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Antiviral Research 56 (2002) 207–217

Antiviral Research

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Effect of antioxidants on apoptosis induced by influenza virus infection: inhibition of viral gene replication and transcription with pyrrolidine dithiocarbamate

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Received 5 June 2002; accepted 12 July 2002

Abstract

Influenza virus (IV) infection induced apoptotic DNA fragmentation and the moderate overproduction of reactive oxygen species (ROS) in primary cultured chorion cells prepared from human fetal membranes, and IV particles were released from the infected cells. The antioxidant pyrrolidine dithiocarbamate (PDTC) inhibited the induced DNA fragmentation, ROS overproduction and IV particle release. Although Trolox inhibited ROS overproduction, it did not inhibit DNA fragmentation or IV production. The inhibitory effect of PDTC on DNA fragmentation was manifested when added up to 3 h after infection or by exposing the infected cells to it for only 1 h after infection. PDTC inhibited IV hemagglutinin (HA) viral (vRNA) and complementary (cRNA and mRNA) RNAs synthesis until 6 h after infection and delayed and decreased HA protein synthesis. However, HA RNA synthesis resumed after 12 h even in the presence of PDTC. These results suggested that PDTC inhibited apoptosis by inhibiting viral macromolecule synthesis rather than through its antioxidant effect, because Trolox did not inhibit apoptosis or IV production, although ROS overproduction was inhibited. The synthesis of specific viral macromolecules at the early stage of infection may play a critical role in the mechanism of apoptosis induction and moderate ROS overproduction may not be involved in the mechanism.

Keywords: Apoptosis; Influenza virus; Reactive oxygen species; Antioxidant; Fetal membrane

1. Introduction

Influenza virus (IV) causes cytocidal infection accompanied by virus production and apoptotic cell death in various types of cultured cell lines such as HeLa, Madin–Darby canine kidney

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0166-3542/02/$ - see front matter
PII: S0166-3542(02)00109-2

(MDCK) and U937 as well as peripheral blood monocytes (Takizawa et al., 1993; Price et al., 1997; Fesq et al., 1994). Infections with defective interfering particles or mutants of IV result in persistence accompanied by virus production and cell survival in HeLa, MDCK and baby hamster kidney (BHK) cell lines (Camilleri and Maassab, 1988; De and Nayak, 1980; Frielle et al., 1984). In addition, we reported that a cytopathogenic strain of IV causes cytocidal infection in primary cultured chorion cells and persistent infection in primary cultured amnion cells prepared from human fetal membranes (Uchide et al., 2002a). That study demonstrated that IV induces apoptosis in chorion but not in amnion cells even during IV replicated, and the antiviral agent ribavirin simultaneously inhibits apoptosis and IV replication in infected chorion cells. Apoptosis induced by IV may require not only viral replication but also a mechanism that is invoked as a consequence of viral replication.

Several lines of evidence support the involvement of reactive oxygen species (ROS) in many models of apoptotic cell death (Payne et al., 1995). Pyrrolidine dithiocarbamate (PDTC) is a radical scavenger, a divalent metal ion chelator, and it acts as an antioxidant in viable cells through alterations in ROS metabolism caused by both of these properties (Meyer et al., 1993). In addition, PDTC regulates the gene expression of antioxidant enzymes such as manganese superoxide dismutase (Borrello and Demple, 1997), heme oxygenase-1 (Hartsfield et al., 1998) and γ-glutamylcysteine synthetase (Wild and Mulcahy, 1999) but, conversely, it may act as a pro-oxidant and modulator of free thiol groups (Nobel et al., 1995; Kim et al., 1999). PDTC blocks the accumulation of ROS and the death of IV-infected A549 cells as well as the apoptotic-like death of IV-infected J774.1 cells (Knobil et al., 1998; Lowy and Dimitrov, 1997). Although PDTC does not block apoptosis in IV-infected MDCK cells (Olsen et al., 1996), the general antioxidant *N*-acetyl-cysteine (NAC) inhibits both ROS accumulation and apoptosis (Lin et al., 2001). Notably, the effect of antioxidants on apoptosis varied between different cell types, indicating that hitherto unknown mechanisms contribute to the inhibitory effect of antioxidant on IV-induced apoptosis.

The present study investigates the role of ROS in apoptosis induced by IV infection. We examined the effect of PDTC on apoptosis, ROS production, IV production and viral macromolecule synthesis in IV-infected apoptotic cells. Furthermore, to understand the nature of the ROS participation in more detail, we also examined the effects of another antioxidant and antiviral agent.

