Lactate Dehydrogenase Leakage as a Marker for Apoptotic Cell Degradation Induced by Influenza Virus Infection in Human Fetal Membrane Cells

Noboru Uchide  Kunio Ohyama  Toshio Bessho  Hiroo Toyoda
Department of Clinical Molecular Genetics, School of Pharmacy, Tokyo University of Pharmacy and Life Sciences, Tokyo, Japan

Introduction

The risk of premature delivery, abortion and stillbirth has increased during the past pandemics of influenza virus type A – the Spanish influenza 1918–1919 [1] and the Asian influenza 1957–1958 [2–4]. Even more recently, a cluster of spontaneous abortions and stillbirths occurred within 2–4 weeks from the onset of influenza infection [5]. Epidemiological studies in Tennessee from 1974 to 1993 estimated that 0.25% of pregnant women were hospitalized for the influenza infection [6]; 6.0% of pregnant women with asthma were hospitalized during influenza season, which was significantly higher than 0.51% of pregnant women without asthma [7]. It is clear that many pregnant women are exposed to the risk of influenza-associated complications of pregnancy. Therefore, it is important to study the mechanism of virulence of influenza virus to pregnant woman and fetus, and the prediction of the risk of influenza-associated complications of pregnancy.

Human fetal membranes are appendages of placenta, forming boundaries between the fetus and the external world. They are composed of fetus-derived amnion and chorion tissues, which play a critical role as a defensive barrier against infection in order to maintain normal pregnancy [8]. The occurrence of intrauterine infection with influenza virus type A has been substantiated [9–12]. A series of our study has demonstrated that the infection with influenza virus type A induces lactate dehydro-

Key Words
Lactate dehydrogenase • Chorion cells • Amnion cells • Apoptosis • Influenza virus • Caspase

Abstract

Objective: In order to elucidate the implication of apoptosis in lactate dehydrogenase (LDH) leakage from influenza virus-infected cells, the effects of a general caspase inhibitor, N-t-Boc-Asp(OMe)-fluoromethylketone (Boc-D-fmk), on LDH leakage, apoptosis induction and virus proliferation were examined. Methods: Cultured human fetal membrane chorion and amnion cells were incubated with or without Boc-D-fmk after influenza virus infection. LDH leakage was estimated by measuring LDH activities in the culture supernatants and cell lysates. The extent of apoptosis was determined by caspase-3 protein cleavage and DNA fragmentation. Virus proliferation was determined by a plaque-forming assay. Results: While virus proliferation was observed in both chorion and amnion cells, the virus infection resulted in LDH leakage, caspase-3 protein cleavage, and oligonucleosomal DNA fragmentation, all of which were observed only in the chorion cells and inhibited by the presence of Boc-D-fmk except for the virus proliferation. Conclusion: LDH level in amniotic fluid is known to be one of markers for predicting fetal membrane damage. Therefore, this study provides a possible diagnostic application of LDH level to predict the extent of tissue damage of fetal membranes via apoptosis induced by influenza virus infection.
reagents, such as 1-deoxy-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (ribavirin), pyrrolidine dithiocarbamate and nordihydroguaiaretic acid [13, 17, 18]. These results clearly suggest that the virus replication is a prerequisite for the induction of apoptotic cell degradation in chorion cells by influenza virus infection.

