Brief Definitive Report

REGULATION OF THE ACUTE PHASE AND
IMMUNE RESPONSES IN VIRAL DISEASE

Enhanced Expression of the β2-interferon/Hepatocyte-stimulating

Factor/Interleukin 6 Gene in Virus-infected Human Fibroblasts

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The human β2-interferon/hepatocyte-stimulating factor/interleukin 6 (IFN-β2/IL-6) gene on chromosome 7p15-p21 codes for a set of at least five secretory glycoproteins of molecular mass in the range from 23–30 kD that mediate acute phase plasma protein secretion by hepatocytes and that regulate several different aspects of the host immune response (1–5). In addition to mediating many of the alterations in acute phase plasma protein secretion by hepatocytes, proteins derived from the IFN-β2/IL-6 gene elicit an antiviral state, enhance proliferation of murine hybridoma cells, enhance proliferation of human B cells recently transformed by EBV, enhance secretion of Igs by human B cell lines, contribute to the proliferation of murine and human thymocytes and T cells, enhance colony formation by hematopoietic progenitor cells, and mediate a pyrogenic response to tissue damage (reviewed in 4–9). This multifunctional cytokine has emerged as a major component in the host response to noxious stimuli, such as bacterial infections and tissue damage, such as that after severe burns (reviewed in 4, 9). It is secreted by fibroblasts, monocytes, keratinocytes, endothelial cells, and various other epithelial and mesenchymal cells (4, 6, 8, 10, 11). Typically, the expression of IFN-β2/IL-6 proteins is enhanced in response to other cytokines such as IL-1, TNF, lymphotoxin, platelet-derived growth factor and other IFN, and to poly(I)·poly(C)(reviewed in 5, 12). Bacterial products such as endotoxin also strongly enhance expression and secretion of IFN-β2/IL-6 by monocytes (4, 6, 8, 10) and fibroblasts (4, 13). Thus, the regulation of expression of the IFN-β2/IL-6 gene is clearly well-adapted to participation of this cytokine in the host response to tissue damage. In the present article, we report that the ubiquitous stromal fibroblast secretes this multifunctional cytokine in response to acute viral infections. These data suggest that IFN-β2/IL-6 is likely to be a component in the host response to viral pathogens.

Materials and Methods

The human diploid foreskin fibroblast strain FS-4 was used throughout; procedures for its growth in cell culture, for IFN-β_2/IL-6 mRNA induction, for RNA blot-hybridization analyses, and for ^{35}S-methionine-labeling of secreted IFN-β_2/IL-6 proteins have been described earlier (4, 12, 13). The human hepatoma cell line Hep3B clone 2 was obtained from the American Type Culture Collection, Rockville, MD (No. HB8064). Hep3B2 cells were grown in T-75 flasks in Eagle's MEM supplemented with 10% (vol/vol) heat-inactivated FCS (Gibco, Grand Island, NY), 0.1 mM pyruvate (Gibco), and "MEM nonessential amino acids" (Gibco). The procedure for assaying hepatocyte-stimulating factor activity (increased synthesis and secretion of α_1-antichymotrypsin) has been described elsewhere (4). Medium from virus-infected FS-4 cell cultures was centrifuged at 40,000 rpm for 90 min in an SW50.1 rotor (Beckman Instruments, Inc., Palo Alto, CA) and then γ-irradiated (90,000 rad) before addition to hepatocyte cultures.

