End joining activity of DNA double-strand breaks in the embryonic mouse brain

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(Received December 20, 2007)

Abstract

Double-strand breaks (DSBs) of DNA are introduced in neurons during embryonic brain development. Here I examine the DNA end joining activity to restore DSBs bearing distinct types of DNA ends in embryonic and adult brains. Embryonic brains contained the end joining activity for cohesive DNA ends. By contrast, adult brains showed no detectable end joining activity. The degree of the end joining activity seemed to be correlated with viability of neurons in culture. Thus, DNA repair for DSBs is likely to be involved in neuronal viability during brain development.

Keywords: DNA double strand breaks, DNA ligase, brain, development, neuronal survival

Introduction

Two lines of evidence have recently suggested that DNA repair plays a critical role in neuronal development and maturation. One is the experimental finding resulting from studies using mice lacking a gene for the DNA repair-associated molecule. For example, mice lacking DNA ligase IV, an enzyme involved in non-homologous DNA end joining, were embryonic lethal and exhibited massive neuronal cell death immediately after neurogenesis in the cerebral cortex (1,2). The second line of evidence is the identification of genes responsible for inherited genetic disease showing progressive neurodegeneration. Ataxia telangiectasia is characterized by progressive impairment of gait and speech, oculomotor apraxia,

oculocutaneous telangiectasia and cerebeller atrophy. These clinical manifestations have been demonstrated to be associated with mutations in the ATM gene, whose product plays a role in non-homologous DNA end joining (3). These findings provide us with idea that defective DNA repair leads to neuronal cell death, resulting in abnormal neuronal development or neurodegeneration (4-6).

DNA lesions are known to be induced by various undesirable stimuli, such as oxidative stress and chemical reagents. Particularly, the nervous system exhibits high aerobic metabolic activities and low anti-oxidative activities, thereby neuronal DNAs are easily damaged. These DNA lesions include single strand break (SSB) and double strand break (DSB) of DNAs (6). The mechanisms of DNA repair have been closely examined in various types of cells, and many factors and enzymes to detect, refine and rejoin SSBs or DSBs have been identified. However, a number of fundamental questions remained unresolved. Expression profiles of these factors in developing and mature brain are unknown. The reason why DSB repair occurs at the timing of neurogenesis is unclear. It is also unknown whether DSB-bearing neurons always die or partially survive. If the latter is the case, are such neurons normal in functions and integrated in the neuronal network?

The final aim of our research focusing on DNA repair in the nervous system is to resolve these questions and contributes to the better understanding of the neuronal development and the cause of neurodegenerative diseases. The present study attempts the measurement of DNA end joining activity during the brain development.

Materials and Methods

Preparation of substrates for end joining assay; The pbluescript/GFP-tubulin plasmid (5059 bp, see Fig. 1) was purified by using a Midi prep kit. The plasmid was then digested with SacI (for generating 3'-overhanging DSBs), XhoI (for 5'-overhanging DSBs), EcoRV (for blunt-end DSBs), or XhoI and EcoRV (for non-matching DSBs). Longer DNA fragments containing the backbone pbluescript and partial sequence of GFP-tubulin gene were separated from the rest of plasmid on agarose gel and purified using an agarose gel extraction kit.

Cell culture; Cerebral corticies from embryonic day-13 mice were dissected and dissociated by trypsin/EDTA (7).

Reaction was stopped by adding fetal calf serum and cells were washed with serum-free Opti-MEM twice. Cells were then plated on poly-lysine-coated 9-cm dishes (3.4×10^6/dish for low density culture, 1.9×10^7/dish for high-density culture) and placed in a CO_2-incubator for 3 hr.

Preparation of nuclear extracts; The cerebral corticies of embryonic mice or postnatal mice were dissected and homogenized in ice-cold homogenization buffer (10 mM Tris-Cl, 10 mM KCl, 10 mM $MgCl_2$, 10 mM dithiothreitol/DTT, pH 7.5). Cultured cells were harvested in phosphate-buffered saline using a scraper, collected by centrifugation at 300 x g for 5 min, and homogenized in the homogenization buffer. The homogenates were centrifuged at 1,000 x g for 5 min, and the pellets were resuspended in homogenization buffer containing 350 mM NaCl and placed on ice for 1 hr. The nuclear suspension was further ultracentrifuged at 65,000 rpm for 30 min. The resultant supernatants were subjected to protein assay and the aliquots stored at –80 °C until use.

DNA joining assay; Nuclear extracts (5 μg) were incubated with digested plasmid (0.5 μg) in 25 μl of ligation buffer (70 mM Tris-Cl, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP) at 14 °C for 12 hours. Reaction mixture was mixed with proteinase K (50 μg/ml) at 37 °C for 30 min, followed by analysis on 1% agarose gel electrophoresis.

