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Editorial Open Access

Identification of New Therapeutic Targets for Cerebral Ischemia by Genome-Wide Analysis

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Editorial

Stroke is a devastating condition afflicting mostly the elderly for which no viable medication exists to improve neuro-rehabilitation. In particular, great clinical benefit may accrue from deciphering and targeting basic neurobiological mechanisms underlying post-stroke CNS recovery both in structural and functional terms.

Clinical trials aimed at improving functional recovery after stroke has uniformly failed. One reason for this may be that very little genetic information is available describing the post-stroke events. Recent advances in genomics and DNA array technology may lead to the discovery of new therapeutic targets by uncovering the mechanisms underlying brain repair and regeneration after stroke. Several such studies have employed these techniques with the aim of identifying new therapeutic targets for stroke treatment. Some of these studies revealed changes in transcriptional activity of a variety of genes related to stress response, inflammation, acute- and delayed cell death in young rats [1,2], while later studies revealed a pathway associated with brain defense and tissue repair in a young mouse model of stroke [3] or neurovascular unit development genes [4]. Some other studies were concerned with the gene expression in the contralateral hemisphere, which suggested the presence of bilateral effects and/or differential regulation [5,6].

An important omission of these studies is that they did not include aged animals. The importance of animal age in the physiological response to stroke is emphasized by a recent study that identified an age-specific sprouting or regeneration transcriptome that differentially regulates the process of brain reorganization after brain infarct in young vs. aged animals [7]. However, the genomic response to stroke is not limited to axonal sprouting [7], but also includes physiologic, metabolic, apoptotic, immunologic, proliferative, developmental, angiogenic and wound healing processes that are of equal importance to neurological rehabilitation. In a study directed at elucidating the role of some of these additional processes, employed custom DNA arrays containing genes related to hypoxia signalling, DNA damage and apoptosis, cellular response to injury, axonal damage and re-growth, cell differentiation, dendritogenesis and neurogenesis [5]. They showed an age-related unfolding of genetic events in the contralateral, undamaged hemisphere of post-stroke aged rats, which differed from that seen in young animals.

Despite the obvious clinical significance of post-stroke angiogenesis in aged subjects, a detailed transcriptomic analysis of post-stroke angiogenesis has not yet been undertaken in an aged experimental model. Therefore the logical next step was to extend previous work by taking advantage of recent developments in rat genomics, by performing a whole-genome transcriptomic analysis of the perilesional infarct during the acute- and recovery phases following stroke [8]. Further, through data mining and one-by-one gene function search in the context of the pathophysiology of stroke, Buga et al., [9] identified 161 new.

Most of the changes observed are active responses, only a smaller group showed inhibitory responses and reduced expression. In the aged brains, four genes related to neuropathic syndrome, stress, anxiety disorders and depression (*Acvr1c, Cort, Htr2b and Pnoc*) may have impaired response to stroke. New therapeutic options in aged rats may also include *Calcrl, Cyp11b1, Prcp, Cebpa, Cfd, Gpnmb, Fcgr2b, Fcgr3a, Tnfrsf26, Adam 17 and Mmp14*. An unexpected target is the enzyme 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 in aged rats, a key enzyme in the cholesterol synthesis pathway. Post-stroke axonal growth was compromised in both age groups [9].

"New-for-stroke" genes that were linked to the increased vasculature density in young animals included *Angpt2, Angptl2, Angptl4, Cib1, Ccr2, Col4a2, Cxcl1, Lef1, Hhex, Lamc1, Nid2, Pcam1, Plod2, Runx3, Scpep1, S100a4, Tgfbi and Wnt4*, which are required for sprouting angiogenesis, reconstruction of the basal lamina and the resolution phase. The vast majority of genes involved in sprouting angiogenesis (*Angpt2, Angptl4, Cib1, Col8a1, Nrp1, Pcam1, Pttg1ip, Rac2, Runx1, Tnp4, Wnt4*); reconstruction of a new basal lamina (*Col4a2, Lamc1, Plod2*) or tube formation and maturation (*Angpt1, Gpc3, Igfbp7, SPARC, Tie2, Tnfsf10*), had however, a delayed upregulation in the aged rats. The angiogenic response in aged rats was further diminished by the persistent upregulation of "inflammatory" genes (*Cxcl12, Mmp8, Mmp12, Mmp14, Mpeg1, Tnfrsf1a, Tnfrsf1b*) and vigorous expression of genes required for the build-up of the fibrotic scar (*Cthrc1, Il6ra, Il13ar1, Il18, Mmp2, Rassf4, Tgfb1, Tgfbr2, Timp1*). Beyond this barrier angiogenesis in the aged brains was similar to that in young brains. It is hoped that some of them may be targets for development of stroke therapies [9,10].

