Influenza a virus induced apoptosis: Inhibition of DNA laddering & caspase-3 activity by zinc supplementation in cultured HeLa cells

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Background & objectives: The pathogenesis of influenza virus infection involves virus replication in epithelial cells of the respiratory tract and the consequent degeneration of infected cells. Influenza virus induces cellular degeneration following infection of cultured cells in vitro, and the cytopathic effect (CPE) occurs principally through apoptotic cell death. This study was undertaken to find out the effect of zinc on influenza virus induced apoptosis in cultured HeLa cells.

Methods: The sub-confluent monolayer HeLa cells were used to study the effect of zinc on influenza virus induced apoptosis. The apoptotic markers viz., caspase-3 activity, phagocytic index, morphological changes, and DNA fragmentation were assayed.

Results: When HeLa cells were infected with a cell adapted pathogenic strain of influenza A (A/Udorn/317/72H3N2) virus, DNA fragmentation was observed in virus infected cells by 24 h post infection and caspase-3 activity was maximum at 4 h post infection after which it reached to plateau. Treatment of cells with 0.15mM concentration of zinc till 8 h post infection inhibited DNA fragmentation and also caspase 3 activity was decreased significantly up to 2 h post infection.

Interpretation & conclusions: When the infected HeLa cells were incubated with adherent macrophages, efficient phagocytosis occurred and the release of virus into the culture medium was inhibited. These results suggested that inhibitory effect on influenza virus induced apoptotic death of cultured cells can be determined at an early stage of the infection by treatment of zinc.

Key words: Apoptosis - caspase activity - DNA fragmentation - influenza virus

Apoptosis is a programmed cell death in which the cell actively participates in its own destructive process. In addition to its physiologic roles, apoptosis plays an important role in the pathogenesis of infectious diseases caused by viruses, bacteria and prions. Many viruses carry genes, which directly influence the apoptotic ability of their host cell thus changing the view about significance of apoptosis in determining the outcome of virus infections. The ability of viruses to control the apoptotic response of their host can influence the pathogenicity of the virus. The virulent influenza A viruses produce devastating disease, and experimental infection by these viruses is frequently used as a model system to examine the mechanism of disease.
The damage caused by influenza virus infection in cultured cells, which permit high levels of replication of influenza A virus exhibited several features characteristic of programmed cell death6-8. Depletion of lymphocytes due to apoptosis has also been described in mice infected with a highly virulent influenza A virus (H1N1) isolated from humans911. All mammalian and avian influenza A and B virus strains induce apoptosis in many different cell types, including lymphocytes and human monocytes12. However, the consequence of this activation for virus replication or host cell defense is not well established12.

Zinc supplementation prevents apoptosis induced by a variety of agents in both, in vitro and in vivo models13. The protective effect of zinc has generally been attributed to inhibition of Ca2+ and Mg2+ dependent endonucleases, thereby causing inhibition of DNA fragmentation, a terminal step and hallmark of apoptosis14. However, many studies have also identified caspase-3 as a site of zinc inhibition in the apoptotic pathway14-16. This protease is frequently activated in mammalian cell apoptosis to cleave key cellular proteins, leading to completion of the apoptotic process once the cell has been committed to die15,17,18. The exact role of zinc in the apoptotic pathway of influenza A virus infected cells has not been fully established.

This study was undertaken to characterize the mechanism of cell damage caused by influenza virus infection in cultured HeLa cells; an extensive time-interval study was performed to elucidate the sequence of hallmark events in apoptosis. The effect of zinc was investigated on both the early and late phase of apoptosis in a time dependent manner in cultured cells and confirmed that caspase-3 was a novel and proximal site of Zn2+ inhibition in the apoptotic pathway.

Material & Methods

Virus and infection of HeLa cells: HeLa cells obtained from National Centre for Cell Sciences (NCCS), Pune, India, were cultivated in either 35-mm cell culture dishes or 25 or 75 cm² culture flasks (Greiner, Germany) with Eagle’s minimal essential medium (Sigma, USA) containing 10 per cent foetal bovine serum (Sigma, USA) and antibiotics (100 U/ml penicillin, 100 mg/ml streptomycin, 0.25 mg/ml fungizone, 25 mg/ml gentamicin; all from BioWhittaker, USA) at 37°C in a 5 per cent CO₂ incubator. A pathogenic strain of influenza virus (IV), A/Udorn/317/72(H3N2), obtained from CDC, Atlanta in lyophilized form was originally propagated in embryonated chicken eggs and further grown in cultured HeLa cells. When HeLa cell cultures were 85-90 per cent confluent, the cultures were pretreated with 199 medium (Sigma, USA) and inoculated with influenza virus at a multiplicity of infection of approximately ten, 50 per cent tissue culture infectious doses (TCID₅₀) per cell for 45 min at 37°C. Control HeLa cells were mock infected with 199 medium alone. The mock and IV-infected HeLa cells were incubated at 37°C in 199 medium with 0.25 per cent BSA (Sigma, USA) and 1 mg/ml L-1-tosylamido-2-phenylethyl chloromethyl ketone-trypsin (Sigma, USA) for various times before analysis. The amount of virus released into the culture medium was determined by haemagglutination test and plaque forming unit (PFU) assays.

