Interleukin-8 can mediate acute-phase protein production by isolated human hepatocytes

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Wigmore, Stephen J., Kenneth C. H. Fearon, Jean P. Maingay, Paul B. S. Lai, and James A. Ross. Interleukin-8 can mediate acute-phase protein production by isolated human hepatocytes. Am. J. Physiol. 273 (Endocrinol. Metab. 36): E720–E726, 1997.—During the course of studies designed to identify the role of cytokines in the reprioritization of hepatic protein synthesis associated with cachexia we detected a hepatocyte-stimulating moiety in the supernatants of pancreatic cancer cells that was unrelated to interleukin (IL)-6. This study identifies that moiety as IL-8 and investigates the role of IL-8 in the induction of acute-phase protein production. The human pancreatic cancer cell line MIA PaCa-2 produced >1 ng/ml of IL-8 per 24 h, and supernatants from this cell line induced C-reactive protein (CRP) production from isolated human hepatocytes. Addition of neutralizing anti-human IL-8 antibody to such supernatants produced almost complete inhibition of CRP production. The addition of recombinant human IL-8 to hepatocytes resulted in a dose-dependent increase in CRP, \( \alpha_1 \)-acid glycoprotein, and \( \alpha_1 \)-antichymotrypsin production and a decrease in the production of transferrin and prealbumin. This study demonstrates that recombinant or tumor-derived IL-8 can modulate acute-phase protein production from isolated human hepatocytes and from human hepatoma cells.

C-reactive protein; pancreatic cancer cells

THE ACUTE-PHASE PROTEINS are produced in response to tissue injury as a result of neoplasia, trauma, or infection (12). The acute-phase proteins have been found to have an essential role in the inhibition of extracellular proteases, blood clotting, fibrinolysis, modulation of immune cell function, and the neutralization and clearance of harmful components from the circulation. The acute-phase response involves a reprioritization of hepatic protein synthesis with elevated production of the acute-phase reactants and simultaneous suppression of normal export proteins. This phenomenon is thought to be principally under the control of the cytokine interleukin (IL)-6 acting on the human hepatocyte. IL-6 induces transcriptional activation of a set of human acute-phase proteins (16, 19). Moreover, IL-6-induced nuclear transcription factors that interact with the C-reactive protein (CRP) promoter have been described. However, additional cytokines with IL-6-like activities have also been discovered. Leukemia inhibitory factor (LIF), oncostatin M, glyliferotrophic factor, and IL-11 can each regulate the production of the same set of hepatic export proteins as IL-6 via multisubunit cytokine receptors (13). In addition, the effect of IL-6 on the production of hepatic proteins can be influenced significantly by other cytokines and by insulin and the counterregulatory hormones (dexamethasone, glucagon, and epinephrine) (18). Indeed, the counterregulatory hormones can influence acute-phase protein production in the absence of IL-6. IL-8 is a proinflammatory cytokine whose principal role in infection and inflammation appears to be the recruitment and activation of circulating and tissue neutrophils to the site of tissue damage (15). It has been demonstrated that IL-8 is produced by a wide variety of cell types in vitro, including vascular and lung endothelium (5, 17), monocytes (4), eosinophils (6), kidney mesangial cells (1), astrocytes (3), and keratinocytes. Studies have demonstrated that IL-1\( \beta \) and tumor necrosis factor (TNF)-\( \alpha \) induce transcriptional activation of the IL-8 gene and synthesis of IL-8 protein (1). There are a number of studies that describe elevated levels of circulating IL-8. In sepsis patients (21), for example, IL-8 concentrations have been reported as being markedly elevated at diagnosis and remaining high during the course of the illness.

During the course of studies designed to identify the role of cytokines in the reprioritization of hepatic protein synthesis associated with cachexia (9, 10), we detected a hepatocyte-stimulating moiety in the supernatants of pancreatic cancer cells that was unrelated to IL-6. This study identifies that moiety as IL-8 and investigates the role of tumor-derived and recombinant human IL-8 in the induction of acute-phase protein production from isolated human hepatocytes.

