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Stroke-Related Translational Research

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Abstract

Stroke-related translational research is multifaceted. Herein, we highlight genome-wide association studies and genetic studies of cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy, COL4A1 mutations, and cerebral cavernous malformations; advances in molecular biology and biomarkers; newer brain imaging research; and recovery from stroke emphasizing cell-based and other rehabilitative modalities.

The many facets of cerebrovascular disease have proven to be very fertile ground for translational research. This research involves many aspects of cerebrovascular disease: risk factors and prevention, diagnosis, prognosis, acute treatment, potential neuroprotection, and recovery. Various disciplines and avenues are being explored, including genetics, molecular biology, animal models, brain and vascular imaging, stem cells, and magnetic and direct current stimulation. This review cannot convey the breadth of these activities; instead, 6 active researchers introduce and eclectically summarize their various areas of research as examples of activity in their fields.

GENETICS

During the last quarter century, there has been a revolution in molecular biology and genetics. Genetics has a large influence in determining who will develop strokes, which
subtypes of stroke will develop, and who may be more vulnerable to neuronal death after vascular occlusions. Genetic analysis of mutations has become very important in the diagnosis and understanding of some specific genetic and mitochondrial diseases. In this section, we will review 3 different avenues of genetic research: (1) genome-wide association studies that aim to map stroke risk loci; (2) progress in understanding cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), the most extensively studied hereditary ischemic stroke condition; and (3) research on collagen genes that seem to be related to brain hemorrhage, aneurysms, and arterial dissections.

**Genome-wide Association Studies**

The availability of high-density microarrays that allow rapid screening of genome-wide sets that range from 100,000 to more than a million single-nucleotide polymorphisms shows promise of revealing important genetic associations with stroke and stroke risk factors. Defining the genetic etiology of and influences on cerebrovascular disease can help patients’ relatives and progeny as well as patients. A family history of stroke increases the risk of ischemic stroke and its major subtypes. Parental history of stroke increases the risk of stroke over all levels of risk defined by classical factors like cigarette smoking. Shared genetic and environmental traits presumably explain this added risk running in families. Several genome-wide association studies have been performed using longitudinal and case-control samples in an attempt to map ischemic stroke risk loci. Table 1 shows some loci posited to increase the risk of ischemic stroke and silent brain infarction. This list is a preliminary draft likely to change considerably in the near future. Loci with smaller effect sizes will be added, and some reported associations may not withstand further scrutiny. For example, the initial report of an association of the chromosome 12p13 locus with ischemic stroke could not be replicated in a case series that included thousands of cases. Additional questions remain. It is not clear why silent brain infarction should harbor different risk loci than ischemic stroke in the same consortium. This may be due to the preponderance of lacunar strokes among patients with silent infarcts and fewer lacunar strokes among patients with ischemic stroke. Alternatively, the disparate findings may be the result of a signal-to-noise problem that can only be overcome with larger sample sizes. Additional studies like the Wellcome Trust Case-Control Consortium 2 and the Stroke Genetics Network Study (which was sponsored by the National Institute of Neurological Disorders and Stroke) should bring further clarification.

The heterogeneity of ischemic stroke is reflected in the heterogeneous effects of genetic risk factors. The chromosome 9p21.3 locus, first identified in coronary artery disease, has been shown to be a risk factor for large-vessel atherosclerotic ischemic stroke independent of myocardial infarction. PITX2 variants, first discovered as risk factors for atrial fibrillation, were later shown to also be risk factors for cardioembolic stroke. There was a suggestion that PITX2 variants might also be associated with cryptogenic stroke, perhaps through unrecognized intermittent atrial fibrillation, but this has not yet been confirmed.

**Cerebral Autosomal Dominant Arteriopathy With Subcortical Infarcts and Leukoencephalopathy**

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy is a relatively newly recognized clinical and pathological entity that is of special interest because it offers a window into genetics and the conditions that infiltrate brain vessels. It is the most common heritable cause of stroke and vascular dementia in adults and is a genetic archetype of nonhypertensive ischemic small-vessel disease of the brain. The clinical presentation and neuroimaging abnormalities closely resemble those of sporadic ischemic small-vessel disease. The key distinguishing pathological alteration is the presence of granular osmiophilic deposits, of yet unknown composition, in the brain and...
peripheral arteries. The disease is caused by dominant mutations in the NOTCH3 gene, which encodes a transmembrane receptor predominantly expressed in vascular smooth muscle cells and pericytes in adults. This receptor is critically required for the structural and functional integrity of small arteries.

More than 500 CADASIL pedigrees have been analyzed, and at least 150 distinct CADASIL mutations have been identified. NOTCH3 has 33 exons, but there is now ample evidence that the pathogenic mutations occur exclusively in exons 2 to 24 encoding the 34 epidermal growth factor–like repeats (EGFRs), which compose the extracellular domain of the NOTCH3 protein, and consistently lead to an odd number of cysteine residues within an EGFR (Figure 1). First, an analysis of very large families has established a perfect segregation of these mutations (commonly called “cysteine mutations”) with the disease phenotype. Second, the occurrence of a de novo noninherited cysteine mutation has been shown in a few patients. Third, these cysteine mutations cause the Notch3 protein to abnormally multimerize and accumulate in vessel walls. Finally and most importantly, expression in the mouse of a NOTCH3 protein with a cysteine mutation is sufficient to recapitulate the pathological features of the disease, including the abnormal aggregation of Notch3 and granular osmiophilic deposits in blood vessels and brain lesions.