PCR; PCR was performed using 0.5 μl of reaction mixture, platinum Taq DNA polymerase and T3 and T7 primers, according to the manufacturer's protocol. The cycling protocol was performed as follows: 60 s at 94°C; 20 to 25 cycles of 30 s at 94°C, 30 s at 56°C, and 90 s at 72°C; and a final extension period of 7 min at 72°C.

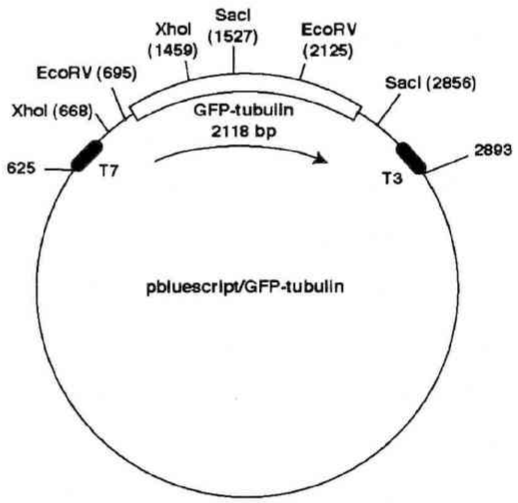

Fig. 1 Map of the pbluescript/GFP-tubulin plasmid. Restriction sites and T3 and T7 primer binding sites are shown. The number is base pair.

Results and Discussion

I used a protocol for an in vitro DNA end joining assay as described previously (8-10). In this assay, the fragments prepared from the digested pbluescript/GFP-tubulin were chosen as substrates for DNA end joining assay. This plasmid contains two restriction sites for SacI, XhoI and EcoRV, and digestion of the plasmid with SacI, XhoI, EcoRV or XhoI/EcoRV generates cohesive 3'-overhanging ends, cohesive 5'-overhanging ends, blunt ends or non-matching ends of DNA, respectively. The linearized fragments are different in length (Fig. 1).

Firstly, I examined whether embryonic brains contained the DNA end joining activity. Incubation of the SacI-digested DNA with nuclear extracts of the cerebral cortex from embryonic day-16 mice resulted in the production of ligated DNA, which appeared as dimers and multimers of fragments (Fig. 2A). The extracts also showed the end joining activities for 5'-overhanging ends of XhoI-digested DNA. However, only small amounts of dimers were detected when DNA with blunt (EcoRV-digested) ends was used. Similarly, DNA with non-matching (XhoI/EcoRV-digested) ends was ligated with low efficiency. In any case, self-ligated products, which would appear near linear DNA fragment,

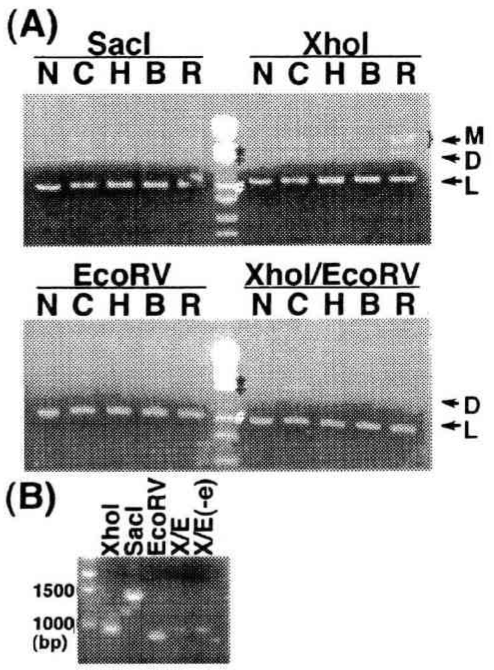

Fig. 2 DNA end joining activity in various regions of embryonic brains. (A) DNA fragments digested with SacI, XhoI, EcoRV or XhoI/EcoRV were mixed with the nuclear extracts from cerebral cortices (C), hippocampus (H), brain stem (B), or rhombic lip (R), and analyzed on gel electrophoresis. *, + and # on DNA ladder marker are 10, 8 and 4 kb. M; multimers, D; dimers, L; linear. (B) The mixture of DNA with cortical extracts was analyzed by PCR. X/E; XhoI/EcoRV digestion, X/E(-e); XhoI/EcoRV-digested DNA treated with no nuclear extracts.

were undetectable. To confirm that digested fragments were correctly ligated, reaction mixtures were subjected to PCR with T3 and T7 primers. Because if ligation was correct, the PCR should produce DNA bands different in length; 2269 bp for non-digested plasmid, 940 bp for SacI-digested plasmid, 1478 bp for

XhoI-digested plasmid, 839 bp for EcoRV-digested plasmid, and 811 bp for XhoI/EcoRV-digested plasmid (see Fig. 1). PCR amplification of the mixtures with SacI-, XhoI- and EcoRV-digested plasmids resulted in the bands corresponding to ligated products (Fig. 2B). However, there was no significant, expected product for non-matching DNA ends, indicating that the dimers of DNA observed in Figure 2A were generated by ligation of two cohesive ends or blunt ends. These results demonstrated that overhanging ends were efficiently ligated. The nuclear extracts of hippocampus, brainstem and rhombic lip also contained the end joining activity with a similar degree to those of cerebral cortex (Fig. 2A), indicating that there was no regional specificity for the activity.

