Cytokine production by endothelial cells infected with human T cell lymphotropic virus type I

Hiroyuki Takashima, Katumi Eguchi, Atsushi Kawakami, Yojiro Kawabe, Kiyoshi Migita, Masahiro Sakai, Tomoki Origuchi, Shigenobu Nagataki

Abstract

Objective—To investigate the ability of human T cell lymphotropic virus type I (HTLV-I) to infect endothelial cells and induce cytokine production by these cells.

Methods—Human umbilical vein endothelial cells (HUVEC) were cocultured with HTLV-I infected T cell line (MT-2 cells) or uninfected T cell line (CEM cells).

Results—Following coculture with MT-2 cells, endothelial cells expressed HTLV-I specific core antigens. Endothelial cells cocultured with MT-2 cells produced significant amounts of several cytokines, including interleukin (IL)-1α, IL-6, granulocyte colony stimulating factor (G-CSF), and granulocyte/macrophage colony stimulating factor (GM-CSF), compared with endothelial cells cocultured with CEM cells. Coculturing of endothelial cells with MT-2 and CEM cells failed to produce detectable amounts of IL-1β and tumour necrosis factor α (TNF-α). The production of cytokines by endothelial cells cocultured with MT-2 cells was more persistent than that by endothelial cells cocultured with CEM cells after several passages. Furthermore, the production was blocked by cocultivation of endothelial cells and MT-2 cells using the Millicell system. Finally, after cocultivation of endothelial cells and MT-2 cells, endothelial cells positive for HTLV-I antigen were stained by anti-GM-CSF antibody.

Conclusions—HTLV-I can infect endothelial cells, resulting in their active production of several cytokines, such as IL-1α, IL-6, G-CSF, and GM-CSF. These findings strongly suggest that the excess production of these cytokines by HTLV-I infected endothelial cells may be involved in the pathogenesis of HTLV-I associated inflammatory diseases.


Human T cell lymphotropic virus type I (HTLV-I) is the causative agent of adult T cell leukaemia/lymphoma (ATLL)1,2 and HTLV-I associated myelopathy/tropical spastic paraparesis (HAM/TSP).3,4 Accumulating evidence suggests that HTLV-I may play a part in the development of various inflammatory disorders including polyarthritis,5 Sjögren’s syndrome,6uveitis,6 polymyositis,6 and alveolitis.6 Furthermore, transgenic mice expressing Tax protein, a transactivator encoded by HTLV-I, develop proliferative synovitis7 and exocrinopathy affecting lacrimal and salivary glands, features similar to those of Sjögren’s syndrome in man.8 Although the underlying pathogenic mechanisms of these HTLV-I inflammatory diseases are not fully understood, several cytokines produced by HTLV-I infected cells, including interleukin (IL)-1β, IL-2, IL-6,9 IL-6,10,11 and granulocyte/macrophage colony stimulating factor (GM-CSF),10,11 are thought to be involved in the development of these diseases. HTLV-I encodes a transactivating factor (Tax) which activates not only the transcription of the viral genome but also the expression of various of cellular genes.12 The target cellular genes of Tax identified up to now include IL-2, IL-3, IL4, tumour necrosis factor α (TNF-α), transforming growth factor β (TGF-β), IL-2 receptor α (IL-2Rα), GM-CSF, and c-fos.13 We reported previously that human synovial cells cocultivated with an HTLV-I producing T cell line expressed HTLV-I viral antigens.14 In the same study, we also showed that HTLV-I infected synovial cells produced GM-CSF, suggesting that these cells might trigger active proliferation of synovial cells.14 In the present study, we extend our investigation to the endothelial cells and show that HTLV-I infects human endothelial cells, resulting in the production of various cytokines.

