Original Article

Extracellular matrix in the CNS induced by neuropathogenic viral infection

Rihito Watanabe and Masatoshi Kakizaki

Department of Bioinformatics, Faculty of Engineering, Soka University, Hachioji, Japan

During the early phase of infection with an extremely neurovirulent murine coronavirus, cl-2, the ER-TR7 antigen (ERag)-positive fibers (ERfibs) associated with laminin and collagen III show a rapid increase in expression levels in the meninges, followed by an appearance of the antigens in the ventricle and brain parenchyma. Then, cl-2 invades the ventricle and ventricular wall along the newly assembled ERfibs after infection, using them as a pathway from the meninges, the initial site of infection. In the lymph nodes and spleen, ERag is mainly produced by fibroblastic reticular cells (FRCs), which play a key role in nursing the ERfibs to form a fibroblastic reticular network (FRN). The FRN functions as a conduit system to transfer antigens, cytokines or leukocytes in the lymphoid organs. In the brain parenchyma, astrocytes were found to produce the main components of mature ERfibs, such as collagen, laminin and ERag, which have been identified in the lymphoid organs. The producibility of these extracellular matrices (ECMs) by astrocytes was further confirmed by primary brain cultures, which disclosed the dissociation of laminin and ERag production, and the close association of ERag production with that of collagen, forming a fibrous structure. The pattern of ECM production in vitro indicated the process of forming mature ERfibs in the brain, that is, fibers made of collagen fibers and ERag are wrapped by laminin prepared as a sheet structure. In addition, the brain parenchymal cells that produce interferon β after infection in spite of their residence away from the sites of viral invasion were surrounded by ERfibs, which were closely associated with astrocytic fibers. These findings indicate that astrocytes play a central role in forming the astrocytic reticular network (ARN) in the brain parenchyma, as FRCs do to form FRN in the lymphoid organs.

Key words: astrocyte, collagen, coronavirus, fibroblastic reticular cell, laminin.

INTRODUCTION

cl-2 virus (cl-2) is an extremely neurovirulent murine coronavirus, replicating rapidly in the brain after infection.1 Infected mice fall into a morbid state within 48 h post-inoculation (hpi), and die within 72 hpi with many infected neurons in the brainstem and cortex. The less virulent viruses srr7 virus (srr7)1 and Mu-3 virus (Mu-3)2,3 have been isolated from cl-2 and srr7, respectively. Most mice infected with srr7 survive for 8 days after infection. The viral antigens are predominantly detected in the white matter, and neurons are not infected.1,4,5 The virulence of Mu-3 is intermediate between that of cl-2 and srr7, showing a mixed pattern of the neuropathologies of cl-2 and srr7.6 The viral antigens are detected in both the white matter and neurons.3 Typical neuropathological change observed in the mice infected with Mu-3, and not with cl-2 or srr7, was apoptosis-induced in the CA2 and CA3 regions of the hippocampus at 4 days post-inoculation (dpi). In spite of these differences in virulence and neuropathological findings, the viruses exhibit a similar pattern of viral spread during the initial phase of infection. Between 12 and 24 hpi, viral antigens are detected only in the meninges, followed by viral spread into the ventricular wall at 24 to 48 hpi before invasion into the brain parenchyma, indicating that the viruses use a passage between the meninges and ventricular wall as an entry route into the brain parenchyma.3–5

