An Immunohistochemical Study of Radial Glial Cells in the Mouse Brain Prenatally Exposed to \( \gamma \)-irradiation

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Abstract. The features of a glial cell population in the developing brain of mice prenatally exposed to \(^{60}\)Co \( \gamma \)-irradiation at the most radiosensitive stage were studied with immunohistochemistry for anti-midkine (MK), anti-vimentin (Vim), and anti-GFAP antibodies. Anti-MK- and anti-Vim-positive radial glial fibers distributed in a similar radial fashion; these fibers were observed primarily in the embryonic period and disappeared after birth. Anti-MK- and anti-Vim-stained radial fibers run perpendicular to the pial surface in controls, whereas such fibers were disorganized 6 hours (h) after irradiation. This finding provided new evidence that the migratory pathways of young neurons were interrupted beginning a few hours after irradiation. By El7 the ectopic cell masses formed so as to replace the parts of the ventricular zone where no anti-MK immunoreactive radial fibers were present, but where anti-GFAP-stained fibrillary astrocytes emerged in the ectopic cell masses from the early postnatal period. The results suggested a twofold source of the generated astrocytes: either directly from a separate precursor of the astrocytes, or due to the transformation of the classic radial glial cells. In the newborn, numerous protoplasmic transitional forms displayed by astrocytes in irradiated brains indicated that reactive gliosis was a powerful response of a brain exposed to irradiation.

Key Words: Astrocyte; Corticogenesis; Glial fibrillary acidic protein; Midkine; Radial glial cell; Vimentin.

INTRODUCTION

The analysis of neuron-glia relationship at the early stages of fetal development has been somewhat tentative because of the difficulty in distinguishing immature radial glial cells from elongated migrating neurons using conventional histological techniques. Thus, the classical Golgi silver impregnation technique, while adequate for the classification of cells at advanced developmental stages, is less useful for analysis of younger embryonic neural tissues (1–4). Although the technique has been used successfully during the middle and late stages of neurogenesis to elucidate the identity of several classes of radial processes as glial cells, the existence and role of radial glial cells in early brain development has remained unclear.

In recent years, radial glial cells were characterized by the advanced immunocytochemical methods such as the glial fibrillary acidic protein (GFAP) immunocytochemical method. Glial fibrillary acidic protein, a major constituent of fine, intracytoplasmic glial fibers, has been localized in astrocytes of a variety of vertebrates (5). The availability of a specific biochemical marker provides a unique opportunity to analyze the formation of glial cells during ontogeny (6–8). Anti-GFAP has been particularly useful in identifying radial glial cells and Bergmann glia in the developing brain. It has been used to demonstrate glial cells in the embryonic chick spinal cord (9), newborn rat cerebellum and cerebrum (10–12), and human fetal cerebrum (13), but the GFAP-immunoreactive glial fibers were observed markedly only after birth in the mouse cerebrum (14). Glial fibrillary acidic protein may not be present or detectable in cells at earlier stages, even though some cells may already be committed to a glial lineage.

Midkine (MK) is a heparin-binding growth factor specified by a retinoic acid–responsive gene. It is the first member of a new protein family of developmentally regulated cytokines with diverse biological activities (15, 16). Midkine is mitogenic for certain fibroblastic cell lines, and enhances neurite outgrowth and survival of various embryonic neuron types (17–20). Increased expression of MK was detected on the processes of radial glial cells in the developing rat cerebral cortex (21). Thus, MK may be available for analysis of gliogenesis in the early stages of the developing central nervous system.

Radial glial cells have been thought to play a role as guides for late-generated neurons that migrate long distances to the superficial layers of primate neocortex (2, 22–28). Previous studies revealed the process of migration to be highly sensitive to ionizing irradiation (14, 63). However, there have been few reports on the morphological features of radial glial cells in fetal brain exposed to \( \gamma \)-irradiation. The present study focuses on identifying the features of a glial cell population in the developing brain of fetal mice exposed to \(^{60}\)Co \( \gamma \)-irradiation at the most radiosensitive stage. An immunohistochemical study of
anti-MK, anti-Vimentin, and anti-GFAP antibodies was employed.