Methods

PREPARATION OF HUMAN ENDOTHELIAL CELLS

Endothelial cells were obtained from human umbilical cord veins by an enzymatic digestion method described previously.15 Briefly, umbilical cord veins were infused with 100 µg ml⁻¹ of collagenase (Sigma) in Hank’s balanced salt solution (HBSS). After 10 min of incubation at room temperature, the solution containing the detached endothelial cells was flushed out with phosphate buffered saline solution (PBS). The cells were suspended in RPMI-1640 supplemented with 20% heat inactivated fetal calf serum (FCS; GIBCO), 25 µg ml⁻¹ of endothelial cell growth supplement (Sigma), 5 units ml⁻¹ of heparin, and antibiotics (100 units ml⁻¹ of penicillin and 100 µg ml⁻¹ of streptomycin). Cells were then cultured in Petri dishes (Falcon 3003, Becton Dickinson) precoated with fibronectin (Sigma). The identity of endothelial cells was established by their characteristic morphology—a cobblestone appearance under phase contrast microscopy—and by an enzyme labelled antibody technique using a mouse monoclonal anti-human von...
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The endothelial cell preparation was more than 99% reactive with this antibody, as shown by an immunohistological method (avidin-biotin-immunoperoxidase technique). The endothelial cells, which proliferated well under these conditions, were used at the second or third passage in our experiments.

Figure 1 Detection of HTLV-I gag protein by immunohistochemical staining. (A) Endothelial cells were cocultured with the HTLV-I infected T cell line (MT-2) for 3 d. After extensive washing, the cells were passaged and then cultured in a chamber slide for 4 d. Following incubation, the cells were treated with anti-human von Willebrand factor antibody and stained by avidin-biotin immunoperoxidase technique. Finally, the cells were stained with haematoxylin. Positive staining is evidenced by the presence of a brown deposit (× 400). Endothelial cells were stained positively. (B) The cells were reacted with GIN14 monoclonal antibody and stained by avidin-biotin immunoperoxidase technique. Note the GIN14 positive endothelial cell (arrow) and a negative endothelial cell (arrowhead).

as an HTLV-I infected T cell line. MT-2 cells contain proviral HTLV-I DNA and produce viral particles. These T cells were treated with 100 μg ml⁻¹ of mitomycin C for 1 h at 37°C.²⁸ After washing five times with PBS, they were cultured with an equal number of human umbilical vein endothelial cells (HUCVC) in RPMI-1640 containing 20% FCS. A total of 6 x 10⁶ of each of the cells was added to culture dishes (Falcon 3003; Becton Dickinson), or 6.5 x 10⁶ of each of the cells were added to 24-well flat bottomed culture plates (Costar). The culture medium was changed on the third day after coculture or passages, and the endothelial cells were passaged on the 10th, 20th, and 30th day. CEM cells were used as the uninfected T cell line. In some experiments, endothelial cells were cultured with mitomycin C treated T cell lines in a Millicell-HA (Millipore Products) equipped with a transparent 0.45 μm pore membrane. In this system, endothelial cells are cultured without contacting T cells, although both cells share the same medium.