At 48 hpi, the passage was found to be constructed by the extracellular matrix (ECM) between the ventricle and meninges at the cerebellopontine angle.7 The ECM includes an antigen recognized by Erasmus University Rotterdam-thymic reticulum antibody 7 (ER-TR7).8 ER-TR7-antigen (ERag)-positive fibers (ERfibs) associated with laminin and collagen III function as a conduit system for viral entry into the brain parenchyma from the meninges.7 Studying the initial phase of infection in our previous report,7 the formation of ECMS other than ERag were detected in the ventricular and paraventricular zone, and around the blood vessels, but it was not prominent in the brain parenchyma in vivo. Therefore, in this report, we studied ECM formation in the brain during the later phase of infection at 72 hpi.
The ERtibs detected by ER-TR7 are the main components as well as a hallmark of the fibroblastic reticular network (FRN) in the lymph nodes\textsuperscript{9,10} and spleen.\textsuperscript{11,12} The FRN as a conduit facilitates the transfer of foreign antigens or immuno-mediating cytokines to their proper sites in order to start or enhance efficient immune reactions, and guides immuno-competent cells for their homing.\textsuperscript{10,12} The main construct of FRN is maintained by ERag-producing fibroblastic reticular cells (FRCs) in the lymphoid organs.\textsuperscript{13} In the CNS, ERag is produced by almost all cell types that constitute the CNS, and the most prominent ERag-producing cells in the brain parenchyma are astrocytes.\textsuperscript{7} However, it has yet to be elucidated what cell type in the brain parenchyma produces the components of ERtibs other than ERag. The ERtibs in the lymphoid organs have collagen fibers as a core, which is wrapped in an ERag-rich matrix, and further by the laminin-rich basement membrane at the outermost surface.\textsuperscript{11,14} These components in lymph nodes are produced by the stromal cells, which include FRCs, lymphatic endothelial cells, blood endothelial cells and contractile pericytes.\textsuperscript{13} In the brain, especially in the brain parenchyma, we propose that astrocytes play a central role, like FRCs in the lymphoid organs, in ERag-positive reticular formation after infection, because we found that astrocytes produce the main components of ERtibs, such as ERag, laminin and collagen, in response to viral invasion.

**MATERIALS AND METHODS**

**Viruses and animals**

A highly neuropathogenic mouse hepatitis virus (MHV) strain JHM virus (JHMV) cl-2 (cl-2)\textsuperscript{15} was propagated and used to inoculate the right frontal lobe of BALB/c mice (Charles River, Tokyo, Japan) under deep anesthesia, as previously described.\textsuperscript{7} The mice were maintained according to guidelines set by Soka University. At scheduled time periods after inoculation, organs including the brains and spleens were removed from mice after exsanguination under deep anesthesia, and were embedded in Tissue Tek OCT (Sakura, Tokyo, Japan) and snap frozen to prepare frozen sections.\textsuperscript{7}

**Immunofluorescence**

Ten-micrometer cryostat sections prepared from unfixed frozen tissues were fixed in ice-cold acetone for 10 min.\textsuperscript{7} Primary mixed neural cell cultures were established from the brains of neonatal mice, as previously described.\textsuperscript{7,16,17} Mixed primary cultures on eight-well plastic chamber slides (Nalge Nunc International, Rochester, NY, USA) were fixed in cold ethanol for 1 min followed by fixation in cold acetone for 5 min. Immunostaining was carried out using antibodies for collagen type I (ColI) (Calbiochem, San Diego, CA, USA), ColIV (SouthernBiotech, Birmingham, AL, USA), ColVI (Abcam, Tokyo, Japan), and interferon β (IFNβ) (PBL Interferon Source, Piscataway, NJ, USA), in addition to the antibodies and reagents previously described,\textsuperscript{5,7} such as antibodies to detect MHV-JHM, ERag, laminin, ColIII, podoplanin, neurons, oligodendrocytes, GFAP and appropriate secondary antibodies, and reagents such as biotin-conjugated streptavidin, and Hoechst 33342 for nuclear staining. Fluorescence was visualized with a confocal laser scanning microscope (Leica Microsystems, Heidelberg, Germany) to obtain images of double or triple immunostaining and three-dimensional constructs, as previously described.\textsuperscript{7} A fluorescence microscope (Keyence, Osaka, Japan) equipped with a BZ analyzer (Keyence) was used for statistical study. Quantification of immunofluorescence staining was performed using 24-bit color images with a 1360 × 1024 pixel resolution, acquired with a fluorescence microscope (Keyence). Three independent approaches were performed to quantify the observed immunofluorescence signal using a BZ analyzer. The background fluorescence intensity (FI) in areas of the field of view was subtracted from total measurements of FI. The average fluorescence intensity (AFI) within a unit area was obtained by dividing its integrated FI by its area.