Virus Stocks and Procedure for Infection of Fibroblasts. Seed stocks of Sendai and Newcastle disease viruses were passaged in embryonated chicken eggs. Egg-grown Sendai and Newcastle disease virus preparations had titers of 8,000 and 4,000 hemagglutination units (HAU) per ml, respectively (1 HAU/ml corresponds to ~10^5 plaque forming units (PFU) per ml). Vesicular stomatitis virus (Indiana strain) was grown in chicken embryo fibroblast cultures (titer in the range of 10^7-to 10^8 PFU/ml). Encephalomyocarditis virus was grown in murine L cell cultures (titer 6 × 10^8 PFU/ml). Influenza virus type A strains WSN and Udorn (titers in the range of 1,000–4,000 HAU/ml) were grown in embryonated chicken eggs. Pseudorabies virus was grown in HeLa cells (titer 4.8 × 10^8 PFU/ml). Adenovirus type 5 grown in HeLa cells was purified through a cesium chloride gradient (titer 6.4 × 10^{12} virus particles/ml). Hyperimmune horse anti-Sendai virus antiserum was used in some of the neutralization experiments.

Confluent cultures of FS-4 cells in T-175 flasks 5–8 d after the previous medium change were rinsed twice with PBS and then incubated with 2 ml/flask of Eagle's MEM containing the virus at an appropriate concentration. The virus was allowed to adsorb for 1–1.5 h at 37°C, the cell sheets were then washed 10 times with PBS, and the flasks were replenished with either Eagle's MEM supplemented with 5% FCS or with the appropriate medium for ^{35}S-methionine labeling.

Measurement of Bacterial LPS (Endotoxin) Levels. Endotoxin levels in the different virus preparations at the highest concentrations used in Fig. 1 were measured using the QCL-1000 Quantitative Chromogenic Limulus Amebecyte Lysate assay kit (Whittaker M. A. Bioproducts, Walkersville, MD) calibrated using an *Escherichia coli* strain 0111:B4 endotoxin standard. In this assay, which was verified to be linear in the range of 0.1–1.0 endotoxin U/ml (10–100 pg/ml), only the pseudorabies virus preparation contained a measurable level of endotoxin (~50 pg/ml). This concentration has already been shown to be insufficient to affect IFN-β_2/IL-6 gene expression in human diploid fibroblasts (13).

Results and Discussion

Enhancement of IFN-β_2/IL-6 mRNA Levels in Virus-infected Fibroblasts. Fig. 1 presents an overview of the ability of RNA- and DNA-containing viruses belonging to different subgroups to induce IFN-β_2/IL-6 mRNA in human fibroblasts. In this experiment, the virus concentration used in each case was the highest that could be added to the cultures given the titers of the various virus stocks (virus stocks were diluted from 1:20–1:100; estimated multiplicities of infection in the range of 0.1–2 PFU/cell). It is clear from Fig. 1 that different virus preparations differ markedly in their ability to induce IFN-β_2/IL-6. The Sendai virus (paramyxoviridae) preparation was the strongest inducer (10-fold enhancement) from among the different RNA-containing virus preparations tested; the pseudorabies virus (herpesviridae) preparation was the strongest inducer (sixfold enhancement) from among the DNA-
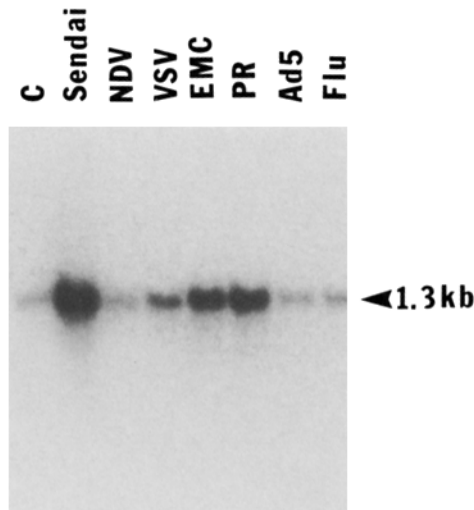
FIGURE 1. Induction of IFN-β_2/IL-6 mRNA in human fibroblasts by virus infection. Confluent monolayers of FS-4 cells in T-175 flasks (2 flasks per group) were exposed for 1.5 h to different viruses, rinsed thoroughly with PBS, and then incubated for another 6 h in Eagle's MEM. The levels of IFN-β_2/IL-6 mRNA were monitored by agarose gel electrophoresis followed by blot hybridization using a human IFN-β_2/IL-6 cDNA probe (pβ_2.15). Lane C is uninfected control; the other lanes illustrate mRNA from virus-infected cell cultures. The virus stock was diluted 1:20 in the case of Sendai, NDV, PR, and Flu (WSN strain); 1:40 in the case of VSV and EMC; and 1:100 in the case of Ad5. By densitometric scanning, the relative content of IFN-β_2/IL-6 mRNA was 9.6-, 1.8-, 3-, 5.3-, 5.7-, 1.1-, and 1.3-fold in Sendai-, NDV-, VSV-, EMC-, PR-, Ad5-, or Flu-infected cultures, compared with that in uninfected cultures.

