IN VIVO ANALYSIS OF RGD-SEQUENCE DEPENDENT CORTICAL HISTOGENESIS IN MOUSE TELENCEPHALON

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The amino acid sequence arginine-glycine-aspartate (RGD) acts as a specific ligand for integrins in cell adhesion. We injected RGD into the telencephalic vesicle of mouse embryos exo utero on E13 and examined the cortical histogenesis of the telencephalon on E15, 16 and 18. RGD induced disorganized cytoarchitecture and reduced cell number in the cortical plate and intermediate zone. In the ventricular zone although there was no difference in cell density between RGD-injected brains and controls, mitotic activity analysis revealed the lower proliferative rate in RGD-injected brains. These results suggest that RGD-proteins or integrins play an important role both in the migration of neuroblasts and the proliferation of neuroepithelial cells during the cortical histogenesis of the telencephalon.

Cell adhesion, such as cell-to-extracellular matrix (ECM) contact and cell-to-cell contact (1, 2), plays the important biological roles. In the developing central nervous system, many cell adhesion molecules (CAMs) mediate these contacts and contribute to the neuronal migration and differentiation (3-8). Recent studies group CAMs into three families, which are the immunoglobulin superfamily, the integrin family and the cadherin family (9, 10). Of these molecules, some contain the amino acid sequence arginine-glycine-aspartate (RGD) (8, 11-14), and RGD sequence is a specific ligand for integrins (10), which is the superfamily of integral membrane proteins that are heterodimers of α and β subunits (15). Recently, functions of integrins in the developing chick brain was studied with use of retrovirus-mediated gene transfer technique (5). In this experiment, antisense β1 integrin RNA was introduced into the optic tectum of chick embryos and contribution of integrins to neuronal migration and survival was evaluated.

Considering the spatial dispersion of migrating neurons, in vivo analysis has a definite advantage over in vitro experiment to study functions of CAMs in the mammalian brain morphogenesis and histogenesis. Exo utero development in mice, which was reported by Mineoka et al. (16), is an useful method in this respect. With this method, it is possible to manipulate mouse embryos directly and to let them develop after the manipulation until the full term of pregnancy. We applied this technique to inject RGD into the left telencephalic vesicle of mouse embryos and its effects on the cortical histogenesis were examined after embryos developed to the indicated stages. The significance of cell adhesion mediated by the RGD-proteins and integrins for both migration of the post-mitotic cells and the proliferation of neuroepithelial cells is discussed.

MATERIALS AND METHODS

Exo utero development and microinjection
The present study was based on exo utero development (16). The animals used were Crl:ICR mice (CLEA Japan Inc., Tokyo). All treatments on experimental animals were performed in accordance with guidelines for animal experiments of Shimane Medical University, Izumo, Japan. A female (8-20 weeks old) was housed with a male over night in the same cage. If the vaginal plug was found on the following morning, the day was designated embryonic day 0 (E0). On E13, dams were anesthetized with 70 mg/kg of pentobarbitol and operated on. Before operation, dams were also injected intraperitoneally with 1.4 mg/kg of ritodrine hydrochloride (lot. 71H0295, Sigma, St Louis, MO) to relax the myometrium (17). The myometrium was cut longitudinally on the opposing wall of the placenta. For microinjection, glass micropipette (10-20 μm in diameter) was made by a magnet puller (PN-3, Narishige Scientific Instrument Lab., Tokyo). The tip of the pipette was sharpened by a pipette grinder (MCC-II, Chatani Limited, Tokyo) and a microforge (MF-79, Narishige Scientific Instrument Lab., Tokyo). The pipette was connected with a Hamilton threaded plungersyringe (0.5 ml, 8100, Hamilton Co., USA). RGD (lot. ZD844, Bachem INC., California) was dissolved in PBS (2 μg/μl). One μl of RGD solution was injected into the left telencephalic vesicle of the E13 embryo through the amnion. PBS without peptide was used as a control. The injection was performed under a surgical microscope (OME-NA, Olympus, Tokyo). The peritoneal cavity of the dam was filled with approximately 1.5 ml of Hank’s solution to avoid the adhesion of the incised myometrium with the peritoneum. After operation, dams were warmed on the hot plate at 37°C. Embryos were sacrificed on E15, 16 or E18. The viability of treated embryos throughout the present study was more than 80%.

