FULL PAPER *Pathology*

Non-Cytopathic Bovine Viral Diarrhea Virus Infection Inhibits Differentiation of Bovine Neural Stem/progenitor Cells into Astrocytes *in Vitro*

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ABSTRACT. Bovine viral diarrhea virus (BVDV) causes fetal brain malformations in ruminants when the fetuses are infected transplacentally in mid-pregnancy. In both cytopathic and non-cytopatic virus infections, viral lytic infection in actively replicating cells and interruption of vascular integrity have been suggested as the pathogenesis, but functional disturbance of infected neural developing cells has been unclear. In this study, we examined the effect of infection with non-cytopathic BVDV2 on the differentiation of neural stem/precursor cells isolated from the bovine fetus. In the process of differentiation to three types of neural cells, neurons, astrocytes and oligodendrocytes, virus infection significantly and selectively inhibited the differentiation of neural stem/precursor cells into the astrocytic lineage. This inhibition is possibly important for the pathogenesis of congenital brain malformations associated with non-cytopathic BVDV infection.

KEY WORDS: astrocyte, bovine viral diarrhea virus, central nervous system, differentiation, neural stem cell.

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In cattle, bovine viral diarrhea virus (BVDV) infections cause a variety of clinical and pathological conditions, including congenital defects of the central nervous system (CNS), according to the virus strain and state of the host [5, 8, 23]. BVDV is a small enveloped virus with a positive-strand RNA genome, classified in the family *Flaviviridae* and genus *Pestivirus*. In BVDV, there are two biotypes designated non-cytopathic (NCP) and cytopathic (CP), depending on their effect on tissue culture cells [13], and also two genotypes, type 1 and 2, according to antigenic and genetic differences [26].

BVDV infection in susceptible pregnant cows frequently results in transplacental spread of virus to the fetus. The outcome for the infected fetus varies, principally depending on the immunological maturity of the fetus at the time of infection [5, 8, 23]. Infections in the second trimester of gestation, when fetal immunocompetence develops and the CNS is in the final stages of organogenesis, cause a variety of congenital CNS malformations [2–4, 7, 16, 17, 22]. In both field and experimental cases of CNS malformations following intrauterine BVDV infection, the lesions have been mainly investigated by histopathological methods, whereas functional disturbances of developing cells in the CNS infected with BVDV have not been examined previously. In this study, we examined the effects of NCP BVDV infection on the differentiation of neural stem/progenitor cells derived from bovine fetuses.

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MATERIALS AND METHODS

Dissociation of brain tissue and culture of bovine neural stem/progenitor cells: Isolation and subsequent culture of bovine neural stem/progenitor cells (BNSP cells) were undertaken by a modification of the neurosphere method [24, 25]. The whole brains were removed aseptically from bovine fetuses of about 15 cm in crown-rump length, which was negative for BVDV. The tissue was homogenized by gentle pipetting in Dulbecco's modified Eagle's medium/F12 medium (DF; Invitrogen, Carlsbad, CA, U.S.A.). After allowing enough time for the undissociated pieces to settle to the bottom, the top cell suspension was transfered to a new tube. Dissociated single cells were harvested by repeating these steps and the cell number counted. The cells were seeded in uncoated plastic dish at a density of 2×10^5 viable cells/m*l* and cultured in DF medium supplemented with a neural supplement mix (NSM), including 80 μg/m*l* human transferrin (Sigma, St. Luis, MO, U.S.A.), 25 μg/m*l* bovine insulin (Sigma), 60 μM putrescine (Sigma), 20 nM progesterone (Sigma), and 30 nM sodium selenite (Sigma), along with growth factors (GF), 20 *n*g/m*l* epidermal growth factor (Peprotech, London, UK) and 10 *n*g/m*l* fibroblast growth factor b (Peprotech), at 37°C in a humidified atmosphere of 5% CO_2. One half of the culture medium was replaced with fresh culture medium every 3 days. In this culture condition, dissociated cells started to form floating cell aggregates similar to microspheres as reported for other mammals (Figs. 1A, B) [6, 9, 19, 24, 25].

Differentiation of BNSP cells: Two to 13 days after seeding, the culture medium, including the floating spheres, was collected. After centrifugation at 110 g for 10 min and removal of the supernatant, the pellet was dissociated to sin-

gle cells or small clumps by gentle pipetting in fresh DF medium. Cells at about 2×10^5 cells were seeded in 35 mm plastic dish pretreated with 0.01% poly-L-ornithine (Sigma). The cells were incubated in DF with NSM and 2% heat-inactivated horse serum at 37°C in a humidified atmosphere of 5% CO_2.