2. Materials and methods

2.1. Chemicals

PDTC (Wako Pure Chemical Industry, Osaka, Japan), Trolox (6-hydroxyl-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water-soluble analog of vitamin E (Calbiochem, CA, USA), ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) (Sigma, MO, USA), 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (Molecular Probes, Inc., OR, USA) were purchased from listed suppliers. Oligo DNA primers corresponding to the nucleotide positions 385–404 (sense primer; 5′-TGA GGG AGC AAT TGA GCT CA-3′) and 815–796 (antisense primer; 5′-TGC CTC AAA TAT TAT TGT GT-3′) of IV hemagglutinin (HA) cDNA (Yamada et al., 1991) and to the internal nucleotide positions 453–472 (nested sense primer; 5′-CCC AAC CAC AAC ACA ACC AA-3′) and 796–777 (nested antisense primer; 5′-TCT CCG GGT TTT AGC AAG GT-3′) (Winter et al., 1981) were custom-prepared by Amersham Pharmacia Biotech (Tokyo, Japan).

2.2. Cells and viruses

Primary cultured chorion and amnion cells were prepared from human fetal membranes as described (Ohyama et al., 2000). IV type A (PR/8/34) was propagated in the allantoic cavity of embryonal chicken eggs and plaque-forming assays in MDCK (NBL-2) cells (Human Science Research Resources Bank, Osaka, Japan) were performed as described (Uchide et al., 2002a). Confluent monolayers of chorion and amnion cells

were infected with IV at desired multiplicities of infection (MOI) as described (Uchide et al., 2002a). Mock-infected control cells were similarly treated, but without virus.

2.3. DNA fragmentation assay

The DNA fraction was extracted from cells and resolved by agarose gel electrophoresis, then DNA fragmentation was analyzed in digitized images as described (Uchide et al., 2002a).

2.4. Measurement of ROS levels

ROS levels were measured as described (Goodman et al., 1994). Cell monolayers in 24 well-plates were rinsed three times with 1 ml of Hanks' balanced salt solution (HBSS) and incubated with 0.5 ml of HBSS containing 5 μM DCFH-DA at room temperature for 20 min. Fluorescence intensity excited at 485 nm in the wells was measured at 535 nm using a FluoroScan Plus (TECAN, Austria).

2.5. Reverse transcriptase-polymerase chain reaction (RT-PCR)/Southern blot analysis

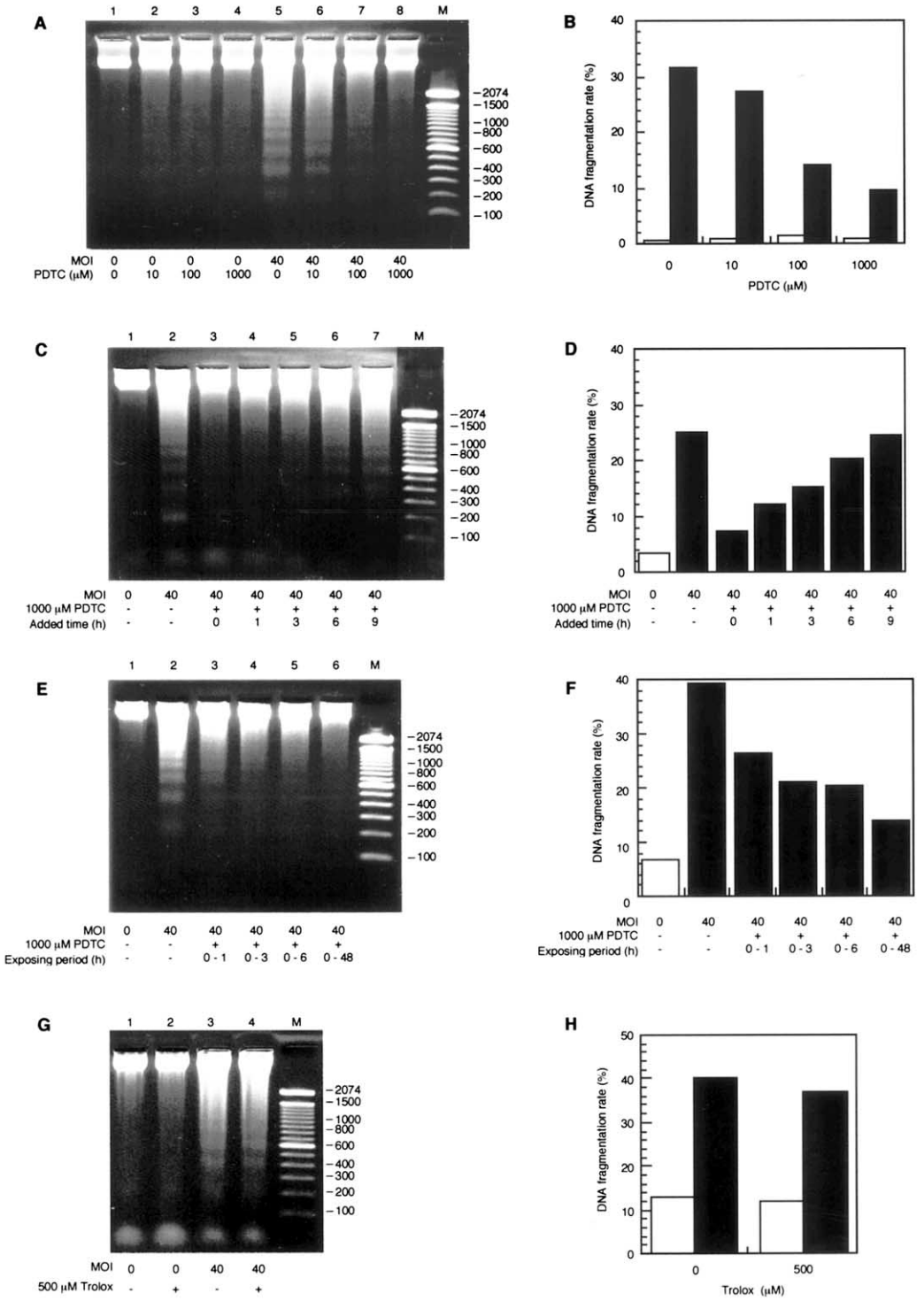
Total RNA was extracted from cells using ISOGEN kits (Wako Pure Chemical Industries) and RT-PCR proceeded as described (Yamada et al., 1991) with some modifications. The cDNAs for HA viral (v) RNA and complementary and/or messenger (c/m) RNA were synthesized using sense and antisense primers, respectively. PCR amplification using the sense and antisense primers was achieved as follows: 15 cycles for HA vRNA and 12 cycles for HA c/mRNA of PCR consisted of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min. The reaction mixture (2.0 μl) was resolved by electrophoresis on 2.0% agarose gels. Biotinylated DNA probe was prepared with nested primers as described (Lo et al., 1988). PCR products related to HA RNAs were detected by Southern blotting with the probe as described (Boener et al., 1999) with some modifications. Streptavidin (Bio-Rad, CA, USA), biotinylated alkaline phosphatase (Bio-Rad) and a BCIP/NBT phosphatase substrate kit (Kirkegaard and Perry Labs. Inc., MD, USA) were used according to the supplied instruction manuals. Bands were quantified in images of the membranes digitized using NIH Image 1.60.

