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Dengue virus infection of human skin fibroblasts in vitro production of IFN-β, IL-6 and GM-CSF

I. Kurane, J. Janus, and **F. A. Ennis**

Division of Infectious Diseases and Immunology, Department of Medicine, University of Massachusetts Medical Center, Worcester, Massachusetts, U.S.A.

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Summary. Dengue virus is transmitted to humans by the bite of infected mosquitos. In our efforts to understand the pathogenesis of dengue virus infection, we examined whether skin fibroblasts can be infected in vitro with dengue viruses. Fibroblasts could be infected with dengue viruses, yellow fever virus and West Nile virus. Dengue virus antigen-positive cells were detected as early as 4 h and the percentage of dengue virus antigen-positive cells reached maximum levels by 24 h after infection. High titers of infectious dengue virus were also detected in the culture supernatants at 20 h after infection. Dengue virus-infected fibroblasts produced interferon-β (IFN-β), and the IFN-β protected uninfected fibroblasts from dengue virus infection. Dengue virus-infected fibroblasts also produced interleukin 6 (IL-6) and granulocyte macrophage colony stimulation factor (GM-CSF).

These results suggest that skin fibroblasts may be one of the cell types which first support dengue virus and other flavivirus infections in vivo after introduction by the bite of infected mosquito, and that production of IFN-β, IL-6, and GM-CSF by these virus-infected fibroblasts may be important host immune responses to control flavivirus infections.

Introduction

Dengue virus infections are serious health problems in many areas of the world; Southeast and South Asia, Central and South America, the Carribean, and Africa [9]. Dengue viruses belong to the family *Flaviviridae*, genus *Flavivirus*, and are transmitted to humans by mosquitos. Dengue virus infections cause two forms of diseases; dengue fever (DF) and dengue haemorrhagic fever (DHF)/dengue shock syndrome (DSS). DF is a self-limited febrile disease. In some situations patients infected with dengue virus develop life-threatening complications such as plasma leakage, haemorrhagic manifestation and shock,

which are called DHF/DSS [8]. The pathogenesis of DF and DHF/DSS has not been elucidated. It has been reported that monocytes are the cells which predominantly support dengue virus infections [10] and dengue virus antigen-positive monocytic cells have been detected in patients with DHF/DSS [1, 2]. However, the cells which are initially infected and propagate dengue virus after a mosquito bite of humans are unknown, although growth of virus in local tissues and in local lymph nodes has been suggested [18].

In this study, we attempt to determine whether primary cultures of human skin fibroblasts can be infected with dengue and other flaviviruses. Fibroblasts are one of the indigenous cells of the dermis [13]; therefore, it is possible that they are first infected with dengue and other flaviviruses shortly after the mosquito bite occurs. It is also known that human fibroblasts can produce cytokines such as interferon-β (IFN-β), interleukin 6 (IL-6) [7], granulocyte macrophage colony stimulating factor (GM-CSF) [14] and interleukin 1 (IL-1) [19], which may effect the virus infection and the immune responses.

Primary cultures of human skin fibroblasts can be infected with flaviviruses and produce high titers of infectious virus. IFN-β is produced by dengue virus-infected fibroblasts and IFN-β protects other uninfected fibroblasts from dengue virus infection. Dengue virus-infected fibroblasts also produce IL-6 and GM-CSF. However, IL-6 and GM-CSF have no effect on dengue virus infection of fibroblasts determined by immunofluorescence staining. These results suggest that fibroblasts may be important for flavivirus propagation at the site of entry. Production of IFN-β, IL-6 and GM-CSF by dengue virus-infected fibroblasts may be important immune responses which control dengue virus infections.

Methods

Human skin fibroblasts

Skin fibroblasts cultures were established from a 3 mm biopsy of skin obtained from the forearm of a healthy adult. A piece of skin was minced and cultured in MEM containing 10% fetal calf serum (FCS) for 2–3weeks. Cells were passed several times in 75 cm^2 flasks before aliquots were cryopreserved. These cells were later thawed and maintained in MEM containing 10% FCS as described previously [3].