MATERIALS AND METHODS

Cell lines. The adherent human pancreatic cancer cell lines, MIA PaCa-2, CFPAC, and PANC-1, and the hepatoma cell line, Hep G2, were obtained from the European Collection of Animal Cell Cultures (Public Health Laboratory Service Centre for Applied Microbiology and Research, Porton Down, UK). The pancreatic cancer cell lines were plated out at a concentration of \( 5 \times 10^4 \) and the Hep G2 hepatoma line at \( 3 \times 10^4 \) cells per well in 96-well tissue culture plates (Costar, High Wycombe, UK) in 200 µl of medium per well. Cells were incubated in Dulbecco’s modification of Eagle’s medium (Life Technologies, Inchinan, UK) supplemented with 5% heat-inactivated fetal calf serum (Life Technologies, Irvine, UK) and 2 mM L-glutamine (ICN Biomedicals, Irvine, UK) at 37°C in 5% humidified air-5% CO\(_2\).

Endogenous cytokine production by human pancreatic cancer cells. Pancreatic cancer cells were incubated as previously described. In time-course experiments, cells were counted at 0, 24, 48, 72, 96, 120, 144, and 168 h after trypsinization and staining with trypan blue, which also permitted evaluation of cell viability. Supernatants were removed at these times and stored at \(-70°C\) until assay.

mRNA extraction and reverse transcription. The reverse transcription (RT)-polymerase chain reaction (PCR) method
was used to detect IL-8 mRNA in pancreatic cancer cell lines. Poly(A)^+ mRNA was purified from Mia PaCa-2 cells using a Fast Track mRNA purification kit (Invitrogen, San Diego, CA). The poly(A)^+ mRNA (1 µg) was then reverse transcribed using an RT kit (Promega, Madison, WI) according to manufacturer's instructions. A 20-µl reaction consisted of 5 mM MgCl_2, 1 mM deoxynucleotides, 0.5 µg oligo(dT) primer, 2 µl 10×RT buffer (500 mM KCl, 100 mM Tris(hydroxymethyl)amino-
methane (Tris)-HCl, 1% Triton X-100), 20 U RNasin ribonu-
clease inhibitor, and 15 U Avian Myeloblastosis Virus RT. The RT mixture was incubated at 42°C for 15 min, and the RT was inactivated by heating the reaction mixture at 99°C for 5 min followed by a 5-min incubation at 0°C. The reaction mixture containing cDNAs was stored at -20°C.

PCR. After RT to cDNA, amplification was carried out by PCR. The sense and anti-sense IL-8 primers were (5'-3') ATGACTTCCAAGCTGGCCGTGGCT and TCTCAGCCCTTTCCAAAACCTTCTC, respectively (Oswell, Southampton, UK). The predominant cDNA amplification product was predicted to be 289 base pair (bp) for IL-8, and 983 and 1,000 bp for glyceraldehyde-3-phosphate dehydrogenase (G3PDH) and B cell lymphocyte (bcl2), respectively, which were used as positive controls. A typical 10-µl PCR reaction mixture contained cDNAs was stored at 70°C until assay for acute-phase proteins.

Measurement of acute-phase proteins IL-6 and IL-8. Sandwich ELISAs were employed for the measurement of CRP, α1-acid glycoprotein, α1-antichymotrypsin (18), IL-6, and IL-8 (11) as described previously. Briefly, 96-well immunoplates were coated with either goat anti-human CRP, α1-acid glycoprotein or α1-antichymotrypsin (Dako, High Wycombe, UK), monodonal anti-human IL-6 (Boehringer-Mannheim), or rabbit anti-human IL-8 (AMS, Biotechnology Ltd, Oxford, UK), respectively. Supernatants were diluted 1:10 for CRP, α1-acid glycoprotein, and α1-antichymotrypsin or 1:5 for IL-6 and IL-8 and were added to wells in triplicate before incubation at 4°C for 18 h. The secondary antibodies were, respectively, rabbit anti-human CRP, α1-acid glycoprotein or α1-antichymotrypsin (Dako), goat anti-human IL-6 (R & D Systems) or goat anti-human IL-8 (R & D Systems). These were detected by peroxidase-conjugated antibody directed against rabbit or goat immunoglobulins, respectively (Sigma), and the substrate 3,3',5,5'-tetramethylbenzidine. The plates were read at 490 nm using a MR5000 ELISA plate reader (Dynatech, Billinghamurst, UK), and concentrations in the samples were calculated using the AssayZap (Biosoft, Cambridge, UK) computer software. The limits of sensitivity of the assays, taking into consideration the sample dilutions, ranged from 1 to 500 pg/ml for the different acute-phase proteins, 40 pg/ml for IL-6, and 190 pg/ml for IL-8.

