expected by the greater amyloid burden found in female mice, insoluble A β 40 levels reveal a greater gender difference than soluble A β 40. This difference, however, is far less than expected, leading to discussion of a missing pool of A β . There were no significant gender differences in the much lower levels of either soluble or insoluble A β 42. In contrast to Hsiao et al,²² the authors find no correlation between plaque load and performance on a behavioral task (Morris water maze), male or female, and suggest that this may be an insufficiently sensitive task to detect a gender difference. King et al³⁰ report gender differences in several behavioral tasks in Tg2576 mice at 3 and 9 months of age, that is, before significant A β and amyloid deposits develop in the

brain. No other reports on the effect of gender on behavior of Tg2576 mice have been published.

These data bring up the debate as to which forms of A β are deleterious: intraneuronal *versus* extracellular, soluble *versus* insoluble, monomers, oligomers, or amyloid fibrils? Because behavioral and electrophysiological abnormalities precede amyloid deposition in brains of Tg2576 and other hAPP tg mice, soluble and oligomeric, and perhaps intraneuronal, A β is implicated in various neurotoxic mechanisms.^{22,29,65,66} Given the more marked gender difference in plaque burden as opposed to soluble A β levels and no effect on behavioral decline (Morris water maze) of Tg2576 mice with aging,⁴⁹ then perhaps the more important pathogenic variable with respect to behavior is soluble A β levels. Amyloid plaques in brain may develop as a threshold or saturation phenomenon and sequester A β peptides in a more inert form that produces only local toxicity. This notion has been suggested in other neurodegenerative diseases with abnormal (intracellular) protein aggregates.

Perspectives

This study raises more questions than it answers and generates new testable hypotheses. Tg2576 mice recapitulate another feature of human AD—the gender effect—despite the behavioral and pathological shortcomings of this tg mouse as a model of human disease.⁴⁹ Although independent confirmation in Tg2576 (or other hAPP tg) mice is lacking, these animal models may be useful in dissecting the mechanism(s) of the gender effect on amyloidogenesis in Tg2576 mice, and perhaps in man. This mechanism may or may not involve the pleiotropic effects of estrogens in the brain. A novel therapy for AD may result from identifying the risk factor(s) in female tg hAPP mice, or conversely, in defining the protective factor(s) in males. Interestingly, testosterone also reduces secretion of A β peptides from cultured cells.⁶⁷ A more gradual decline in testosterone levels with andropause (progressive androgen deficiency of the aging male) may increase the risk of developing AD in aging men, but replacement therapy again engenders an increased risk of cancer.

Whatever the mechanism, these data provide an important clue as to the variables influencing age-dependent amyloid plaque deposition in Tg2576 mouse brain. These data also warn that all future investigations of Tg2576 mice, and perhaps other hAPP tg mice, must include gender as a variable in pathological mechanisms as well as treatment outcomes, particularly in studies measuring amyloid burden in brain. Prevention of age-dependent amyloid deposition and behavioral/cognitive decline in tg hAPP mice and in man will be the ultimate test of the amyloid hypothesis.⁴⁷ The current race between various pharmacological *versus* immunological, and, perhaps ultimately, genetic strategies for AD will benefit millions of future patients, the majority of whom will be women.

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