2.6. Western blot analysis

Cell layers were rinsed twice with phosphate buffered saline (PBS) and lysed in boiling 2-fold concentrated sample buffer. Samples (20 μg of protein) were resolved by electrophoresis in 12% sodium dodecyl sulfate-polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes according to standard procedures. HA protein on the membrane was detected by Western blotting using a mouse anti-influenza virus type A (HA) antibody (HyTest, Finland) and the ECL^{TM} Western blotting detection reagents kit (Amersham Pharmacia Biotech) according to the recommendations of the supplier.

3. Results

The effect of PDTC on apoptosis in IV-infected chorion cells was examined DNA fragmentation assays showed that PDTC inhibited apoptosis induced by IV infection (MOI = 40) in a dose-dependent manner (Fig. 1A). At concentrations up to 1000 μM, PDTC did not induce DNA fragmentation in mock-infected chorion cells (lanes 2–4). The DNA ladder resulting from IV infection was considerably decreased by 100 and 1000 μM PDTC (lanes 7 and 8). The DNA fragmentation was inhibited 14, 58 and 72% by 10, 100 and 1000 μM PDTC, respectively (Fig. 1B). Fig. 1C shows that the inhibitory effect of PDTC on apoptosis was manifested when added up to 3 h after infection. When 1000 μM PDTC was added at 0, 1 and 3 h after IV infection, the density of the DNA ladder was considerably decreased on the gel (lanes 3, 4 and 5). Fragmentation was inhibited 82, 60, 46, 21 and 9% by PDTC added at 0, 1, 3, 6 and 9 h, respectively (Fig. 1D). Fig. 1E shows that IV-induced apoptosis was inhibited by incubating the cells with PDTC for only 1 h after infection. The amount of the DNA ladder of IV-infected cells was considerably decreased by exposure to 1000


A
1 2 3 4 5 6 7 8 M
2074
1500
1000
800
600
400
300
200
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MOI 0 0 0 0 40 40 40 40
PDTC (μM) 0 10 100 1000 0 10 100 1000
B
DNA fragmentation rate (%)
0 10 100 1000
PDTC (μM)
C
1 2 3 4 5 6 7 M
MOI 0 40 40 40 40 40 40
1000 μM PDTC - - + + + + +
Added time (h) - - 0 1 3 6 9
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DNA fragmentation rate (%)
MOI 0 40 40 40 40 40 40
1000 μM PDTC - - + + + + +
Added time (h) - - 0 1 3 6 9
E
1 2 3 4 5 6 M
MOI 0 40 40 40 40 40
1000 μM PDTC - - + + + +
Exposing period (h) - - 0 - 1 0 - 3 0 - 6 0 - 48
F
DNA fragmentation rate (%)
MOI 0 40 40 40 40 40
1000 μM PDTC - - + + + +
Exposing period (h) - - 0 - 1 0 - 3 0 - 6 0 - 48
G
1 2 3 4 M
MOI 0 0 40 40
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H
DNA fragmentation rate (%)
0 500
Trolox (μM)

Fig. 1

μM PDTC from 0–1 h after infection (lanes 3). Fragmentation was inhibited 40, 56, 59 and 78% by exposure to PDTC from 0–1, 0–3, 0–6 and 0–48 h, respectively (Fig. 1F).

