Differentiation of monocytes to macrophages induced by influenza virus-infected apoptotic cells

Noboru Uchide,1 Kunio Ohyama,1 Bo Yuan,1 Toshio Bessho2 and Toshio Yamakawa1

1Department of Biochemistry, School of Pharmacy, Tokyo University of Pharmacy and Life Science, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan
2Yoneyama Maternity Hospital, 2-12 Shin-machi, Hachioji, Tokyo 192-0065, Japan

The effect of the culture supernatant of influenza virus (IV)-infected apoptotic and non-apoptotic cells on the differentiation of monocytes to macrophages was investigated. IV infection induced apoptotic DNA fragmentation in cultured chorion cells but not in amnion cells prepared from human foetal membrane tissue. To examine the differentiation of monocytes to macrophages, an adhesion assay was employed using the human monocytic leukaemia THP-1 cell line. THP-1 cells became adherent to a substrate by incubation with the culture supernatant of IV-infected chorion cells, but not with that of amnion cells. The spreading THP-1 cells were morphologically characteristic of macrophages and they phagocytosed latex particles. RT–PCR analysis revealed that the expression of class A scavenger receptor mRNA was induced in THP-1 cells by incubation with the culture supernatant of IV-infected chorion cells. These results suggested that monocytic THP-1 cells were morphologically and functionally differentiated to macrophages by IV-infected apoptotic cells due to a soluble factor released from the apoptotic cells.

Influenza virus (IV) causes the apoptotic cell death of cultured cells as well as cells in the tissues of infected animals (Mori et al., 1995). Cells infected with IV undergoing apoptosis were phagocytosed by dendritic cells and macrophages in an apoptosis-dependent and phosphatidylserine-mediated manner and these reactions induced antigen presentation to T lymphocytes and the abortion of virus growth in vitro (Albert et al., 1998; Fujimoto et al., 2000; Shiratsuchi et al., 2000). These studies suggest that the phagocytosis of infected cells undergoing apoptosis leads to the initiation of specific immune responses and to pathogen elimination in infected organs. The earlier studies used mature dendritic cells and activated macrophages to phagocytose apoptotic cells. To phagocytose apoptotic cells by macrophages in vivo, immature monocytes in the bloodstream should be attracted to virus-infected apoptotic cells and differentiate to mature macrophages. Whether or not monocytes are differentiated to macrophages by IV-infected cells undergoing apoptosis remains unknown. Here we investigated the effect of culture supernatant of

![Image]
IV-infected apoptotic and non-apoptotic cells on monocyte differentiation to macrophages.

Primary cultured chorion and amnion cells were prepared from human foetal membranes obtained by caesarean section during the month of normal parturition and the cells were cultured in 80% DMEM/F12 (Gibco BRL), 20% heat-inactivated foetal bovine serum (FBS), 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 described previously (Ohyama et al., 2000). IV stock suspension was diluted to the desired concentrations using serum-free medium. Confluent cell monolayers were inoculated with the diluted IV (A/PR/8/34, H1N1) suspension at the desired m.o.i. as described (Fujimoto et al., 2000). Mock-infected control cells were treated similarly but without virus.

Apoptosis induction was examined by DNA fragmentation assays based on agarose gel electrophoresis as described (Ohyama et al., 1998). After mock and IV infection at an m.o.i. of 40, chorion and amnion cells were cultured for various periods. Fig. 1 shows that, in chorion cells, DNA fragmentation into oligonucleosomes was detected at 24 h after IV infection and increased gradually with increased incubation time. DNA laddering occurred in infected chorion cells but not in either mock-infected chorion cells or mock- and IV-infected amnion cells. Since DNA laddering is a key feature of apoptotic cell death (Wyllie et al., 1980), these results indicated that IV infection induced apoptosis in chorion cells, but not in amnion cells.

