

Changes of Neurofilament Content of Neurons as Consequences of Double-Strand DNA Breaks in Aging

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Abstract

Neurofilaments are the major structural proteins of neurons. Since DNA damage and repair are important factors in the transcriptional process the present study was aimed to examine age-related changes of Neurofilaments and double-strand DNA breaks in the neuronal preparations from 3 days, 3 months and 30 months rat brain. The relative integrity of the cells was confirmed by lactate dehydrogenase activity. The concentrations of neurofilament in the frozen-thawed cells were measured by enzyme-linked immunosorbent assay. The rate of double-strand DNA breaks was determined directly using an ethidium bromide solution as a binding fluorescent dye. The activity of neuronal lactate dehydrogenase activity was similar for all ages. The neurofilament content of neurons increased during development of rat brain and decreased in the neuronal preparations of aging rats, whereas the amount of double-strand DNA breaks increased only in the neurons of aging animals. The opposite relationship between the amount of double-strand DNA breaks and the neurofilament content of the neurons in aging is consistent with the susceptibility of the neuronal DNA integrity in aging brain. It is suggested that the decrease of the neurofilament content of the neurons may be a causative factor of neuronal DNA damage in aging.

Keywords: Aging; Brain; DNA damage; Neuron; Neurofilament

Introduction

Neurofilament proteins (NFPs) are the main constituent of intermediate filaments that form some parts of the neuron cytoplasmic cytoskeleton. NFs support neuronal shape and facilitate the transport of particles and organelles within the cytoplasm. These proteins are important both for the maturation of axons and the maintenance of axonal integrity [1,2]. NFPs are particularly abundant in neurons with large diameter axons such as those of periphery motor neurons controlling skeletal muscle, where fast impulse conduction velocities are crucial for proper functioning [3]. Alterations of NFPs are known as a common pathologic feature of a variety of human neurodegenerative diseases particularly those related to aging [4-6]. These cytoskeletal elements have been shown to contribute to the formation of the neurofibrillary changes observed during normal aging [4-6]. Numerous publications have documented increased levels of damaged DNA and mutation frequency in aged organs [7,8]. Age-related changes in the metabolism of nucleic and gene regulation and the occurrence of DNA damage and repair have been extensively investigated. A number of evidences are in favor of significant changes in process of temporal chromatin disassembly and DNA interruptions, mainly double strand DNA breaks [9]. It is widely claimed that the aging process is characterized by a decrease in the ability to repair DNA damages. Errors in DNA repair can cause mutations and chromosome instability that lead to cell death [10]. While most DNA damages can undergo DNA repair, such repair is not relatively well-organized and a number of un-repaired DNA damages have been shown to accumulate in aging brain, including double-strand breaks and modified bases in non-replicating cells [11,12]. Furthermore, changes in the cellular content of tRNA are demonstrated to disturb protein synthesis in aged brain [14]. Although Neurodegenerative disorders are characterized by extensive neuron death that leads to functional decline, but the

neurobiological correlates of functional decline in normal aging are less well defined, thus, it is of great interest to clarify the influence of double strand DNA breaks on NFPs of aging brain. Since NFPs have been shown to be exclusively concentrated in the neuronal fraction [15] the present study undertaken to explore the effects of aging on the number of double-strand DNA breaks and the concentrations of NFP, at the cellular level of the neuronal fraction isolated from rat brain.

Materials and Methods

Animals

Male Wistar rats new born 3 days old, adults 3 months old and aged 30 months old were used and fed ad libitum; drinking water was always available. Animals were maintained with respect to the animal welfare regulation in animal house to the desired age was attained.

Chemicals

Polyvinylpyrrolidone (PVP, mol. Wt. 40,000), NADH, pyruvate, Tween-20, Triton X-100, neurofilaments (low molecular weight, BM68), DNA and ethidium bromide were purchased from Sigma Chemical Company (U.K.). Anti-neurofilament antibody and anti-mouse IgG peroxidase were obtained from Pierce Company (France). Immunoplates were purchased from NUNC (France). 5-Amino-salicylic acid was obtained from OSI Distributor (France). All other reagents used were of the highest purity commercially available and double glass-distilled water was used in the preparation of all solutions.

Isolation of Neuron

Neurons were isolated from rat brain, according to the method of Johnson and Sellinger [16]. In each experiment, 2-6 rats (about 2g wet. tissue) were killed by decapitation and the brains were removed on an ice-cooled glass plate over crushed ice and chopped into the consistency of a mince. The tissue was disrupted in 8 ml of suspending medium (7.5% (w/v) PVP, 1% BSA and 10mM CaCl₂) by passing the suspension up and down 15 times through a 1 mm nylon mesh cemented to the end of a 10 ml syringe from which the needle support had been removed. After this procedure, the disrupted brain tissue was suspended in 40 ml of suspending medium. The cell suspension was then passed three times through 80 μ m stainless steel screen using the disposable syringe. After 20 min, 5 ml of the resulting crude cell suspension was layered over a two-step gradient consisting of 5 ml 1.75M sucrose from the bottom-up and 6 ml of 1.0M sucrose containing 1% (w/v) BSA above, then centrifuged at 41 000g for 30 min. Purified neuron was obtained as a pellet in 1.75 M sucrose. The cells were washed three times using 0.32 M sucrose solution. The morphological characteristics of the cell preparations were confirmed by Immunocytochemistry (Figure 1), and the structural integrity of the cell was assessed by the neuronal lactate dehydrogenase (LDH) activity.