Histological observation
The embryos were decapitated on E15 and 16 and fixed in a mixed solution of 4% paraformaldehyde and 0.2% of picric acid in 0.1 M phosphate buffer (pH 7.3) by immersion. The brains of E18 embryos were removed carefully and fixed in the same solution mentioned above by immersion. Specimens were
embedded in paraffin and sectioned at 5 μm in the horizontal plane on E15 and 16 and coronary or horizontally on E18. Sections were stained with hematoxylin-eosin (HE).

Quantitative studies using image analyzer

Cosmozone LS (Nikon, Tokyo) and NEC PC9801 VM personal computer (NEC, Tokyo) were used. Five-μm-thick paraffin sections of E15 embryos were stained with hematoxylin (RGD: n = 10; PBS: n = 9). Cell densities of the cortical plate (CP), intermediate zone (IZ), and ventricular zone (VZ) were obtained in the dorsolateral roof of the telencephalon. In the same region, the thickness of each layer and its proportion to the total thickness of the telencephalic wall were measured. An average of data in three serial sections was obtained for each embryo. Mitotic activity in the VZ of the dorsolateral wall was calculated as the ratio of the number of cells at M phase to the number of total cells faced on the ventricular lumen. Mann-Whitney U test was used for the statistical analyses.

RESULTS

Cortical cytoarchitecture is disorganized in RGD-injected brains

In E18 embryos injected with RGD on E13, an irregular surface of the dorsolateral roof of the telencephalon was characteristic (Fig. 1, and Table I). In E15 and 16 embryos injected with RGD on E13, such an irregular surface of the telencephalic roof was less apparent than in the E18 embryos. Disturbed cytoarchitecture, however, in the CP and IZ was remarkable in E15, 16 and 18 embryos injected with RGD. Common histological features of the telencephalon in the RGD-injected group were: (1) the intercellular space was enlarged and neuroblasts were fewer in the CP (Figs. 2, 3, and 4 for E15, 16 and 18, respectively). Neuroblasts associated with radially oriented fibers in the IZ were remarkably fewer in E16 and 18 embryos.

Figure 2. Loose fibrous architecture of the MZ was characteristic in the E18 telencephalon injected with RGD (a). Cell composition in the CP was scarce and a disrupted fibrous structure was observed (arrowheads, b) in the RGD-injected brain. In contrast, the CP of PBS-injected brain demonstrates the fine fibrous network in the MZ (c) and the densely packed CP (d). Panels a to d are in the same magnification. Bar: 25 μm.

Figure 3. The scarcely populated CP (a) was observed in the E16 RGD-injected brain. Neuroblasts were densely packed in the CP of control embryos (c). Radially oriented fibers with a few migrating neuroblasts were observed in the RGD-injected brains (arrowheads, b). In the control, a lot of neuroblasts attached to the radially oriented fibers (d). Panels a to d are in the same magnification. Bar: 50 μm.
RGD dependent cortical histogenesis

Figure 4. Cell density of the CP on E15 was already different histologically between the RGD-injected telencephalon (a) and control (b). Panels a and b are in the same magnification. Bar: 50 μm.

Table 1. IRREGULAR SURFACE OF THE TELENCEPHALIC ROOF OBSERVED IN THE RGD-INJECTED EMBRYOS

<table>
<thead>
<tr>
<th>Injection*</th>
<th>No. of Embryos</th>
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<tbody>
<tr>
<td></td>
<td>Total Irregularity (+)</td>
</tr>
<tr>
<td>RGD (2 μg)</td>
<td>7               4</td>
</tr>
<tr>
<td>PBS</td>
<td>3               0</td>
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*: Embryos were injected on E13 and analyzed on E18.

than in controls (Fig. 3b vs. d for E16). In controls, the CP was densely packed with neuroblasts (Figs. 2d, 3c, and 4b). In addition, (2) radially oriented fibers were disrupted in the CP (Figs. 2b and 3a) and (3) the fibrous network in the marginal zone (MZ) was loose when compared with that in controls (Fig. 2a vs. c). Histological difference in the VZ was not remarkable between the RGD-injected and control brains.

Pyknotic cells in the telencephalic wall were quite rare throughout this experiment and there was no significant difference in the number of pyknotic cells between experimental and control groups.