IMMUNOHISTOCHEMICAL STAINING

Immunohistochemical staining was performed using the avidin-biotin-immunoperoxidase method developed in our laboratory,²⁷ and by the indirect immunofluorescence method. First, the cells were cultured in eight-chamber glass slides (eight-well Lab-Tek; Nunc). Then 1.5 x 10⁶ cells were incubated in each well for 3 d, after which the cells were fixed with cold acetone (4°C) for 10 min. For immunohistochemical staining, endogenous peroxidase was inactivated by placing the slide into 0.3% H₂O₂ in HBSS for 10 min at room temperature. The cells were then incubated with a diluted solution of the primary antihuman monoclonal antibodies, anti-CD3 (Coulter Immunology), anti-human von Willebrand factor, and GIN14 (monoclonal mouse antibody reactive with the HTLV-I core proteins p19 and p28) (Fujirebio),²⁸ using the Histofine staining reagents kit (Nichirei). The sections were treated with biotinylated goat anti-mouse IgG for 12 min. After careful washing, the treated sections were incubated with streptavidin-peroxidase conjugates, after which they were incubated in 0.5 mg ml⁻¹ of diaminobenzidine (Sigma) prepared in 5 mM Tris buffer solution, pH 7.6, containing 0.03% H₂O₂, then stained with haematoxylin. The control sections were treated routinely with a mouse IgG (Coulter Immunology) instead of a specific monoclonal antibody. The cells were analysed by a double immunofluorescence method as described in detail previously.²⁹ Briefly, the cells were exposed to a fluorescein isothiocyanate conjugated polyclonal rabbit anti-human GM-CSF (Genzyme) and a rhodamine conjugated GIN14 monoclonal antibody for 1 h at 4°C. A rabbit antibody against human α fetoprotein (Dako) and mouse IgG were used for control antibodies. After washing each section, positive cells were identified by fluorescence microscopy (Micro System, Zeiss LSM).
passage). The results of the cytokine production by endothelial cells cocultured with MT-2 or CEM cells are shown in Figure 2. Human umbilical vein endothelial cells (HUVEC) were cocultured with mitomycin C-treated MT-2 or CEM cells. After 3 d of cocultivation, the supernatants were harvested and used for assay. IL-1α and IL-1β, TNF-α, IL-6, G-CSF, and GM-CSF were measured by an immunoenzymatic assay. Briefly, plates precoated with monoclonal antibody to each cytokine were incubated with samples, incubated further with polyclonal rabbit anti-cytokine antibody, and then reacted with goat anti-rabbit Ig conjugated to horseradish peroxidase. Between each step, excess reactants were removed by washing three times with 0.01 M phosphate buffer (pH 7.4) containing 1% bovine serum albumin. Absorbance of a chromogenic compound produced by the addition of an enzyme substrate was measured at 490 nm. The sensitivity of cytokine determination was 10 pg ml⁻¹ for IL-1α and 20 pg ml⁻¹ for IL-1β, TNF-α, IL-6, G-CSF, and GM-CSF.

**STATISTICAL ANALYSIS**
The statistical significance of any difference was examined using the Student t test.

**Results**

**DETECTION OF HTLV-I ANTIGENS IN ENDOTHELIAL CELLS COCULTURED WITH HTLV-I INFECTED CELLS**

HUVEC were cocultured with either MT-2 or CEM cells. After cocultivation for 3 d, the supernatant was obtained from each dish, and HUVEC were washed extensively and exchanged with fresh medium. After the cells were cultured for 7 d, they were washed thoroughly and then passaged. After several passages, the HUVEC were harvested for assessment by immunohistochemical staining for expressing HTLV-I viral antigens. While specimens after the second passage of HUVEC contained less than 0.5% CD3-positive MT-2 cells, the HUVEC of the third passage and beyond contained less than 0.1% CD3-positive MT-2 cells, and more than 99% of the cells reacted with anti-von Willebrand factor monoclonal antibody, observed by immunoenzymetric methods (fig 1A). All cells had cobblestone appearance morphologically, confirming that they were endothelial cells. As shown in fig 1B, HUVEC cocultured with MT-2 cells reacted strongly with GIN14. The frequency of endothelial cells positive for GIN14 was 2-5/100. In contrast, endothelial cells cocultured with CEM cells were never found to react with GIN14, although more than 10 000 cells were carefully examined (data not shown).

**CYTOKINE PRODUCTION BY ENDOTHELIAL CELLS COCULTURED WITH MT-2 OR CEM CELLS**

It is now clear that HTLV-I infected T cells are capable of producing various cytokines through the transactivation of cytokine genes by the Tax protein.²⁻¹⁰⁻²¹⁻²²⁻²³ Therefore we investigated the production of cytokine by endothelial cells cocultured with MT2 or CEM cells. Figure 2 summarises the results of a representative experiment on cytokine production by endothelial cells. Small amounts of cytokines, such as IL-1α, IL-6, G-CSF, and GM-CSF, were detected in the culture supernatant on the third day after coculture with or without mitomycin C treated CEM cells. Endothelial cells cocultured with mitomycin C-treated MT-2 cells released considerable amounts of IL-1α, IL-6, G-CSF, and
GM-CSF (fig 2 upper panel). Neither IL-1β nor TNF-α were detected in the culture supernatant of endothelial cells cocultured with or without MT-2 cells. We also measured the level of different cytokines 30 d after coculture. As shown in the lower panel of fig 2, only endothelial cells cocultured with MT-2 cells released considerable amounts of IL-1α, IL-6, G-CSF, and GM-CSF. Cytokines were not detected in the media of mitomycin C treated MT-2 and CEM cells. The same experiments were repeated as shown in the table. Endothelial cells cocultured with mitomycin C treated MT-2 cells released IL-1α, IL-6, G-CSF, and GM-CSF but not IL-1β and TNF-α. However, there was a wide variability between experiments in the level of each cytokine released by endothelial cells cocultured with MT-2 cells. It was noted that the release of cytokines was diminished when endothelial cells were passaged several times.