Plaque forming unit (PFU): Virus particles in HeLa cell culture suspensions following IV infection were quantified by PFU enumeration using standard procedures as described by Kurokawa et al19. Briefly, confluent monolayers of Madin-Darby canine kidney cells were incubated with the culture supernatants collected at various time points after virus infection serially diluted in phosphate buffer saline (PBS) containing 1 per cent bovine serum albumin (pH 7.2) for 1 h at room temperature. The cells were then overlaid with Eagle’s minimal essential medium (EMEM) supplemented with 0.2 per cent bovine serum albumin, 0.1 per cent DEAE Dextran, 1 µg trypsin/ml and 0.8 per cent agar, and maintained in a humid atmosphere containing 5 per cent CO₂ for 3 days. The agar media were then removed and the cells were fixed with 5 per cent formalin solution and stained with 0.03 per cent methylene blue solution. Visualized plaques were counted and the virus titre was expressed as log₁₀ PFU/ml.

Cell viability assays: Cells were transferred to a slide and stained with 0.5 per cent trypan blue. After 1 min staining, cells were observed under the microscope (Nikon E600, Japan). Dead cells were stained blue, whereas living cells remained unstained. The percentage of cell death was obtained by calculating the ratio of dead cells to total cells.

DNA fragmentation assay: To assess the fragmentation of cellular DNA into the characteristic apoptotic ladder, IV-infected HeLa cells were pelleted at 6, 12, 18 and 24 h post infection (pi) and mock infected cells were pelleted at 24 h pi. DNA samples were extracted using a similar method as described by Gong et al20 with slight modifications. Briefly, HeLa cells were lysed in
ice-cold cell lysis buffer (10 mM Tris, pH 7.5; 1 mM EDTA; 0.5% Triton X-100). Samples were then treated with 0.1 per cent sodium dodecyl sulphate (SDS) (GIBCO-BRL, USA) and 300 mg/ml of Proteinase K (Sigma, USA) at 56°C overnight. Lysates were phenol-chloroform extracted and the DNA samples were cold ethanol precipitated in the presence of 300 mM sodium acetate (pH 5.2). DNA was further treated with RNase A (Sigma, USA) (1 mg/ml) for 1 h at 37°C and subjected to electrophoresis (Submarine System, Sub Cell GT, Bio-Rad, USA) using 1.8 per cent agarose in Tris-EDTA buffer. DNA was visualized by staining with ethidium bromide and photographed in gel documentation system (AlphaImager™ 2200, USA).7

Caspase-3 assay: The Caspase-3 assay was performed in a multiwell plate using a kit obtained from BD Biosciences Pharmingen (USA). Cell lysates (1x10⁶ cells/ml) were prepared at 0, 1, 2, 3, 4, 5, 6, and 7 h post infection. For each reaction, 5µl fluorogenic substrate, Ac-DEVD-AMC (acetyl-Asp-Glu-Val-Asp/7-amino-4-methylcoumarin) was added to a well containing 0.2 ml of 1X HEPES buffer; 50 µl of cell lysate was added to each well containing HEPES buffer. Caspase-3 blocking assays were set up in parallel to each reaction mixture by adding 5 µl Ac-Asp-Glu-Val-Asp-CHO. The reaction mixtures were incubated for 1 h at 37°C. The amount of fluorescence emitted was measured using a spectrofluorometer with an excitation wavelength of 380 nm and an emission wavelength range of 440 nm.21

Annexin V assay: Detection of flipping of phosphatidylserine was done by annexin V assay by using a commercially available TACS™ Annexin V-FITC Apoptosis Detection kit (R & D, USA).20 At 0, 3, 6, 9, 12, and 15 h pi, HeLa cells were pelleted and specific binding of annexin V was achieved by incubating 10⁵ HeLa cells in 60 µl of binding buffer with a saturated concentration of annexin V for 15 min at 4°C in the dark. To discriminate between early apoptosis and necrosis, the cells were simultaneously stained with annexin V and propidium iodide (PI) before analysis. The binding of FITC-annexin V (FL1) and PI (FL2) to the cells was measured by flow cytometry (FACSCalibur; BD Biosciences, USA) using Cell Quest software from BD Biosciences, (USA). At least 10,000 cells were counted in each sample.22