Cell density is lower in the cortical plate and intermediate zone in RGD-injected brains

To confirm quantitatively the histological finding that the number of neuroblasts was affected by RGD-injection, cell density in the CP and IZ was analyzed in theleft dorsolateral telencephalic wall of E15 embryos (Fig. 5). This region has been chosen because on E15 the neopallial cortex is being differentiated and stratified into well defined layers which can be clearly observed on the horizontal sections. The RGD-injected brains had significantly lower cell density than controls in the CP (RGD: 0.296 ± 0.034 cells/μm³, control: 0.365 ± 0.035 cells/μm³; p < 0.005) and in the IZ (RGD: 0.234 ± 0.028 cells/μm³, control: 0.307 ± 0.02 cells/μm³; p < 0.005). On the other hand, there was no statistical difference in cell density of the VZ between the RGD-injected and control brains. These quantitative results confirmed the histological findings of the

Figure 5. Cell density in the CP, IZ, and VZ of the left telencephalon on E15. In the RGD-injected brain, cell density is significantly lower in the CP (p < 0.005) and intermediate zone (p < 0.005). These data supported the histological findings shown in Figs. 2, 3, and 4. Note that cell density in the VZ in RGD-injected brain is not different from the control. Mean ± standard error.

Figure 6. Thickness of the dorsolateral wall of the left telencephalic roof and each layer on E15. There are no significant differences between the RGD-injected telencephalon and control. Mean ± standard error.

Figure 7. Mitotic activity in the RGD-injected telencephalon of E15 embryos. Frequency of mitotic cells was significantly lower in the RGD-injected group than control (p < 0.05) in the dorsolateral roof of telencephalon. RGD: n = 10; PBS: n = 9. Mean ± standard error.

scarcely populated CP and IZ in the RGD-injected brains (Figs. 2, 3 and 4). The thickness of the total telencephalic wall, CP, IZ, and VZ of E15 embryos was measured in the same region. There were no statistical differences in thickness either of the total telencephalic wall or of each layer between the RGD-injected group and the control (Fig. 6). These data thus suggest that
the total cell number of CP and IZ in the RGD-injected brains is lower than in controls.

**Proliferative activity is reduced in the ventricular zone in RGD-injected brains**

Next, to examine the proliferative activity of progenitor cells, the frequency of mitotic cells in the most superficial layer of the left ventricular zone was obtained in the RGD-injected group and the control (Fig. 7). Our data showed the significantly lower mitotic activity of the neuroepithelial cells in the RGD-injected group (RGD: 8.1 ± 0.8%, PBS: 11.1 ± 0.9%: *p < 0.05*).

Thus, although there was no decrease in cell density in the VZ, proliferation of the neuroepithelial cells was reduced in RGD-injected brains. This suggests that there may be migration disturbance in addition to reduced proliferation, maintaining the overall cell density in the VZ (see discussion).

**DISCUSSION**

The present observations revealed both a decrease in cell density in the CP and IZ and reduced proliferative activity of the neuroepithelial cells in the VZ in RGD-injected brains. These data suggest that RGD inhibits the migration of post-mitotic cells and/or mitosis of the neuroepithelial cells in the telencephalic cortical histogenesis.

**RGD-proteins and integrins play roles in neuronal migration**

Of many CAMs which are involved in organizing the nervous system, laminin, fibronectin, L1 and tenascin have RGD sequence (13, 18-20). Recently, localization of laminin and fibronectin was demonstrated in the IZ and VZ of the developing telencephalon (21, 22), and moreover, laminin is also localized in the basement membrane of the neural tube (6). The radially oriented processes attach to contribute to the glial limiting membrane. In this attachment, laminin is the critical adhesive factor. The most widely supported hypothesis for the migration of neuronal cells has been the radial unit hypothesis in the developing telencephalon (23-25). In this hypothesis the radial glial cell is thought to act as the important guide for neuronal migration mainly based on the data from primates (24), although negative evidences of radial glial guidance for neuronal migration have been also reported in rodents (26, 27). The present observations show that fibrous architecture is scanty in the marginal zone of RGD-injected brain. Further radial fibrillary structures were disorganized in association with fewer neuroblasts in the cortical plate and the intermediate zone in RGD-injected brains. These changes may reflect the inhibition of attachment of the processes to the basement membrane by RGD exposure, or a decrease in the number of cells whose processes contribute to the glial limiting membrane. This disorganized architecture may suggest a primary inhibition of the RGD sequence-dependent guidance for neuronal migration. Tenascin has been reported to have RGD sequences at least in chicken and humans (18) and to mediate cell attachment through an RGD-dependent integrin receptor (28). In mice, however, it has been demonstrated recently to have no RGD sequence (29). The L1 molecule has been demonstrated on the cell surface of post-mitotic neuroblasts but not on neuroepithelial cells (7) and mediates cell-to-cell attachment by homophilic binding (7), and neuron-glia interaction (3). However, it is questionable whether or not the RGD sequence functions in L1-mediated cell adhesions (12) and it is also unclear whether L1 molecule-mediated cell adhesion was affected or not by exposure to RGD in the present study.

