ORIGINAL

Expression of neural cell adhesion molecule L1 in the brain of rats exposed to X-irradiation in utero

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Abstract : To gain insight to the cellular and molecular mechanisms involved abnormal neuronal migration induced by irradiation, we investigated expression of neuronal cell adhesion molecule L 1 and neuronal migration in the brains through comparison between rats prenatally exposed to X-ray and controls. To observe the pattern of neuronal migration, bromodeoxyuridine (BrdU) was chosen as a marker to label migrating cells. The results showed some of the labeled cells remained in the lower of the cortical plate in the irradiated rats, suggesting that neuronal migration was disrupted by X-ray. To study change of expressing neural cell molecule L1, rat brains were analyzed by SDS-PAGE after isolation of L 1 by immunoaffinity chromatography. In the all brain membrane fraction, immunoaffinity purified L1had bands at 200, 180, 140 and 80 kDa. However, the bands in the irradiated group were very weak when compared with the control. Taking these results into account, abnormal neuronal migration and reduction of expression L1 found in the irradiated brain indicated that migration of neural cells may be largely dependent on radial glial fiber as well as neural cell molecules like L1. A decrease in L1expression may be one of reasons of abnormal neuronal migration. J. Med. Invest. 50 : 187-191, 2003

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INTRODUCTION

The developing brain is one of the fetal structures most susceptible to ionizing radiation. The high vulnerability of the fetal brain is a distinctive teratological characteristic commonly recognized in all mammalian species including humans (1, 2). Studies of survivors exposed to atomic bombing in Hiroshima and Nagasaki (3) or to X-rays during medical procedures (4) indicate that microcephaly with typical heterotopic gray matter in the lateral ventricles and mental retardation are prominent findings. Although these biological effects of radiation on embryos have been considered to be responsible for cell death and abnormal migration (5), the mechanisms involved in cell death and abnormal migration have remained unclear.

Development of the mammalian brain requires the integration of many cellular processes. Neuron migration is the critical cellular process which initiates histogenesis of neocortex, because the neurons that are born progressively later in gestation must travel increasingly long distances to reach their correct location. It is now accepted that glial radial fibers guidance provides a primary mechanism for the positioning of young neurons in the developing brain (6, 7). To study cell-cell interactions, neural cell adhesion molecule L1, that appears to mediate cell-cell adhesion, has been found to be implicated in several morphogenic processes, especially in neuronal migration (8 -10). The relationship between neuronal migration and neural cell adhesion

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molecule L1 has been tested by two different *in vitro* assay system (microwell and explant culture systems) (11-14), but few study concerning such relationship has reported *in vivo* assay system. To gain insight to the cellular and molecular mechanisms involved abnormal neuronal migration induced by X-ray, we investigated changes of L1 expression and neuronal migration in the brains through comparison between rats prenatally exposed to X-irradiation and controls.

MATERIALS AND METHODS

Animals

The animals used were commercially supplied SLc : Wistar rats (Hamamatsu, Shizuoka, Japan). They were housed in an are-conditioned room (23±2°C) with a relative humidity of 55±5% under an alternating 12h light/dark schedule (7 : 00 A.M.-7 : 00 P.M.). Food and water were provided ad libitum. Nulliparous females about 10 weeks old were caged with potent males in pairs overnight. Pregnancies were dated as embryonic day 0(E0) when copulation plugs were found following overnight mating. Rat with positive plugs were housed in individual cages.

Three dams were randomly selected for study in each experimental period, and 6 offspring were examined in each group.

Treatment

Pregnant females were exposed to a single whole-body X-irradiation at a dose of 1.5 Gy On E15. The physical factors of the X-rays used were 200kVp, 15mA, 0.5 mm Cu+0.5mm Al filter, 90cm distance, and 0.45Gy/ minute exposure rate. Control pregnant rats were sham-treated. To observe the pattern of migrating neurons, bromodeoxyuridine (BrdU) was chosen as a marker to label migrating cells. Animals of both groups were injected intraperitoneally with 30mg/Kg BrdU (Becton Dickinson, San Jose, Calif., USA) on E 17.

HE-staining and Immunohistochemical staining

The brains of the fetuses were obtained at 6h (E15) of exposure, E17 and postnatal day 4 (P4). The samples were fixed in Bouin's solution, dehydrated, embedded in paraffin, and serially sectioned in frontal plane at 5 μm. The sections were stained with hematoxylin and eosin, or by immunohistochemical staining using monoclonal antibody specific for BrdU (Becton Dickinson, San Jose, Calif., USA). The visualization of BrdU in brain sections was performed basically according to the method of Roberts et al. (15). Sections were denatures in 4N HCl for 20 min at room temperature, neutralized with 0.1 N sodium borate buffer (pH 8.5), washed in phosphate-buffered saline (PBS), and reached with anti-BrdU monoclonal antibody (1:100 in PBS with 1mg/ml bovine serum albumin) for 60 min at room temperature. The sections were then sequentially incubated with goat anti-rat immunoglobulin (Ig) biotinylated second antibody, horseradish peroxidase (HRP)-conjugated streptavidin, and colored with 3, 3-diaminobenzidine (DAB), resulting in the formation of a brown precipitate in the nuclei of BrdU-labeled cells.

