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Research paper

Expression of CGRP neurotransmitter and vascular genesis marker mRNA is age-dependent in superior cervical ganglia of senescence-accelerated prone mice

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ABSTRACT

Calcitonin gene-related peptide (CGRP) is a neurotransmitter that is released from the superior cervical ganglion (SCG) and causes head and neck pain. The morphological properties of human SCG neurons, including neurotransmitter content, are altered during aging. However, morphological changes in CGRP in the SCG during aging are not known. Therefore, we investigated CGRP and other markers in the SCG during aging in an aging model of senescence-accelerated prone mouse (SAMP8) and senescence-accelerated resistant mice (SAMR1) using real-time RT-PCR mRNA analyses and *in situ* hybridization. The abundance of neurotransmitter (CGRP, NPY, TRPV1), vascular genesis marker (CD31, LYVE-1), and cytochrome C mRNA differed between 12-week-old and 24-week-old SAMP8 and SAMR1. Abundance of TRPV1, CD31 and cytochrome C mRNAs of SAMP8 decreased between 12- and 24-week-old. The ratio of CGRP mRNA positive cells and CGRP mRNA abundance levels of the SCG of aging mouse such as SAMP8 have already been also higher than that of SAMR1 at 12-week-old. The CGRP positive shrunken ganglion cells was increased from 12- to 24-weeks-old mouse in SAMR1 and SAMP8. The SCG primarily affected the internal and external carotid arteries, larynx thyroid gland, and pharyngeal muscle during aging.

1. Introduction

Senescence-accelerated prone mice (SAMP) are a model of accelerated senescence [40]. SAMP8 exhibit accelerated aging with a shortened life span and increased amyloidosis [28]. Various reports using the senescence-accelerated model mouse clarified that the underlying accelerated senescence and etiopathogenic mechanisms in age-associated pathobiologies [35,41] include proteasome activation [3] and increased oxidative stress [26]. The morphological properties of human intracardiac ganglia are altered during aging [14]. The mean frequency of respiratory rhythms is not significantly different during aging in rats [16]. Stellate ganglion neuron properties are not altered significantly [18], but superior cervical ganglion (SCG) neurons are not constant [23]. Transient receptor potential vanilloid 1 (TRPV1), a ligand-gated ion channel, is expressed in nociceptor nerve terminals and plays a major role in detecting noxious stimuli [31]. TRPV1 upregulates CGRP expression in neurogenic inflammation in DRGs [12,32]. Neuropeptide Y (NPY) is an important sympathetic neurotransmitter, but two rat strains exhibited no NPY response from 12 to 24 months in a previous study [8]. Therefore, whether the morphological properties of SCG neurons are altered during aging is not clear. The SCG sends numerous branches in various directions and organs in elderly humans and contains numerous shrunken neurons and various sizes of blood vessels [27]. Stellate ganglion blocks are performed for chronic pain avoidance *via* sympathetic tone. Dysfunction of the sympathetic autonomic nervous system is important during aging. Neurites containing numerous vacuoles were confined to the SCG and increase in frequency with aging [38]. Age-related morphological and neurochemical changes in the human SCG include larger neuron body, cytoplasm and nucleus areas, an increased amount of lipofuscin, a greater number of large-sized neurons, and decreased numbers of neurofilament-positive neurons and myelinated fibers. These morphological changes influence the deterioration of neuronal functional capacity, neuronal plasticity, and regenerative characteristics in the human SCG [21]. The number of neurons in the SCG and the density of noradrenergic innervation in target organs, such as the iris and the submandibular gland, were not significantly different in 4- and 24-month-old rats [36]. Significant reductions in soma size, total dendritic length, number of branch points

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and total area of dendritic arborization were also found in old age [1]. Therefore, we need to clarify the morphological properties of the SCG with stellate ganglion neurons during aging. The localization of calcitonin gene-related peptide (CGRP) is similar to neurotransmitters, and its release from the SCG produces head and neck pain in the area innervated by the SCG. CGRP is localized near nociceptive receptors, and it affects vessels [37] and tensor tympani muscles [44]. CGRP-like immunoreactivity in mesenteric and femoral arteries and increased vasodilation were found during age-related alterations in circulatory hemodynamics [20]. Various morphogenesis markers, such as angiogenesis markers vascular endothelial growth factor-A (VEGF-A), PECAM (CD31), and lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1), regulate formation of the masseter muscle (MM) during gestational stages. CGRP affects the capability of VEGF-A [45]. Therefore, CGRP may also affect ganglion cells, chronic pain and vascular smooth muscles of the SCG during aging. Expression of vanilloid receptors in sympathetic neurons decreases substantially during development in the rat nodose ganglion of the vagus nerve, spinal ganglia, autonomic neurons, sympathetic ganglia of cranial cervical nerves and stellate and celiac sympathetic ganglia containing calbindin [17,24]. Other neurotransmitters and vascular genesis in the SCG are also altered during aging. Therefore, we need to investigate changes in CGRP and other neurotransmitters in the SCG during aging.

2. Materials and methods

2.1. Animals

All laboratory animals were purchased and processed according to the provisions of the experimental animal committee of Genostaff Co., Ltd. (Tokyo, Japan). SAMP8 and senescence-accelerated resistant mice (SAMR1) were used at 12 and 24 weeks of age. Fresh SCGs were prepared from these two groups. Tissue from each stage (n = 6) was used for light microscopy studies and mRNA analyses using real-time RT-PCR and *in situ* hybridization.