**RESULTS**

**ECM expression in primary brain tissue cultures**

In primary brain tissue cultures, many cells in the uninfected culture were found to produce ERag (Fig. 1A). This effect may be due to physical stress during cell preparation for culture, because physical treatment causes an increase in ERag expression in vivo.\textsuperscript{18} However, the fibrous extracellular structure of ERag was not as clear in the uninfected culture as in the JHMV cl2-infected culture (Fig. 1B–F) and a deposition of bulky ERag\textsuperscript{+} fibers was found to surround the infected areas (Fig. 1B). Thus, the expression levels of ERag were increased in the infected culture when analyzed by the average fluorescence intensity (AFI) (Fig. 2). Immature forms of ERtibs, detected in vivo\textsuperscript{7} as solitary ERag expression without an association with other components of ECM such as laminin and collagen, were also observed in the brain mix culture. Especially, the co-localization of ERtibs and laminin\textsuperscript{+} fibers occurred infrequently (Fig. 1C). Also, a large population of laminin-producing cells was found to surround a small cluster of ERag-producing cells, whereas there were only a few laminin-producing cells in the areas with a large cluster of ERag-producing cells (Fig. 1D). In addition, laminin was found to be extracellularly secreted to form a sheet-like structure, which was observed when an image was taken in different focal planes (Fig. 1E4). In the deepest plane (Fig. 1E1) among the images shown in Figure 1E, laminin...
Fig. 1 Seven days after seeding a CNS cell suspension obtained from newborn mice on eight-well chamber slides, the culture cells were infected with cl-2, or left uninfected (V(−)). At 24 and 48 hours post-infection (hpi) (V (24 hpi) and V (48 hpi), respectively), culture slides were processed to detect ER-TR7 antigen (ERag) (Er), mouse hepatitis virus strain JHM virus (JHMV) (V), laminin (La), and collagen III (Col). Hoechst 33342 (H) was used for nuclear counter-staining. E: A Z-stack study with a 0.5-μm step size, as illustrated in E4, revealed La-positive (La⁺) lace curtain-like structures in E3 at the 12th step (z12), which is located 4 μm above z8 (E1). At a higher resolution in E than that in C and D, only a minor population of La⁺ fibers was found to co-localize with ERag-positive fibers (ERfib), in contrast to the high numbers of Col⁺ fibers co-localized with ERag (F). G: A cluster of cells producing both ERag and Col is shown, which also contrasts with an image of La production (D). Long arrows indicate a fiber with co-localization of ERag and laminin. Short arrows in E3 indicate holes in the laminin sheet. The arrowhead indicates the nucleus. Single and double bars indicate 50 and 20 μm, respectively.

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expression is not prominent except for as a partial colocalization with the bold ERfib indicated by long arrows. At a position 2 μm above the image shown in Figure 1E1, ERag+ laminin+ sheets (Fig. 1E3), with small-diameter holes (short arrows) compared with the size of the nucleus (Fig. 1E2), appeared to cover underlying ERtibs. The ERag+ laminin+ sheets showed a lace curtain structure hanging from the mature ERag+ laminin+ ERtibs (arrows in Fig. 1E1–3). In contrast, collagen fibers were associated with ERtibs more closely (Fig. 1F) than laminin. Furthermore, an area of clustered cells that produced both collagen and ERag was observed (Fig. 1G).

**In vivo expression of laminin and collagen**

Before identifying the type of brain parenchymal cells in primary brain culture that produces ECMs, we studied in vivo ECM expression during the late phase of infection, at 72 hpi, because laminin and collagen expression was not marked during the early phase of infection between 12 and 48 hpi in the brain parenchyma.7 As expected, at 72 hpi, laminin and collagen were detected more intensively in the brain parenchyma (Fig. 3) than during the earlier phase of infection,7 when they were examined in the area where tissue destruction was not marked (Fig. 3A). ERag appeared in the brain parenchymal area close to the meninx or ventricle (Fig. 3B), and was found at a distance from the viral antigens (arrows in Fig. 3B1, B2), as we previously reported.7 However, laminin expression was observed in wider areas compared with ERag expression, with fine fibrous structures in the brain parenchyma (Fig. 3C1–3). The colocalization of laminin and GFAP was observed in the areas near the meninges and ventricle (Fig. 3C3–5), where structures of blood vessels were accompanied by laminin expression (Fig. C3). Some of them were wrapped by laminin-positive (La+) astrocytic fibers (Fig. 3C3, C4), but many were free of La+ foot process of astrocytes at a low magnification (Fig. 3C3). However La+ GFAP+ dot-like structures on the outer surface of blood vessels were noted at a higher magnification (Fig. 3C5).