Isolation of L1molecule by immunoaffinity chromatography

The purification of the L1molecule from rat brain was performed according to the method of Rathjen and Schachner (16). The crude membrane fraction were prepared from rat brains of E17 and solubilized in a buffer containing 0.5% NP-40, 20mM Tris, 150mM NaCl, 1mM EDTA and 4U aprotinin/ml, pH 7.2. Protein concentrations were adjusted to1mg/ml. Non-solubilized material was removed by centrifugation at 100000g and 4°C for 1h. The supernatant was added first to a column containing immunobilized non-specific rat-IgG antiserum. The flow-through of this column was connected directly to the L 1 antibody-containing colum(5 mg IgG/ml CNBr sepharose, Pharmacia). The bound protein from anti-L 1 antibody column was eluted with an alkaline buffer containing 0.1M diethylamine, 0.5% deoxycholate (DOC), pH 11.5. Collecting volumes were neutralized by the addition of 0.5 M Tris-HCl, pH 6.8. The protein was then dialyzed against 20mM Tris,0.1%DOC, pH8.5.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis

After dialysis, the protein was concentrated by microdialyzer system (Pierce Chemical Company) for the SDS-PAGE analysis. SDS-PAGE analysis was carried out on 7% polyacrylamide gels according to Laemmli (17). Staining of gels was performed with Coomassie brilliant blue (CBB).

RESULTS

Pattern of neuronal migration:distribution of BrdU-labeled cells

Histological examination showed that extensive cell death (pyknotic cells), which were extremely dark and often had fragmented nuclei, occurred 6h after X-irradiation, and dead cells disappeared by 48h (E17)

after exposure. On E17, at 6 h after BrdU injection, BrdU-labeled cells (yellowish-brown color) were observed only in the bottom of the ventricular zone in the control brain. In the irradiated brain, most labeled cells were observed in the ventricular zone, while some labeled cells had appeared in the middle of the brain mantle. By P4, the labeled cells has further migrated from the ventricular zone to the cortical plate, almost labeled cells were reached in the upper part of the cerebral mantel in the control rat (Fig.1a). In the irradiated group, a high proportion of the labeled cells were located in the superficial layers of the cortical plate, but some of the labeled cells were remained in the lower of the cortical plate (Fig.1 b).

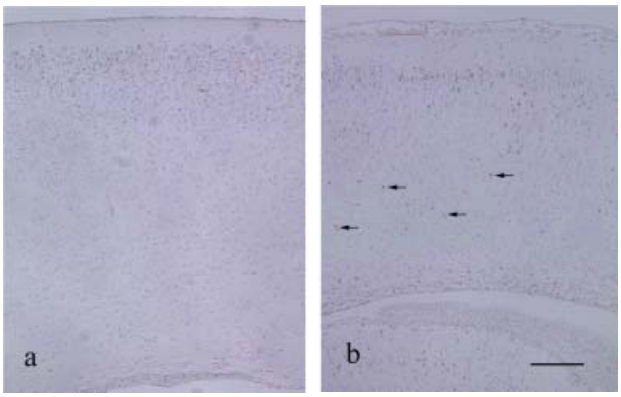

Fig.1. Micrographs of the rat brain mantle (parietal region) at postnatal day 4 showing distribution of BrdU-labeled cells.(a). Almost labeled cells have arrived the superficial layer in the control. (b). Some labeled cells (arrows) are remained in the lower of the cortical plate, although a high proportion of the labeled cells have reached in the superficial layer in the irradiated brain. Scale bar=150μm.

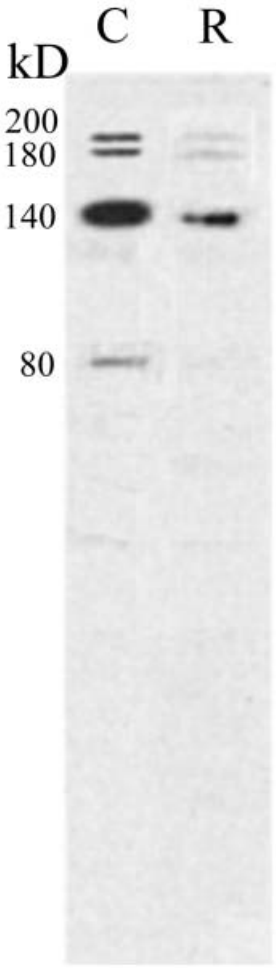

Fig.2. SDS -PAGE showing expression of neuronal cell adhesion molecule L1. L1 was purified by immunoaffinity chromatography using monoclonal antibody from brains of embryonic day 17. The 7% SDS-polyacrylamide gel was stained by Coomassie brilliant blue (CBB). In the all brain membrane fraction, immunoaffinity purified L 1 had bands at 200, 180, 140 and 80kDa. However, the bands in the irradiated group were very weak when compared with the control. C : Control ; R : Irradiation.