The literature on gene expression profiles after stroke in humans is limited. In this regard, Vikman and Edvinsson [11] have shown similarities in gene expression profiles between human strokes and those in animal models, and reported new genes that support the dynamic changes that occur in the middle cerebral artery branches supplying the ischemic region. Also, promising results of blood genomic profiling in human stroke have been obtained in pilot studies [12-14]. These results argue for the utility of proangiogenic therapies in stroke, space given the potential beneficial effects consisting of increasing blood flow, decreasing infarct size, and supporting the restoration and recovery of neurovascular networks after ischemia 15]. However, recently it

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has been reported that the aged human brain is capable of mounting a vigorous angiogenic response after stroke, which most likely reflects the remaining brain plasticity of the aged brain [10].

Methods

RNA integrity for the the hybridization experiments of RNA pools was assessed with the RNA 6000 nano kit using the Bioanalyzer 2100 instrument (Agilent, Böblingen, Germany).

RNA integrity numbers ranged between 6.5 and 8.2. Two hundred nanograms of each sample were processed with the whole transcript (WT) expression kit (Ambion, Darmstadt, Germany), i.e., subjected to RNA amplification via reverse transcription to double-stranded cDNA and subsequent *in vitro* transcription; this was followed by another round of reverse transcription yielding single-stranded DNA in sense orientation. Hybridization cocktails were produced after fragmentation and biotin labeling of target DNAs following the protocol of the GeneChip WT terminal labeling kit (Affymetrix, Santa Clara, CA, USA). Microarray hybridization to GeneChip Rat Gene arrays (Affymetrix) was performed according to the manufacturer's protocol using the Fluidics Station 450 with the program FS450_0007. CEL files from scanned microarrays were produced with the expression console (Affymetrix).

Microarray Evaluation

Consistently high quality microarray data was ensured by visual inspection of scanned images for hybridization artifacts and correspondence analysis (COA) of rawand normalized microarray data. Normalizations were performed with the Quantiles method [16]; background correction and probe set summary were achieved with robust microarray average (RMA) [17]. Differentially expressed genes were determined by comparing 3 days post-stroke vs. naive and 14 days post-stroke vs. naïve. These comparisons were done separately for young and aged animals. The false discovery rate (FDR) of differential expression for the described comparisons was estimated with an empirical Bayes methodology employing lognormal normal data modeling [18] as previously described [9].

Results were confined to probe sets belonging to gene ontologies (GO) related to pathophysiological events after stroke. Expression values thereof were subjected to agglomerative hierarchical clustering and results were displayed as a heat map. The effect of stroke, post-stroke time, and age of rats was quantified by the Eigenvalues determined from a COA. In brief, COA is an ordination method that performs its ordination simultaneously on column (sample) and row (probe set) scores such that dependencies between data points become evident. The COA visualization shows samples and probe sets in the same coordinate system, typically made up of the two most informative axes describing independent variation in the dataset. Due to the unsupervised nature of the COA, there is no predefined axis notation. All analyses were Q4 performed in R version 2.14.01 along with Bioconductor2 packages affy (for data import and pre-processing), made (for COA and heatmap), and EBarrays (for detection of differential expression). Genes of interest were checked by RT-PCR.

Conclusions

To date, all mono-therapeutic attempts to prevent or lessen brain damage following stroke have failed. In view of our findings that stroke impacts a wide range of systems in an age-dependent manner, from CNS physiology to CNS regeneration and plasticity, the failure of therapies aimed at only a single target system is perhaps inevitable. Recent data suggests that a multi-stage, multimodal treatment in aged animals may be more likely to produce positive results. Such a therapeutic approach should be focused on tissue restoration but should also address other aspects of patient post-stroke therapy such as neuropathic syndrome, stress, anxiety disorders, depression, neurotransmission and blood pressure.

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