containing virus preparations tested. EMC (picornaviridae) and VSV (rhabdoviridae) also clearly enhanced IFN-β_2/IL-6 mRNA levels by 6–8 h after virus infection in this experiment (Fig.1).

At 6 h after infection, an enhancement in IFN-β_2/IL-6 mRNA levels is seen in fibroblasts infected with Sendai virus at 1 HAU/ml (multiplicity of infection ~0.002 PFU/cell) (not shown). The induction of IFN-β_2/IL-6 by Sendai virus can be blocked by incubating the virus with hyperimmune antiserum to the virus, suggesting that it is virus infection that triggers the enhancement of IFN-β_2/IL-6 gene expression (not shown). An enhancement in IFN-β_2/IL-6 mRNA levels is also seen using pseudorabies (PR) virus at a multiplicity of infection as low as 0.001 PFU/cell (not shown). Thus infection of as few as 10,000–100,000 cells per culture (a T-175 flask contains ~5 × 10^7 cells) with Sendai or pseudorabies virus is sufficient to yield a detectable increase in accumulation of IFN-β_2/IL-6 mRNA.

Secretion of IFN-β_2/IL-6 Proteins by Virus-infected FS-4 Cells. Cytokine- or endotoxin-induced human fibroblasts secrete two different sets of IFN-β_2/IL-6 proteins: N- and O-glycosylated species of molecular mass 28, 29, and 30 kD, and O-glycosylated species of molecular mass 23 and 25 KD (4; our unpublished data). These can be detected by electrophoresis through highly resolving gels of immunoprecipitates of ^{35}S-methionine-labeled proteins from the culture medium of induced cells using a rabbit antiserum to human IFN-β_2/IL-6 protein expressed in *E. coli* (4). Figs. 2 and 3 summarize the observation that FS-4 cell cultures infected with Sendai, EMC, VSV, Flu, PR, or Ad5 viruses synthesize and secrete enhanced levels of IFN-β_2/IL-6 into the culture medium (2–15-fold increase). In these gels, the 28–30-kD species are clearly resolved from the 23–25-kD proteins. The relative levels of IFN-β_2/IL-6 secretion observed approximately correlate with the RNA blot-hybridization data in Fig. 1. It is remarkable that even as the EMC-infected cells are undergoing pathological changes (note the additional high molecular mass protein bands that contaminate the immunoprecipitate, particularly at the higher multiplicity of infection), the cultures continue to secrete high levels of IFN-β_2/IL-6 (Fig. 2).
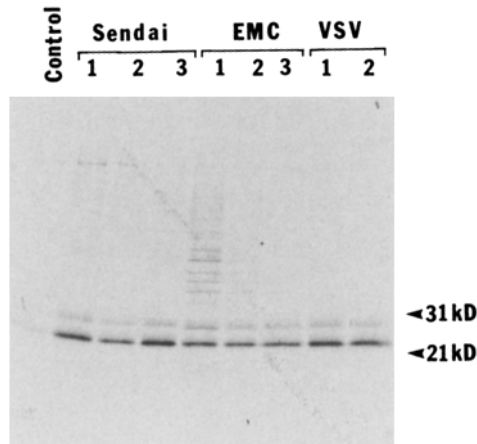
Figure 2. Synthesis and secretion of IFN-β_2/IL-6 proteins by human fibroblasts infected with Sendai, encephalomyocarditis, and vesicular stomatitis viruses. ^{35}S-methionine-labeled IFN-β_2/IL-6 proteins secreted over a 6-h period by virus-infected FS-4 cells were analyzed by immunoprecipitation using rabbit antiserum to *E. coli*-derived IFN-β_2/IL-6 followed by SDS-PAGE and autoradiography. Different dilutions of each of the three viruses were evaluated: Sendai lane *1*, 1:80 dilution of virus stock; lane *2*, 1:320; lane *3*, 1:1,280; EMC lane *1*, 1:80; lane *2*, 1:320; lane *3*, 1:1,280; VSV lane *1*, 1:80; lane *2*, 1:320. By densitometric scanning, there is up to a 15-, 9-, and 13-fold increase in IFN-β_2/IL-6 synthesis and secretion by Sendai-, EMC-, or VSV-infected FS-4 cells, respectively.