BVDV infection: Non-cytopathic BVDV2, strain KZ-91 NCP [20], was used for infection. Viruses were propagated in Madin-Darby bovine kidney cells and titered by endpoint dilution in these cells. We inoculated viral supernatant, including 2×10^5 50% tissue culture infective dose ($TCID_{50}$) at a multiplicity of infection (MOI) of 1, when the dissociated BNSP cells were induced to differentiate.

Immunocytochemistry: For the quantative estimation of four classes of neural cells, dissociated BNSP cells at the start of differentiation and those harvested with 0.25% trypsin-0.1 mM EDTA at 3 and 7 days post-differentiation (DPD) were fixed in 4% paraformaldehyde for 15 min at room temperature, attached to silane-coated glass slides and used for immunocytochemistry by an indirect fluorescent method. The first antibodies used to identify cell classes were mouse monoclonal antibodies against nestin (clone Rat-401, Chemicon, Temecula, CA, U.S.A.) for neural stem/progenitor cells, beta III tubulin (TUB; clone TU-20, Chemicon) for neurons, oligodendrocyte-specific protein (OSP; clone CE-1, Chemicon) for oligodendrocytes and rabbit polyclonal anti-glial fibrillary acidic protein (GFAP; DakoCytomation, Glostrup, Denmark) for astrocytes. For the detection of BVDV antigen, mouse monoclonal antibody 46/1 against BVDV NS3 protein [18] and rabbit serum immunized with BVDV NS3 protein were used. Secondary antibodies were Alexa Fluor 488-labeled donkey anti-mouse IgG (Molecular Probes, Eugene, OR, U.S.A.), Alexa Fluor 555-labeled donkey anti-rabbit IgG (Molecular Probes) and fluorescein-conjugated donkey anti-mouse IgM antibodies (Jackson ImmunoResearch, West Grove, PA, U.S.A.). Monomeric cyanine nucleic acid (TO-PRO-3, Molecular Probes) was used for nuclear staining.

For morphological examination, undissociated spheres were collected, fixed, attached to the coated slide and stained for nestin. Differentiating cells at 7 DPD adhering to the plastic dish with or without viral infection were fixed and immunostained for the antigens described above. Analyses were performed using confocal laser scanning microscopy (Carl Zeiss, LSM5 PASCAL).

Quantitative determination of differentiation of bovine neural stem/progenitor cells: Differentiation rates in both uninfected and infected cells were analyzed at 0, 3 and 7 DPD. The rates were scored by counting cells positive for nestin, TUB, OSP or GFAP in over 500 cells in random fields from 3 dishes, respectively. The examination was performed independently at least three times and data are expressed as the means ± SD. Statistical significance of the composition rates of four cell classes between uninfected and infected cells were evaluated using Student's *t*-test ($P<0.05$).

RESULTS

Evaluation of bovine neural stem/progenitor cells: Dissociated cells obtained from fetal brain tissue formed floating cell aggregates known as neurospheres and these spheres gradually enlarged (Figs. 1A, B). The cells composing these spheres expressed nestin (Fig. 1C). Using the differentiation procedure, individual cells from the spheres adhered to the bottom surface and differentiated into TUB-expressing cells with a spherical cytoplasm and long, slender cell processes (Fig. 1D), GFAP-expressing cells with relatively broad cytoplasm and processes (Fig. 1E), or OSP-expressing cells with an ellipsoid cytoplasm and long, slender processes (Fig. 1F). In the culture conditions used in this study, we were able to maintain the spheres for up to 6 months while maintaining the expression of nestin, but cells did not show favorable proliferation properties.

Effect of bovine viral diarrhea virus infection on differentiation of bovine neural stem/progenitor cells: We inoculated BVDV strain KZ-91 NCP into neurosphere-comprising cells at the induction of diffentiation and investigated the changes in the composition rates of cells expressing each cell class-specific antigen, and then compared the rates with those in uninfected cultures (Table 1). At the start of differentiation, cells were composed of 35.5 ± 11.7% of nestin-expressing cells, 14.6 ± 6.1% of TUB-expressing cells, 10.1 ± 9.4% of GFAP-expressing cells and 33.7 ± 12.1% of OSP-expressing cells. In uninfected cultures under the differentiation condition, nestin-expressing cells decreased and cells positive for the three differentiated cell markers increased.

In cultures inoculated with BVDV, GFAP-expressing cells did not increase and the decrease of nestin-positive cells detected in uninfected cells was inhibited by the same amount. The percentages of nestin- or GFAP-expressing cells were statistically different between BVDV-infected and uninfected cells at 7 DPD. There were no significant differences in the proportions of differentiated cells expressing TUB or OSP between uninfected and infected cells. There were no differences in cell morphology and total cell number between uninfected and infected cultures (Figs. 1G-I). Virus antigens were not detected in uninfected cells.

