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Age-specific transcriptional response to stroke

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ABSTRACT

Increased age is a major risk factor for stroke incidence and post-ischemic mortality. To develop age-adjusted therapeutic interventions, a clear understanding of the complexity of age-related post-ischemic mechanisms is essential. Transient occlusion of the middle cerebral artery—a model that closely resembles human stroke—was used to induce cerebral infarction in mice of 4 different ages (2, 9, 15, 24 months). By using Illumina cDNA microarrays and quantitative PCR we detected a distinct age-dependent response to stroke involving 350 differentially expressed genes. Our analyses also identified 327 differentially expressed genes that responded to stroke in an age-independent manner. These genes are involved in different aspects of the inflammatory and immune response, oxidative stress, cell cycle activation and/or DNA repair, apoptosis, cytoskeleton reorganization and/or astrogliosis, synaptic plasticity and/or neurotransmission, and depressive disorders and/or dopamine-, serotonin-, GABA-signaling. In agreement with our earlier work, aged brains displayed an attenuated inflammatory and immune response (Sieber et al., 2011) and a reduced impairment of post-stroke synaptic plasticity. Our data also revealed a distinct age-related susceptibility for post-ischemic depression, the most common neuropsychiatric consequence of stroke, which has a major influence on functional outcome.

1. Introduction

Worldwide, the population is aging. As more people live to old age, the prevalence of age-related diseases will increase. Increased age is a major risk factor for stroke incidence, post-ischemic mortality, and severe and long-term disability (Chen et al., 2010; Denti et al., 2009; Liu et al., 2009). Although stroke remains an important health issue, most pharmaceutical companies have terminated their research programs because of the failure of previous studies (Gladstone et al., 2002; O'Collins et al., 2006). The preferred use of young rodents in preclinical stroke research might partly explain the inability to transfer promising experimental results to the clinic (Chen et al., 2010). Thrombolysis with recombinant tissue plasminogen activator is currently still the only accepted therapeutic option and, moreover, only applicable in a narrow time window following stroke (Murray et al., 2010; Wahlgren et al., 2008).

Fortunately, in the last decade, more preclinical studies focused on stroke outcome have incorporated aged animals. Post-ischemic aged rodents exhibit marked neurologic deficits and limited functional recovery (Brown et al., 2003; DiNapoli et al., 2008). Aged brains respond to stroke with an altered infarct progression, glial reaction, axonal sprouting, and inflammation, less edema formation, an increased blood-brain barrier permeability, and a diminished anti-oxidant capacity (Buga et al., 2008; Chen et al., 2010; Dinapoli et al., 2010; Liu et al., 2009; Petcu et al., 2008; Sieber et al., 2011). Although post-ischemic mechanisms are complex, most previous studies focused on one specific age-related aspect of the disease. Furthermore, in general, investigations were centered on only one young versus one aged group. To overcome the barriers between preclinical and clinical studies and to develop age-adjusted therapeutic interventions following stroke, a clear understanding of the complexity of age-related post-ischemic mechanisms is necessary.

In this study, we aimed to identify age-dependent cerebral transcriptional responses following stroke in mice. By incorporating 4 different ages (2, 9, 15, and 24 months), we monitored transcriptional changes to stroke over the lifespan of the mouse.

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Occlusion of the middle cerebral artery (MCAO)—a model closely resembling human stroke—was used to induce cerebral infarction in mice (Carmichael, 2005; Sieber et al., 2008). Ischemic brain regions at 2 different reperfusion times (2 and 7 days) were subjected to microarray analysis.

2. Methods

2.1. Stroke induction

Stroke was induced in male C57Bl/6 mice at different ages [adult, 2-month-old; middle-aged, 9-month-old; and aged 15 to 24 month-old] by a transient (30 minutes) occlusion of the middle cerebral artery as previously described (Sieber et al., 2008). Sham-operated mice of equivalent ages underwent the surgical procedure without occlusion of the middle cerebral artery. All surgeries were performed under deep anesthesia with 2.5% isoflurane in a N_2O:O_2 (3:1) mixture. All animal procedures were approved by the local government (Thueringer Landesamt für Lebensmittelsicherheit und Verbraucherschutz, Germany) and conformed to international guidelines on the ethical use of animals.

2.2. Sample preparation

Mice were sacrificed by cervical dislocation with subsequent brain removal on day 2 and 7 after reperfusion. Using a Precision Brain Slicer (BS-2000C Adult Mouse; Braintree Scientific, Inc, USA), coronal sections (2-mm thickness) comprising the infarct (bregma +0.8 to −1.2 mm) (Paxinos and Franklin, 2001), were separated into the ipsi- and contralateral hemisphere (Supplementary Fig. 1). Adjacent slices were used for infarct validation by vital staining (2% TTC in 0.9% NaCl at 37 °C for 10 minutes). Young and aged mice involved in this study exhibited similar lesion sizes. Each age (2, 9, 15, and 24 months) and reperfusion group (2 and 7 days) comprised 4 individual biological samples, which were separately processed. Therefore, 64 samples were hybridized on Illumina complementary DNA (cDNA) microarrays (4 ages × 2 reperfusion times × 4 mice × 2 hemispheres). Different littermates as well as mice of different cages were taken.