Trolox is a radical scavenger and a metal ion chelator that acts as an antioxidant like PDTC (Sommer et al., 2000). We therefore examined the effect of Trolox on apoptosis in infected chorion cells. DNA fragmentation assays showed that Trolox did not inhibit apoptosis induced by IV infection (Fig. 1G). Trolox (500 μM) neither induced DNA fragmentation in mock-infected cells (lane 2) nor decreased the density of the DNA ladder resulting from IV infection (lane 4) on the gel. Fig. 1H shows that Trolox inhibited only 1% of the fragmentation.

Because we found that IV infection induces apoptosis in chorion but not in amnion cells, and that IV replicates in both types of cells (Uchide et al., 2002a), we examined the effect of IV infection on ROS production in these cell types. In comparison with mock-infected control cultures (MOI = 0), IV infection increased and decreased ROS production by 31% in chorion cells (Fig. 2A) and by 43% in amnion cells (Fig. 2B), respectively. IV infection increased ROS production by $19.0 \pm 7.6\%$ in chorion cells (ranging from 8.8 to 31.1%, $n = 11$); ROS production decreased by $33.6 \pm 13.3\%$ in amnion cells (ranging from 18.5 to 50.2%, $n = 5$). These data are calculated from independent experiments repeated n times and are expressed as means and standard deviations. These results indicated that IV-induced ROS production is associated with the induction of apoptosis but that the cause is not simply IV replication.

Whether IV-induced ROS production is responsible for the induction of apoptosis remains unclear. The effects of PDTC and Trolox on ROS production were then examined to ascertain the role of ROS in apoptosis. To gain a more thorough understanding, the effect of the antiviral agent ribavirin was examined. Table 1 shows that IV infection at MOI = 40 moderately increased ROS production by 11–15%. Trolox and PDTC at 500 μM significantly repressed basal ROS production by 40 and 30% in mock-infected chorion cells, respectively, and IV infection did not increase ROS production. These results indicated that both PDTC and Trolox function as antioxidants in mock- and IV-infected chorion cells. ROS production was not increased in the presence of ribavirin (1000 μM) without repression of basal ROS production.

Many investigators have proposed that IV replication is associated with apoptotic event. To understand the antiviral activity of antioxidants, we examined the effect of PDTC and Trolox on IV production in chorion cells. Cells were infected with IV at MOI = 4 because PDTC decreased the DNA ladder resulting from infection under these conditions to the same extent as that at MOI = 40 (data not shown). Viral particles were released from the infected chorion cells in a time-dependent manner (Fig. 3A). The high virus titers ($> 10^3$ PFU/ml) at 0 h after infection may result from virus particles that were not internalized. Six hours after infection, the virus yield in culture supernatants increased, reaching a plateau at 12 h (closed circles). PDTC at 1000 μM repressed virus yield to less than 5% of that without PDTC at 12–48 h after IV infection (open circles). Up to 3 h after infection, 1000 μM PDTC repressed virus yield to 5% of that without PDTC (Fig. 3B). In addition, 1000 μM PDTC at a higher MOI (= 40) also repressed virus yield to less than 7–28% at

Fig. 1. Effects of PDTC and Trolox on DNA fragmentation resulting from IV infection. A After mock and IV infection, chorion cells were cultured for 48 h in the absence or presence of PDTC. C After mock and IV infection, PDTC was added or not at 0, 1, 3, 6 and 9 h. All chorion cells were then cultured for 48 h in total after infection. E After mock and IV infection, culture medium without or with PDTC was added to chorion cells at 0 h after infection. Medium was remoed at 1, 3, 6 and 48 h after infection. After washing cells with PBS, fresh culture medium without PDTC was added, then cells were cultured for 48 h in total after infection. G After mock and IV infection, chorion cells were cultured for 48 h after infection in the presence or absence of Trolox. Panels A, C, E and G show profiles of agarose gel electrophoresis of DNA fractions extracted from the cells. Lane M shows DNA size marker. Sizes of DNA fragments in lane M are shown as base pairs. Panels B, D, F and H show DNA fragmentation rates. Open and closed columns indicate mock- and IV-infection.