DISCUSSION

The fetal bovine brain-derived cells used in this study formed floating cell aggregates in the culture medium that were morphologically similar to those reported in other mammals [6, 9, 19, 24]. These cell aggregates are called neurospheres and are reported to contain neural stem/progenitor cells. The floating cell aggregates expressed nestin, an intermediate filament of neural stem/progenitor cells and, under the differentiation condition, nestin-expressing cells decreased and the proportions of the three types of neural differentiated cells increased. Therefore, we considered that the spheres formed in our culture contained neural stem/progenitor cells and were useful in investigating the differenti-

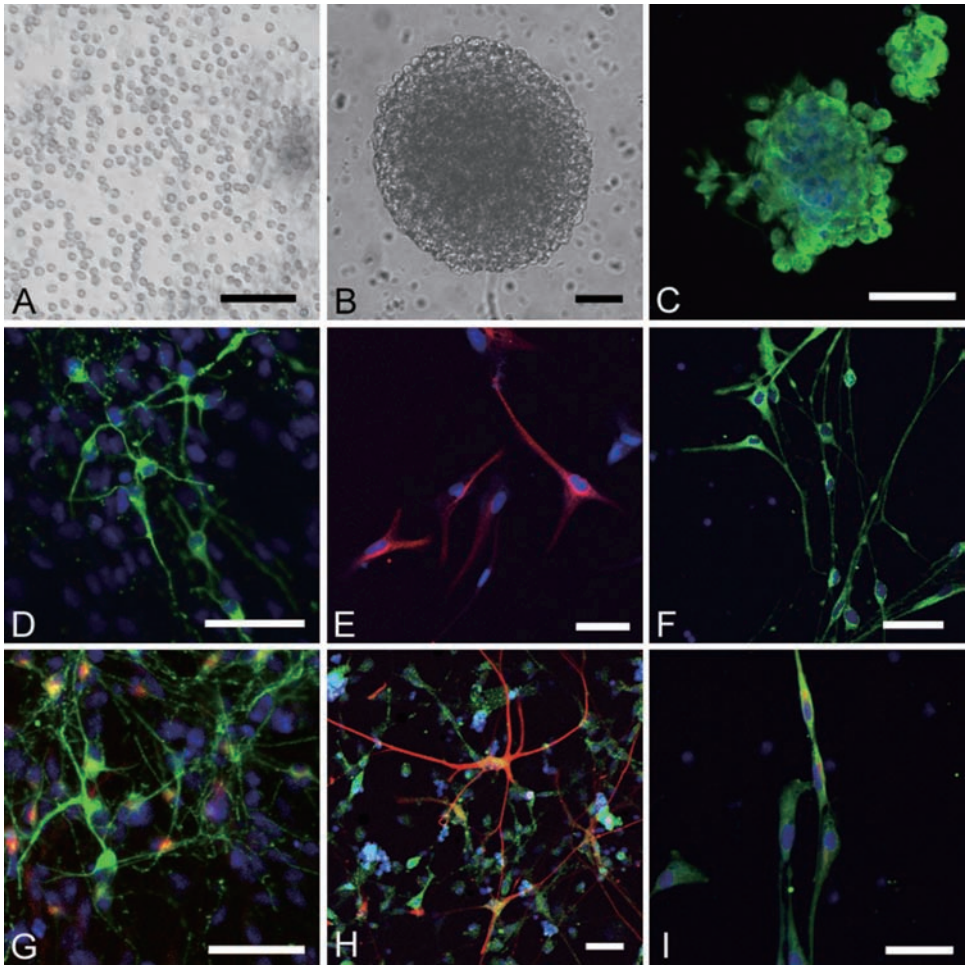

Fig. 1. Morphology and immunocytochemistry of bovine fetal brain-derived neural cells. Bars=50 μm. A) Dissociated cells floating in the medium at the start of culture. B) Floating cell aggregate (neurosphere) at 13 days in culture. C) Nestin expression (green) in neurosphere at 7 days in culture. D–F) Uninfected differentiating cells at 7 DPD. D) TUB-expressing cells with a spherical cytoplasm and long, slender cell processes (green). E) GFAP-expressing cells with relatively broad cytoplasm and processes (red). F) OSP-expressing cells with an ellipsoid cytoplasm and long, slender processes (green). G–I) BVDV-infected, differentiating cells at 7 DPD. G) Virus antigen (red), TUB (green). H) Virus antigen (green), GFAP (red). I) Virus antigen (red), OSP (green). (C–I) Nuclei were stained blue.