There is clearly a degree of variability between different liver resection specimens and the hepatocytes prepared from these samples. There are few published studies on acute-phase protein production from human hepatocytes, and these data are generally presented as percentage change compared to normal liver.
with control (18, 23) and no absolute values are given. However, there is some information available on protein production from different hepatocyte preparations that have been allowed 24 h to attach to collagen-coated plates before commencing experiments (23).

Statistics. Analysis of significance between variables was performed using the paired two-tailed t-test. A difference was considered significant using 95% confidence intervals (P < 0.05).

RESULTS

Endogenous cytokine production by pancreatic cancer cell lines. Measurement of the production of IL-8 by the three human pancreatic cancer cell lines, MIA PaCa-2, CFPAC, and PANC-1, demonstrated that MIA PaCa-2 produced substantial quantities (>1 ng/ml culture medium per 24 h) of IL-8 (Fig. 1A). Titers of IL-8 increased sequentially with time to a maximum of 50 ng/ml by day 7. However, correction of cytokine production for cell count indicated a constant rate of IL-8 production. Measurement of production of IL-6 demonstrated that CFPAC cells were the highest producers of this cytokine (>1 ng/ml of culture medium per 24 h) (Fig. 1B). The quantity of IL-6 secreted by CFPAC cells increased with time to a peak of 7.3 ng/ml on day 7. However, correction of cytokine production for cell count indicated a constant rate of IL-8 production (0.252 pg·cell⁻¹·24 h⁻¹). The CFPAC cell line demonstrated an intermediate amount of IL-8 (0.5–0.99 ng/ml culture medium per 24 h), and the PANC-1 cell line was the lowest producer of IL-8 (<0.2 ng/ml culture medium per 24 h).

Measurement of production of IL-6 demonstrated that CFPAC cells were the highest producers of this cytokine (>1 ng/ml of culture medium per 24 h) (Fig. 1B). The quantity of IL-6 secreted by CFPAC cells increased with time to a peak of 7.3 ng/ml on day 7. The MIA PaCa-2 and PANC-1 cell lines secreted less IL-6 (0.11 ng/ml culture medium per 24 h).

RT-PCR demonstration of message for IL-8 in the MIA PaCa-2 cell line. The cDNA amplification product for the IL-8 primers (Fig. 2) was a single band of the predicted 289 bp. Other lanes contained the predicted 1,000 bp for bcl2 and 983 bp for G3PDH, which were used as positive controls. IL-8 message was also detected in CFPAC and PANC-1 (data not shown). IL-6 message was detected in CFPAC and a lesser amount in PANC-1 and MIA PaCa-2 (data not shown).

Stimulatory activity of supernatants from pancreatic cancer cell cultures on hepatocyte CRP production. Addition of 24-h supernatants from the three pancreatic cancer cell lines, MIA PaCa-2, CFPAC, and PANC-1, to primary cultures of isolated human hepatocytes (Fig. 3) resulted in significant increases in CRP production. Supernatants from the MIA PaCa-2 cell line had the greatest stimulatory effect on CRP production, resulting in 150 ± 39 ng/ml from a 48-h hepatocyte culture. Supernatants from CFPAC and PANC-1 induced the production of 80 ± 13 and 50 ± 24 ng/ml of CRP, respectively, from a 48-h hepatocyte culture. The basal production of CRP from isolated hepatocytes in these experiments was 28 ± 3 ng/ml over a 48-h period.