Next, we examined collagen (Col) expression, especially Col type one (ColI), ColII, ColIV and ColVI, because they are reportedly components of FRN associated with ERag.13 In the brain parenchyma, the expressions of ColII, II and IV were identified (Fig. 3D–F) but not that of ColVI (data not shown). The co-localization of GFAP and ColII or ColIII was clearly detected apart from in the structures of blood vessels (Fig. 3D, E). However, ColIV was not co-localized with GFAP (Fig. 3F), and was mainly detected in the structures of blood vessels (Fig. 3F1, 3F3) and arachnoid cells (Fig. 3F1, 3F3). Col and ColIII were distributed in a similar manner except for their association with blood vessels. Intensive expression of ColIII in the wall of blood vessels was observed (Fig. 3E1, E3, E4), whereas the association of CoII with the structure of blood vessels was not marked (Fig. 3D). A low-level expression of laminin and collagen in the brain parenchyma of sham-infected mice has been reported.7 Here for a comparison, we present the results of studies on ColIII and ColIV in the sham-infected mice (Fig. 3G), which were not shown in our previous report. In the brains of sham-infected mice, low-level expression of laminin and collagen was not marked (Fig. 3D). A low-level expression of laminin and collagen in the brain parenchyma of sham-infected mice has been reported.7 Here for a comparison, we present the results of studies on ColIII and ColIV in the sham-infected mice (Fig. 3G), which were not shown in our previous report. In the brains of sham-infected mice, low-level expression of ColIII was detected in a line of arachnoid cells. Activated astrocytes that produced CollIII were not observed, and CollIII+GFAP+ figures were obtained only as dot-like structures (Fig. 3G1) in the sham-infected mice. Coll expression in the brain parenchyma of sham-infected mice was also not marked (Fig. 3G2). In the present study, we did not perform a statistical analysis of the relationship between viral antigens and ECM expression, because the areas we examined are not the main viral entry site into the brain parenchyma.3,5,7 However, we found a tendency whereby a higher ECM expression occurred in the areas where viral antigens were detected (Fig. 3H).

**ERag-producing cells in primary brain tissue cultures**

Similar to the in vivo observation,7 we also found a variety of ERag-producing cells, including astrocytes (Fig. 4A) and oligodendrocytes (Fig. 4B). Podoplanin-positive (Pod+) cells that did not appear as characteristic fibroblasts (Fig. 4C) were also found to produce ERag. We could not characterize the cell type of Pod+ cells in a way we did in our in vivo study as ependymal or arachnoid cells,7 because these cells did not form tissue structures in the primary culture.
Fig. 3  Frozen sections were prepared from the hypothalamic area of mice infected with cl-2 at 72 hours post-infection (hpi) except when otherwise indicated. A sham-infected mouse is indicated as V(−). Sections were stained by HE or immunofluorescence for ER-TR7 antigen (ERag) (Er), mouse hepatitis virus strain JHM virus (JHMV) (V), laminin (La), GFAP (Gf), collagen type I (Coll), CollIII and CollIV. B2, C2, C4 and C5, E2 and E3, and F2 and F3 are higher magnifications of B1, C1, C3, E1 and F1, respectively. Asterisks indicate the third ventricle. The bold arrows indicate the blood vessels. Triangles in C indicate dot-like co-localization of laminin and GFAP. Short arrows in D, or E and G indicate the positions of GFAP-positive cells with Coll or CollIII expression, respectively. Long arrows in F indicate arachnoid cells. Single and double bars indicate 100 and 20 μm, respectively.
CD11b+ cells in the culture were ERag+ (Fig. 4D), as we found in an in vivo study. However, although we reported ERag+ neurons in the in vivo study, we could not obtain convincing pictures of ERag+ neurons in the primary culture, using anti-neurofilament (Nf) and anti-neuronal nuclei (NeuN) antibodies (data not shown).