To investigate the effect of culture supernatants of IV-infected apoptotic and non-apoptotic cells on monocyte differentiation to macrophages, culture supernatants of chorion and amnion cells were collected from live IV-infected chorion cells and IV-infected amnion cells and heat-inactivated IV diluted 10-fold (m). THP-1 cells adhered to plastic substrate after incubation with culture supernatants collected from chorion (shaded columns) and amnion (unshaded columns) cell culture at 48 h after mock-infection (m.o.i. = 0) and IV infection at m.o.i. = 0, 4 and 40 (Fig. 2). THP-1 cells adhered to chorion cell culture supernatant at m.o.i. = 0, 4 and 40 (shaded columns). THP-1 cells significantly adhered to wells after exposure to the culture supernatant of IV-infected chorion cells, depending on the incubation period of the chorion cells after IV infection (shaded columns, Fig. 2A) and to a much lesser extent after exposure to the supernatant of mock-infected chorion cells (unshaded columns, Fig. 2A), but not with that of mock- and IV-infected amnion cells (unshaded and shaded columns, respectively, Fig. 2B). In addition, THP-1 cells adhered to a substrate in a chorion cell-m.o.i.-dependent manner (shaded columns, Fig. 2C) but did not in the case of amnion cells (unshaded columns, Fig. 2C). Moreover, THP-1 cells did not adhere after incubation with IV stock solution heated at 56 °C for 30 min (data not shown). These results showed that THP-1 cells adhered to substrate in the presence of culture supernatants of chorion cells responding to IV infection. The
activity was stable at 56 °C for 30 min. Therefore, chorion cells may have released a factor with the adhesion-inducing activity whereas amnion cells did not, even though the cells were infected with IV. The putative factor may be associated with differences in cell types and/or apoptosis induction. However, in either case, IV-infected apoptotic but not non-apoptotic cells should release the putative factor with adhesion-inducing activity. To further investigate these results, THP-1 cells were incubated with various dilutions of culture supernatant of chorion cells following either mock infection, infection with live IV or infection with heat-inactivated (56 °C for 30 min) IV. As shown in Fig. 2(D), THP-1 cells adhered depending on the content of culture supernatants, and the adhesion level of THP-1 cells treated with culture supernatant of live IV-infected chorion cells (d) was much higher than mock infection (s) or infection with heat-inactivated IV (m). These results indicated that the presence of adhesion-inducing factor was substantiated by the titration assay, and virus replication was required to release the factor from chorion cells responding to IV infection.

Since the ability to adhere THP-1 cells to a substrate in the culture supernatant of chorion cells was highest at 48 h after IV infection at an m.o.i. of 40, IV-infected chorion cell supernatant was collected at 48 h. The supernatant was designated IV-C-sup and used for all following experiments after an incubation at 56 °C for 30 min.

The features of THP-1 cells that caused adherence to a substrate after exposure to IV-C-sup were analysed. THP-1 cells (1·25 × 10⁶ cells) were incubated for 4 days with 50% IV-C-sup on glass coverslips in 24-well plates in a total volume of 0·5 ml. Alternatively, non-treated THP-1 cells were smeared on to glass slides. Firstly, the morphology of cells stained with Giemsa’s solution was observed under an Eclipse E600 microscope (Nikon). Non-treated THP-1 cells were round, the nucleocytoplasmic ratio was decreased to highly basophilic with a few vacuoles (Fig. 3A). The THP-1 cells exposed to IV-C-sup on coverslips were irregularly shaped, the nucleocytoplasmic ratio was increased to < 1, and the cytoplasm was weakly basophilic with many vacuoles (Fig. 3B). Next, we examined the phagocytic function of IV-C-sup-treated THP-1 cells on coverslips. On day 4, the cells were incubated with 1 × 10⁷ particles/ml of 2 µm fluorescent latex particles (PolySciences, PA, USA) at 37 °C or 0 °C for 4 h as described (Oda & Maeda, 1986). Fluorescence microscopy revealed many fluorescent latex particles in IV-C-sup-treated THP-1 cells at 37 °C (Fig. 3C), while particles were virtually absent after the incubation at 0 °C (Fig. 3D). Furthermore, the number of fluorescent latex particles per cell (200 cells per sample) was counted visually in phase contrast views under the fluorescence microscope. The percentage of cells carrying fluorescent latex particles was 78·0 ± 7·2 (P < 0·001) after a 4 h incubation at 37 °C, and 7·8 ± 6·7 after a 4 h incubation at 0 °C. The number of fluorescent latex particles per cell was 5·2 ± 2·1 (P < 0·005) after a 4 h incubation at 37 °C, and 0·1 ± 0·1 after a 4 h incubation at 0 °C. The data are shown as means and standard deviations calculated from four independent experiments. Statistical analysis using the t-test (n = 4) showed significant differences between incubations at 37 °C
and 0 °C. These observations demonstrated that the morphology of IV-C-sup-treated THP-1 cells on the coverslips was characteristic of macrophages (Tsuchiya et al., 1982), and that the fluorescent latex particles were phagocytosed by the cells in a temperature-dependent manner, suggesting that the THP-1 cells were morphologically and functionally differentiated to macrophages by IV-C-sup. Furthermore, THP-1 cells were cultured with mock-inoculated chorion cell culture supernatant (mock-C-sup) and IV-C-sup for 4 days on glass coverslips. THP-1 cells that adhered on the coverslips were incubated with fluorescent latex particles at 37 °C for 4 h. In treatments with both mock-C-sup and IV-C-sup, the percentage of cells carrying fluorescent latex particles was between 86.5 and 87.0%, and the mean latex particle number per cell was between 6.1 to 6.3. No significant difference in percentages of THP-1 cells carrying fluorescent latex particles or mean of fluorescent latex particle number per cell was observed between treatments with mock-C-sup and IV-C-sup. Next, THP-1 cells adhered to a coverslip in a microscopic view were counted (magnification × 400) after staining with Giemsa’s solution. The number of THP-1 cells that adhered to a coverslip by treatment with mock-C-sup was 61.6 ± 15.9, while the number with IV-C-sup was 221.8 ± 51.5 (t-test, n = 10, P < 0.001). A significant difference in the number of THP-1 cells adhered to the coverslips was observed between the treatments. As expected, the total number of fluorescent latex particles phagocytosed with IV-C-sup-treated THP-1 cells was 3.7 times greater than that of mock-C-sup-treated THP-1 cells, indicating that IV-C-sup significantly enhanced phagocytotic reaction by increasing the number of differentiated macrophages.