Figure 1: Immunocytochemistry of isolated neurons. Neurons were fixed with paraformaldehyde and then immunostained by specific antibodies.

Assay of LDH

Lactate dehydrogenase latency was estimated before and after the neuronal suspension was exposed to Triton X-100 (0.5% v/v). Reaction was made by the addition of pyruvate and oxidation of NADH (0.2 mM) at 340 was monitored against a blank containing all compounds except pyruvate [17]. Proteins were determined by the method of Lowry, *et al.* [18] with BSA as the standard.

Determination of double strand DNA breaks

Double strand DNA breaks were investigated using the method described by Daniel, *et al.* [19], which linked the number of strand breaks by the rate of unwinding. The cell lysates were exposed to alkaline solution and the rate of strand unwinding was determined directly using an ethidium bromide solution (6.7 ug/ml of 13.3 mM NaOH) as a specific double strand DNA binding fluorescent dye. The fluorescence was monitored at excitation of 520 nm and emission of 590 nm (Perkin-elmer, LSE spectrophotofluorimeter).

ELISA determination of neurofilament

Enzyme-linked immunosorbent assay (ELISA) was performed to assess the NF contents of the neuronal preparations [20]. The frozen-thawed sample was diluted to 25 ug protein/ml in 50 mM bicarbonate buffer pH 8.5. In each immunoplate well 200 ul of the homogenate was added and allowed to bind to the plate overnight at 4°C. The following day the wells were washed three times with the buffer and then incubated with 1 % BSA for 1 h to block excess protein binding sites. The wells were washed with 0.1% Tween-20 in the bicarbonate buffer. Anti-neurofilament antibody was added at an optimal dilution (1:100) and allowed to stand at room temperature. After 3 hours the wells were washed three times with bicarbonate/Tween-20 and then incubated with a 1:500 dilution of rabbit anti-mouse IgG peroxidase conjugated for 2 h at room temperature. After three final wash steps, the wells were incubated with 50 mM 5-amino-salicylic acid and 5 mM H₂O₂ in a 0.1 M sodium acetate solution pH 5.5. The reaction was stopped with 100 ul sodium hydroxide and the absorbance of each well was determined at 450 nm in a micro-ELISA reader. The absorbance was linear with the time for the first 20 min of the peroxidase-catalyzed reaction and also with the protein concentrations of 100 ug/ml protein. Duplicate wells were performed for each sample, and the mean absorbance was used in subsequent calculations. On each plate, standards of NF in a concentration range of 50 to 1000 ng/ml were used.

Results

The data summarized in Table 1 can be analyzed from three different perspectives: (a) the relative integrity of the neuronal preparations, (b) percentage of double strand DNA breaks of the neuronal suspensions of different ages, and (c) the NF content of the cell preparations in different ages. As shown in Table I the LDH latency, given by apparent LDH activity in the present of Triton X-100/apparent LDH activity in its absence, is similar for all neuronal preparations of different ages. The results included in Table I also show that there is no significant changes in percentage of double strand DNA breaks in the neurons of new born (3 days old) and adult (3 months old) animals, whereas the percentage of unwinded DNA in the neurons of aged animals (30 months old) was approximately threefold as compared to that of adult animals (P < 0.005). The NF content of the neuronal preparations showed significant alteration during aging. The highest concentrations of the NFps proteins were recorded in the adult rat brain (6.5 ± 0.3 ug/mg neuronal protein), whereas NFps concentrations in the neuronal preparations from newborn and aged animals were about 40% and 60% of that of adult animals respectively.

Age	Ratio of LDH activity	% double strand DNA breaks	Neurofilament content ug/mg protein
3 days	19.1 ± 1.7	7.4 ± 0.6	2.4 ± 0.4•
3 months	18.8 ± 1.4	7.9 ± 0.6	6.5 ± 0.3
30 months	18.5 ± 1.9	21.0 ± 1.6 •	4.1 ± 0.3 •

Table 1: Effects of aging on double strand DNA breaks and Neurofilament concentrations in isolated rat brain neurons.

The ratio of LDH activity, percentage of double strand DNA breaks and neurofilaments content were assayed in aliquots of neuronal suspensions isolated from rat forebrain of different age. Results are mean \pm SEM of 6 separate experiments. The significance is expressed relative to adult rat (3 months old) neuron, • $P < 0.05$.

Discussion

The present data show that maximum levels of NF are present in the neurons isolated from adult rat brains. The lower amount of the proteins in the neuronal preparation of newborn animals is in good agreement with the developmental profile reported by Shaw and Weber [21]. Since NFPs play a crucial role in the formation of axons and dendrites during neuronal development and seem also to be important for the maintenance of the mature neuronal shape and function [1-3], the decline of the NFPs in the neurons of aging animals may reflect a kind of neuronal degeneration [6,22]. This is consistent with an increase of double strand DNA breaks in the neurons of aging animals (Table 1). Although there may have been some kind of cell damage during cell separations, the similar LDH activity of the neuronal preparations of different ages indicates similar integrity for all preparations. It is therefore quite unlikely that the preparation procedure had affected the rate of double strand DNA breaks. Because DNA damage may have serious consequences for a cell, the greater extent of double strand DNA breaks in the neurons of aging animals may be proposed as a causative factor for the lower levels of NFPs concentrations in the neurons of aging animals. In conclusion, the decreased neuronal NFPs content of aging animals is consistent with the age-related decline in brain function.

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