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

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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 IV-infected chorion cells on the differentiation of monocytes to macrophages.

Fig. 1. Induction of chromosomal DNA fragmentation into oligonucleosomes by influenza virus (IV) infection. After IV infection at an m.o.i. of 40 and mock-infection, chorion and amnion cells were cultured for 0, 12, 24 and 48 h. Profiles of agarose gel electrophoresis of 10 µg DNA extracted from the cells are shown. Lane M shows 100 bp DNA Ladder (GibcoBRL) as DNA size markers. Sizes of DNA fragments in lane M are shown as base pairs at the right of panel.
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 (GibcoBRL), 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 at various times after mock and IV infection at an m.o.i. of 40 by centrifugation at 450 g for 5 min at 4 °C to remove cell debris. The supernatants were stored at −80 °C. They were thawed for use and incubated at 56 °C for 30 min to inactivate the virus and then centrifuged at 14000 g for 10 min at 4 °C to remove a very small amount of insoluble substance prior to use. Human monocytic leukaemia THP-1 cells differentiate to macrophages in the presence of 12-O-tetradecanoylphorbol 13-acetate (TPA), and the TPA-treated THP-1 cells adhere to a substrate and phagocytose yeasts and IgG-coated sheep red blood cells (Tsuchiya et al., 1982). THP-1 cells (Health Science Research Resources Bank, Osaka, Japan) were cultured in 95% RPMI 1640 (GibcoBRL), 5% heat-inactivated FBS, 100 units/ml penicillin G sodium and 100 µg/ml streptomycin sulfate. THP-1 cells (1 x 104 cells) were incubated for 4 days with 50% of the culture supernatants of chorion and amnion cells in 96-well plates (0.1 ml total volume). The adherence of THP-1 cells to the well was measured as described (Hirano et al., 1989). Briefly, wells were rinsed twice with PBS, then the remaining cells were fixed with 0.25% glutaraldehyde, stained with 0.2% crystal violet and lysed in 0.1% SDS. Absorbance of the cell lysate at 550 nm was measured with an MTP-32 microplate reader (Corona Electric, Ibaragi, Japan). Fig. 2(A, B) shows that 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
whereas amnion cells did not, even though the cells were
may have released a factor with the adhesion-inducing activity
activity was stable at 56 °C for 30 min. Therefore, chorion cells
activity. To further investigate these results, THP-1 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
infected 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
1 cells (1·25 x 10⁶ cells) were incubated for 4 days with 50% IV-
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 > 1 and the cytoplasm was
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 decreased to < 1, and
the cytoplasm was weakly basophilic with many vacuoles (Fig.
3B). Next, we examined the phagocytotic function of IV-C-
sup-treated THP-1 cells on coverslips. On day 4, the cells were
incubated with 1 x 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 inde-
pendent experiments. Statistical analysis using the t-test (n =
4) showed significant differences between incubations at 37 °C

Fig. 3. Morphology, phagocytotic function and change of phenotype of
THP-1 cells incubated with culture supernatant of influenza virus (IV)-
injected apoptotic cells. After 48 h infection at m.o.i. = 40, IV-infected
chorion cell-supernatants (IV-C-sup) were incubated at 56 °C for 30 min.
(A) and (B) show morphology of non-treated (A) and IV-C-sup-treated (B)
THP-1 cells (on day 4) stained with Giemsa’s solution (x 740). (C) and
(D) are phase-contrast views under a fluorescence microscope showing
IV-C-sup-treated THP-1 cells (on day 4) incubated with fluorescent latex
particles at 37 °C (C) or 0 °C (on ice) (D) for 4 h (x 740). Scale bar,
10 μm. (E) shows induction of class A scavenger receptor (SR-A) mRNA
in THP-1 cells by treatment with culture supernatant of IV-infected
apoptotic cells. After 48 h of infection at m.o.i.

expression of SR-A and glycerol-3-phosphate-dehydrogenase (G3PDH)
mRNAs in non-treated THP-1 cells and IV-C-sup-treated THP-1 cells was
analysed by RT-PCR assay as described in the text. Lanes 1 and 3; non-
treated THP-1 cells. Lanes 2 and 4; IV-C-sup-treated THP-1 cells on day
4. Lanes 1 and 2; SR-A mRNA-related PCR product (432 bp). Lanes 3
and 4; G3PDH mRNA-related PCR product (983 bp). Lane M; 100 bp
DNA Ladder (GibcoBRL) as DNA size markers. Size of DNA fragments in
lane M is shown as base pairs at the right of panel.
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 phagocyted 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-infected 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 and 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 phagocyted 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 phagocytic 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., 1995) 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 CCT CTC AT 3’ (residues 230–249, sense strand) and 5’ GGT ATT CTC TTG GAT TTT GCC 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 mRNA was amplified as a control in RNA samples. PCR products, glycerol-3-phosphate dehydrogenase (G3PDH) purity of the RNA sample considerably influence the yield of 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,β integrin, thrombospondin, phosphatidylserine receptor, CD36 and CD1D3 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 (D-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


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