Phagocytosis of influenza virus-infected cells by macrophages: Macrophages were isolated from peritoneal fluids of thioglycollate-treated eight weeks old BDF₁ mice obtained from the animal house of V.P. Chest Institute, Delhi.23 The macrophages were maintained in RPMI 1640 (Sigma, USA), 10 per cent foetal bovine serum.22 1x10⁶ HeLa cells infected with influenza A virus were collected at 0, 6, 9, 12, 18, 24 h post infection, labelled with biotin and added to the macrophage culture at a ratio of one HeLa cell to two macrophages. The mixed culture was incubated for 1 h at 37°C, washed extensively by pipetting with PBS and then with trypsin (0.5 µg/ml) to remove HeLa cells free from or lightly attached to macrophages. The remaining cells were further fixed with PBS containing 2 per cent paraformaldehyde, 0.5 per cent glutaraldehyde, and 0.05 per cent Triton X-100 and then supplemented with FITC-conjugated avidin (Vector Laboratories, USA). The number of macrophages containing engulfed cells was determined using fluorescence and phase-contrast microscopy (Nikon E600, Japan) and expressed relative to the total number of macrophages, this ratio was termed the phagocytic index.24,25

Effect of zinc on DNA fragmentation: To assess the ability of Zn²⁺ ions to block endonucleolytic DNA cleavage ZnSO₄ (Sigma, USA) (0.15 mM,) was added to the infected monolayer cultures at 0, 4, 8, 12, 16, and 20 h post infection. At 24 h pi, cellular DNA was extracted from virus infected control, mock infected and infected cells with treatment and analyzed by gel electrophoresis.26

Effect of zinc on caspase-3 activity: Infected HeLa cells were treated with 0.15 mM of ZnSO₄ and the caspase-3 activity was observed at 0, 1, 2, 3, 4, 5 and 6 h post infection. The activity at 4 h p.i. was compared to that of mock infected treated and untreated infected HeLa cells at 4 h.26

Statistical analysis: The data were analyzed with one and two way analysis of variance (ANOVA) followed by bonferroni’s multiple comparison tests. All the results were expressed as mean ± SEM.

Results

Effect of influenza virus infection on cell death: The inoculated plates were subjected to virus titration at 6 hourly intervals and it was observed that the virus titre increased with time. A significant increase in the virus titre was observed at 12 h (P<0.05) post infection. At 24 h pi, the maximum virus titre was observed after which it reached a plateau. At 4 hourly intervals post-infection, the infected HeLa cells were stained with trypan blue and assessed for cell viability. It was
observed that the percentage of cell viability decreased as the time of infection increased and at 24 h post infection, it was less than 10 per cent.

Detection of DNA laddering in influenza virus infected HeLa cells: DNA laddering is commonly accepted as an important biochemical hallmark of apoptosis. Thus it was critical to test whether or not influenza virus infection induced DNA laddering in cultured HeLa cells. Agarose gel electrophoresis analysis of DNA extracted from virus infected cells showed distinct laddering of DNA fragments increasing in size by multiples of 180 bp started to appear at 12, 18 and the classical ladder pattern of apoptosis was observed at 24 h post infection whereas no DNA fragmentation was shown in DNA from the 6 h pi and mock infected cells. The results suggested activation of endonuclease by influenza virus infection (Fig. 1).

Fig. 1. DNA ladder assay showing virus-induced DNA fragmentation. HeLa cells were infected with A/Amn1/317/72 (H3N2). Equal numbers of total cells, harvested at 6, 12, 18 and 24 h p.i. examined for apoptotic DNA. Mock infected cells (MO) used as controls. DNA fragments were visualized by ethidium bromide staining on 1.8% agarose gels. Lane 1 - DNA ladder; Lane 2 - DNA from Mockinfected cells; Lane 3-6 - DNA ladder at 6, 12, 18, 24 h p.i.

Kinetics of activation of cysteinyl proteases; caspase-3 on influenza virus infection in cultured cells: To study the activation of caspase-3 time-interval study was performed, cell lysates were prepared at various time points after inoculation of virus i.e., 0, 1, 2, 3, 4, 5, 6, and 7 h respectively and the amount of AMC (7-amino-4-methylcoumarin) liberated from Ac-DEVD-AMC was measured using a spectrofluorometer. Apoptotic cell lysates containing active caspase-3 were estimated by a considerable emission as compared to non-apoptotic cell lysates. A significant increase in fluorescence occurred 2 h after infection (P<0.001). Maximum caspase-3 activity was observed at 4 h post-infection (Fig. 2).