Viruses and titration of dengue virus

Dengue virus type 1, Hawaii strain; dengue virus type 2, New Guinea C strain; dengue virus type 3, CH53489 strain and 904 strain; dengue virus type 4, 814669 and Thai 286 strain; yellow fever virus (YFV), 17D strain; and West Nile virus (WNV), E101 strain were used. Dengue virus type 1, Hawaii strain and dengue virus type 2, New Guinea C strain were supplied by Dr. Walter Brandt of the Walter Reed Army Institue of Research, Washington, D.C., U.S.A. Dengue virus type 3, CH53489 strain was supplied by Dr. Bruce L. Innis of Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand. Dengue virus type 3, 904 strain, and dengue virus type 4, Thai 286 strain were supplied by Dr. Srisakul Kliks of University of California, San Francisco, California, U.S.A. Dengue virus type 4, 814669 strain was supplied by Dr. Kenneth Eckels of the Walter Reed Army Institute of Research, Washington, D.C., U.S.A. Yellow fever virus, 17D strain was supplied

by Dr. Jacob Schlesinger of University of Rochester School of Medicine and Dentistry, Rochester, New York, U.S.A. West Nile virus, E101 strain was supplied by Dr. Margo A. Brinton of Georgia State University, Atlanta, Georgia, U.S.A.

Viruses were propagated in mosquito cells (C6/36) as previously described [17]. The amount of infectious virus was determined by plaque assays in Vero cells as previously described [16].

Infection of fibroblasts with viruses

10^5 fibroblasts were cultured in 24 well flat bottom plates in MEM containing 10% FCS for 2 days. The number of cells were about 2–4 × 10^5 at the time of infection. Cells were washed 2 times with MEM containing 2% fetal calf serum (FCS), and incubated with 0.1 ml of viruses at 37 °C for 2 h. The multiplicity of infection (m.o.i.) was 5–10 p.f.u. per cell as determined by p.f.u. on Vero cells. Fibroblasts were washed twice and cultured in MEM containing 2% FCS. Culture supernatants were collected at various times, and virus titers were determined. Cells were stained for the presence of viral antigens by indirect immunofluorescence (IF) using hyperimmune mouse ascitis fluids to dengue-2 virus or a flavivirus-crossreactive monoclonal antibody, 4G2 [11] as previously described [17].

Reagents

Antisera to IFN-α and IFN-β were purchased from Enzo Biochemicals, New York, NY, U.S.A. Antiserum to IFN-γ was purchased from Interferon Sciences, New Brunswick, NJ, U.S.A. Recombinant IL-6 and GM-CSF were purchased from Genzyme, Cambridge, MA, U.S.A.

Interferon assay

Interferon was assessed by a cytopathic effect-reduction assay as previously described [15]. Two fold serial dilutions of supernatant fluids obtained from dengue virus-infected fibroblasts were incubated on human amnion cells (WISH cells) for 20 h at 37 °C and then challenged with vesicular stomatitis virus. International interferon standards were included in each assay, and the titers were read after 24 h at 37 °C.

Assays for IL-1α, IL-1β, IL-6, GM-CSF, IFN-γ and TNFα

Levels of IL-1α were measured using a commercial ELISA from Endogen, Boston, MA, U.S.A. Levels of IL-1β was measured using a commercial ELISA from Cistron, Pine Brook, NJ, U.S.A. Levels of IL-6 and GM-CSF were measured using commercial ELISA from Genzyme, Boston, MA, U.S.A. Levels of IFN-γ and TNFα were measured using commercial radioimmunoassay (RIA) purchased from Centocor Diagnostics, Malvern, PA, U.S.A.

Results

Infection of human fibroblasts with dengue-2 virus

Human skin fibroblasts were infected with dengue-2 virus, and the percentage of dengue virus antigen-positive cells and the infectious virus titers in the culture supernatant fluids were examined. Figure 1 shows immunofluorescence staining of dengue-2 virus-infected fibroblasts. Dengue virus antigen-positive cells were detected as early as 4 h after infection and the percentage of dengue virus antigen-positive cells reached maximum levels by 24 h after infection (Fig. 2). High titers