Genetic testing with direct sequencing of exons 2 to 24 of the NOTCH3 gene is now the gold standard for the diagnosis of CADASIL, with 100% specificity when a cysteine mutation is detected. The sensitivity is near 100%. With extensive use of this test, reports of patients carrying gene sequence variants not involving a cysteine residue are now emerging. However, these mutational events should be regarded as variants of unknown significance, and genetic diagnosis should not be offered until additional studies have established a causal relationship between the disease and the variant. Ultrastructural examination of a skin or muscle biopsy, with careful analysis of a sufficient number of arterioles, is needed to determine whether the disease is CADASIL or another small-vessel disease. Analysis of the messenger RNA (mRNA) of the NOTCH3 gene is needed to test the possibility that the variant ultimately leads to an odd number of cysteine residues. Nucleotide substitutions predicted to cause missense substitutions alter exonic splice enhancer sites to cause aberrant splicing. For variants associated with a small-vessel disease distinct from CADASIL and for variants that definitely do not affect a cysteine, experimental works using, for example, cultured cells or genetically engineered mice are needed to decipher their effect on NOTCH3 signaling and cerebrovascular function.

Our understanding of how CADASIL-associated NOTCH3 mutations lead to vascular lesions is still incomplete. In vitro studies and genetic studies of mice that combine Notch3 null mice and transgenic mice expressing representative CADASIL-associated mutant NOTCH3 have shown that a change in cysteine residue number, but not the effect of the mutation on NOTCH3 function and canonical signaling, is the common denominator in patients who develop CADASIL. The data suggest a model involving novel pathogenic roles for the mutant NOTCH3 receptor rather than a compromised NOTCH3 function as the primary determinant of clinical CADASIL. Analysis of genotype-phenotype correlations suggests that the loss of function of NOTCH3 associated with mutations located in EGFRs 10 to 11, which are required for binding to the ligand, may be associated with a better clinical outcome, including better cognitive performances and lower disability, when compared with common mutations located in the mutational hot-spot region, including EGFRs 2 to 5. Additional studies in humans and mice are needed to further confirm these results and elucidate the novel function likely gained by the mutated receptor.
Modeling CADASIL in the mouse is quite laborious.\(^ {25,34}\) At present, there is only 1 mouse model that recapitulates the presymptomatic stage of the disease with vascular lesions and white matter abnormalities but no lacunar infarcts or clinical findings\(^ {24}\) (Figures 2 and 3). A P1-derived artificial chromosome–based transgenesis approach was used to overexpress a mutant Notch3 (Arg169Cys) in an endogenous-like expression pattern. Studies in these transgenic mice, despite being an incomplete model of CADASIL, provide fresh insights into specific aspects of CADASIL pathogenesis. In particular, the data show, contrary to common belief, smooth muscle cell loss and fibrosis of the arterial wall do not contribute to disease initiation. Rather, they suggest a model in which white matter abnormalities result from chronic hypoperfusion caused by cerebrovascular dysfunction and capillary reduction.\(^ {24}\) Capillaries are heavily involved in the pathology. These mice will be valuable for further exploring the mechanisms of CADASIL. However, mouse models that better replicate the full human disease are needed to test the efficacy of therapeutic interventions, although it is unclear how to achieve these, given the potential problems arising from overloading cells with Notch proteins. An international, placebo-controlled, double-blind, randomized parallel-group trial\(^ {35}\) has recently been conducted in CADASIL patients with cognitive impairment to test the efficacy of the cholinesterase inhibitor donepezil hydrochloride. This multicenter study, although negative, emphasizes the feasibility of randomized trials in patients with CADASIL.\(^ {35}\)

There is ample evidence that hypertension, which has deleterious effects on brain vessels, is important in sporadic ischemic small-vessel disease of the brain.\(^ {36}\) Given the importance of NOTCH3 in the cerebrovasculature, it is tempting to speculate that the NOTCH3 signaling pathway might be also involved in sporadic hypertensive ischemic small-vessel disease.\(^ {12-14}\)

**Genetics of Hemorrhagic Stroke and Arterial Dissections**

Effective prevention of intracerebral hemorrhage (ICH), a condition for which therapeutic tools are still very limited, requires identifying individuals at high risk and understanding the mechanisms that underlie its occurrence. Several mendelian (also called monogenic) inherited conditions are known causes of brain hemorrhages. The identification of genes involved in mendelian stroke conditions, in vitro analyses, and in vivo studies of animal models of those mendelian diseases have proven to be valuable tools for (1) patients’ clinical care, (2) deciphering the involved signaling pathways, and (3) identifying potential therapeutic targets. Recent gains have been made concerning 2 autosomal dominant conditions that cause ICH. This progress shows promise of translating soon into better clinical care.

