REGULATION OF THE ACUTE PHASE AND IMMUNE RESPONSES IN VIRAL DISEASE

Enhanced Expression of the \( \beta_2 \)-interferon/Hepatocyte-stimulating Factor/Interleukin 6 Gene in Virus-infected Human Fibroblasts

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The human \( \beta_2 \)-interferon/hepatocyte-stimulating factor/interleukin 6 (IFN-\( \beta_2 \)/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-\( \beta_2 \)/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 IgG 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-\( \beta_2 \)/IL-6 proteins is enhanced in response to other cytokines such as IL-1, TNF, lymphotxin, 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-\( \beta_2 \)/IL-6 by monocytes (4, 6, 8, 10) and fibroblasts (4, 13). Thus, the regulation of expression of the IFN-\( \beta_2 \)/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-\( \beta_2 \)/IL-6 is likely to be a component in the host response to viral pathogens.

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Materials and Methods

The human diploid foreskin fibroblast strain FS-4 was used throughout; procedures for its growth in cell culture, for IFN-β/IL-6 mRNA induction, for RNA blot-hybridization analyses, and for 35S-methionine-labeling of secreted IFN-β/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 α1-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 10^5 to 10^6 PFU/ml). Encephalomyocarditis virus was grown in murine L cell cultures (titer 6 × 10^5 PFU/ml). Influenza virus type A strains WSN and Udorn (titers in the range of 1,000 to 4,000 HAU/ml) were grown in embryonated chicken eggs. Pseudorabies virus was grown in HeLa cells (titer 4.8 × 10^6 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 35S-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 Amebocyte Lysate assay kit (Whittaker M. A. Bioproducts, Walkersville, MD) calibrated using an Escherichia coli strain 011: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/IL-6 gene expression in human diploid fibroblasts (13).

Results and Discussion

Enhancement of IFN-β/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/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/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-
Induction of IFN-β/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/IL-6 mRNA were monitored by agarose gel electrophoresis followed by blot hybridization using a human IFN-β/IL-6 cDNA probe (pβ15). 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 Ads. By densitometric scanning, the relative content of IFN-β/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.

At 6 h after infection, an enhancement in IFN-β/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/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/IL-6 gene expression (not shown). An enhancement in IFN-β/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 x 10⁷ cells) with Sendai or pseudorabies virus is sufficient to yield a detectable increase in accumulation of IFN-β/IL-6 mRNA.

Secretion of IFN-β/IL-6 Proteins by Virus-infected FS-4 Cells. Cytokine- or endotoxin-induced human fibroblasts secrete two different sets of IFN-β/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 35S-methionine-labeled proteins from the culture medium of induced cells using a rabbit antiserum to human IFN-β/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/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/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/IL-6 (Fig. 2).
FIGURE 2. Synthesis and secretion of IFN-β/IL-6 proteins by human fibroblasts infected with Sendai, encephalomyocarditis, and vesicular stomatitis viruses. \^{3}S-methionine-labeled IFN-β/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/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/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/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/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/IL-6 protein secretion (not shown). The induction of IFN-β/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/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 α1-antichymotrypsin secretion by human hepatoma Hep3B2 cells; secretion of α1-antichymotrypsin by hepatocytes is enhanced by IFN-β/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 α1-antichymotrypsin by Hep3B2 cells exposed to culture media from FS-4 cells infected with Sendai, NDV,
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 α1-antichymotrypsin was evaluated by labeling the Hep3B2 cells with 35S-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 α1-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/IL-6 proteins by the ubiquitous stromal fibroblast. The secreted IFN-β/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/IL-6 has been reported to be highly pyrogenic; see reference 9). The development of assays that detect IFN-β/IL-6 in body fluids (9), the cloning and expression of the murine IFN-β/IL-6 gene, and the availability of serologic reagents that neutralize IFN-β/IL-6 (4) now permit an evaluation of the biological importance of IFN-β/IL-6 in human and murine virus infections.

Summary

We have defined the expression of the mRNA for, and secretion of, IFN-β/hepatocyte-stimulating factor/IL-6 (IFN-β/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/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/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/IL-6 was used in immunoprecipitation experiments to monitor the secretion of 35S-methionine-pulse-labeled IFN-β/IL-6 proteins by fibroblasts up to 7 h after the beginning of infection. Enhanced levels of secretion of IFN-β/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/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 (α1-
antichymotrypsin) by human hepatoma Hep3B2 cells. These observations make it likely that IFN-β/IL-6 mediates, in part, the host response to acute virus infections.

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