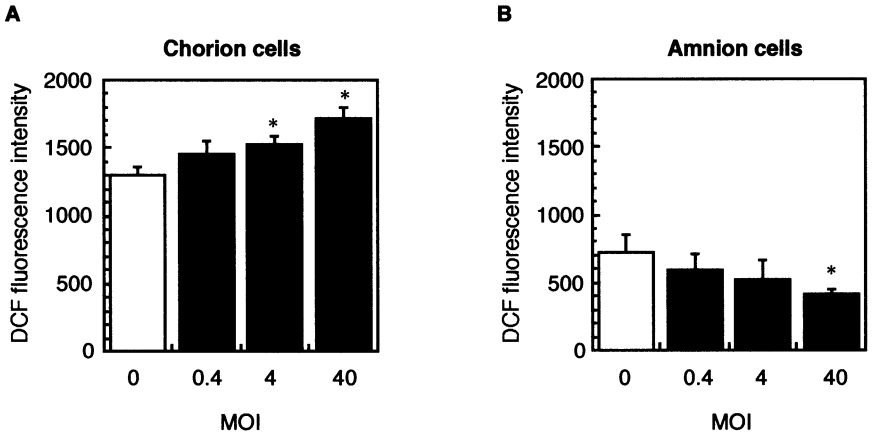

Fig. 2. Alterations of ROS production by chorion and amnion cells after IV infection. Chorion (A) and amnion cells (B) were infected with IV at MOI = 0, 0.4, 4 and 40 and cultured for 24 h. Levels of ROS produced by the cells were measured using ROS reactive fluorescence probe DCFH-DA. Data are shown as means and standard deviations ($n = 4$). Statistical analyses using the t-test showed significant differences between mock and IV infection (* $P < 0.05$).

Table 1
Effects of PDTC and Trolox on ROS production by chorion cells after mock and IV infection

Additive	Fluorescence intensity of dichlorofluorescein (DCF)	
	Mock (MOI = 0)	IV (MOI = 40)
Vehicle (0.05% PBS)	2106±219	2428±73*
PDTC (500 μM)	1475±134[a]	1502±112[b]
Vehicle (0.1% EtOH)	1936±111	2156±75*
Trolox (500 μM)	1141±51[b]	1188±92[b]
Vehicle (0.1% PBS)	1993±137	2258±108*
Ribavirin (1000 μM)	1843±120	1803±160[a]

Chorion cells were pre-incubated with vehicle or ribavirin for 24 h prior to infection and cultured with vehicle or ribavirin for 24 h after mock and IV infection (MOI = 40). Alternatively, chorion cells were cultured with vehicles, PDTC or Trolox for 24 h after infection. Levels of ROS produced by the cells were measured using ROS reactive fluorescence probe DCFH-DA. Data are shown as means and standard deviations ($n = 4$). Statistical analyses using the t-test showed significant differences between mock and IV infection (* $P < 0.05$) and between vehicle and drug ([a] $P < 0.05$, [b] $P < 0.01$).

12–48 h after infection (data not shown). Furthermore, Table 2 shows that 500 μM PDTC significantly repressed virus yield to less than 5% of that observed in the absence of PDTC in both infected chorion and amnion cells; in contrast, 500 μM Trolox did not repress virus yield in infected chorion cells. These results indicated that PDTC inhibited IV production, whereas Trolox did not. Moreover, the ability of PDTC to inhibit IV production is evident in amnion cells where infection does not induce apoptosis.

To investigate the inhibitory effect of PDTC on IV production, we examined the effect of PDTC on the replication and transcription of viral gene and on viral protein synthesis. Fig. 4A and B show that in controls, HA vRNA was scarcely discernible at 0 h, then increased from 3 to 48 h, and that HA c/mRNA was virtually undetectable at 0 h, but increased from 3 to 12 h then gradually decreased from 24 h. In the presence of 1000 μM PDTC, HA vRNA was barely detectable until 6 h after infection, but increased from 12 h, and HA c/mRNA increased from 12 h after infection. These results showed that PDTC completely inhibited HA vRNA and c/mRNA synthesis until 6 h after IV infection, whereas syntheses resumed from 12 h even in the presence of PDTC. When added before and at 6 h after IV infection, the inhibitory effect of PDTC on HA vRNA and c/mRNA synthesis was, respectively, intensified and diminished to zero (Fig. 4C and D). It was no longer inhibitory effect when PDTC was added at 6 h after IV infection. Fig. 4E shows that HA protein in IV-infected chorion cells appeared from 12 h after infection and further increased till 48 h (control).