**ICH and COL4A1 Mutations**

Mutations of *COL4A1*, a gene encoding the a1 chain of type IV collagen, a major component of vascular basement membranes, have been shown recently to cause ICH in mice and humans.\(^ {37,38}\) The clinical presentation of *COL4A1*-mutated patients is very heterogeneous.\(^ {39-42}\) Intracerebral hemorrhage is one of the most severe manifestations of the disease. It can occur during prenatal and postnatal life, in infants, children, and adult patients. Intracerebral hemorrhage can be the only clinical manifestation, or it can be accompanied by various neurological and systemic vascular and nonvascular features (including lacunar infarcts, cerebral aneurysms, infantile hemiparesis associated with porencephaly, retinal arteriolar tortuositities, cataracts and congenital defects of the anterior eye segment, hematuria and bilateral kidney cysts, and muscle cramps). Most patients have a diffuse white matter hypersignal on T2-weighted magnetic resonance imaging (MRI) scans, and some patients have microbleeds. Figure 4 is a montage of brain images that show the major clinical lesions found in patients with *COL4A1* mutations. The severity of the condition is highly variable, even within a given family; an asymptomatic adult carrier may
be at risk of having a severely affected infant or child. This variability may be associated with environmental factors and modifying genes. COL4A1-mutated patients are highly sensitive to factors favoring ICH, such as birth trauma and antithrombotic agents. Analysis of the various mouse models carrying COL4A1 mutations should clarify the mechanisms leading to hemorrhagic stroke and the mechanisms modifying the genes.

These data have important implications for the clinical care of patients and for their relatives. First, preventive measures should be implemented in COL4A1 mutation carriers to decrease the risk of brain hemorrhage. Second, identification of COL4A1 mutation carriers has very important implications for genetic counseling. Table 2 lists COL4A1 mutations reported to date. Prenatal diagnosis is often required by young parents who already have an affected child. Neurologists who usually care for adult patients must be aware that these patients and their relatives should benefit from genetic counseling.

The proportion of nonhypertensive patients with ICH who carry a mutation of the COL4A1 gene is unknown. Ongoing prospective studies on large series of patients with ICH should help solve this question as well as help define the evolution and prognosis of this condition and so improve clinical care. Many questions remain unanswered, including how to manage the cerebral aneurysms encountered in some patients. Additional work is also needed to identify the mutated genes in patients who present with the same clinical features but do not carry mutations within COL4A1. The ongoing progress in genotyping and sequencing will help us to identify those genes and novel targets for hemorrhagic stroke prevention and treatment.

Collagen-related genes are also prime suspects rendering susceptibility to arterial dissections. Patients with Ehlers-Danlos syndrome type IV (the vascular type) are known to have arterial dissections, and their genetic defect is a mutation in the COL3A1 gene. Type I collagen is an important structural protein that accounts for more than half of the collagen in most arterial walls. Mutations in 1 of 2 genes that code for proteins that combine to form type I collagen (COL1A1 and COL1A2) account for many of the defects in type I collagen found in osteogenesis imperfecta. Two patients with osteogenesis imperfecta have been reported with multiple cervical arterial dissections. Exploration of collagen-related genes shows promise of uncovering new information about a large group of poorly understood vascular conditions, including fibromuscular dysplasia, arterial dolichoectasia, and cerebral aneurysms.

ICH in Patients With Cerebral Cavernous Malformation

Hereditary cerebral cavernous malformations (CCMs) are characterized by multiple lesions and a high but unpredictable risk of ICH. Lesions located within the basal ganglia, brainstem, and spinal cord are particularly difficult to treat surgically, and there is a strong need for other nonsurgical approaches for these lesions.

Three CCM genes have been identified, KRIT1, CCM2, and PDCD10. These genes encode for 3 unrelated proteins whose role in angiogenesis was previously unsuspected. Important progress has been made in the last 3 years in understanding the functions of proteins encoded by these genes. A plethora of in vitro and in vivo data strongly suggest that CCM proteins play an essential role in endothelial cells and regulate vessel morphogenesis and blood vessel stability. All 3 proteins have also been shown to be involved in the integrity of the blood-brain barrier. In vitro, loss of the KRIT1 and CCM2 genes leads to the activation of Ras homolog gene family, member A (RhoA), a guanosine triphosphate protein; an increased number of actin stress fibers; and enhanced permeability of endothelial cell layers. The use of statins reduces the availability of geranylgeranyl diphosphate, a lipid needed for the isoprenylation of RhoA. The favorable effects of statin use have been
observed on some of the in vitro and in vivo consequences of KRIT1 and CCM2 loss, raising the question of their possible usefulness in patients with CCM.\textsuperscript{67,70,71}

To our knowledge, there has been no study of the effect of statin use on bona fide CCM lesions because all published CCM animal models in which KRIT1 or CCM2 genes have been totally nullified did not develop CCM lesions because the animals died in utero. A very recently developed CCM2 postnatal mouse model mimicking human CCM lesions in both the retina and the brain is now available and will be a very useful tool for preclinical trials.\textsuperscript{72}

Based on the very rapid progress in our understanding of the effect of CCM proteins on endothelial cells, preclinical and clinical trials for CCM will most likely begin very soon.

**MOLECULAR BIOLOGY AND BIOMARKERS**

Much information has been gathered about the microscopic anatomy and physiology of the brain, but more is still to be learned. We need to know more about how blood flow is regulated by various cells (neurons, astrocytes, and endothelial cells) and various chemical substances, and how the cells and substances interact. Most relevant from a translational medicine standpoint is how to apply in a clinical setting what is known about the molecular biology of the brain and vascular function.