It has been demonstrated that higher levels of LDH activity in amniotic fluids are associated with chorioamnionitis [19] or preterm deliveries [20], suggesting that the LDH levels in amniotic fluids are a useful marker for predicting the extent of fetal membrane damage. Therefore, LDH levels in amniotic fluid may be used to predict the extent of fetal membrane damage during influenza virus infection. However, in virological fields, earlier studies have used LDH leakage as an indicator for evaluating antiviral activities of compounds against influenza virus [21, 22]. It has been speculated that the release of virus particles is responsible for the LDH leakage from influenza virus-infected cells, because several antiviral reagents, such as ribavirin, 3-deazaguanine, pyrazofurin and carbodine, inhibited the LDH leakage [22]. On the other hand, later studies have used LDH leakage as an indicator for evaluating the extent of cell damage not only in chorion cells [13] but also in Madin-Darby canine kidney (MDCK) [23, 24] and human histiocytic leukemia U937 cells [23] after influenza virus infection. Thus the biological significance of LDH leakage from influenza virus-infected cells seems to be inconsistent between the earlier and later studies. In each type of cells, such as MDCK, U937 and chorion cells, apoptotic cell degradation is consistently observed after the virus infection [13, 23, 24]. It should be noted that apoptotic cell degradation or LDH leakage was not observed in a case of persistent infection of cultured amnion cells, yet viruses were replicated in the cells [13]. Therefore, it seems that LDH leakage is associated with the apoptotic cell degradation irrespective of virus proliferation.

In influenza virus-infected cells, caspases are implicated in various cellular functions, such as apoptotic cell degradation [25, 26], the post-transcriptional processing of cellular cytokine and viral component proteins [27, 28], and efficient virus proliferation [29]. Caspase-3 plays a key role for apoptotic cell degradation, and the cleavage of pro-caspase-3 protein into active forms is an essential process for apoptotic cell degradation induced by various stimuli [30]. Based on these results, in order to elucidate the implication of apoptosis in LDH leakage from influenza virus-infected chorion cells, we examined the effects of a general caspase inhibitor, N-t-BOC-Asp(OMe)-fluoromethylketone (Boc-D-fmk) [25], on LDH leakage, apoptosis (i.e., DNA fragmentation and pro-caspase-3 protein cleavage) and virus proliferation.

Materials and Methods

Reagents

Boc-D-fmk and ribavirin were purchased from Sigma-Aldrich (St. Louis, Mo., USA) and dissolved in dimethyl sulfoxide (DMSO) and saline, respectively. Each solution was diluted at desired concentrations with culture medium for use.

Cells

Human fetal membrane tissues were obtained during elective cesarean section in the month of normal parturition. The study protocol has been approved by the Institutional Review Board Committee at Tokyo University of Pharmacy and Life Sciences. Informed consent was obtained from patients at the time of surgery. Primary cultured chorion and amnion cells were prepared from chorionic and amniotic tissues, respectively, and grown in a medium [80% DMEM/F12, 20% heat-inactivated fetal bovine serum, 120 μg/ml kanamycin sulfate, 120 units/ml penicillin G sodium, 120 μg/ml streptomycin sulfate, 16 μg/ml gentamicin sulfate, and 0.3 μg/ml amphotericin B] as previously described [31].

Viruses and Infection

Influenza virus type A (Puerto Rico/8/34, H1N1) was propagated in the allantoic cavity of embryonal chicken eggs as previously described [13]. Plaque-forming capacity was assayed on confluent monolayers of MDCK (NBL-2) cells (Health Science Research Resources Bank, Osaka, Japan) as previously described [13]. Chorioallantoic fluid contained infectious virus particles at 8 × 10^5 plaque-forming units (pfu)/ml. The chorioallantoic fluid was diluted with serum-free culture medium for use. Confluent monolayers of chorion (8 × 10^4 cells/cm²) and amnion cells (20 × 10^4 cells/cm²) were rinsed with phosphate-buffered saline (PBS) and inoculated with 3.2 × 10^4, 32 × 10^4, and 320 × 10^4 pfu of infectious virus particles in a volume of 0.04 ml/cm² for 1 h at 37° as previously described [31]. Mock-infected control cells were prepared by inoculation with 0.04 ml/cm² of serum-free cul-
ture medium alone in the absence of viruses for 1 h at 37°C. After removal of the inoculants, cells were rinsed with PBS and incubated with a culture medium containing 20% heat-inactivated fetal bovine serum (0.2 ml/cm²) for desired periods at 37°C in a humidified atmosphere containing 5% CO₂ in air.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis
Cells adhered on flasks were rinsed with PBS. RNA fractions were obtained from the cells using an RNA extraction kit, Isogen (Wako Pure Chemical Industry, Osaka, Japan). Influenza virus hemagglutinin virion RNA (HA vRNA) and cellular glycerol-3-phosphate dehydrogenase (G3PDH) mRNA in RNA samples were amplified using RT-PCR techniques as previously described [32, 33]. Oligo DNA primers for HA vRNA (sense 5’-TGA GGG AGC AAT TGA GCT CA-3’; antisense 5’-TGC CTC AAA TAT TAT TGT GT-3’) and G3PDH mRNA (sense 5’-TGA AGG TCG GAG TCA ACG GAT TTG GTG-3’; antisense 5’-CAT GTG GGC CAT GAG GTG CAC CAC-3’) were used for PCR amplification [32, 33]. After resolving on agarose gels, G3PDH mRNA-related PCR products were detected by ethidium bromide staining. HA vRNA-related PCR products were detected by the Southern blot method as previously described [33].