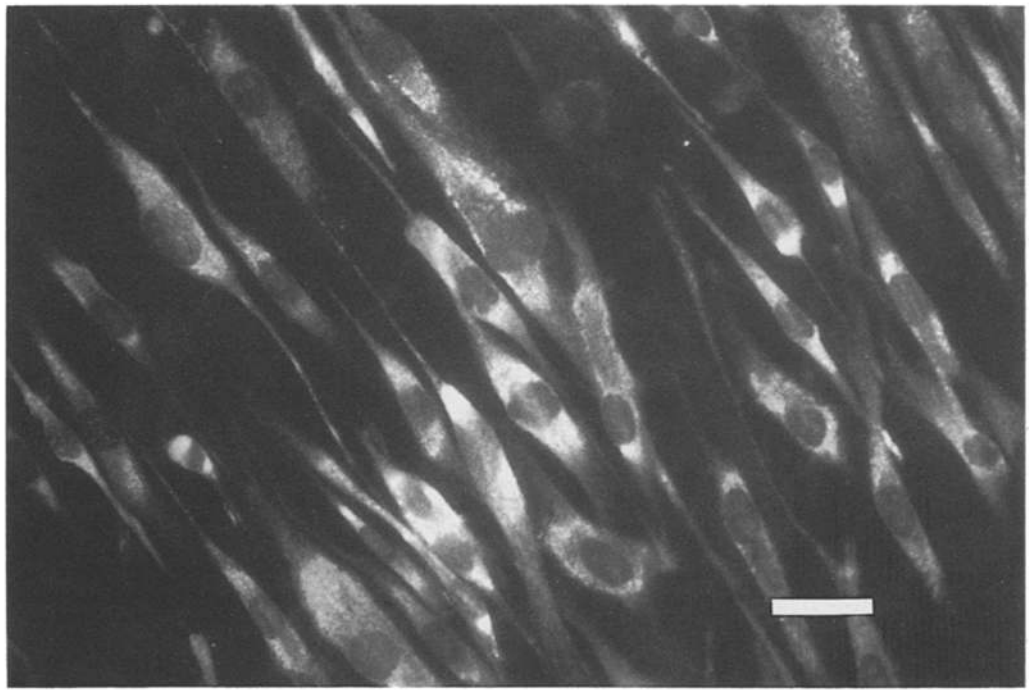

Fig. 1. Immunofluorescence staining of dengue-2 virus-infected human skin fibroblasts. Fibroblasts were infected with dengue-2 virus at an m.o.i. of 5 pfu/cell. Cells were stained for cytoplasmic dengue virus-antigens at 24 h after infection. Bar: 10 μm

of infectious dengue virus were detected at 24 and 44 h after infection. These results indicate that fibroblasts can be infected with dengue virus.

Production of IFN-β by dengue-2 virus-infected fibroblasts

The supernatant fluids of dengue virus-infected fibroblasts were examined for interferon (IFN) activity. High levels of IFN activity were detected at 24–48 h after infection (Table 1). IFN was characterized as IFN-β, because specific antibody to IFN-β neutralized the activity, but antibodies to IFN-α or IFN-γ did not.

Protection of dengue-2 virus infection of fibroblasts by IFN-β

We examined whether IFN-β produced by dengue virus-infected fibroblasts protects other fibroblasts from dengue virus infection. Supernatant fluids of dengue virus-infected fibroblasts were exposed to ultra violet light at 4 °C for 20 min to inactivate dengue virus and used in the next experiment. Pretreatment of fibroblasts with IFN-β or with the culture supernatants of dengue virus-infected fibroblasts prevented dengue virus infections (Table 2). Addition of anti-IFN-β antibody abrogated the ability of culture fluids to protect other fibroblasts from infection. These results indicate that IFN-β is included in protection of fibroblasts from dengue virus infection.

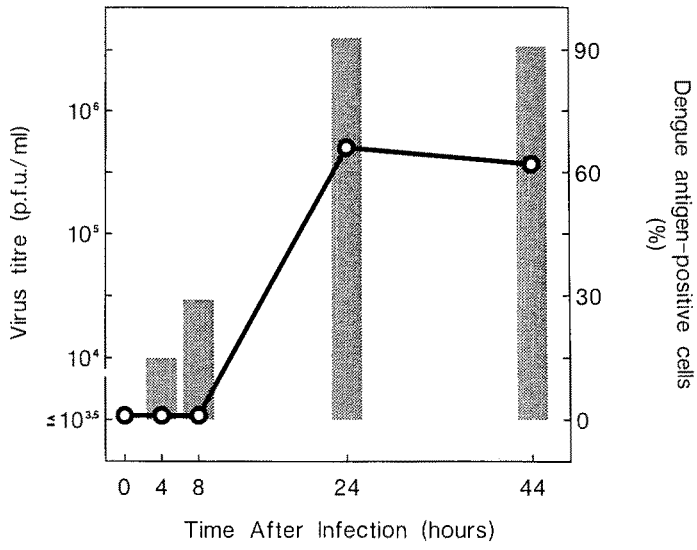

Fig. 2. Time course of the appearance of infectious dengue virus in the culture supernatant fluid and cytoplasmic dengue virus antigens in infected fibroblasts. Fibroblasts were infected with dengue-2 virus at an m.o.i. of 10 pfu/cell, and cultured at 2×10^5/ml in MEM containing 2% FCS. Titers of infectious dengue virus in the culture fluids and the percentage of dengue virus antigen-positive cells were examined at various times after infection. ○ Titers of infectious dengue virus in the culture fluids. ■ The percentage of dengue virus antigen-positive cells