**Therapeutic Targets, Animal Models, and Testing**

Progress has been made in understanding the basic molecular and cell biology of how neurons die in various central nervous system diseases, including stroke. However, translation of this fundamental knowledge into clinically effective neuroprotective therapies remains elusive. In recent years, 4 ideas have emerged that might explain and help overcome some of these translational barriers: (1) the necessity of salvaging function in the entire neurovascular unit, (2) the possibility that many stroke targets may be biphasic, (3) the improvement of quality control in animal models, and (4) the utility of novel “human models” that can bridge experimental findings to clinical stroke patients.

The concept of the neurovascular unit suggests that a purely neurocentric focus may not be sufficient and that all elements of the neurovascular unit (ie, cells from neuronal, glial, and vascular compartments) must be considered.\textsuperscript{73} Without cell-cell signaling between neurons and astrocytes, neurotransmitter dynamics cannot be sustained. Communication between astrocytes, pericytes, and the endothelium is required for the optimal function of the blood-brain barrier. Without multicell signaling between neurons, glia, and vascular cells, hemodynamic coupling between brain activation and cerebral blood flow cannot take place. Preventing neuronal death alone may not be enough. Clinically effective stroke therapies must rescue functional cross talk between all cell types in the neurovascular unit.\textsuperscript{74}

Because novel mechanisms continue to be analyzed for stroke, emerging data now strongly suggest that many targets may be biphasic in nature. The same mediator or molecule can play different roles under different physiologic and pathophysiologic conditions. For example, overactivation of the N-methyl-D-aspartate receptor clearly mediates excitotoxic neuronal death during hyperacute stages of brain ischemia. During stroke recovery, however, N-methyl-D-aspartate signaling may be essential for neurogenesis and neuronal plasticity.\textsuperscript{75} The same is true for the class of matrix metalloproteinases (MMPs) that have been implicated in blood-brain barrier damage. During early phases of brain injury, inhibition of MMPs is beneficial; during later times, however, MMP activity is required for angiogenesis, vasculogenesis, and neurovascular remodeling.\textsuperscript{76,77} The standard target for many years has been to save brain cells in the penumbra; however, it is now clear that the penumbra is not only dying but also actively trying to recover.\textsuperscript{78} As we seek therapies to block cell death pathways, it will be important to understand how, where, and when these
signals and substrates transition from initial injury to repair, so as not to interfere with the endogenous mechanisms of recovery.

The molecular cascades triggered by brain ischemia and hemorrhage are highly complex. Multifactorial cascades are activated in neurons, glial, vascular, and inflammatory cells. Candidate targets and therapies must be tested in experimental animal models of stroke before these therapies can be used by humans. But how reliable are our animal models, and how reproducible are our preclinical therapeutic data? Meta-analyses suggest that much of the data on animal models is not as rigorous as it should be. Increased attention to blinding, randomization, proper statistical powering, and overall study quality are critical if we are to find the best candidate therapies for testing in clinical trials.

Animal models will always remain experimental, with many caveats and limitations. Animal models mimic many aspects of stroke and are critically important for clarifying mechanisms, but no single animal model can truly replicate stroke patients in all their complexity. Leading candidate therapies should be tested in multiple animal models in multiple laboratories. More recently, novel “human models” have been posited to provide new opportunities to supplement our experimental platforms. For example, human brain endothelial cells in culture have been used to proteomically screen for candidate neurovascular mediators that can be confirmed in the plasma of stroke patients. Samples can be obtained from patients undergoing carotid endarterectomy for potential biomarkers during vessel occlusion and reperfusion. These human models may prove useful in linking experimental data with clinical measurements in stroke patients.

**Blood Stroke–Related Biomarkers**

Blood biomarkers have the potential of becoming a novel and powerful tool for assessing stroke pathophysiology in vivo and in real time. To contribute to important advances, stroke blood biomarkers should provide information about the following problematic aspects related to stroke diagnosis, treatment, and prognosis: (1) Data should be obtained on the ultra-early positive diagnosis of ischemic stroke, which includes the distinction between stroke and conditions that mimic a stroke and the discrimination between ischemic and hemorrhagic stroke. This may widen access to ultra-fast reperfusion therapies and therefore increase the proportion of patients receiving thrombolytic treatments, substantially improving stroke outcome. (2) Data should be obtained on the dynamics of brain ischemia, the markers to monitor brain ischemia and necrosis, the biochemical signatures of ischemic penumbra and infarct core, and the assessment of extent and severity of ischemic injury. (3) Data should be obtained on in vivo monitoring of basic mechanisms and processes that have a crucial effect on stroke outcome, such as inflammatory infiltration and activity, blood-brain barrier disruption, characteristics of thrombi and their potential to resist the effects of thrombolytic drugs, and processes involved in neurorepair. The 2 latter aspects have major potential to identify new therapeutic targets. (4) Data should be obtained on the monitoring of response to therapies, the assessment of successful reperfusion, and other treatment end points. (5) Data should be obtained on the prediction of stroke risk in primary and secondary prevention by in vivo evaluation of the causal conditions of stroke.