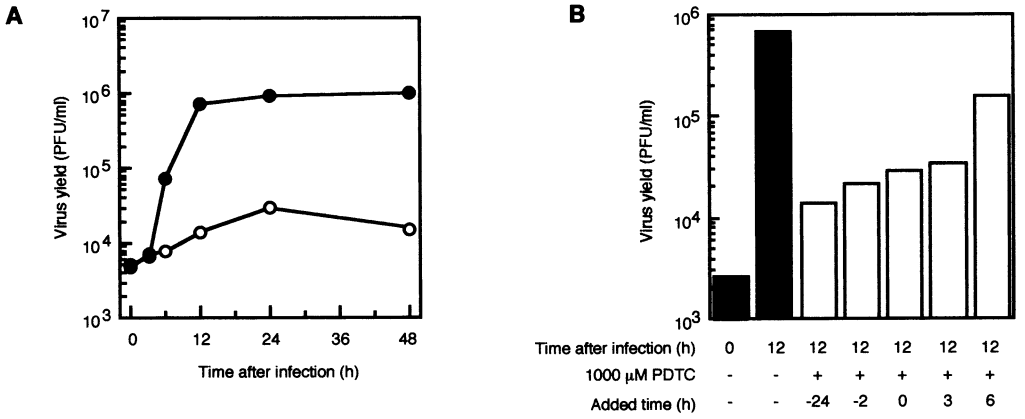

Fig. 3. Effect of PDTC on IV particle release. (A) After IV infection at MOI = 4, chorion cells were cultured for various periods in the absence (closed circles) or presence of 1000 μM PDTC (open circles). (B) Chorion cells were incubated with PDTC for 2 and 24 h before IV infection, then infected with IV at MOI = 4 and cultured for a further 12 h with PDTC. Alternatively, PDTC was added or not at 0, 3 and 6 h. All cells were then cultured for 0 and 12 h. Virus yields in culture supernatants were measured by plaque-forming assays and are indicated as plaque-forming units (PFU)/ml. Closed and open columns indicate absence and presence of PDTC, respectively.

However, 1000 μM PDTC delayed and decreased HA protein synthesis. These results showed that PDTC inhibited viral macromolecule synthesis at the early stage of infection.

Ribavirin reduces IV ribonucleoprotein (RNP) synthesis (Wray et al., 1985). We compared the effect of PDTC on viral RNA synthesis with that of ribavirin. The results showed that ribavirin inhibited HA vRNA and c/mRNA synthesis throughout infection (Fig. 4A, B), indicating that PDTC and ribavirin inhibit viral RNA synthesis at different periods.

4. Discussion

The precise mechanism of apoptosis induced by IV is presently unclear. However, a high level of ROS participates as a possible mediator of apoptosis induced by IV (Lin et al., 2001). Our results indicated that PDTC blocks IV-induced apoptosis (Fig. 1). The present study demonstrated that ROS production is moderately increased in IV-infected apoptotic cells and decreased in IV-infected non-apoptotic cells (Fig. 2), and that PDTC represses ROS overproduction in IV-infected apoptotic cells

Table 2
Effects of PDTC and Trolox on IV particle release

Additive	Time after infection (h)	Virus yield (PFU/ml)	
		Chorion cells	Amnion cells
None	0	2020 ± 950	5700 ± 479
None	24	448 000 ± 91 600	705 000 ± 85 400
PDTC (500 μM)	24	16 300 ± 3590*	14 150 ± 866**
Trolox (500 μM)	24	383 000 ± 84 000	Not tested

Chorion and amnion cells were infected with IV at MOI = 4, then cultured for 0 and 24 h in the absence (none) or presence of PDTC or Trolox. Virus yields in culture supernatants were measured by plaque-forming assays and are indicated as plaque-forming units (PFU)/ml. Data are shown as means and standard deviations ($n = 4$). Statistical analysis using the t-test showed a significant difference between none and PDTC (* $P < 0.005$, ** $P < 0.001$).