Estimation of Virus Particle Release Rate
After influenza virus infection at 32 × 10⁴ pfu/cm², chorion and amnion cells were incubated in triplicate until 48 h changing culture medium at 12 and 24 h. Virus yields in culture supernatants during 0–12, 12–24 and 24–48 h were determined by plaque-forming assay. The rate of virus particle release was calculated by dividing the virus yield of segmental periods by the duration time.

Western Blot Analysis
Cells adhered on flasks were rinsed with PBS and dissolved in the sample buffer (62.5 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate, 10% glycerol and 0.005% bromophenol blue) as previously described [34]. Protein concentrations in cell lysates were determined by the Lowry method using bovine serum albumin as a protein standard [35]. Samples (50 μg of protein) from cells were resolved on 14% sodium dodecyl sulfate-polyacrylamide gels under a reducing condition and transferred to nitrocellulose membranes according to standard procedures [34, 36]. Caspase-3 and β-actin proteins on the membrane were detected with monoclonal rabbit anti-caspase-3 antibody (StressGen, Victoria, B.C., Canada) and monoclonal mouse anti-β-actin antibody (Sigma-Aldrich, respectively, and an ECL™, Western blotting detection reagents kit (Amersham Pharmacia Biotech, Little Chalfont, Bucks., UK). Chemiluminescence was detected with a lumino image analyzer, LAS-1000plus (Fujifilm, Tokyo, Japan), and quantitated with the software Image Gauge (Fujifilm).

DNA Fragmentation Analysis
The extent of apoptosis was determined by agarose gel electrophoresis as previously described [13]. After staining with ethidium bromide, the photographs of gel on UV transilluminator were obtained. The DNA fragmentation rate was calculated from digitized images employing the software NIH Image 1.60 as previously described [13].

Immunohistochemical Analysis
Influenza virus nucleoprotein (NP) in cells was detected with immunohistochemical techniques using monoclonal mouse anti-influenza virus NP antibody (HyTest, Turku, Finland) and polyclonal rabbit anti-mouse immunoglobulin G antibody conjugated with tetramethylrhodamine isothiocyanate (Sigma-Aldrich) as previously described [31]. Stained cells were observed under a fluorescence microscope, Axiosvert 200 (Carl Zeiss, Göttingen, Germany). Furthermore, total cells under phase contrast views (>200 cells/sample) and NP-positive cells under fluorescence views were counted visually on photomicrographs, and the percentage of NP-positive cells was calculated.