On the other hand, antisense $\beta 1$ integrin RNA was introduced into the optic tectum in chick embryos and pathological changes similar to our present results were demonstrated (5). In this respect, lower cell density in the RGD-injected brain in the present study may be the results of inhibition of integrin-mediated migration in addition to inhibition of guidance of the radially oriented process mentioned above.

Another possible explanation is that the cell death was caused by inhibiting migration induced hypoplastic plate. In fact, Galileo et al. (5) described that the cell death could be caused in the neurons with interfered migration several weeks later after birth. However, the pyknotic cells in the telencephalon were not evident throughout the experimental periods (E15 - E18) in the present study and therefore the cell death induced by RGD-injection is not likely to be the prior causative reason for the present phenotype.

Irregular surface morphology was the characteristic feature of the RGD-injected brains of E18, but not E15, embryos. Wood et al. (30) reported that most cortical neurons in the telencephalon were generated from E14 (the day of vaginal plug was designated E0, which corresponds to E0 in our study) and that neurons generated before E13 migrated to the subplate and did not contribute to the thickness of the CP. The estimated time of migration of neurons from the VZ to their final destination in the CP formation is about two days in mice or rats (31). On E15, even control brains had thin CHs which were mainly composed from neurons generated on E13 and E14. Considering the continuous inhibitory effects of the peptide since E13, the temporal factor mentioned above might be the reason why the telencephalon on E18 has more remarkable external changes than on E15 in the present study.

**RGD peptide affects the neurogenesis in the VZ**

Although RGD peptide inhibited the cortical histogenesis, interestingly, cell density in the VZ was not lower in the RGD-injected than in the control brains. If solely migration of the post-mitotic cells was inhibited, interfered cells should be remaining in the VZ and increase the total number of cells in the VZ. However, there was no obvious difference in cell density and thickness of the VZ, which reflected the total number of cells composing the VZ, between the RGD-injected group and the control. We analyzed the mitotic activity in the VZ and revealed the low frequency of mitosis in the VZ of the RGD-injected brain. Therefore the inhibitory effects of RGD both on migration and neurogenesis may underlie the
present observations. RGD may primarily inhibit neuronal proliferation. Galileo et al. (5) demonstrated the inhibitory effect of integrins on proliferation of the neuroepithelial cells in chick embryos and suggested the integrin-mediated interactions as the survival factors in neuronal development. Alternatively, loss of cell-to-cell contact by RGD exposure may secondarily affect neuronal proliferation, since it is well evidenced that proliferation is anchorage-dependent in non-transformed cells (32). In some cases, contact itself is the primary inducible factor for mitosis (33).

An observed lower proliferative rate may be limited to neuronal progenitors or may include the glial cell population in M phase (27). In the hypothesis of the radial unit and clonal column of neurons, a radial glial cell guides the migration of numerous clonal neurons. Therefore the influence by the possible lower frequency of radial glial mitosis should be noted during analyzing data.

Possible interaction between neuroblast migration and progenitor proliferation

"Niche" hypothesis of hemopoiesis by Schofield (34) may offer another explanation for the inhibition both in the neuroblast migration and the progenitor proliferation. According to his hypothesis, of two daughters divided from a hemopoietic stem cell, only one occupies the niche as the stem cell and the other differentiates. During retaining in the niche, the stem cell fails to mature and maintains its capacity to proliferate. In such an environment, specific cell-to-cell contact is important. This hypothesis has been also introduced for the fixed cells in the tissue (34). The kinetics of generating neurons is similar to that in this hypothesis. The progenitors of neurons in S phase are fixed in the deep area of VZ where they have contacts with their neighbors, and then they move to face the ventricular lumen in M phase without cell-to-cell contact and become round in shape. After cell division, one remains in the undifferentiated state to generate more neurons and the other becomes the neuroblast (35). In the present study, post-mitotic neuroblasts exposed to RGD might be inhibited to migrate and remain in the VZ, failing to make a vacant space for the progenitors. In the "niche" hypothesis that neuronal progenitors behave as the stem cells, the proliferative capacity of the progenitor may be affected by the migration activity of the neuroblast that makes a new space for division of other progenitors.

In vitro studies in parallel with in vivo direct injection experiments are indispensable to further analyze these possibilities. As in the discrepancy in the radial glial guidance hypothesis, the comparative studies from evolutionarily low species to primates should also be required.

REFERENCES

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