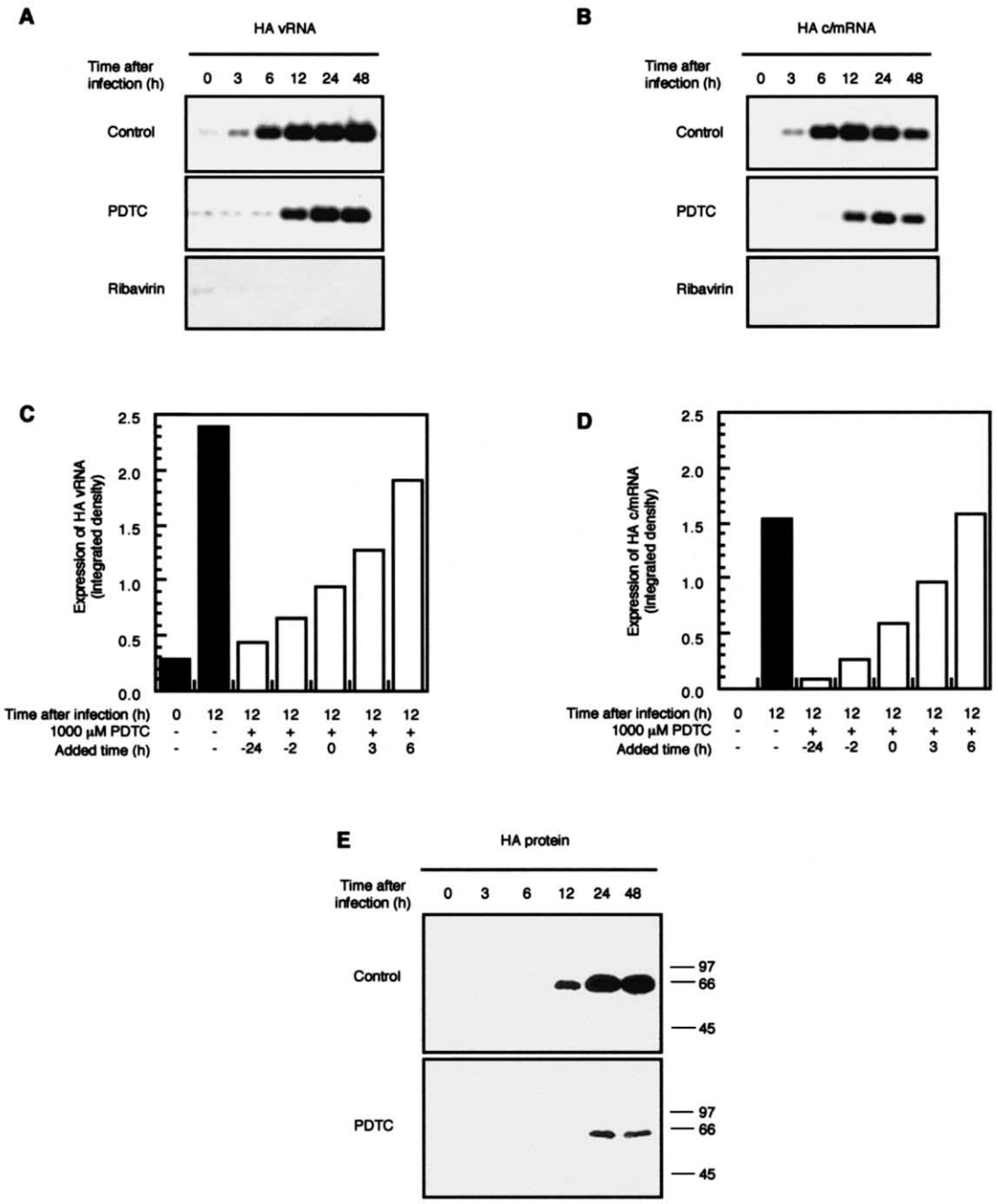

Fig. 4

(Table 1). Therefore, PDTC seemed to block apoptosis induction through repressing ROS overproduction. However, Trolox repressed ROS overproduction (Table 1) but did not block apoptosis (Fig. 1), suggesting that the inhibition of ROS overproduction is not accompanied by a blockade

of apoptosis. Therefore, a moderate level of ROS may not be responsible for apoptotic events.

We suggested elsewhere that ribavirin blocks IV-induced apoptosis through the inhibition of IV replication (Uchide et al., 2002a). The present study showed that ribavirin repressed IV-induced ROS overproduction without affecting basal ROS production (Table 1), indicating that ribavirin did not act as an antioxidant under our conditions. Ribavirin may have repressed ROS overproduction by blocking apoptosis. Therefore, we propose that moderate ROS overproduction results from the induction of apoptosis by IV infection.

The question is then raised as to how PDTC blocks IV-induced apoptosis. Many findings suggest that IV production is essential for the induction of apoptosis (Price et al., 1997; Lowy and Dimitrov, 1997; Fesq et al., 1994; Morris et al., 1999). Miyamoto et al. (1998) reported that the metal chelator thujaplicin–copper complex simultaneously inhibited apoptosis in IV-infected MDCK cells and IV particle release from the infected cells, and that these effects are maintained for up to 2 h after infection. Takizawa et al. (1993) indicated that the critical period for the induction of apoptotic cell death in IV-infected HeLa cells is 2–4 h after infection. The present study also found that PDTC inhibits apoptosis when added at times up to 3 h after IV infection (Fig. 1C, D) or by exposure for only 1 h after IV infection (Fig. 1E, F), indicating that PDTC inhibited IV infection-induced apoptosis at the early stage of IV infection. Moreover, PDTC added until 3 h after infection simultaneously inhibited IV production (Fig. 3B). All of these studies suggested that IV replication at the early stage of infection plays a critical role in the induction of apoptosis by IV infection. This notion appears to hold among different cell types. Accordingly, PDTC appeared to block IV-induced apoptosis through inhibiting IV production rather than its antioxidant effect.