Brain ischemia is characterized by several cellular and molecular alterations (such as excitotoxicity, oxidative stress, inflammation, cell death, and apoptosis) that are accompanied by biochemical changes in the peripheral blood. The first approach in biomarkers development was to test the applicability of single predetermined molecule candidates derived from the molecular pathways known to be involved in stroke. An example of this approach was the identification of the plasma level of MMP-9 measured before thrombolytic therapy as a powerful predictor of posttreatment hemorrhagic complications. The high predictive value of MMP-9 was later confirmed by a different
group,\textsuperscript{86} which also identified cellular fibronectin as a predictor of hemorrhagic transformation of ischemic stroke after thrombolysis. Because both molecules are known to be related to blood-brain barrier disruption and endothelial damage, the authors inferred that their plasma level may indicate the severity of deleterious processes that were taking place in the brain.\textsuperscript{86} Following a translational approach, immunostaining of brain samples obtained from patients who died after hemorrhagic transformation of ischemic stroke revealed strong MMP-9–positive neutrophil infiltration surrounding brain microvessels associated with severe basal lamina type IV collagen degradation and blood extravasation.\textsuperscript{87}

A similar approach was used to identify markers for ischemic stroke. Given the molecular complexity of the ischemic cascade, the search for a single biomarker was replaced by the evaluation of multiple proteins simultaneously (a biomarker panel).\textsuperscript{88} The diagnostic accuracy of a biomarker panel including D-dimer, brain natriuretic peptide, MMP-9, and astroglial protein S100B was evaluated in a prospective multicenter trial.\textsuperscript{89} The multivariate model only moderately differentiated between stroke and stroke mimics. The reasons for this apparent failure are related to the complexity of basic mechanisms and biologic events that precede and follow ischemic stroke, including genetic predisposition, comorbidity, and the effect of treatment.\textsuperscript{90} The same processes have different meanings and effects depending on the time or phase after stroke onset. Levels within the blood biomarker profile may not adequately reflect the presence and intensity of remote processes that have such a great inter- and intraindividual (in time) variation. Translational research may allow a more refined search for valid biomarkers.

The “omics” approach (genomics, transcriptomics, and proteomics) may achieve a more robust moment-to-moment physiologic profile. Recent translational research in stroke biomarkers combined the application of omics to a variety of methodological settings and samples, such as brain specimens (obtained from animal models and from human necropsies), cerebrospinal fluid, brain microdialysate, and blood samples. Promising results have been reported for genomic analyses of circulating white blood cells, in which genetic expression changes rapidly in neutrophils and monocytes after stroke in humans.\textsuperscript{90} Brain ischemia causes extensive transcriptional and posttranslational processing; the use of relative mRNA production is a valid method of identifying potential biomarkers of brain ischemic injury. Animal models are useful because they provide access to tissue. For example, using gene array analyses on a mouse model to screen for biomarkers preferentially and abundantly produced in the brain, Laterza et al\textsuperscript{91} identified a promising candidate, visinin-like protein 1, and then detected it in both the plasma of ischemic stroke patients and the cerebrospinal fluid of rats after middle cerebral artery occlusion. Regarding proteomics, proteome-wide screening of human cerebral microdialysate or cerebrospinal fluid holds promise in the identification of novel and more brain-specific biomarkers.\textsuperscript{92} Finally, microRNAs (miRNAs; small noncoding RNAs) have recently been reported as useful biomarkers in cancer and diabetes. Initial studies in young ischemic stroke patients suggest that peripheral blood miRNAs and their profiles can be developed as stroke diagnostic and prognostic biomarkers.\textsuperscript{93}

Molecular brain imaging may represent the ideal bench-to-bedside tool capable of in vivo, noninvasively monitoring brain ischemic injury and the response to stroke therapies. Direct visualization of basic processes involved in brain tissue destruction and repair, such as inflammation, is becoming feasible and may help the search for blood biomarkers that reflect changes in the intensity and direction of these processes. In vivo assessment of these processes may allow identification of the right time window and the way (augmentation vs inhibition) in which they should be targeted. The clinical applicability of novel candidate blood biomarkers could be tested using molecular imaging monitoring as a robust indicator of the instantaneous activity of the processes that they are posited to inform.
An initial approach to imaging inflammation in acute brain ischemia was iron oxide nanoparticle-enhanced MRI. Ultra-small paramagnetic iron oxide (USPIO) nanoparticles can be injected intravenously and are taken up by cells of the mononuclear phagocyte system, whose local deposition results in regional MRI–magnetic field inhomogeneities. Increasing USPIO enhancement on T1-weighted images after stroke indicates brain infiltration by USPIO-laden macrophages and could serve as a surrogate marker of inflammation in stroke. A more refined approach to delineating inflammatory areas in the ischemic brain is the tagging of infiltrating immunocompetent cells with contrast agents. Recent studies in murine models of brain ischemia showed the feasibility and safety of using biochemically inert nanoemulsions of perfluorocarbons as positive MRI contrast agents to image inflammation at high local resolution.