2.2. Real-time RT-PCR mRNA analyses

2.2.1. Isolation of total RNA

SCGs were immediately removed from sacrificed mice *via* scraping and stored at −80 °C. The muscles were cut into small pieces, and total RNA was isolated from the samples (0.1 g). Total RNA was extracted using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Contaminating DNA was removed using RNase-free DNase (DNA-free; Ambion, TX, USA), and total RNA absorbance at 260 nm was quantified using a spectrophotometer (Biowave S2100; Cambridge, UK). The samples were stored at −80 °C until use. Total RNA was converted to cDNA using 0.4 μM random hexamers (N808-0127; Applied Biosystems, CA, USA) in a mixture containing 1 mM of each dNTP, 20 units of RNase inhibitor (2311A; TaKaRa, Tokyo, Japan), 5 units of AMV reverse transcriptase XL (2620A; TaKaRa), 25 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM DTT, and 5 mM $MgCl_2$. The following thermal PCR cycling conditions were used: 30 °C for 10 min, 42 °C for 30 min, 90 °C for 5 min, and 5 °C for 5 min.

2.3. Quantitative real-time RT-PCR

Quantitative real-time RT-PCR was performed using the Applied Biosystems 7300 Fast Real-Time PCR System (Applied Biosystems, CA, USA) according to the manufacturer's instructions. Each amplification mixture (50 μl) contained 100 ng of cDNA, 900 nM forward primer, 900 nM reverse primer, 250 nM fluorogenic probe, and 25 μl of Universal PCR Master Mix (Applied Biosystems, CA, USA). The PCR cycling parameters were 50 °C for 2 min, 95 °C for 10 min, 50 cycles at 95 °C for 15 s, and 60 °C for 1 min. The following sequences were amplified: CGRP (calcitonin/calcitonin-related polypeptide alpha; Mm00801463 g1, Applied Biosystems, CA, USA), TRPV1 (transient receptor potential cation channel, subfamily V, member 1; Mm01246282_m1, Applied Biosystems), NPY (neuropeptide Y; Mm01410146_m1, Applied Biosystems), VEGF-A (vascular endothelial growth factor A, Mm00437304_m1, Applied Biosystems, CA, USA), platelet/endothelial cell adhesion molecule 1 (CD31) (Mm01242584_m1, Applied Biosystems, CA, USA), LYVE–1 (Mm00475056_m1, Applied Biosystems) and cytochrome C (cytochrome c oxidase, subunit Vb,Mm00833840_g1 Applied Biosystems). TaqMan Rodent GAPDH Control Reagents with VIC Probe were also used (Applied Biosystems). The abundance of the amplified mouse cDNA was normalized to GAPDH (rodent GAPDH primers and probes were obtained from "Assays-On-Demand", Applied Biosystems).

2.4. Statistical analysis

Differences in SCG measurement data were assessed using two-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test with one categorical independent variable and one continuous variable (the independent variable may consist of a number of groups). The level of significance was set as $p < 0.05$. The results are reported as the means ± SD. Statistical analyses were performed using the IBM SPSS Statistics Base, version 22.

2.5. Histological observations

The total length of the SCG was defined by the presence or absence of the origin of nerve axons from sagittal serial sections of the SCG. Paraffin-embedded blocks and sections of the SCG for histochemistry were obtained from Japanese donor cadavers. SCGs were fixed with Tissue Fixative (Genostaff Co., Ltd.), embedded in paraffin using Genostaff's proprietary procedures and sectioned at approximately 5 μm. The median sagittal and horizontal tissue sections were deparaffinized in xylene and rehydrated through a series of ethanol solutions in PBS. The sections were stained with Mayer's Hematoxylin and Eosin (Muto, Tokyo, Japan), dehydrated and mounted with malinol. The stained sections were evaluated using microscopy (DM-2500; Leica Microsystems, Germany). We analyzed SCG neuron bodies (long axis) and shrunken neurons in each section using image-analyzing computer software, which modified the measurement methods of Mituoka et al. [27].

2.6. In situ hybridization

A 272-bp DNA fragment corresponding to nucleotide positions 624-895 of mouse CGRP (GenBank accession number NM001033954.3) was sub-cloned into the p-GEMT-Easy vector (Promega, USA) to generate sense or anti-sense RNA probes. Paraffin-embedded intestine sections (6 μm) of 12 and 24 weeks of age SAMP8 and SAMR1mice were obtained from Genostaff Co., Ltd. They were dissected, fixed with Tissue Fixative (Genostaff Co., Ltd.), embedded in paraffin according to their proprietary procedures, and sectioned at 6 μm. Tissue sections were deparaffinized with xylene and rehydrated through a graded ethanol series and PBS. The sections were fixed in 10% NBF (10% formalin in BS) for 15 min at RT and washed with PBS. The sections were treated with 4 μg/ml proteinase K in PBS for 10 min at 37 °C, washed with PBS, re-fixed with 10% NBF for 15 min at RT, washed again with PBS, and placed in 0.2 N HCl for 10 min at RT. Sections were washed with PBS and placed in 1 x G-WASH (equal to 1 x SSC). Hybridization was performed with probes at 300 ng/ml in G-Hybo (Genostaff Co., Ltd.) for 16 h at 60 °C. Sections were washed in 1 x G-WASH for 10 min at 60 °C and 50% formamide in 1 x G-WASH for 10 min at 60 °C. The sections were washed twice with 1 x G-WASH for 10 min at 60 °C, twice with 0.1 x G-WASH for 10 min at 60 °C, and twice with TBST (0.1% Tween 20 in TBS) at RT. Sections were treated with 1 x G-Block (Genostaff Co.,