MATERIALS AND METHODS

A closed colony of Slc:ICR mice (Japan SLC Inc., Hamamatsu, Japan) was maintained on a mouse pellet diet (CE-2; CLEA Japan Co., Tokyo) and water ad libitum in a temperature-controlled room and was kept on a 12 hour (h) light/dark cycle. Nulliparous females approximately 8 weeks old were mated with males of the same age in cages overnight, and the following morning was called day 0 of gestation if a vaginal plug was found. Pregnant mice were exposed to a single dose (1.5 Gy) of whole-body 60Co γ-irradiation on embryonic day 13 (E13). The dose rate was 0.04 Gy/min. Pregnant control mice were sham-treated. Some mice were allowed to give birth and rear their litters. At least two were randomly selected for evaluation at each experimental stage when about 4–6 fetuses and pups were examined. Between 6 h after irradiation and postnatal day 7 (P7), the brains from fetuses and pups were extracted and removed for analysis as follows.

For MK immunohistochemical examination, samples were fixed with Zamboni’s solution (4% formaldehyde and 0.2% picric acid in 0.1 M phosphate buffered saline [0.1 M PBS, pH 7.4]) at 4°C overnight. The brains were immersed in 20% sucrose at 4°C for 1 day and embedded in 10% gelatin at 4°C for 2 days, then snap-frozen by carbon dioxide gas, cut in the frontal plane (5–15 μm thickness) through the entire cerebrum by cryostat (CM 1800, Leica, Germany), and thaw-mounted onto poly-L-lysine-coated glass slides. Particular attention was given to the dissection and orientation of the tissue blocks in this study. The tissue blocks through the full thickness of the cerebrum cortex were dissected as nearly as possible perpendicular to the longitudinal axis of the cerebral hemisphere. For each brain, 3 sections were selected from the serial sections treated with 0.6% hydrogen peroxide in 80% methanol for 5 minutes (min) to block endogenous peroxide activity, then rinsed 3 times with 0.1 M PBS containing 0.3% Triton X-100. The sections in each group were incubated with affinity-purified rabbit anti-MK antibody in 0.1 M PBS (1:200, containing 1% normal goat serum, 1% Bovine serum albumin and 0.3% Triton X-100) at 4°C for 2 days. The development of polyclonal antibody against MK protein was generated in a rabbit. A rabbit was immunized against recombinant MK produced in L cells and resulting antibody was affinity purified using MK-glutathione S-transferase fusion protein as a ligand. After rinsing 3 times with 0.1 M PBS, the sections were incubated subsequently with biotinylated anti-rabbit goat IgG at 4°C for 1 day, then with ABC kit (Vector Laboratories, Inc. USA) for 3 h at room temperature. Immunopositive structures were visualized with a freshly prepared solution of 0.02% 3, 3-diaminobenzidine-4HCl and 0.005% hydrogen peroxide in 0.05 M Tris-HCl buffer (pH 7.6). After being rinsed in distilled water, the sections were dehydrated and mounted with resin. In negative control experiments, the specimens not incubated with anti-MK antibody were processed and stained as described above. No-immune goat serum was substituted for the primary antibody.

For GFAP-immunohistochemical examination, samples were snap frozen in isopentane in liquid nitrogen, and cut in the frontal plane (15 μm thickness) through the entire cerebrum. The dissection and orientation of the tissue blocks were performed as described above. For each brain, three sections were selected from the serial sections. After mounting onto poly-L-lysine-coated glass slides, the sections were air-dried and then fixed by 4% paraformaldehyde in 0.1 M PBS (pH 7.4). This method proved to be the most satisfactory for preservation of both tissue structure and immunoreactivity. Following three 0.1 M PBS washes, endogenous peroxidase activity was exhausted by incubation for 15 min in 0.3% H2O2. Sections were washed again and then incubated for 30 min at room temperature in bovine serum albumin (BSA) in 1:20 dilution, and then incubated 4°C overnight in a 1:500 dilution of rabbit anti-GFAP. The antiserum to GFAP, raised in the rabbit against GFAP prepared from the human spinal cord, was described by Woodhams et al (29). Its specificity to the astroglia was tested on sections of adult rat cerebellum, and it also showed a characteristic intracellular fibrillary staining pattern in cultured astrocytes. Goat anti-rabbit γ-immunoglobulin (IgG) and rabbit peroxidase-antiperoxidase were both obtained from DAKO Co. and used at a dilution of 1:50. All antisera contained 0.3% Triton X-100. Following three 10-min PBS washes, the sections were incubated for 30 min in goat anti-rabbit IgG and after washing, were incubated again in rabbit peroxidase-antiperoxidase. Sites of immunoreactivity were visualized by reaction for 5 min in 0.05% diaminobenzidine (DAB) in 0.05 M Tris buffer (pH 7.6) containing 0.005% H2O2; the reaction being terminated by washing in distilled water.