The use of proton magnetic resonance spectroscopy may aid in the discovery of metabolic biomarkers that are specific for determined cell types, thus serving as a “metabolomic” technique. Metabolomics has recently emerged as a scientific field of study aiming to investigate metabolites as possible biomarkers within the cell, tissue, and organism, thus providing functional insight into the biochemical status of a tissue, which results from the environmental effects on its genome background. Using this approach, Maletić-Savatić et al described a metabolic biomarker of neural stem/progenitor cells. Optical brain imaging is a novel technique with a great potential applicability in stroke translational research. Noninvasive near-infrared fluorescence imaging may represent an ideal tool to monitor basic processes such as blood-brain barrier disruption.

The combination of biomarker research and multimodal imaging is also needed to improve the prediction of stroke risk by assessing in vivo the dynamics of stroke and its causes, such as atherosclerosis. Noninvasive characterization of the vulnerable atherosclerotic plaque in both extracranial and intracranial atherosclerosis represents an enormous challenge for future research. Multicolor computed tomography, high-resolution MRI, contrast MRI (with nanoparticles able to molecularly target desired molecules such as MMPs), optical imaging of human atheromata, and other sophisticated imaging techniques provide a potentially powerful research armamentarium.

RECOVERY

The process and mechanisms by which patients recover from strokes, both ischemic and hemorrhagic, have been receiving more research attention and will receive even more attention in the future. Newer imaging and diagnostic stimulation techniques have made it possible now to understand both the qualitative and the quantitative aspects of brain function after vascular insults. Newer restorative therapies have also surfaced and are being investigated. Translational research in stroke recovery has gained considerable impetus during the past decade.

Cell-Based Therapies

Cell-based therapy has emerged as a new investigational approach to improve recovery after stroke. Cellular products with therapeutic activity in animal models of stroke are being developed from a range of tissues, including embryonic and fetal tissue, neural tissue, bone marrow, peripheral blood, umbilical cord, placenta, amniotic fluid, menstrual blood, dental pulp, and adipose. Many of these cellular preparations have the properties of multipotent stem cells, whereas others are composed of heterogeneous populations of cells such as umbilical cord blood. The use of cells rather than drugs is a novel approach to stroke treatment. Cellular therapies have been shown to improve outcome when administered in the first few days to weeks after stroke in animal models. The mechanisms appear multifactorial. Some cell types release cytokines that dampen the postischemic inflammatory
response and reduce ongoing injury in the peri-infarct regions. Other cell types may stimulate endogenous repair processes such as neurogenesis and angiogenesis. Figure 5 is a cartoon that shows the brain targets of stem cell treatment.

As basic science studies continue to delineate the precise mechanisms by which cellular therapies enhance stroke recovery, investigators are tackling a number of translational barriers, including the therapeutic time window and optimal dosing regimens. Other important steps in successful translation concern the delivery route and biodistribution of injected cells in the human body. An intravenous delivery route is least invasive, but many types of stem cells aggregate and are trapped in the lungs after intravenous administration. Intra-arterial injection into a brain-supplying artery bypasses the first pass filter of the lungs but might cause microvascular obstruction, which has already been reported for at least 1 type of mesenchymal stem cell. More direct routes to the brain, such as stereotactic injections, are also being explored, but they carry their own risks, such as infection, hemorrhage, and seizure. Monitoring the fate and survival of injected cells in patients would facilitate a better understanding of the behavior of injected cells (ie, their presence and activity). Many approaches are now being developed in which cells are labeled with magnetic resonance–compatible tracers such as iron, but these approaches have not reached the stage of clinical testing.

With the wealth of preclinical studies supporting the safety and efficacy of cell-based therapies in animal models of stroke, clinical trials have already commenced. The first pilot trials occurred in the late 1990s and involved stereotactic injections of neural cells in patients with chronic stable infarcts of the basal ganglia. These studies collectively suggested feasibility but were too small to draw definitive conclusions about safety or efficacy. During the past decade, clinical research in this area has shifted approaches and is now focused on adult cells derived from bone marrow. The first studies are using autologous mixed cell populations in which bone marrow is extracted from the iliac crest of stroke patients and separated; the bone marrow cells are reinjected back into the patient. The University of Texas is conducting the first intravenous study testing the safety and feasibility of bone marrow harvest and autologous intravenous reinfusion of mononuclear cells in stroke patients within 24 to 72 hours after symptom onset. Other stem cell centers are assessing the effects of intracarotid injections of bone marrow–derived mononuclear cells or other types of bone marrow cells in longer time windows after stroke. Although autologous cell administration has become the main approach globally, researchers are on the verge of introducing a new paradigm. Purified allogeneic cells are being developed from various tissues that carry the advantage of being “off the shelf” products, and many of these cellular products have immunomodulatory effects with low risks for rejection. Several clinical trials will begin soon to test allogeneic cells in stroke patients. We are witnessing the dawn of a new era that exemplifies the extensive efforts to translate a new therapeutic approach for stroke.

Stroke Recovery and Rehabilitation

A new stroke sets in motion a number of events in the brain. Many of these involve acute injury effects, such as the ischemic cascade, inflammation, delayed neuronal loss, and apoptosis. A stroke also initiates brain events that are related to repair. A new stroke is also followed by increased expression of growth-associated genes, increased levels of growth factors, angiogenesis, expansion of endogenous neural stem cell proliferation, increased dendritic arborization, synaptogenesis, and more. These repair-related events represent biological targets for an emerging class of restorative therapies.