Possible contribution of astrocytes to reticular network formation in the brain parenchyma

Next, we examined the production of ECMs other than ERag by astrocytes in the primary culture to confirm the in vivo observations described above. Laminin and Coll were produced in the GFAP+ cells in the primary culture (Fig. 5A,B), not contradicting the in vivo findings. However, ColIII+GFAP+ cells were not detected in the cultured cells (Fig. 5C), although the co-localization of ColIII and GFAP was clearly detected in the in vivo study. The ColIII+ astrocytes observed in vivo might be a small and/or specialized population among astrocytes (see Discussion). ColIV-positive astrocytes were not found in the primary culture (Fig. 5D), which agreed with the in vivo observation.

A question that arises is why these astrocytes are producing several kinds of ECMs after infection. In order to answer this, we focused on the expression of IFNβ, a type I interferon, among several cytokines. Reportedly, MHV infection in vitro inhibits IFNβ production, although the level of IFNβ is elevated in the brain after infection with neuropathogenic MHV strains. A reason for this discrepancy was clarified by our in vivo study. The infected cells in the ventricle and ventricular wall during the early phase of infection did not produce IFNβ, but the cells surrounding the infected cells did (Fig. 6A1). Furthermore, deep in the brain parenchymal area of the pons where no viral antigens were detected, IFNβ-positive cells were found (Fig. 6A2). How did these cells, away from the infected site, get the information on infection? We propose that astrocytes form an astrocytic reticular network (ARN), which creates a conduit system in a similar fashion to FRN found in lymphoid organs, by producing ECMs including ERag, a maker of FRN. In support of this, a fine astrocytic network was found to surround the IFNβ-producing cells deep in the pontine parenchyma (Fig. 6B), where no viral antigens are found during the early phase of infection, and tissue destruction has yet to occur (Fig. 6C). Furthermore, spotty ERag+ areas were connected by astrocytic foot processes (Fig. 6C).

DISCUSSION

Using the brain primary culture, we confirmed the findings of a previous report that showed the in vivo production of ERag by many types of cells in the CNS during the early phase of infection with cl-2. In addition, we showed that the neuro-virulent viruses can induce the production of ECMs other than ERag in the brain parenchymal cells, especially astrocytes, producing major components of mature
ERfibs including ERag, laminin and Col. Studies in vivo on
the later phase of infection at 72 hpi, where an enhanced ex-
pression of ECM in the brain appeared compared with that
in the earlier phase, enable us to see more clearly that the
areas of laminin expression were more widely spread than
the ERag-positive areas, compared with our previous study.7
Although the interaction of astrocytic laminin and surround-
ing conditions, mainly in the areas of the blood brain barrier
(BBB),22–24 or in the developmental brain,25 has been stud-
ied, the interaction of laminin and ERag expression has not
been clarified, because astrocytic ERag production has not
been reported except in our previous study.7 The earlier pro-
duction of laminin by astrocytes prior to ERag expression7
might have stimulated ERag production through autocrine
effects on the function of astrocytes, because astrocytic lam-
inin is necessary for some of their functions, shown by the
fact that a lack of the laminin α2 subunit produced by astro-
cytes induces hypertrophic endfeet of astrocytes and ob-
structs appropriate polarization of aquaporin 4 expressed
on astrocytes in the BBB.22 Furthermore, the modulation
and organization of astrocytic laminin levels mediate glia-
nervous commitment.23 Another possibility is that astro-
cytic laminin restricts ERag expression, leading to narrower
areas of ERag expression compared with laminin-positive
areas. Astrocytic laminin prevents the differentiation of
pericytes, which have been shown to express ERag in the