Table 1. Compostition rates of cells expressing cell class-specific antigens after differentiation with or without BVDV infection

Antigens	BVDV infection	Days post differentiation		
		0	3	7
Nestin	Uninfected	35.5 ± 11.7	18.6 ± 6.0	9.8 ± 2.5
	Infected		27.9 ± 4.4	27.6 ± 5.0[a)]
TUB	Uninfected	14.6 ± 6.1	25.4 ± 11.7	23.9 ± 5.3
	Infected		25.7 ± 12.1	22.6 ± 6.2
GFAP	Uninfected	10.1 ± 9.4	12.8 ± 7.2	22.3 ± 6.6
	Infected		10.8 ± 7.4	10.3 ± 4.1[b)]
OSP	Uninfected	33.7 ± 12.1	40.1 ± 14.7	44.4 ± 10.4
	Infected		38.5 ± 10.2	42.2 ±12.0

Values represent the percentages of each antigen-positive cell (means ± SD).
a,b) Significant difference between uninfected and infected cells (P<0.05).
TUB, beta III tubulin, GFAP, glial fibrillary acidic protein, OSP, oligodendrocyte-specific protein.

ation processes of bovine neural cells. To our knowledge, this is the first report of culturing BNSP cells by the neurosphere method; however, this procedure should be modified to promote cell proliferetion, such as by using bovine specific growth factors, for future research.

On the basis of studies of natural and experimental cases, fetal brain malformations associated with BVDV infection could be caused by both CP and NCP biotypes. A variety of congenital lesions have been described, including microencephaly, porencephaly, internal hydrocephalus, hydranencephaly, cerebellar hypoplasia and hypomyelination [2–4, 7, 16, 17, 22]. To establish these CNS malformations, the timing of infection in the dam, i.e., the second trimester of gestation, is considered to be important, because this period coincides with the development period of fetal immunocompetence and the final stages of CNS organogenesis when the bovine cerebellum shows a maximum rate of increase in mass [8, 17].

Histopathological investigations have been mainly undertaken on cerebellar hypoplasia (degeneration). Cerebellar lesions in fetuses maternally infected with the CP biotype of viruses consisted of necrosis of undifferentiated cells in the external germinal layer, moderate to severe edema of the white matter, focal hemorrhage and leptomeningitis [3, 22]. These lesions were interpreted as resulting from viral affinity for mitotically active cells and interruption of normal vascular integrity.

In NCP BVDV infection, lesions were composed of varying degrees of deficiency of cortical layers, depletion of granule cells, ectopia of Purkinje cells and small to extensive areas of cavitation in the white matter [4]. These lesions are thought to be due to viral destruction of mitotically active cells in the external germinal layer and vascular disturbance, whether by direct vascular damage or by the induction of intravascular coagulation [4, 5, 17]. Brownlie [5] additionally suggested viral inhibition of normal cell division and differentiation as a possible cause of congenital maldevelopment.

Retardation of myelin production resulting from interference of oligodendrocyte differentiation has been suggested as a mechanism in the pathogenesis of congenital hypomyelination [2]. Astrocytes have long been considered as structural- and metabolic-supporting cells for neurons, but recent evidences have shown that they have versatile roles in the development and function of the CNS [10, 14, 15]. Astrocytes and their precursors, radial glias, interact with developing neurons and control neuronal development, including proliferation, differentiation, migration, neurite extention and synapse formation. In addition, the onset of initial myelination by oligodendrocytes has been shown to be linked to astrocytic differentiation [21].

In this study, during the differentiation process of BNSP cells, NCP BVDV infection inhibited the decrease in the percentage of nestin-expressing cells and the increase in that of GFAP-expressing cells. Thus, the virus selectively inhibited differentiation of BNSP cells into the astrocytic lineage. Given the present results and the important roles of astrocytes in the development of the CNS, inhibition of differentiation of BNSP cells into astrocytes, along with a disturbance of normal astrocytic functions, may provide part of explanation for the congenital CNS lesions associated with NCP BVDV2 infection. Despite the antigenic and genetic differences, both BVDV1 and BVDV2 were isolated from aborted fetuses [11, 12] and no differences have been reported in the pathogenesis of CNS malformations between two genotypes. Therefore, the present findings might be applied not only to BVDV2 but also to BVDV1.

Recently, Bielefeldt-Ohmann and colleagues described the decreased number of GFAP-immunoreactive astorocytes in bovine fetus persistently infected with NCP BVDV, which might be an *in vivo* collaboration of our in vitro observations [1]. Further studies will be required to elucidate the molecular interaction between viral infection and astroglial differentiation.

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