**COCULTURE OF ENDOTHELIAL CELLS AND T CELL LINES USING MILLICELL SYSTEM**

In the next series of experiments we cocultured cells using the Millicell system equipped with a transparent 0.45 μm pore membrane. This setup allowed examination of the effect of cytokines released from T cell lines. As shown in fig 3, endothelial cells cocultured with HTLV-I infected T cells in the absence of Millicell released considerable amounts of G-CSF and GM-CSF. In contrast, cytokines were not detected in supernatants of endothelial cells with MT-2 cells using the Millicell system and no GIN14 staining on endothelial cells was detected in these cells (data not shown). Similar results were obtained from three other experiments. No cytokines were detected in the culture medium containing endothelial cells and CEM cells, with or without the use of the Millicell system. These results indicate that the production of cytokines by endothelial cells requires a contact of these cells with HTLV-I infected T cells.

**PRODUCTION OF GM-CSF BY HTLV-I INFECTED ENDOTHELIAL CELLS**

Finally, cocultured endothelial cells were stained by the double immunofluorescence method. Figure 4 shows that endothelial cells reacting with rhodamine conjugated GIN14 were also stained by fluorescein isothiocyanate conjugated anti-GM-CSF polyclonal antibodies. The percentage of single GIN14 positive cells was 2.7(0.3)%, of single GM-CSF positive cells, 3.5(0.5)%, and of both GIN14 and GM-CSF positive cells, 2.6(0.4)% from five individual experiments, suggesting that GM-CSF was mainly produced by HTLV-I infected endothelial cells. These findings were confirmed up to the fourth passage, and no cultures contained anti-CD3-positive MT-2 cells.

**Discussion**

This study showed that the HTLV-I genome can be transmitted into endothelial cells from an HTLV-I producing T cell line. Immunohistochemically, the monoclonal antibody GIN14 detected HTLV-I gag proteins within endothelial cells cocultivated with mitomycin C treated MT-2 cells. In this method, endothelial cells

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**Cytokine production by endothelial cells cocultured with CEM cells or MT-2 cells.** HUVECs were cocultured with mitomycin C treated CEM cells or MT-2 cells. After 3 d of cocultivation, we measured the level of cytokines in the supernatant. The cultured endothelial cells were washed extensively and passaged on the 7th day and 17th day. The supernatant on the 10th and 20th day was used to measure the level of cytokines. Values are pg ml⁻¹.

<table>
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**Figure 4 Detection of HTLV-I gag protein and GM-CSF by double immunofluorescence staining.** Endothelial cells were cocultured with MT-2 cells for 3 d. After incubation, the cells were passaged three times. The cells on the chamber slide were stained with rhodamine conjugated GIN14 monoclonal antibody and fluorescein isothiocyanate (FITC) conjugated anti-GM-CSF polyclonal antibody. Endothelial cells positive for GIN14 (reactive with HTLV-I gag proteins p19 and p28) were strongly stained with FITC conjugated anti-GM-CSF antibody (×400). Yellow colour denotes double positive staining.
could easily be distinguished morphologically from MT-2 cells. The efficiency of infection by this method was relatively low since only one to five endothelial cells were infected in every 100 cells, as shown by immunohistochemical examination. However, the rate of infection of endothelial cells was higher than that reported for synovial cells.42

HTLV-I has tropism to CD4+ cells, but non-lymphoid cells—such as vascular endothelial cells, fibroblasts,20-21 synovial cells,21,22 glial cells,26-29 dendritic cells,30 and monocytes31-36—are infected by HTLV-I when they were cocultivated in vitro with HTLV-I producing T cells. Vascular endothelial cells infected by HTLV-I appear as multinucleated syncytial giant cells and show positive immunofluorescence for specific viral antigens.37 In the present study, we were able to detect the viral antigen, but not syncytia formation. The discrepancy between our results and those of previous studies with regard to syncytia formation may be due to the use of HTLV-I producing cells in our study, while C91PL cells were used by the other investigators as the HTLV-I producing cells.30,31