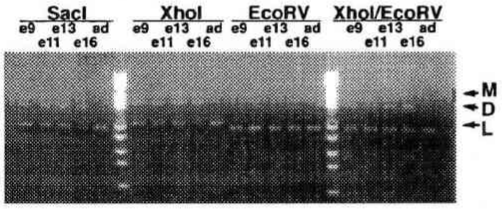

Fig. 3 DNA end joining activity in the embryonic and adult brains. DNA fragments digested with SacI, XhoI, EcoRV or XhoI/EcoRV were mixed with the nuclear extracts from cerebral cortices prepared from embryos (e9~e16) or adults (ad). M; multimers, D; dimers, L; linear.

The DNA end joining activity was observed as early as embryonic day-9 (Fig. 3). In contrast, cerebral cortex of the adult mice showed no detectable activities in all types of digested plasmid (Fig. 3). Investigation on the activity in postnatal brains (P1 to adult) is the next subject.

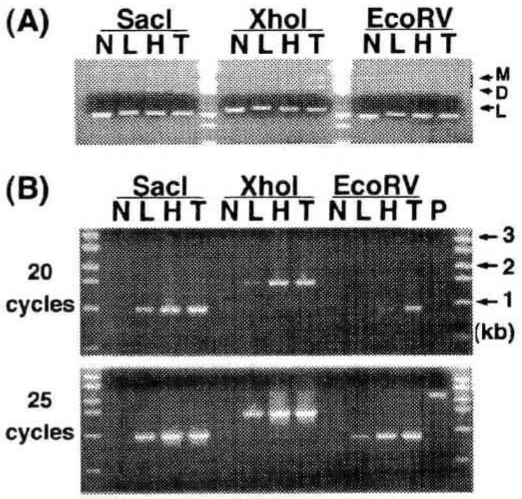

Fig. 4 DNA end joining activity in neuronal cultures. (A) DNA fragments digested with SacI, XhoI or EcoRV were mixed without (N) or with the nuclear extracts from low- (L) or high-density (H) cultures or cerebral cortices (T). M; multimers, D; dimers, L; linear. (B) The reaction mixture was analyzed by PCR at 20 or 25 cycles. P; non-digested plasmid was used as a PCR template.

It is well-known that the viability of neurons in cultures depends on the cell density plated on a culture dish (7,11,12). Although the reason why neurons in low-density culture are vulnerable is unclear, DNA damage induced by oxidative stress during neuronal cell preparation may not be

effectively restored, resulting in neuronal cell death. Thus, it is possible that the DNA end joining activity is reduced in low-density neuronal cultures. To address this issue, the activity was examined in low- and high-density cultures. The neuronal survival ratio in the high-density cultures at 24 hours of culturing is 5-fold higher than that in low-density cultures, although there is no difference between them at 3 hours of culturing (7,11,12). Nuclear extracts from high-density neuronal cultures at 3 hours after plating of cells showed the DNA end joining activity, comparable to that of the cerebral cortical tissue (Fig. 4A). By contrast, the activity in low-density cultures was remarkably reduced (Fig. 4A). This was also confirmed by PCR amplification with T3 and T7 primers (Fig. 4B). These results indicated that the viability of neurons was correlated with the DNA end joining activity.

The present study revealed that embryonic brains contained DNA end joining activity, which was likely to be related with neuronal survival. Considering massive neuronal cell death immediately after neurogenesis in DNA ligase IV-deficient mice (1), DNA end joining activity observed here might be required for restoring DSBs produced during neurogenesis. Whether DNA end joining activity detected in this study is attributed to DNA ligase IV or other molecules remains to be cleared.

DNA end joining activity was measured by detecting ligated DNA on agarose gel, combined with PCR for confirmation. This assay is easy, but not quantitative nor sensitive. On the other hand, investigation from other laboratories employs the similar assay combined with southern blot analysis probed with radio-labeled DNA (8-10). This is more sensitive to detect the end joining activity even in adult brains, but complicated. Easier and more sensitive assay is needed to more closely examine the end joining mechanisms in embryonic and adult brains.

Recent studies have demonstrated that DNA end joining activity is reduced in the brains of Alzheimer disease patients (13). These observations imply that DSBs are still introduced into DNA and restoring mechanisms play a role in adult brains and failure of DNA repair as well as increase of oxidative stress is related with neurodegenerative disease. Further investigation on DNA repair in the nervous system should help us contribute to the better understanding of the neuronal development and the cause of

neurodegenerative diseases.

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