Fig. 2. Caspase-3 activity during influenza virus induced cell death in mock infected, infected, infected with treatment and mock infected with treatment HeLa cells. Values are the means ± SD of three separate experiments. The level of significance was **P<0.05 and ***<0.001, NS-not significant (Two-way ANOVA, Bonferroni post tests).

Fig. 3. Flipping of plasma membrane by annexin V staining of phosphatidylserine (PS) externalization at 0, 3, 6, 9, 12, 15 h p.i. (a, b, c, d, e and f respectively).
**Flipping of plasma membrane in response of virus induced cell death:** Monolayers of HeLa cells were infected with the virus for various time periods (0, 3, 6, 9, 12, 15 h respectively), and cells were examined for flipping of plasma membrane by measuring phosphatidylserine (PS) externalization. Annexin V assay at various time points showed that maximum phosphatidylserine externalization occurred after 9 h of infection (Fig. 3).

**Phagocytosis of infected HeLa cells:** The extent of phagocytosis of normal HeLa cells and of HeLa cells that had been infected with influenza virus for various periods (0, 6, 9, 12, 18, 24 h respectively) was determined. It was observed that phagocytosis became evident at significant levels at 9 h post infection, and the phagocytic index continued to increase thereafter (Fig. 4). At 18 h p.i., more than 30% of the macrophages appeared to incorporate virus-infected cells, whereas these did not react with normal uninfected cells.

**Effect of zinc supplementation on DNA fragmentation in influenza virus infected HeLa cells:** To determine the effect of zinc on influenza A/Udorn/317/72 (H3N2) virus infected HeLa cells, 0.15 mM, 0.2 mM, and 0.25 mM of ZnSO4 was added to the infected monolayer cultures at 0, 3, 6, 9, 12, and 18 h post infection and cellular DNA was analyzed by gel electrophoresis. It was observed that ZnSO4 at a concentration of 0.15 mM, and 0.2 mM added to the medium just after infection effectively blocked the DNA fragmentation in HeLa cells. The inhibition of DNA fragmentation was still evident when ZnSO4 was added at 3 and 6 h p.i. It was also observed that lower concentration of zinc i.e. 0.10 mM and higher concentration 0.25 mM were unable to cause inhibition of DNA fragmentation (data not shown here). To study the effect of zinc treatment on DNA fragmentation with time, 0.15 mM zinc concentration was used and it was observed that treatment of zinc till 8 h p.i. completely protected cells from DNA fragmentation (Fig. 5).

**Effect of zinc on caspase-3 activity during influenza virus infection in cultured cells:** The cells treated with 0.15 mM zinc showed significantly less fluorescence (*P*<0.05) in contrast to the untreated cells at 2 h and the maximum decrease in fluorescence after zinc treatment was observed at 4 h p.i. (*P*<0.001) (Fig. 2).

Fig. 4. Phagocytic index of infected HeLa cells: phagocytic macrophage is represented relative to total macrophages. Error bars indicate standard deviations. The level of significance was **P**<0.05 and ***<0.001, compared with NS- not significant (Two-way ANOVA, Bonferroni post tests).

Fig. 5. DNA ladder assay showing inhibition of virus-induced DNA fragmentation by zinc treatment. HeLa cells were infected A/ Udorn/317/72 (H3N2). Cells were treated by zinc at 0, 4, 8, 12, 16, 20 h p.i harvested at 24 h p.i., were examined for apoptotic DNA laddering pattern. Mock infected cells (MO) and virus infected cells without treatment (MT) used as negative and positive controls of apoptosis. DNA fragments were visualized by ethidium bromide staining on 1.8 per cent agarose gels. Lane 1 - Mock infected cells (MO); Lane 2-7 - DNA ladder at 0,4,8,12,16 and 20 hr p.i.; Lane 8 - Virus infected cells without treatment (MT); Lane 9 - DNA ladder (M).
Discussion