Table 1. IFN production by dengue-2 virus-infected fibroblasts*

	Time after infection (h)	IFN (U/ml)	% Dengue Ag-positive cells
Exp. 1	0	< 25	0
	4	< 25	15
	24	800	93
	44	1200	91
Exp. 2	0	< 25	0
	24	200	55
	48	2400	50

* Fibroblasts were infected with dengue-2 virus at an m.o.i. of 10 pfu/cell in experiment 1 and 5 pfu/cel in experiment 2, and were cultured at 2×10^5/ml. IFN activity in the culture fluids and the percentage of dengue antigen-positive cells were assessed at various times after infection

Production of IL-6 and GM-CSF by dengue-2 virus-infected fibroblasts

We examined whether dengue virus infection induces IL-6, GM-CSF, IL-1α, IL-1β, TNFα and IFN-γ. High levels of IL-6 and GM-CSF were detected in the supernatant fluids of dengue virus-infected fibroblasts, and IL-6 and GM-CSF were detected at very low levels in the culture fluids of uninfected fibroblasts

Table 2. Protection of dengue-2 virus infection of fibroblasts by IFN-β[a]

Treatment of fibroblasts	Anti-IFN-β antibody	% Dengue Ag-positive cells
Culture supernatant of dengue-2 virus-infected fibroblasts	−	1
	+	53
IFN-β (100 U/ml)	−	1
	+	46
None (medium)	−	47
	+	49

[a] Fibroblasts were incubated with IFN-β or with UV-treated supernatant fluids of dengue-2 virus-infected fibroblasts, which contained 100 U/ml of IFN activity, for 24 h in the presence (+) or absence (−) of anti-IFN-β antibody. Fibroblasts were then infected with dengue-2 virus at an m.o.i. of 5 pfu/cell and the percentage of dengue antigen-positive cells was assessed using indirect immunofluorescence

Table 3. Detection of IL-6 and GM-CSF in the culture fluids of dengue-2 virus-infected fibroblasts[a]

	Dengue-2-infected		Uninfected	
	24 h	48 h	24 h	48 h
IL-6 (ng/ml)	20.0	31.6	0.7	0.7
GM-CSF (pg/ml)	2.7	10.5	0.2	0.4
IL-1α (pg/ml	124	116	102	104
IL-1β (pg/ml)	<2	<2	<2	<2

[a] Fibroblasts were infected with dengue-2 virus at an m.o.i. of 5 pfu/cell and cultured at 1.5×10^5/ml for 24 and 48 h. Culture supernatant fluids were collected and examined for IL-6, GM-CSF, IL-1α and IL-1β. Percentage of dengue virus-antigen positive cells determined by IF staining was 53% at 24 h after infection

(Table 3). Similar levels of IL-1α were detected in the culture fluids of infected and uninfected fibroblasts. IL-1β, TNFα, or IFN-γ were not detected in the culture fluids of infected or uninfected fibroblasts. These results indicate that dengue virus-infected fibroblasts produce IL-6 and GM-CSF.

The effects of IL-6 and GM-CSF on dengue virus infection of fibroblasts were examined. Fibroblasts were incubated with recombinant human IL-6 at 1 to 10000 U/ml, recombinant human GM-CSF at 1 to 2500 U/ml or mixtures of IL-6 and GM-CSF for 48 h, and infected with dengue-2 virus. The percentage of dengue virus antigen-positive cells were similar between untreated fibroblasts and those pretreated with IL-6, GM-CSF, or mixtures of IL-6 and GM-CSF (data not presented). These results indicate that IL-6 and GM-CSF do not have antiviral or augmenting effects on dengue virus infection of human skin fibroblasts.