Endothelial cells cocultured with MT-2 cells produced significant amounts of IL-1α, IL-6, G-CSF, and GM-CSF, but not IL-1β and TNF-α. HTLV-I transformed T cells have been reported to produce a multitude of cytokines, including IL-1α,2-4 IL-2,14-15 IL-3,10 IL-6,16-19 TNF-α,39 IFN-γ40 GM-CSF,40 transforming growth factor β (TGF-β),41 and leukemia inhibitory factor.42 HTLV-I infected non-lymphoid cells have also been reported to produce various types of cytokines. In this regard, we showed previously that synovial cells infected by HTLV-I produce significant amounts of GM-CSF.43 However, the synovial cell clones integrated by HTLV-I Tax gene were able to express IL-1β and IL-6 mRNA.43 Furthermore, the glial cells that carry the HTLV-I Tax gene can produce IL-1, IL-6, GM-CSF, and TGF-β.44 Infection with HTLV-I enhances the production of IL-6 and TNF-α in human microglial cells and human monocytes.45 These findings suggest that the cytokine profile differs among various HTLV-I infected target cells.

It is possible that MT-2 cells may produce various amounts of inflammatory cytokines and, in turn, these cytokines stimulate the endothelial cells to produce more cytokines. However, the production of cytokines by endothelial cells cocultured with MT-2 cells in the Millicell system was significantly lower than that observed in standard cultures. The finding suggests that cell to cell contact with HTLV-I infected T cells is necessary for cytokine production by endothelial cells. It is known that HTLV-I genome transmission occurs only through cell to cell contact, and not through free materials,46 and we showed that no GIN14 staining was detected on endothelial cells cocultured with MT-2 cells using the Millicell system. Therefore active cytokine production by endothelial cells may relate to HTLV-I infection. To confirm our speculation that HTLV-I infection of endothelial cells caused the active production of cytokines by these cells, the persistence of such production was examined. Our results showed that endothelial cells actively produced cytokines even after several passages. Since we excluded the effect of mitomycin C treated MT-2 cells and their products in this experiment, it appears, therefore, that HTLV-I infection of endothelial cells may play a role in the enhanced production of cytokines.

Transcription of GM-CSF is also regulated by Tax protein in HTLV-I infected T cells.45 The expression of the GM-CSF gene is induced through the action of specific cellular transcription factors that can interact with Tax protein.44,45 These findings suggest a possible operation by the Tax protein of GE-CSF transcription in endothelial cells in a manner similar to that in HTLV-I infected T cells.

To our knowledge, HTLV-I viral antigens have not been identified at endothelial cells in inflammatory tissues due to the extremely low copy number of HTLV-I DNA. However, recent studies of necropsied HAM/TSP cases have shown chronic inflammatory changes in the central nervous system, particularly in the spinal cord. These studies showed perivascular infiltration of mononuclear cells in both the grey and the white matter of the spinal cord, together with changes in vascular walls, including fibrous thickening of the adventitia and hyalinised changes in small vessels.46-47 We found previously that adherence of T cells to endothelial cells increases significantly in HAM/TSP patients compared with seronegative control subjects and anti-HTLV-I antibody carriers. Based on these findings, we suggest that infection of endothelial cells by HTLV-I may serve as a reservoir to further propagate the virus or may represent the initial step of a process that alters the production of IL-1α, IL-6, G-CSF, and GM-CSF. These cytokines may enhance the expression of adhesion molecule on endothelial cells and stimulate endothelial cells proliferation, resulting in mononuclear cell infiltration into the inflammatory lesion.48

In summary, endothelial cells may be infected by HTLV-I. This process promotes the production of inflammatory cytokines by the infected cells. Such process may be involved in the pathogenesis of HTLV-I associated inflammatory diseases, such as HAM/TSP, Sjögren’s syndrome, and polyarthritis.

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