The control mice sections were processed in the same manner as those of the γ-irradiated animals in the different experiments.

RESULTS

MK-stained fibers in the telecephalic wall were primarily detected in the embryonic period (examination from E13) and were not observed in the postnatal cerebral cortex. On E13, the cerebral wall already consisted of 3 cardinal embryonic zones, the ventricular, subventricular, and intermediate zones, as well as a thin cortical plate and a marginal zone. In this embryonic stage, MK-immunoreactive fibers were clearly observed, and radially traversed the distance between the ventricular and the pial surface. They were straight and perpendicular to the pial surface, oriented in the direction of neuronal migration (Fig. 1A). In the thinner sections (8 μm), numerous MK-stained fibers could be traced from the ventricle to the pial surface, even though following their entire length was difficult. Some of the stained cell somas were seen in the bottom of the ventricular zone (Fig. 1A'). Six h after irradiation (E13), MK-stained fibers were scattered throughout the brain mantle; they were no longer regularly distributed or perpendicularly oriented to the pial surface. The majority of the ventricular zone was destroyed (Fig. 1B). In 8-μm sections, numerous pyknotic cells were present among preserved neuroepithelial cells. Pieces of the radial fibers were also visible (Fig. 1B'). On E14, the brain mantle became thicker and MK-stained fibers were regularly distributed in the control brain, their courses initially parallel to each other and...
always perpendicular to the pial surface. In the irradiated brain, the pyknotic cells had decreased. Because the curved fibers passed out of the depth of focus, only segments of disorganized radial fibers were seen in the fields observed. The destroyed ventricular zone had not recovered yet. On E15, the individual radial fibers across the entire distance from the ventricular zone to the pial surface were more easily recognized in the control. In the intermediate zone, horizontally distributed arborizations appeared and ran through the longitudinal radial fibers. The fibers in 2 directions formed a network (Fig. 2A). In the irradiated brain, because of acute edema and some cell death, low cell density was noted in some neocortical areas. The curved fibers could be observed randomly (Fig. 2B). On E16, the fascicles of the radial fibers remained approximately parallel to each other and fanned out as they approached the cortical plate. Such morphological features were readily observable in the control cortical plate (Fig. 3A), whereas in the irradiated brain, many cells aggregated to form rosettes (Fig. 3B). In 8-μm sections, the central hollow margins of the rosettes were intensely stained with the anti-MK complex, and the MK-immunoreactive fibers in the rosettes radially projected from the central hollow to the outer boundary of the rosettes. The other fibers in the cortical plate outside of the rosettes formed several fascicles which changed their course in the different directions. On E17, MK-stained radial fibers were still visualized in the control, although their density in most areas seemed diminished (Fig. 4A). The outline of rosettes in the irradiated brain became unclear, and ectopic cell masses formed so as to replace part of the ventricular zone. In the ectopic cell masses, no anti-MK-positive fibers were observed (Fig. 4B). By E18, there was a dramatic decrease in the number of radial fibers and in the intensity of staining for MK both in the control and the irradiated brain. After birth, the number of radial fibers that spanned the entire cerebral wall became progressively lower. Midkine-reactive fibers almost disappeared with the postnatal stages.