Class A scavenger receptor (SR-A) mRNA is induced during monocyte differentiation to macrophages (Shirai et al., 1999). As a differentiation marker for macrophages, the expression of SR-A mRNA in THP-1 cells was analysed using RT–PCR as described (Nagafuji et al., 1999) with minor modifications. PCR primers were originally designed according to the published SR-A cDNA sequences (Matsumoto et al., 1990), namely 5′ GCA GTT CTC ATC CTT CTC AT + 3′ (residues 230–249, sense strand) and 5′ GGT ATT CTC TGG GAT TTT GGC 3′ (residues 661–641, antisense strand). For amplification of SR-A mRNA, 30 cycles of PCR consisted of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min and extension at 72 °C for 1 min, using a Takara Thermal Cycler MP. Since differences in the extent of degradation and purity of the RNA sample considerably influence the yield of PCR products, glycerol-3-phosphate dehydrogenase (G3PDH) mRNA was amplified as a control in RNA samples. PCR primers for G3PDH mRNA were purchased from Clontech and 21 cycles of PCR proceeded according to the manufacturer’s protocol. The PCR solution (20 μl) was resolved by electrophoresis on a 2.0% agarose gel and stained with ethidium bromide. Fig. 3(E) shows that the level of the G3PDH mRNA-related PCR product (983 bp) was not altered between non-treated (lane 3) and IV-C-sup-treated (lane 4) THP-1 cells, and that an SR-A mRNA-related PCR product (432 bp) appeared in IV-C-sup-treated cells (lane 2), but not in non-treated THP-1 cells (lane 1). These results showed that SR-A mRNA was significantly induced in THP-1 cells after a 4 day incubation with IV-C-sup, suggesting that the monocytic phenotype of THP-1 cells was changed to the phenotype of macrophages by IV-C-sup.

In conclusion, the present study has shown that IV-infected cultured human chorion cells undergoing apoptosis released a soluble factor, which was stable at 56 °C for 30 min and which could make monocytes differentiate to macrophages. Furthermore, the expression of SR-A mRNA was induced by factors derived from IV-infected chorion cells undergoing apoptosis. SR-A is implicated in the recognition and clearance of apoptotic cells (Peiser & Gordon, 2001), in addition to α,β3 integrin, thrombospondin, phosphatidylserine receptor, CD36 and α1D3 antigen (McCarthy & Evan, 1999). Therefore, IV-infected apoptotic cells may lead to recognition and clearance of self cells by mature macrophages with SR-A protein. A fibroblast-derived differentiation inducing factor (F-DIF), identical to interleukin (IL)-6, induces the differentiation of monocytic leukaemia cell lines including THP-1 to macrophages, and this activity is synergistically enhanced by respective combination with tumour necrosis factor (TNF)-α, interferon (IFN)-γ, granulocyte macrophage colony-stimulating factor (GM-CSF), IL-1 and IL-4 (Noda et al., 1991; Takeda et al., 1988). Because IV infection was found to induce the mRNA expression of IL-1β, IL-6, TNF-α, IFN-γ and GM-CSF in cultured human chorion cells but not in amnion cells (unpublished data), some of these cytokines might be associated with the differentiation-inducing activity in the culture supernatant of IV-infected chorion cells undergoing apoptosis. Since it is known that TNF-α and IL-6 are heat-stable at 56 °C for 30 min (Ruff & Gifford, 1980; Fujibayashi & Matsuda, 1991), these cytokines become the default candidates for the proposed differentiation-inducing factor at present. We are now investigating whether or not cytokines released from IV-infected chorion cells act as putative factors in the differentiation-inducing activity.

References


Monocyte differentiation by apoptotic cells


Received 29 August 2001; Accepted 27 November 2001