Therapies that target repair-related processes after stroke are of interest for several reasons. Preclinical studies suggest a time window often measured in days rather than minutes. Most
patients with a new stroke do not receive acute reperfusion therapies, often owing to late arrival at the hospital. Repair-based therapies could increase the proportion of patients offered this type of therapy. Among those who do receive acute therapy, many nonetheless have significant long-term disability; for example, in the third European Cooperative Acute Stroke Study, 47.6% of patients treated with tissue plasminogen activator in the first 4.5 hours had a modified Rankin score of greater than 1 at 90 days in the intent-to-treat population.

A number of therapeutic approaches that aim to promote brain repair after stroke are being investigated. Examples include growth factors, other large molecules such as monoclonal antibodies, cell-based therapies, physiotherapy-based interventions, robotic devices, electromagnetic brain stimulation, neuroprosthetics, and small molecules. Numerous small molecules have been examined, including drugs that modulate activity within specific brain neurotransmitter systems (such as serotonin or dopamine), niacin, phosphodiesterase type 5 inhibitors, inosine, and 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors. Some drug- and cell-based therapies have been examined in early-phase human clinical trials, and at least some physiotherapy- and robot-based approaches were examined in phase III trials.

When translating brain repair studies to human trials, lessons from translating acute stroke therapies can be valuable. For example, preclinical results will most likely extrapolate to human studies if variables such as treatment time window, route of administration, or dosing schedule are well matched between preclinical and clinical studies. For acute stroke interventions, methods to measure the treatment target, such as diffusion-perfusion mismatch, may guide treatment. The same principle might prove useful in repair-based trials, in which the treatment target might be measured, for example, as the activity or integrity of the surviving cortical area, neurochemical system, or white matter tracts that are related to the therapeutic target (Figure 6). Such an approach might allow for effective patient stratification, which might be useful to maximize treatment effects.

Dose-response relationships are as important to repair-based studies as they are to acute stroke studies. This was well shown by the results of the VECTORS (very early constraint-induced movement during stroke rehabilitation) trial, in which a higher dose of early physiotherapy was associated with poorer behavioral outcome. Dose-response relationships can change as repair-based biological targets evolve in the weeks or months that follow a stroke.

There are also considerations for repair-based studies that are beyond the domain of most acute stroke trials. One major difference is that acute stroke therapies exert their effect rapidly (reperfusion or neuroprotection), around the time of therapy, whereas for a repair-based trial, many of the biological events that affect final behavioral outcome occur days or weeks after treatment (regrowth and repair). Another major difference is that effects of repair-based therapies are experience-dependent. An individual’s experiences, training, and environment interacts with the effects of a repair-based therapy and influences the final outcome. For repair-based therapies, if experience and environment cannot be controlled (which is often the case for practical reasons), the study design must measure such factors. This approach provided useful insights in one recent repair-based stroke clinical trial, in which the amount of “outside” physiotherapy (ie, physiotherapy occurring in parallel with trial participation but prescribed by private physicians outside of the trial’s jurisdiction) was found to differ significantly between active and placebo treatment arms. Such measures can then be treated as planned covariates of interest in statistical analyses.
A number of covariates can also influence the effects of a repair-based therapy. These include socioeconomic factors, caregiver status, and depression/affective state, each of which might be considered less pertinent when examined in a short-acting, acute stroke therapy. Genetic factors might also be important to recovery after stroke, and to the therapeutic attempts to improve it.

Repair-based trials might also benefit from expanding end points. Acute stroke trials often rely on global outcome measures, but many repair-based stroke clinical trials found favorable behavioral effects using domain-specific end points. There are several reasons why a repair-based trial might focus on the measurement of specific behavioral domains, in addition to traditional global end points. First, stroke affects multiple neurological and behavioral domains, and each of these domains can recover to differing extents. The time course of recovery in one neurological domain can be independent of recovery in a second domain of neurological function. Such differential gains might be better detected with domain-specific end points as compared with global outcome measures. The coming years are likely to see increased focus on repair-based therapies after stroke and on the clinical trial structure by which they are assessed.

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72. Boulday, G.; Rudini, N.; Maddaluno, L., et al. The effects of CCM2 invalidation are restricted to intense angiogenesis periods both in the central nervous system and in the eye; Paper presented at: 16th International Vascular Biology Meeting; Los Angeles, CA. June 20-24, 2010;