LDH Leakage Assay
LDH leakage was estimated by measuring LDH activities in culture supernatant and cell lysate using a LDH-Cytotoxic Test Wako kit (Wako Pure Chemical Industry, Osaka, Japan) as previously described [13]. Culture medium served as the background control. Mock-infected cells were lysed in culture medium containing 0.2% Tween 20, and the cell lysate after centrifugation at 12,000 g for 10 min was used as the non-damaged control. Culture supernatants were centrifuged at 12,000 g for 10 min at 4°C in order to remove insoluble materials. The supernatants were diluted 32-fold with PBS, then 50 μl of diluted samples was added into the wells of a 96-well plate and then mixed with 50 μl of ‘substrate solution’ from the kit. The mixture was stirred and incubated at room temperature for 30 min. The reaction was stopped by adding 100 μl of ‘stopping solution’ provided with the kit. Absorption at 550 nm in the reaction mixture was measured using an MTP-32 microplate reader (Corona Electric Co., Ibaraki, Japan). LDH leakage was calculated using the following function: LDH leakage (%) = 100 × (CS – BC)/(NDC – BC). CS, BC and NDC mean absorbance of culture supernatant, background control and non-damaged control, respectively.

Statistical Analysis
Statistical analysis was performed using the Student’s t test method. p values <0.05 were considered significant.

Results

HA vRNA Expression and Virus Particle Release
The levels of HA vRNA expression increased in both chorion and amnion cells with time after infection, which reached a highest level in both cells during 24–48 h after influenza virus infection (fig. 1a). Cellular G3PDH mRNA was amplified from all samples (fig. 1a). The rate of virus particle release from the chorion and amnion cells during segmental periods was estimated (fig. 1b). The rate of virus particle release from the virus-infected chorion cells was the same as that from the virus-infected amnion cells during 0–12 h. The rate in the chorion cells decreased during 24–48 h as compared with that during 0–12 h, whereas that in the amnion cells increased during 12–24 and 24–48 h.
Cleavage of Pro-Caspase-3 Protein after Virus Infection

The expression and cleavage of caspase-3 protein were examined by Western blot analysis (fig. 2). In the chorion cells, pro-caspase-3 protein (32 kDa) was detected between 0 and 48 h after both mock and influenza virus infections. A cleaved form of caspase-3 protein (19 kDa) was weakly detected at 24 h and strongly at 48 h after influenza virus infection but not after mock infection. In the amnion cells, the cleaved form of caspase-3 protein was not detected after either mock or virus infection, although pro-caspase-3 protein was detected.

Inhibition of Virus Infection-Induced Apoptosis with Boc-D-fmk

DNA ladder was detected in the chorion cells at 48 h after influenza virus infection in the absence of Boc-D-fmk (fig. 3a, lane 4) but not after mock infection (lane 1). The extent of DNA laddering was decreased by 85% in the presence of 50 μM Boc-D-fmk (lane 6). No effect of DMSO alone was observed (lane 5). An inhibitory effect of Boc-D-fmk on DNA ladder formation was observed in a dose-dependent manner (fig. 3b). DNA ladder was not detected in the amnion cells after mock and influenza virus infections (lanes 7–12, fig. 3a). As shown in figure 3c, the cleaved form of caspase-3 protein (19 kDa) was detected in the virus-infected chorion cells in the absence of Boc-D-fmk (lane 2) but not in the presence of Boc-D-fmk (lane 3).

No Effect of Boc-D-fmk on Virus Infection and Proliferation

The number of viral NP-positive cells increased in both chorion and amnion cell cultures in a time-dependent manner after influenza virus infection (fig. 4a). The percentages of viral NP-positive cells were 79 ± 17% of the chorion cells (n = 3) and 49 ± 32% of the amnion cells (n = 3), respectively, at 24 h post-infection at 320 × 10⁴ pfu/cm².

The effect of Boc-D-fmk on influenza virus infection was examined by determining the number of viral NP-positive cells. Viral NP-positive cells in the chorion cell cultures were 62 and 64% in the absence (fig. 4b) and presence of Boc-D-fmk (fig. 4c), respectively, and those in the amnion cell cultures were 26 and 23%, respectively (fig. 4d, e). In the virus-infected chorion cells, viral NP
protein was localized in the nucleus in the absence of Boc-D-fmk (fig. 4b) but diffused into the cytoplasm in the presence of Boc-D-fmk as indicated with arrows (fig. 4c). On the contrary, in the virus-infected amnion cells, viral NP protein was localized in the nuclei both in the absence (fig. 4d) and in the presence of Boc-D-fmk (fig. 4e). The effect of Boc-D-fmk on virus proliferation was further examined. The treatment with Boc-D-fmk did not alter the virus yields in the culture supernatants of the virus-infected chorion cells (table 1).