Expression of neural cell adhesion molecule L1

To study change of expressing neural cell molecule L1 both in control and irradiated brain, rat brains were analyzed by SDS-PAGE after isolation of L1 by immunoaffinity chromatography. In the all brain membrane fraction, immunoaffinity purified L1had bands at 200, 180, 140 and 80kDa. However, the bands in irradiated the group were very weak when compared with the control, even at the place of 80kDa protein marker, the band had disappeared (Fig.2).

DISCUSSION

According to a schedule of brain development, E15 for rats is critical stage for histogenesis of the cerebral cortex, which corresponds to a time when an apparent dramatic surge normally results in neurogenesis along with the establishment of architectonic stratification of the cerebral wall (18). Therefore, X-irradiation resulted in extensive cell death in the rat cerebral cortex on E15. That no dead cells were observed 48h after exposure indicated developing brain had a high recovery capacity. Thus, BrdU injection on E17 may be the proper time to label actively migrating cells.

Examination with immunohistochemistry for anti-BrdU antibody showed a clear course of histogenesis of the neocortex. Neuronal cells in the neocortex were born in a region of proliferating cells as seeing BrdU-labeled cells in the ventricular zone at 6h after BrdU injection, and later these cells migrated to the superficial region of the cortical plate to establish neuronal lamina and settle onto the outmost layer. However, that some of the labeled cells were remained in the lower of the cortical plate in the irradiated rats suggested that neuronal migration was disrupted by X-ray.

Neuronal migration is a remarkable complex process. Normal neuronal migrating needs a guidance of radial glial fiber (6, 7) and also needs proper receptors, ligands, neuronal cell adhesion molecules (NCAM, L1, etc.) (19). Studies on neuronal cell adhesion molecules has identified that L1 is a 200kDa integral membrane

glycoprotein and it plays various important roles in the dynamic phases of neural development, neuronal cell migration, neurite elongation and fasciculation of axons (14, 16, 20). It has been believed that the important roles of L1are related to its biological characterization. Expression of L1is one of the first signs of neuronal differentiation. Following L1expression, other NCAMs are always coexpressed on the L 1-positive neuron. It has been demonstrated that NCAM enhances L1-dependent cell-cell interaction (21). Moreover, L1 first appears in the subplate (the first neurons in the cortex) coexpressed with NCAM during the development of the neocortex, and it is predominantly expressed on axons and scarcely on cell bodies or dendrites. Antibodies to L1efficiently perturb neurite elongation and fasciculation (22, 23). Furthermore, L1 is restrictedly expressed in particular to post-mitotic neurons in the central nervous system, but no on glia or several non-neural tissues tested. If L1 functions as a ligand in cell-cell adhesion and if the corresponding receptors are also localized on neurons, the L1may be responsible for adhesion between neuronal cells only, and not between neurons and glia. It is conceivable that L1 mediates adhesion among neurons in the form of a "self-aggregation" (24). Alternatively, L1 may mediate adhesion of neurons to other cell types carrying different receptors. This mode of interaction may lead to an association between post-mitotic pre-migratory neurons with radial glial fibers which have been postulated to guide migrating neurons (25).

As mentioned above, neuronal migration is a complex process and also highly sensitive to various physical including to irradiation. Any disturbance of the normal process may result in neuronal migration disorder. Previous study has confirmed that prenatal irradiation caused a disturbance of radial glial fibers (26). By immunohistochemistry for anti-BrdU antibody and the SDS-PAGE analysis, we have presented here the first direct evidence that relationship between neuronal migration disorder and expression of L1 molecule in the brain following prenatal exposure to irradiation. Neuronal migration disorder and reduction of expression L1 were found in the irradiated brain when compared with the control, suggesting that migration of neural cells may be largely dependent on radial glial fiber as well as neural cell molecules like L1. A decrease in L1 expression should be one of reasons of disruption of neuronal migration. As to mechanisms caused reduction of L 1 expression by prenatal exposed to irradiation, neuron death and/or inhibition of any step of L1 expressing has been considered. Further studies are needed to clarify these points.

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