The present study found that PDTC is likely to inhibit replication and transcription of viral gene as well as viral protein synthesis at the early stage of infection (Fig. 4). The inhibition of IV production by PDTC may result from the inhibition of viral macromolecule synthesis. Dithiocarbamates can chelate various metal ions, leading to the formation of a lipophilic dithiocarbamate–metal complex, and rapid transport via a lipophilic complex by PDTC has been proposed to explain the intracellular recruitment of zinc and copper ions from the extracellular medium (Kim et al., 1999). Oxford and Perrin (1974) demonstrated that zinc and copper ions inhibit IV RNA-dependent RNA polymerase activity, and that the inhibitory effect of metal chelator bathocuproine–copper and bathocuproine–zinc complexes is greater than the effect of bathocuproine itself. PDTC could inhibit viral RNA synthesis through the inhibition of IV RNA-dependent RNA polymerase activity by increasing the amount of intracellular copper and zinc ions or intracellular PDTC–copper and PDTC–zinc complexes.

Ribavirin is a guanosine analogue that reduces IV RNP synthesis through reducing the size of the cellular guanosine 5′-triphosphate pool and by directly affecting viral replicative enzymes (Wray et al., 1985). Furthermore, Eriksson et al. (1977) found that ribavirin triphosphate, a cellular metabolite of ribavirin, selectively inhibits IV RNA-dependent RNA polymerase. Our results indicated that ribavirin inhibited viral RNA synthesis throughout infection, whereas PDTC inhibited only at the early stage of infection (Fig. 4). If PDTC acted only as an inhibitor of IV RNA-dependent RNA polymerase, viral RNA synthesis

Fig. 4. Effect of PDTC on iral macromolecule synthesis. A and B After IV infection at MOI4, chorion cells were cultured for 0, 3, 6, 12, 24 and 48 h in the absence Control or presence of 1000 M PDTC. Alternatiely, chorion cells were incubated with 1000 M ribairin for 24 h then cultured with ribairin after IV infection. C and D Cells were incubated with PDTC for 2 and 24 h before infection. Alternatiely, PDTC was added or not to the culture medium at 0, 3 and 6 h. All cells were then cultured for 0 and 12 h after infection in the presence or absence of PDTC. Expression of HA iral RNA and complementary andor messenger cm RNA in cells was analyzed by RT-PCRSouthern blot assays. Closed and open columns indicate absence and presence of PDTC, respectiely. E After IV infection at MOI4, chorion cells were cultured for 0, 3, 6, 12, 24 and 48 h without Control or with 1000 M PDTC. Expression of HA protein in cells was analyzed by Western blot assays. Sizes of protein molecular weight markers are shown as kDa at the right.

stopped by PDTC would not resume in its presence and prior exposure of the cells to PDTC would not intensify its effect. However, our results demonstrated that HA RNA synthesis that was stopped by PDTC resumed from 12 h after IV infection even in the presence of PDTC (Fig. 4A and B), and that prior exposure of the cells to PDTC intensified its inhibitory effect on HA RNA synthesis (Fig. 4C and D). Cellular proteins such as karyopherin, RNA polymerase regulatory factor (PRF), RNA polymerase activating factor (RAF) and NS1-binding protein (NS1-BP) are associated with the processes of IV gene replication and transcription (Whittaker et al., 1996; Shimizu et al., 1994; Wolff et al., 1998). PDTC regulates the gene expression and/or activity of some cellular factors such as antioxidant enzymes and transcription factor (Borrello and Demple, 1997; Hartsfield et al., 1998; Wild and Mulcahy, 1999). Therefore, PDTC is likely to act not only as an inhibitor of IV RNA-dependent RNA polymerase but also as a modulator of cellular factors associated with the process of IV gene replication and transcription.

In conclusion, the present study shows that PDTC inhibits apoptosis through the inhibition of viral macromolecule synthesis rather than through its antioxidant effect, because Trolox did not inhibit apoptosis or IV replication, although it did inhibit ROS overproduction. Specific viral macromolecules synthesized at the early stage of infection may play a critical role in the mechanism of apoptosis induction. Moderate ROS overproduction may result from apoptosis induction and may not be causative in apoptosis in IV-infected chorion cells.

Finally, we will present the rationale with respect to selection of primary cultured cells of human fetal membranes as our study model. A cytopathogenic strain of IV (A/PR/8/34) causes both cytocidal and persistent infections in different types of cells prepared from human fetal membrane tissue (Uchide et al., 2002a). Interestingly, IV-infected chorion cells undergoing apoptosis induce inflammatory cytokines and putative monocyte differentiation-inducing factor(s) that would regulate the host immune response to eliminate viral pathogens, which IV-infected non-apoptotic amnion cells do not (Uchide et al., 2002b,c). A series of comparative studies employing these cells will provide further information regarding virus–host cell interactions and antiviral responses of the host.

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