**Inhibition of Virus Infection-Induced LDH Leakage with Boc-D-fmk and Ribavirin**

The effect of Boc-D-fmk on LDH leakage was examined (fig. 5). LDH leakage (30%) was induced in the virus-infected chorion cells in the absence of a caspase inhibitor, but not in the amnion cells. The treatment of chorion cells with 50 μM Boc-D-fmk resulted in approximately 10% of the LDH leakage. The treatment with ribavirin decreased the extent of virus infection-induced LDH leakage from the chorion cells in a dose-dependent manner (table 2).

**Discussion**

Our previous study has demonstrated that HA vRNA synthesis is accompanied by virus particle release from the chorion cells until 12 h after influenza virus infection, whereas cumulative amounts of virus particles released into culture supernatants reached a plateau at 24 h even though the level of HA vRNA synthesis is increased [33]. Based on the results, we hypothesized that the virus particle release from influenza virus-infected chorion cells is restricted at 24 h post-infection. In this study, we further measured the rate of virus particle release during segmental periods in order to substantiate the hypothesis. Although considerable amounts of HA vRNA was expressed in both chorion and amnion cells during 24–48 h after influenza virus infection, the rate of virus particle release from the virus-infected chorion cells was decreased. In contrast, the rate in the virus-infected amnion cells was increased. The restriction of virus particle release is also observed in other types of cultured cells undergoing apoptosis after influenza virus infection [37, 38]. Accordingly, our results substantiate that virus particle release is restricted in the virus-infected chorion cells undergoing apoptosis but not in the...
Fig. 3. **a** Chorion (lanes 1–6) and amnion cells (lanes 7–12) were preincubated, respectively, for 6 h in the absence (lanes 1, 4, 7 and 10) or presence of either 50 μM Boc-D-fmk (lanes 3, 6, 9 and 12) or 0.25% DMSO alone (lanes 2, 5, 8 and 11). After mock (lanes 1–3 and 7–9) and influenza virus infection at 320 × 10^4 pfu/cm^2^ (lanes 4–6 and 10–12), cells were incubated for 48 h in the absence or presence of either 50 μM Boc-D-fmk or 0.25% DMSO alone. **b** Chorion cells were preincubated for 6 h in the absence (lanes 1 and 5) or presence of Boc-D-fmk at 25 (lanes 2 and 6), 50 (lanes 3 and 7), and 100 μM (lanes 4 and 8). After mock (lanes 1 and 4) and influenza virus infection at 320 × 10^4 pfu/cm^2^ (lanes 5–8), chorion cells were incubated for 48 h in the absence or presence of Boc-D-fmk at 25, 50 and 100 μM. DNA extracted from the cells was resolved on agarose gels. The profiles of DNA are shown in the panels. Lane M shows DNA size markers. DNA sizes are indicated as basepairs (bp). **c** Chorion cells were preincubated for 6 h in the presence of either 0.25% DMSO alone (lanes 1 and 2) or 50 μM Boc-D-fmk (lane 3). After mock (lane 1) and influenza virus infection at 320 × 10^4 pfu/cm^2^ (lanes 2 and 3), chorion cells were incubated for 48 h in the presence of either 0.25% DMSO alone or 50 μM Boc-D-fmk. Caspase-3 and β-actin proteins in the cells were detected by Western blotting. The profiles are shown in the panels. The expression of cleaved caspase-3 protein was corrected with that of β-actin. The ratios versus mock infection was further calculated and indicated at the base of the panels.
Fig. 4. **a** After influenza virus infection at \(320 \times 10^4\) pfu/cm\(^2\), chorion (closed circles) and amnion cells (open circles) were incubated, respectively, for 0.5, 3, 6, 12, or 24 h. Viral NP was detected using immunohistochemical techniques, and percentages of NP-positive cells were calculated as described in Materials and Methods. **b–e** Chorion (**b, c**) and amnion cells (**d, e**) were preincubated for 6 h in the presence of either 0.25% DMSO alone (vehicle) (**b, d**) or 50 \(\mu\)M Boc-D-fmk (**c, e**). After influenza virus infection at \(320 \times 10^4\) pfu/cm\(^2\), cells were incubated for 24 h in the presence of either 0.25% DMSO alone or 50 \(\mu\)M Boc-D-fmk. Viral NP protein was detected using immunohistochemical techniques. Arrows indicate cells with NP diffused in the cytoplasm.