To confirm the possibility that abnormalities of migration could be related to abnormal guidance by radial glial fibers, radial glial fibers were also examined by means of

Fig. 1. Telencephalic wall stained with anti-midkine (MK) at embryonic day 13 (E13). A, A': Control. MK-immunoreactive fibers radially traverse the distance between the ventricular zone and the pial surface. These fibers can be traced from one to the other in the thinner sections (8 μm, A'). B, B': Irradiation. MK-immunoreactive fibers are scattered throughout the brain mantle. In the 8-μm section (B'), numerous pyknotic cells (arrowheads) are present among preserved neuroepithelial cells. Parts of the radial fibers can also be seen. Thickness of sections: A, B = 15 μm; A', B' = 8 μm. Scale bar = 50 μm.
vimentin (Vim) immunocytochemistry. The characteristics of this antibody have been described in detail by Dellagi et al (62). Radial fibers stained with anti-Vim antibody were similar to those fibers stained with anti-MK antibody in a distinct fashion in the embryonic specimens from E13. On E13, the parallel arrays of the fibers stained with anti-Vim radially traversed the ventricular to the pial surface in the control brain (Fig. 5A); 6 h after exposure to irradiation, numerous pyknotic cells were present in the telecephalic wall, and some radial fibers could be found near the preserved portion of the ventricular zone (Fig. 5B). Three days later, at E16, full-length fibers were observed running radially from the ventricular zone up to the pial surface (Fig. 5C). In the irradiated brain, rosette formations appeared in the neocortical areas. The radial fibers in the rosettes showed a radial distribution from the same circular margins of the central hollow to the outer boundary of the rosettes. In the other neocortical areas, radial fibers could be traced for a short distance in different orientations (Fig. 5D). On E17, many radially oriented fibers ran in parallel arrays through the intermediate zone and cortical plate to the marginal zone in the control. Ectopic cell masses formed beneath the thin cerebral mantle in the irradiated brain, creating the potential for ectopic gray matter to form in the white matter of the adult brain (14). In the ectopic cell masses, no anti-Vim-stained fibers were observed. By E18, only a small number of lightly stained fibers could be found both in the control and irradiated brains.

Positively stained radial fibers with anti-GFAP antibody were first visualized in the cortical plate at E18. On P4, the developing neocortex contained large numbers of radial fibers that were stained completely and could be followed from the ventricular zone up to the pial surface. In the control brain, these glial fibers were thin and perpendicular to the surface of the cortex (Fig. 6A), while in the irradiated brain, the fibers were hypertrophied and crumpled (Fig. 6B). By P7, radial glial cells which spanned the entire cerebral wall became progressively fewer, and cells began to increase in number. Such displacements between radial glial cells and cells were found at various distances from the ventricular zone, but the number of cells in the irradiated brain was greater than in the control (Fig. 6C). Many GFAP-positive astroglial cells were also observed in the ectopic cell masses (Fig. 6D).

**DISCUSSION**

Midkine was primarily expressed in the embryonic period and disappeared after birth. This pattern is unusual
Fig. 3. Telencephalic wall stained with anti-MK at E16. A: Control. Radial glial fibers keep approximately parallel to each other and fan out as they approach the cortical plate. B: Irradiation. Many cells aggregate to form several rosettes. The fibers stained with anti-MK in the rosettes radially projected from the central hollow to the outer boundary of the rosettes. The other fibers, out of rosettes, formed several fascicles that changed regular orientation. Thickness of sections = 15 μm. Scale bar = 100 μm.

Fig. 4. Telencephalic wall stained with anti-MK at E17. A: Control. MK-reactive fibers become weaker than before. B: Irradiation. The ectopic cell masses formed so as to replace part of the ventricular zone. MK-stained fibers cannot be found in the ectopic cell masses (arrowheads). Thickness of sections: A, B = 15 μm. Scale bar = 100 μm.