Thus, although the RNA data in Fig. 1 did not reveal a clear enhancement in IFN-β_2/IL-6 mRNA levels after Ad5 or Flu infection (this experiment represents a comparison of mRNA levels at one particular time after virus infection), monitoring of the cumulative synthesis and secretion of IFN-β_2/IL-6 over a 6-h period (Fig. 3) reveals increased production of the cytokine (also see Fig. 4).

In additional experiments, we have observed that stimulation of FS-4 cells with different concentrations of Sendai or PR viruses combined with bacterial LPS in the concentration range of 100–10 ng/ml did not display any synergistic or even an additive enhancement of IFN-β_2/IL-6 protein secretion (not shown). The induction of IFN-β_2/IL-6 proteins by the viral stimuli was the dominant determinant in these experiments.

Hepatocyte-stimulating Activity of Medium from Virus-infected FS-4 Cell Cultures. The outstanding biological effect of IFN-β_2/IL-6 is its ability to elicit the major acute phase alterations in plasma protein secretion by hepatocytes. We have evaluated medium from virus-infected FS-4 cell cultures for its ability to enhance α_1-antichymotrypsin secretion by human hepatoma Hep3B2 cells; secretion of α_1-antichymotrypsin by hepatocytes is enhanced by IFN-β_2/IL-6 but not by other cytokines such as TNF (6). Fig. 4 illustrates an experiment showing the enhanced synthesis and secretion of the positive acute phase protein α_1-antichymotrypsin by Hep3B2 cells exposed to culture media from FS-4 cells infected with Sendai, NDV,
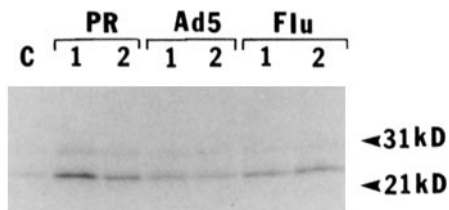

Figure 3. Synthesis and secretion of IFN-β_2/IL-6 proteins by human fibroblasts infected with pseudorabies virus, adenovirus type 5, and influenza (Udorn) viruses. The experiment was carried out as mentioned in legend to Fig. 2; only the 21–31-kD region of the autoradiogram is illustrated. Different dilutions of each of the three viruses were evaluated: PR lane *1*, 1:10 dilution of virus stock; lane *2*, 1:100; Ad5 lane *1*, 1:100; lane *2*, 1:1,000; Flu lane *1*, 1:10; lane *2*, 1:100. By densitometric scanning, the synthesis and secretion of IFN-β_2/IL-6 is increased 6-, 2.3-, and 4-fold by PR-, Ad5-, or Flu-infected FS-4 cells, respectively.
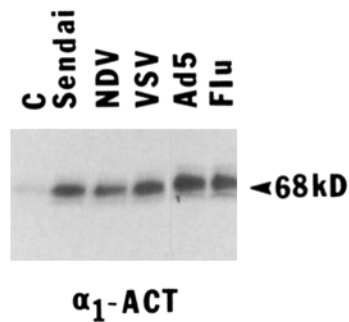
Sendai
NDV
VSV
Ad5
Flu
C