Table 1. Effect of Boc-D-fmk on influenza virus particle release

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Treatment</th>
<th>Virus yield, pfu/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chorion</td>
<td>Control</td>
<td>128,000 ± 7,640</td>
</tr>
<tr>
<td></td>
<td>0.25% DMSO</td>
<td>125,000 ± 22,900</td>
</tr>
<tr>
<td></td>
<td>50 (\mu)M Boc-D-fmk</td>
<td>147,000 ± 25,700</td>
</tr>
<tr>
<td>Amnion</td>
<td>Control</td>
<td>307,000 ± 20,800</td>
</tr>
<tr>
<td></td>
<td>0.25% DMSO</td>
<td>230,000 ± 49,200</td>
</tr>
<tr>
<td></td>
<td>50 (\mu)M Boc-D-fmk</td>
<td>202,000 ± 20,200*</td>
</tr>
</tbody>
</table>

Chorion and amnion cells were preincubated for 6 h in the absence (control) or presence of either 50 \(\mu\)M Boc-D-fmk or 0.25% DMSO alone. After influenza virus infection at \(320 \times 10^4\) pfu/cm\(^2\), cells were incubated for 48 h in the absence or presence of either Boc-D-fmk or DMSO alone. Virus yield in the culture supernatants was measured in quadruplicate by plaque-forming assay. Data are shown as means and standard deviations. Statistical analysis using the Student’s t test (\(n = 4\)) shows a significant difference in virus yield between the absence and presence of Boc-D-fmk in amnion cells (*p < 0.05).

Table 2. Effect of ribavirin on LDH leakage induced by influenza virus infection

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Ribavirin (\mu)M</th>
<th>Mock infection, %</th>
<th>Virus infection, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>10.9 ± 1.4</td>
<td>35.7 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>11.9 ± 2.7</td>
<td>35.7 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>11.4 ± 1.1</td>
<td>19.2 ± 0.7††</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>9.9 ± 0.4</td>
<td>38.2 ± 2.4</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>9.8 ± 0.5</td>
<td>30.3 ± 2.4†</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>11.6 ± 1.4</td>
<td>21.6 ± 4.2**</td>
</tr>
</tbody>
</table>

Chorion cells were preincubated in quadruplicate for 24 h in the absence or presence of ribavirin at 100, 200 and 1,000 \(\mu\)M. After mock infection and influenza virus infection at \(320 \times 10^4\) pfu/cm\(^2\), cells were incubated for 48 h in the absence or presence of ribavirin. LDH leakage was estimated as described in Materials and Methods. Data are shown as means and standard deviations calculated from two independent experiments. Statistical analysis (\(n = 4\)) using the Student’s t test shows significant differences between the absence and presence of ribavirin (**p < 0.01; †p < 0.005; ††p < 0.001).
virus-infected amnion cells without accompanying with apoptosis.