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Fig. 6  Frozen sections were prepared from the pons of mice infected with cl-2 at 48 hours post-infection (hpi) when viral invasion into the brain parenchyma is still not prominent, and were processed to detect mouse hepatitis virus strain JHM virus (JHMV) (V), interferon β (IFNβ), GFAP (Gf) and ER-TR7 antigen (ERag) (Er). IVv and Mx indicate the fourth ventricle and meninx, respectively. IFNβ production was observed either in the ventricle near viral antigens (A1), in the area away from the ventricle where no viral antigens are detectable at 48 hpi shown as dotted area in A1 and at a higher magnification (A2), or in the middle of the pons in the area of the gigantocellular reticular nucleus away from both the ventricle and meninx (B). C: Long arrows indicate blood vessels surrounded by astrocytes with ERag expression (C1), which do not show any connection with one another in the image with ERag-staining alone (C2). The areas with low expression levels of GFAP or ERag indicated by boxed areas in C1 with an arrowhead and short arrows are shown at higher magnifications in C3 and C4, respectively, which revealed that ERag expression near (C4) and away from blood vessels (C3) is connected with fine networks of astrocytic fibers. Single and double bars indicate 100 and 20 μm, respectively.
brain after infection,7 from the resting stage to contractile stage.24 It is noteworthy that the contractile pericytes are one of the ERag-producing cell types in lymph nodes.13

In contrast to laminin expression, Col expression was more closely associated with that of ERag, taking the form of a fibrous structure. The ERfib in the lymphid organs have collagen fibers as a core that are wrapped by ERag-positive ECM, and finally surrounded by laminin at the outermost surface.26 The findings in the primary cultures indicated the process of forming mature ERfib in the brain. That is, immediately after the secretion of collagen and ERag from astrocytes, or before the secretion, they form intimate contact with each other, and form a fibrous structure. Subsequently, the fibers are packed in the astrocytic laminin prepared in the form of a membranous sheet structure.

Although many of the findings in vivo were confirmed by in vitro studies, there were some discrepancies between them. We documented the in vivo expression of ERag in neurons.7 However, we could not obtain convincing pictures showing the co-localization of ERag and Nf, or ERag and NeuN in the primary tissue cultures. It might be due to the more rapid destruction of neurons in tissue cultures after infection compared with that in in vivo infection. In brain primary cultures, there are clusters of neuronal cells, which disappear after infection leaving scattered neuronal cells.17

Another discrepancy between in vivo and in vitro studies is that we did not find ColIII-producing astrocytes in vitro, while astrocytic ColIII was clearly detected in vivo after infection. However, Heck et al. demonstrated that astrocytes secrete fibrillar Col, III and V in culture, while they found no astroglial collagen expression in vivo in normal rats.27 They adopted different procedures from ours for primary culture, obtaining a large population of astrocytes in the culture, and continued cultures for a longer period than we did. The finding that they did not detect astrocytic Col in vivo using immunofluorescence, which led to their interpretation of why previous neuroscientists had concluded that the brain is the only organ that lacks fibrillar elements such as collagen fibers,28 corresponds to our previous report7 that showed ColIII expression was low-level in the brain except for the meninges and ventricles during the early phase of infection. Since Col expression in the astrocytes was more intensive than that of ColIII, ColII may be a better marker for studying ECM production in the brain parenchyma after brain injury.

Like laminin, collagen is considered to be an indispensable ECM for CNS functions and morphogenesis, including that of astrocytes, neurons28,29 and oligodendrocytes.30 If so, Col and laminin should exist in the brain parenchyma besides the meninges or perivascular areas of animals in the normal state. A limitation to detect a small amount of ECM using the available techniques, including a light microscopical approach, had led to the misinterpretation by neuroscientists as Heck et al. pointed out and as described earlier.28 The reticular fiber that comprises the FRN in the lymph nodes is around 1 μm in diameter.26 We detected the fine network of astrocytic fibers and ERfib only at a high magnification of a confocal laser scanning microscope, which has insufficient resolution to trace the initial conduit that transfers the message of viral invasion deep into the brain parenchyma. One thin line of ERfib might function as a conduit to activate astrocytes deep in the parenchyma after the recognition of viral invasion at the ventricular wall or brain surface facing the arachnoid membrane. Although it is important for medical treatment to handle an initial immune response after infection in the brain, little is known about how the immune network is constructed in the brain. Our experimental model using an extremely neurovirulent viral clone, cl-2, and an attenuated mutant, Mu-3, provides an opportunity to investigate reticular network formation after infection in the brain.

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