A current challenge in the field of influenza A virus pathogenesis is to understand how virus infection leads to specific tissue damage and disease or death of the host. A few previous studies, have shown that all mammalian and avian influenza A strains induce apoptotic death of cultured cell lines as well as that of tissues in infected animals. In this in vitro study cultured HeLa cells were used, which permitted low levels of replication of influenza A/Udorn/317/72 (H1N1) virus and show strong evidence for the influenza virus induced apoptosis in cultured HeLa cells. Virus infection resulted in condensation of cytoplasm, DNA fragmentation and nuclear disintegration and collapse. All these cellular changes are stereotypic hallmarks of apoptosis in both animal and plant cells. The virus titre of the culture medium of infected HeLa cells determined by pfu enumeration at various time intervals post infection indicated that about 100 per cent infection was achieved and that virus release began at approximately 6 h post-infection, and it reached a maximum at 24 h of infection. However it is reported that the completion of apoptosis as determined by various biochemical assays, required almost 36 h. This suggested that peak of virus replication was preceding the arrest of host cell macromolecular synthesis due to apoptosis.

A biochemical confirmation of the occurrence of apoptosis due to influenza A virus infection was done by DNA fragmentation assay. The assay revealed that DNA fragmentation began at 12 h p.i. and classical fragmentation occurred at 24 h p.i. The uninfected cells, however, did not exhibit fragmentation of chromosomal DNA. Takizawa et al. reported that chromosomal DNA in influenza A virus-infected MDCK cells exhibited fragmentation by 6 h p.i. The delayed appearance in HeLa cells may reflect a slower progression and lower degree of cell lysis than that of MDCK cells.

The study was further extended to understand the kinetics of the phenomenon. The central component of the apoptotic machinery is a proteolytic system consisting of a family of cysteiny1 proteases, termed caspases. The activity of caspase-3, where both the pathways, extrinsic and intrinsic converge in the infected HeLa cells was measured at various time points and a significant increase in the activity was observed after 2 h infection with the maximum activity at 4 h p.i. There was no significant increase in caspase-3 activity observed in uninfected cells. Concurrent with earlier reports by using various influenza virus strains and cell types, it was confirmed that caspase-3 activation at the onset of apoptosis was a crucial event during influenza A virus induced apoptosis in cultured cells.

The flipping of some components of the plasma membrane principally phosphatidylserine, from the inside surface to the outside surface is a fairly early event in apoptosis. Annexin V acts as a positive identification tag for cells undergoing apoptosis. Assay of annexin V in the infected cells at various time periods indicated that membrane phospholipid phosphatidylserine (PS) appeared on the surface of virus-infected cells 6 h post infection. This externalization of phosphatidylserine induces the phagocytosis of infected cells, which are committed to apoptosis. The extent of phagocytosis was also determined using HeLa cells that had been infected with influenza A virus for various time periods. The results suggested that the virus together with its host cells was digested in macrophages. These findings add to our understanding that apoptosis of influenza virus-infected cells induces inhibition of virus growth and limits the progression of disease by playing an initial defense against viral invasion. However, findings from the in vivo studies showed that virus infection invokes hyperactivation of immune response which in turn induces apoptosis in bystander uninfected cells and also contributes to the inciting the disease. It is thus anticipated that apoptosis of the influenza virus-infected respiratory epithelial cells controls the tissue load of cells at the inflamed site by eliminating the virus infected cells thereby playing a very important role in the pathogenesis of disease.

Pandey et al. found that DNA fragmentation in nuclei isolated from 5123 tc rat hepatoma cells required the presence of calcium and magnesium ions. Zinc supplementation has been known to inhibit apoptosis induced by a variety of agents both in vitro and in vivo models. The inhibition of influenza virus induced DNA laddering by zinc supplementation in our study is probably based on the same mechanism. These results led to a postulation that in HeLa cells the terminal step of the apoptotic pathway, that is DNA fragmentation, is one of the sites on which zinc exerts inhibitory effect possibly via preventing activation of endonuclease.

However, David et al. have identified caspase-3 as one of the site of zinc inhibition in the apoptotic pathways.
pathway. When infected HeLa cells were treated with Zn\(^{2+}\) and examined for DNA fragmentation, it was observed that Zn\(^{2+}\) at a concentration of 0.15 mM, added to the medium just after infection effectively blocked the fragmentation in HeLa cells. The inhibition of DNA fragmentation was still evident when Zn\(^{2+}\) was added at 4 and 8 h p.i. our study also demonstrated that when infected HeLa cells were treated with 0.15 mM, of Zn\(^{2+}\), significantly less caspase-3 activity was observed as compared to the untreated cells. These results suggested that caspase-3 was a novel and proximal site of Zn\(^{2+}\) inhibition in the apoptotic pathway suggesting a regulatory role of Zn\(^{2+}\) in modulating the upstream apoptotic machinery in a time dependent manner.

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**References**


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