Table 4. Infection of human skin fibroblasts with dengue, yellow fever, and West Nile viruses[a]

Viruses	Strain	% Antigen-positive cells
Dengue-1	Hawaii	40
Dengue-2	New Guinea C	78
Dengue-3	CH53489	53
Dengue-3	904	32
Dengue-4	814669	17
Dengue-4	Thai 286	55
Yellow fever virus	17D	17
West Nile virus	E101	59

[a] Human skin fibroblasts were infected with each virus at an m.o.i. of 10 pfu/cell. The percentage of antigen-positive cells was examined 24 h after infection by IF staining using a flavivirus-crossreactive monoclonal antibody 4G2

Infection of fibroblasts with other dengue viruses, YFV and WNV

To determine whether infection of human skin fibroblasts is a common feature of flaviviruses, we used 7 strains of other mosquito-borne flaviviruses, including dengue-1, dengue-3, dengue-4, YFV and WNV in addition to dengue-2 virus, New Guinea C strain (Table 4). Fibroblasts were infected with viruses at an m.o.i. of 10 p.f.u./cell, and the percentage of antigen-positive cells were examined by IF staining using a flavivirus-crossreactive monoclonal antibody 4G2. All 7 viruses examined infected fibroblasts, although the percentage of antigen-positive cells varied depending on the virus strains. These results suggest that most, if not all, strains of mosquito-borne flaviviruses can infect primary cultures of human skin fibroblasts.

Discussion

In this report we have shown that primary cultures of human skin fibroblasts can be infected with dengue viruses, YFV and WNV and produce high levels of infectious virus in the culture supernatant fluids. Dengue viruses are transmitted to humans by mosquito bites. It has been suggested that dengue viruses replicate in local tissues and in regional lymph nodes, and that viruses are carried via lymphatics to the thoracic duct and into the blood stream [18]. It has been reported that monocytic cells are the cells which mainly support dengue virus infection in vitro [10] and dengue virus antigen-positive monocytic cells have been detected in vivo in patients with DHF/DSS [1, 2]. However, it is not known whether monocytes are the cells which first support dengue virus infection after the mosquito bite. The observations that primary human skin fibroblast cultures can be infected with dengue virus in vitro and produce high levels of infectious virus suggest that skin fibroblasts may be one of the cells which support dengue virus in local tissues after mosquito bite.

Dengue virus-infected fibroblasts produce high levels of IFN-β. Culture supernatant fluids of dengue virus-infected fibroblasts which contain IFN-β protect other uninfected fibroblasts from dengue virus infection. Therefore, production of IFN-β by dengue virus-infected fibroblasts may be important to limit spread of dengue viruses in the microenvironment of the skin. We also detected high levels of IL-6 and GM-CSF in the culture fluids of dengue virus-infected fibroblasts. IL-6 is known to have multiple modulatory functions on T cell and B cell responses [21]. IL-6 has been reported to have weak antiviral activity [22]. IL-6 induces B cell differentiation and stimulates IgG secretion [12]. IL-6 also activates T cells to produce IL-2 [5], and differentiates cytotoxic T lymphocytes [20]. Production of IL-6 would probably stimulate immune responses to dengue viruses during the early stages of infection. We have also detected GM-CSF activities in the culture fluids of dengue virus-infected fibroblasts. GM-CSF stimulates proliferation of the colonies of granulocytes and monocytes [4] and activates the functions of monocytes [6]. Although recombinant IL-6 and GM-CSF had no effects on dengue-2 virus infection of fibroblasts in vitro, we intend to define in more detail the effects of IL-6 and GM-CSF on immune responses to dengue viruses in future experiments.

We have already reported that dengue virus-infected monocytes and DR^+ lymphocytes which contacted dengue virus-infected monocytes produce high levels of IFN-α, and the produced IFN-α prevented other monocytes from becoming infected with dengue virus [15, 16]. These results along with the observations reported in this paper suggest that production of IFN-β and IFN-α may be an important protective mechanism against dengue virus infections.

Because infection of cells in culture is often different from in vivo, it is important to examine in vivo whether dengue virus antigen-positive fibroplasts are observed at the site of dengue virus infection. Dengue virus antigen-positive cells were detected with immunoperoxidase staining in the epidermis and dermis of monkeys infected by the bite of dengue virus-infected mosquitos. Some of the infected cells in the dermis were fibroblast like (A. King and B.L. Innis, pers. comm.). Therefore, our observations in vitro seem to be consistent with the results of in vivo infection experiments. Furthermore, the possibility that other types of cells in the dermis support dengue virus infection in vivo remains to be determined. Understanding the early events of dengue virus infections will be important to elucidate the pathogenesis of dengue virus infections.

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Authors' address: Dr. F. A. Ennis, Division of Infectious Diseases and Immunology, Department of Medicine, University of Massachusetts Medical Center, Worcester, MA 01655, U.S.A.

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