◀68kD

α_1-ACT

FIGURE 4. Culture media from virus infected human fibroblasts enhance acute phase plasma protein secretion by hepatocytes. Human Hep3B2 cells in 24-well multiwell plates were exposed to a 1:5 dilution of ultracentrifuged and irradiated samples of media from FS-4 cell cultures infected with Sendai, NDV, VSV, Ad5, and Flu as described in legend to Fig. 1. The synthesis and secretion of α_1-antichymotrypsin was evaluated by labeling the Hep3B2 cells with ^{35}S-methionine followed by immunoprecipitation of the labeled protein from the culture medium; the immunoprecipitates were analyzed by SDS-PAGE and autoradiography. By densitometric scanning, there was an 8–15-fold increase in α_1-antichymotrypsin secretion in this assay when Hep3B2 cells were exposed to media from virus-infected fibroblasts. Hep3B2 cells treated with media from EMC- or PR-infected FS-4 cells rapidly lifted off the substratum.

VSV, Ad5, or Flu viruses (8–15-fold enhancement). Culture media from EMC and PR-infected fibroblasts had cytocidal effects on Hep3B2 cells; thus these assays are not shown.

In this article, we report that virus infection strongly enhances the expression and secretion of IFN-β_2/IL-6 proteins by the ubiquitous stromal fibroblast. The secreted IFN-β_2/IL-6 may mediate aspects of the host response (acute phase and immune) to viral infections and may itself contribute to the symptoms of viral disease such as pyrexia (recombinant *E. coli*–derived human IFN-β_2/IL-6 has been reported to be highly pyrogenic; see reference 9). The development of assays that detect IFN-β_2/IL-6 in body fluids (9), the cloning and expression of the murine IFN-β_2/IL-6 gene, and the availability of serologic reagents that neutralize IFN-β_2/IL-6 (4) now permit an evaluation of the biological importance of IFN-β_2/IL-6 in human and murine virus infections.

Summary

We have defined the expression of the mRNA for, and secretion of, IFN-β_2/hepatocyte-stimulating factor/IL-6 (IFN-β_2/IL-6) in human diploid fibroblasts (FS-4 strain) infected with different RNA- and DNA-containing viruses. RNA blot-hybridization analyses carried out 6–8 h after the beginning of infection showed that the RNA-containing Sendai virus (paramyxoviridae) enhanced IFN-β_2/IL-6 mRNA levels 10-fold, followed, in decreasing order, by encephalomyocarditis (EMC, picornaviridae), vesicular stomatitis (VSV, rhabdoviridae), Newcastle disease virus (NDV, paramyxoviridae), and influenza A (Flu, myxoviridae) viruses. The DNA-containing pseudorabies virus (PR, herpesviridae) enhanced IFN-β_2/IL-6 mRNA levels sixfold, while the effect of adenovirus type 5 (Ad5, adenoviridae) was considerably less and comparable with that of NDV or Flu. A rabbit antiserum raised against *E. coli*–derived human IFN-β_2/IL-6 was used in immunoprecipitation experiments to monitor the secretion of ^{35}S-methionine-pulse-labeled IFN-β_2/IL-6 proteins by fibroblasts up to 7 h after the beginning of infection. Enhanced levels of secretion of IFN-β_2/IL-6 (2–14-fold) were observed in every instance evaluated (Sendai, EMC, VSV, Flu, PR, Ad5 viruses). A biological consequence of enhanced secretion of IFN-β_2/IL-6 was the ability of media from infected FS-4 cell cultures to enhance by 8–15-fold the synthesis and secretion of a typical acute phase plasma protein (α_1-

antichymotrypsin) by human hepatoma Hep3B2 cells. These observations make it likely that IFN-β_2/IL-6 mediates, in part, the host response to acute virus infections.

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