Figure 1.
Schematic drawing of the NOTCH3 receptor and cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) mutations. A, The mature NOTCH3 receptor contains all canonical Notch motifs, including 34 epidermal growth factor-like repeats (EGFRs), 3 Lin12-Notch repeats (LNRs), a single pass transmembrane (TM) domain, and 7 Ankyrin repeats (ANKRs). NOTCH3 is cleaved between the LNRs and the TM domain and is expressed at the cell surface as an heterodimer. B, Schematic drawing of a normal EGFR with its 6 cysteine (Cys) residues (red circles) and a mutated EGFR with its odd number of cysteine residues in patients with CADASIL.
Figure 2.
Generation of the P1-derived artificial chromosome (PAC)–R169C Notch3 transgenic mice. The schematic drawing shows the PAC clone that was used to generate the transgenic mice. The rat PAC clone contains the entire rat genomic Notch3 gene plus about 60 kilobase (kb) of 5′ flanking and 45 kb of 3′ flanking regulatory sequences. The cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy amino acid substitution R169C was introduced into this clone by homologous recombination.
Figure 3.
Phenotype of the P1-derived artificial chromosome–R169C Notch3 transgenic mice. The neuropathological features in the mutant mice are shown. The brain artery (A [scale bar, 50μM]) and brain capillary (B [scale bar, 20μM]) were immunostained with the 5E1 antibody specific to the Notch3 extracellular domain showing the characteristic granular immunostaining indicative of microscopic aggregates of the Notch3 extracellular domain. An electron micrograph of a pial artery (C [scale bar, 1μM]) demonstrates abundant granular osmiophilic deposits (red arrowheads). Glial fibrillary acidic protein staining in the internal capsula of a 12-month-old mutant mouse (D [scale bar, 70μM]) shows astrogliosis in the white matter bundles. Hematoxylin-eosin staining in the corpus callosum (E [scale bar, 70μM]) and Klüver-Barrera Luxol fast blue staining in the internal capsula (F [scale bar, 70μM]) of a 20-month-old mutant mouse reveal vacuolization in white matter bundles as well as pallor and disorganization of the myelin. EC indicates endothelial cell; SMC, smooth muscle cell.
Figure 4.
Brain magnetic resonance imaging scans of 2 COL4A1-mutated patients. Fluid-attenuated inversion recovery images (A) show diffuse and confluent white matter changes in both caudate nuclei in a 39-year-old patient. T2-gradient-echo images (B) show microbleeds and hemorrhages in both caudate nuclei in the same 39-year-old patient. C, Magnetic resonance angiography in a 47-year-old patient shows an 8-mm aneurysm on the left distal carotid artery (arrow). D, T2-gradient-echo image displays an acute hemorrhage in the left putamen in another 47-year-old patient.
Figure 5.
Cartoon showing the potential brain targets of stem cells.
Figure 6.

A, B, Brain scans of 2 patients. A, This patient had 37.5% of the corticospinal tract injured by stroke and had a gain of 11 points on the Fugl-Meyer (FM) scale across the period of therapy. B, This patient had 93.4% of the corticospinal tract injured by stroke and had a gain of 1 point on the FM scale across the period of therapy. C, Injury to the corticospinal tract correlates ($r=-0.65$, $P<.002$) with the change in score on the arm motor FM scale induced by robotic therapy among patients with hemiparesis in the chronic phase of stroke. Patients with milder corticospinal tract injury had greater gains from treatment. Reproduced with permission from Riley et al.123
### Table 1

<table>
<thead>
<tr>
<th>Associated Region</th>
<th>Nearest Gene(s), Gene Symbol</th>
<th>Mechanism of Action</th>
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<tr>
<td><strong>Ischemic Stroke</strong></td>
<td></td>
<td></td>
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<tr>
<td>4q25</td>
<td>Paired-like homeodomain 2, PITX2</td>
<td>Involved in cardiac development; required for asymmetric morphogenesis of the heart and development of pulmonary myocardium.</td>
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<td>8p23</td>
<td>Rho guanine nucleotide exchange factor (GEF) 10, ARHGEF10</td>
<td>A gene product specifically activates RhoA.</td>
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<td>9p21.3</td>
<td>Antisense noncoding RNA in INK4/ARF locus, NRIL; cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4), CDKN2A; cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4), CDKN2B</td>
<td>The locus is intergenic but thought to be involved in regulating the expression of many genes. The locus has been associated with platelet reactivity and forearm vasodilator reactivity.</td>
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<td>11q12</td>
<td>Angiotensin receptor like-1, AGTR1</td>
<td>Has a role in counter-regulating the rennin-angiotensin system.</td>
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<td>12p13</td>
<td>Ninjurin 2, NINJ2</td>
<td>Homophilic cell-cell adhesion molecule that interacts with matrix metalloproteinase.</td>
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<td>14q22</td>
<td>Protein kinase C, eta, PRKCH</td>
<td>May relate to symptomatic atherosclerosis; expression in vascular endothelial cells and foamy macrophages.</td>
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<td>Zinc finger homeobox 3, ZFHX3</td>
<td>Involved in cardiac development.</td>
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<td>22q13</td>
<td>Cadherin, EGF LAG seven-pass G-type receptor 1 (flamingo homolog, Drosophila), CELSR1</td>
<td>Homologue of CELSR2, which is associated with plasma LDL levels.</td>
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<td><strong>MRI-Defined Brain Infarct</strong></td>
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<td>20p12</td>
<td>MACRO domain containing 2, MACROD2 fibronectin leucine rich transmembrane protein 3, FLRT3</td>
<td>Macro domains bind ADP-ribose. Fibronectin leucine-ric transmembrane protein 3 is expressed in the brain and can promote neurite outgrowth after axonal injury in experimental models.</td>
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Abbreviations: ADP, adenosine diphosphate; LDL, low-density lipoprotein; MRI, magnetic resonance imaging; RhoA, Ras homolog gene family, member A.
<table>
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<tr>
<th>Nucleotide</th>
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