2.3. Gene expression profiling using Illumina cDNA microarrays and data processing

RNA was isolated with the RNeasy Lipid Tissue Mini Kit (Qiagen, Germany). RNA was quantified spectrometrically with an ND-1000 (NanoDrop, Thermo Scientific, Germany) and RNA integrity was determined on a denaturating agarose gel. RNA quality was further examined on an Agilent 2100 Bioanalyzer using the RNA 6000 LabChip Kit (Agilent Technologies, USA). A RNA Integrity Number value of >7 was considered to be of good quality for array profiling. RNA samples were reversely transcribed and amplified using the Illumina TotalPrep RNA Amplification Kit (Ambion, USA). All cRNA samples were quantified with the NanoDrop spectrophotometer. Hybridization to microarrays containing more than 45,200 bead types (unique probe sequences) (MouseWG-6 Expression BeadChip [MouseWG-6_V2_0], Illumina, USA) was performed according to Illumina's Gene Expression on Sentrix Arrays Direct Hybridization System Manual. Hybridized arrays were stained with Streptavidin-Cy3 (FluoroLink Cy3, GE Biosciences, Germany). BeadChips were scanned (Illumina BeadArray Reader) according to the protocol described in Illumina's Whole Genome Gene Expression for BeadStation Manual v3.2, Revision A. Data obtained by the BeadStudio data analysis software (BeadStudio Gene Expression Module v3.2) were processed to .txt files, which were then imported into the statistical software R using Bioconductor package (Du et al., 2008).

Microarray data was processed using the statistics software R (Team, 2008) and Bioconductor (Gentleman et al., 2004). After careful quality control of raw bead level and bead summary data, the raw bead summary data were normalized by variance-stabilizing normalization (Huber et al., 2002) leading to log2-transformed, normalized expression values. In the next step the 18,561 detected bead types (detection p value <0.01 on at least 2 beadarrays) were selected and statistically significant differentially expressed genes (DEGs) were identified by repeated measures 2-way analyses of variance with interaction (false discovery rate-corrected p-values smaller than 0.01) at day 2 as well as at day 7 of reperfusion. Afterwards fold changes were calculated between the ipsi- and contralateral hemisphere of MCAO-treated mice ($n = 4$ per age) for the significant bead types.

Ratios (MCAO ipsi vs. contra) were correlated with age (2, 9, 15, and 24 months; Spearman correlation). Subsequently, genes which showed a mean fold change of at least 1.5 and 0.6 were assorted as age-independently ($p \geq 0.05$ and $0.6 \geq R \geq -0.6$) or age-dependently ($p \leq 0.05$ and $0.6 \leq R \leq -0.6$) regulated. Transcripts were assigned as age-dependently regulated when the required criteria were met at one reperfusion time, which means that age-independently regulated genes are not correlated with age at any reperfusion time. Genes responsible for the age-independent "universal" reaction and genes responsible for the age-specific response after stroke were ascribed to different functional categories based on Ingenuity Pathway Analysis, Gene Ontology terms (biological processes and molecular functions) as well as on the biomedical literature (NCBI, PubMed).

2.4. Quantitative polymerase chain reaction

Equal amounts of total RNA (same samples as used for Illumina cDNA microarrays, n = 4 mice) were transcribed into cDNAs (Fermentas). PCR was performed with Brilliant III SYBR Green QPCR Mastermix (Agilent Technologies) and specific mouse primers at a final concentration of 500 nM (Supplementary Table 1). Amplification was performed using Qiagen's Rotor-Gene 6000 cycler applying the following cycle conditions: 3 minutes polymerase activation, 40 amplification cycles (95 °C for 10 seconds, 60 °C for 15 seconds). In a previous study (Sieber et al., 2008), we confirmed *Gapdh* and *Hmbs* as suitable housekeeping genes in aged ischemic mice. Transcript ratios (ipsi vs. contra) were calculated using the Pfaffl equation (Pfaffl, 2001) and correlated with age (2, 9, 15, and 24 months; Spearman correlation: $p \leq 0.05$ and $0.6 \leq R \leq -0.6$).

3. Results

3.1. Age-related gene expression after stroke

Preprocessing of the data of mice whole-genome beadarrays generated a list of 18, 561 cerebral-expressed transcripts. Subsequent analysis of variance detected 2305 (day 2) and 2873 (day 7) DEGs, representing 5.1% and 6.3% of all bead types on the beadarray, respectively. When calculating the identified DEGs with respect to the complete mouse genome 11% (2305 DEGs/day 2) and 14% (2873 DEGs/day 7) were changed (Church et al., 2009). The dendrogram displayed a clear effect of the MCAO to ipsilateral gene expression by hierarchical clustering (Supplementary Fig. 2).

In agreement with previous studies (Buga et al., 2012; Mitsios et al., 2007), most of the affected transcripts (~85%) were upregulated after stroke.

We revealed a distinct age-dependent response to stroke involving 350 DEGs, of which 81% were upregulated (Fig. 1A, Supplementary Table 2). The transcriptional response changed with