The present study demonstrated that influenza virus infection induced the cleavage of pro-caspase-3 protein into an active form and apoptotic DNA fragmentation in the chorion cells. A general caspase inhibitor, Boc-D-fmk, inhibited both caspase-3 cleavage and DNA fragmentation in the virus-infected chorion cells, while the treatment with Boc-D-fmk did not interfere virus infection and proliferation. Therefore, these results suggest that Boc-D-fmk treatment inhibits apoptotic cell degradation in the influenza virus-infected chorion cells by inhibiting caspase-3 cleavage process irrespective of virus proliferation. That is, the pro-caspase-3 cleavage process is prerequisite for apoptosis induction in the chorion cells infected with influenza virus. In the influenza virus-infected amnion cells, pro-caspase-3 protein cleavage and DNA fragmentation were not observed, yet the virus proliferation occurred in the cells. Apoptosis is a tightly regulated process involving several checkpoints before irreversible cellular degradation begins. The process consists of initiation, commitment and degradation phase [39, 40]. Since the pro-caspase-3 cleavage process is prerequisite for apoptosis induction, it may be said that the absence of pro-caspase-3 protein cleavage process in the amnion cells is implicated in the mechanism of persistent infection without commitment of apoptosis induction. Since our data indicated that the expression of immunoglobulin heavy-chain binding protein, one of the major molecular chaperons in the lumen of endoplasmic reticulum, was increased in the chorion, but not amnion, cells at later stages of the virus infection [41], a biochemical change in the endoplasmic reticulum would seem to relate to apoptosis induction by influenza virus infection as well as other viruses [42, 43].

Viral NP was retained in the nucleus of the virus-infected chorion cells in the absence of Boc-D-fmk, but diffused into the cytoplasm in the presence of Boc-D-fmk, suggesting that the viral ribonucleoprotein (vRNP) complex associated with NP was accumulated within the cytoplasm of the virus-infected chorion cells by treatment with Boc-D-fmk. The present study suggests that the intracellular localization of vRNP was modulated by caspases. Earlier studies have suggested that viral NP is cleaved by caspases in infected cells, and that the vRNP complex associated with the cleaved form of NP is not as-

**Fig. 5.** Chorion (a) and amnion cells (b) were preincubated, respectively, in triplicate for 6 h in the absence or presence of either 50 μM Boc-D-fmk or 0.25% DMSO alone. After mock (open columns) and influenza virus infection at 320 × 10⁴ pfu/cm² (black columns), cells were incubated for 48 h in the absence or presence of either 50 μM Boc-D-fmk or 0.25% DMSO alone. LDH leakage was estimated as described in Materials and Methods. Data are shown as means and standard deviations. Statistical analysis using the Student’s t test (n = 3) shows a significant difference between Boc-D-fmk and DMSO alone (†† p < 0.001).
The inhibitory effect of ribavirin on viral gene replication and transcription is attributed to the inhibition of LDH leakage, because the process of virus replication is a prerequisite for the apoptosis induction [13, 17, 18]. Our results demonstrated that the treatment with Boc-D-fmk inhibited both LDH leakage and apoptotic cell degradation, although the virus infection and proliferation were not inhibited. Therefore, this study using a caspase inhibitor clearly suggested that LDH leakage from influenza virus-infected cells occurred as a result of secondary necrosis [46] after apoptotic cell degradation rather than virus particle release.

Taken together, our results demonstrate that Boc-D-fmk inhibits LDH leakage from influenza virus-infected cells through inhibiting caspase-mediated apoptotic cell degradation irrespective of virus proliferation (fig. 6). That is, apoptosis induction is closely associated with the LDH leakage from the virus-infected chorion cells. LDH level in amniotic fluid is known to be one of markers for predicting fetal membrane damage [19, 20]. Consequently, the present study provides a possible diagnostic application of LDH level using clinical samples, such as amniotic fluid and vaginal washings, to predict the extent of tissue damage of fetal membranes via apoptosis induced by influenza virus infection.

Acknowledgements

This work was supported in part by grants from the Ministry of Education, Culture, Sports, Science, and Technology, and by the Promotion and Mutual Aid Corporation for Private Schools of Japan.

References

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Interventional 2009;52:164–173