for the growth factor family, since most known growth factors, e.g. fibroblast growth factors, increase in amount as neural development proceeds (30, 31). Therefore, MK may be involved in developmental events such as neural differentiation and formation of the neural network, which take place primarily in the embryonic and prenatal periods. Previous works have proved that MK exists on the cell surface only and is one of the membrane-bound
extracellular matrix proteins (21). Neural differentiation and general structural organization in the brain depend on the temporal and spatial association with extracellular matrix molecules. Fibronectin and tenasin are both extracellular matrix molecules and usually regulate cell-cell interactions by altering the adhesiveness between two cells (32–35). Fibronectin was associated with radial glial fibers and with preplate cells with a punctate appearance in the embryonic cerebral brain (32). Interestingly, the staining pattern of MK was quite similar to those of fibronectin in the embryonic period, albeit exhibiting stronger intensity. The possible association of MK protein with radial glial fibers in the telencephalic wall has suggested that MK may be related to cell migration (27) and axon guidance (36). The localization of Vim in embryonic glia was in agreement with such a relationship between MK protein and radial glial fibers. This was not surprising, since it is well known that Vim is a major cytoskeletal component of immature glia in the developing brain (37) and could provide a reference for radial glial fiber development (38, 39). Radial fibers stained with anti-Vim antibody were similar to fibers stained with anti-MK antibody in a distinct fashion in the fetal cerebral cortex of mouse. Both expression patterns of the proteins were developmentally regulated, so the radial fibers stained with anti-MK antibody should be radial glial fibers.

Vim is particularly useful as a mark of glial differentiation, but its immunoreactivity for the radial fibers was weaker than anti-MK for the embryonic cerebral cortex. Since we wished to know the characteristic expression of MK in the normal and abnormal developing processes through this experiment, we selected both antibodies in this study.

The present observations, taken together with the previous studies, demonstrated that radial glial cells are a

Fig. 5. Telencephalic wall stained with anti-vimentin (Vim) at different embryonic days. Panels correspond to control and irradiated brains of the same age. A: E13, Control. Vim-stained radial fibers are straight and perpendicular to the pial surface. B: E13, Irradiation. 6 hours (h) after irradiation, many pyknotic cells (arrowheads) are present, and crumpled radial fibers appear near the preserved ventricular zone. C: E16, Control. Full-length radial fibers can be seen from the ventricular zone up to the pial surface. D: E16, irradiation. Fibers in the rosettes show radial distribution from the same circular margins of the central hollow to the outer boundary; the other fibers not in the rosettes can be traced for a short distance in different orientations. Thickness of sections = 15 μm. Scale bar = 50 μm.
Fig. 6. Telencephalic wall stained with anti-GFAP at different postnatal days. Panels correspond to control and irradiated brains of the same age. A: P4, Control. The glial fibers are thin and perpendicular to the surface of the cortex. B: P4, Irradiation. The glial fibers are hypertrophied and crumpled. C: P7, Control. Radial glial cells become progressively fewer, and astroglial cells begin to increase in numbers. D: P7, Irradiation. Numerous astroglial cells in the irradiated brain outnumber those in control. Many GFAP-positive astroglial cells are also observed in the ectopic cell mass (arrowheads). D': Higher magnification of GFAP-positive astroglial cells in the area of the ectopic cell mass of Figure 6D. Thickness of sections = 15 µm. Scale bar: A, B = 50 µm; C, D = 500 µm; D' = 50 µm.
specialized class of astroglial cells which appear transiently during the development of the central nervous system. Their numbers increase dramatically during the neurogenic stage of brain development, peaking during neuronal migration (1, 40). Anti-MK– and anti-Vim–stained radial glial fibers appeared from at least E13 to the early postnatal period. The appearance of anti-MK– and anti-Vim–positive radially-oriented fibers was consistent with the developmental stage of the brain when proliferated ventricular cells start to migrate to the cortical area. Around E15, it became more conspicuous that the long radial glial fibers spanning the cerebral wall from the ventricular zone to the pial surface were consistently oriented in the direction of neuronal migration and traversed the cortical plate itself in a straight line. These radial fibers guided migrating neurons from their sites of origin to their final positions in the cerebral cortex, and also provided a scaffolding to preserve the cell-cell relationship established in the proliferative zone (23, 41–46). However, the radial fibers were disorganized in brains following 1.5 Gy irradiation. These fibers could not recover completely until mice were born, when the neuronal migration was almost finished. Already on E13, the radial fibers were crumpled 6 h after irradiation. This fact provided new evidence that the migratory pathway of young neurons had been interrupted a few hours after exposure to irradiation. A large number of young neurons migrated along a preserved unpassable, only partially passable, or disoriented pathway. Obviously, some could not move far from the place of their origin around the lateral cerebral ventricle. Instead, if they remained in inappropriate positions in the cortical area, they caused several congenital and/or acquired brain abnormalities such as a four-layered “lissencephalic” cortex (63); if they remained in the white matter, they formed a large mass of ectopic gray matter (14).

One notable finding of the present study was that no anti-MK– and anti-Vim–stained fibers were observed in the ectopic cell masses, whereas they were present in the rosettes. These radial fibers were distributed among the compartments of aggregating cells and showed a mosaic of rosettes. In a developing process known as rosette formation, after large numbers of cells were killed, the surviving primitive neural cells in the periventricular proliferative zone soon began to rebuild the brain. These cells tended to aggregate together and form rosettes, which consisted of cells grouped concentrically around a lumen (47, 48). As development proceeded, these structures were transformed into confluent, subcortical ectopic cell masses in postnatal mice or rats (14, 51). To date, we can not give a complete answer as to what roles MK and Vim proteins play in forming rosettes and why only the germinal cells in rosettes expressed MK and Vim proteins.

As the development of the cortex progresses, around E15, the intermediate zone was further complicated by the arrival of horizontally oriented fibers heavily stained with anti-MK. According to the timetable of the histogenesis of the cerebral cortex, these fibers were generally assumed to be horizontally disposed afferent axons, originating in the diencephalon (52–54). It has been reported that MK has a neurite outgrowth-promoting effect and that MK protein is involved in afferent axon development (55). In the present study, the time of MK protein expressions in the neocortex correlated with that of the afferent axon development. This finding might suggest that MK played an important role in the development of a neural network. However, in the irradiated brains, anti-MK–immunopositive afferent axons showed an irregular distribution as compared with the horizontally disposed axons in controls. It is thus conceivable that the nervous pathway might be affected due to the abnormal network of axons following irradiation. Such structural disorders of nervous pathways had to bring about irreversible functional disorders.

If astrocytes indeed secrete MK (56), there might not be fibrous astroglial cells in the ectopic cell masses in the embryonic period, because no MK immunoreactive cells were found in the ectopic cell masses as described above. However, later in the early postnatal period, anti-GFAP–stained fibrillar astrocytes emerged in the ectopic cell masses. The results suggested a twofold source of the generated astrocytes: either directly from a separate precursor of the astrocytes, or from the transformation of the classic radial glial cells.

We have demonstrated the dramatic change in glial cell labeling in which there was a transformation from anti-Vim–positive and anti-GFAP–negative cells at E13 to nearly the reverse at P7. From the present study, anti-Vim–stained cells had the morphology of classic radial processes in the prenatal brain. In the postnatal brain, some astrocytes stained with anti-GFAP also had radially oriented processes, but the profuse density of lamellate expansions suggested that they were mature astrocytes that had retained some of the original processes of their parent radial glial cells. That transitional forms displaced radial glial cells may prove the hypothesis that radial cells are immature glial cells, and that some astrocytes come from radial cells (1, 44). In the newborn, the numerous protoplasmic transitional forms displaced by astrocytes indicated that reactive gliosis was a powerful response of a brain exposed to irradiation (14, 57). Some experiments with cultured astrocytes have demonstrated several factors implicated in the initiation of reactive gliosis, including growth factors (EGF, FGF, PDGF), cytokines (interleukin-1, tumor necrosis factor), and myelin basic protein (49). These factors have been reported to become more influential following CNS injury (50, 58). Moreover, astrocytes in the neocortex acquired the GFAP immunoreactivity during the early postnatal development. GFAP is present in the cytoskeleton of protoplasmic and
fibrous astroglial cells only (59–61). Thus, it was clear that most astrocytes started to differentiate after birth, and the replacement between Vim and GFAP immunoreactivities might be thought to reflect the differentiation of glioblasts into astrocytes.

On the other hand, glial cells were found to express GFAP and Vim simultaneously during the transition period, but it could not be decided whether both Vim and GFAP proteins were expressed in the same cells simultaneously. Our data indicating the replacement between anti-Vim- and anti-GFAP immunoreactivities suggested that the 2 proteins might not coexist in equal amounts throughout the cell. This might indicate the existence of different intermediate filament systems or different protein constituents within the same filament system. Experiments involving immunofluorescence double-staining of cells with 2 antibodies may help to clarify these questions.

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