Inhibition of the stimulatory effect of MIA PaCa-2 cell culture supernatant on hepatocyte CRP production by addition of anti-human IL-8 and anti-human IL-6 antibody. Addition of neutralizing anti-human IL-8 antibody to pancreatic cancer cell supernatants produced almost complete inhibition of CRP production from isolated hepatocytes. This effect was most marked (Fig. 4) on hepatocytes stimulated with MIA PaCa-2 cell culture supernatant (24-h supernatants contained 2,519 ± 224 pg/ml of IL-8). Neutralizing anti-human IL-6 antibody had no significant effect on hepatocyte CRP production in the presence of supernatants from MIA PaCa-2 cell cultures (24-h supernatants contained 110 ± 15 pg/ml of IL-6). Neither neutralizing antibody had a significant effect on CRP production in the absence of pancreatic cancer cell supernatant (data not shown).

Effect of recombinant human IL-8 on hepatocyte acute-phase protein production. The addition of recombinant human IL-8 to hepatocytes resulted in a dose-dependent increase in CRP, α1-acid glycoprotein, and α1-antichymotrypsin production (Fig. 5, A–C). Recombinant human IL-8 (1 ng/ml) increased hepatocyte CRP.
production by 180% compared with basal production, and 10 ng/ml increased CRP production by 300%. Addition of a fixed dose (1 ng/ml) of recombinant human IL-6 simultaneously with addition of IL-8 (Fig. 5D) demonstrated an additive effect with concentrations of IL-8 $>$ 0.1 ng/ml, but there was no additive effect at lower concentrations. There did not appear to be any synergy between the IL-6- and IL-8-induction of CRP production.

Recombinant IL-8, in common with recombinant IL-6, had a significant effect on the production of the negative acute-phase proteins transferrin and prealbumin (Fig. 6, A and B). A dose of 0.1 ng/ml of IL-8 was sufficient to reduce the production of prealbumin to baseline and to attenuate the production of transferrin from isolated human hepatocytes. These effects of IL-8 were not significantly different from the effect of an equivalent amount of IL-6.

Effect of recombinant human IL-8 on hepatoma acute-phase protein production. Although there was no evidence of Kupffer cell or monocyte contamination in the isolated hepatocyte cultures (potential sources of IL-6), this possibility could not be excluded entirely. Hepatoma cell lines were therefore investigated for their ability to respond to IL-8 by the production of acute-phase proteins. The use of hepatoma cell lines is not without problems because certain acute-phase proteins are not produced by many of these cell lines. Hep G2 cells for example cannot produce CRP possibly through damage to the CRP gene locus or through disruption of transcriptional regulation of this gene. In Hep G2 cells production of the acute-phase protein $\alpha_1$-acid glycoprotein was therefore chosen as an indicator of response to recombinant IL-8 and IL-6. The addition of recombinant human IL-8 to the Hep G2 hepatoma cell line resulted in a dose-dependent increase in $\alpha_1$-acid glycoprotein production (data not shown). Addition of a fixed dose (10 ng/ml) of recombinant human IL-6 (Fig. 7) simultaneously with the addition of a fixed dose of IL-8 (10 ng/ml) demonstrated an additive effect but no synergistic effect between the two cytokines. IL-6 and
IL-8 at this concentration had similar effects on the production of α₁-acid glycoprotein.

**DISCUSSION**

This study has demonstrated for the first time that both recombinant human IL-8 and tumor-derived IL-8 can modulate acute-phase protein production from isolated human hepatocytes and from human hepatoma cells. The implications of this finding for control of the acute-phase response during an inflammatory response may be profound. It has been argued that IL-8 could have little activity in vivo because it is almost completely bound in the circulation to erythrocytes that possess high affinity IL-8 binding sites (it has been estimated that there are $2 \times 10^3$ per cell) (7). However, many actions of cytokines appear to occur at a local level rather than through the systemic effects of circulating cytokines. In the local microenvironment of the liver IL-8 may be a potent regulator of hepatic protein metabolism. There are a number of resident cell types...
within the liver that are capable of producing both IL-8 and IL-6. These include Kupffer cells (4), endothelial cells (8), and possibly hepatocytes. Certain human hepatoma cell lines (22) have been shown to be capable of producing IL-8 in response to TNF or IL-1 stimulation. In addition, there is a constant passage of circulating mononuclear and polymorphonuclear cells through the liver sinusues, and many of these cells may secrete IL-8 and IL-6 as they transit the liver, particularly during an inflammatory response to a local or systemic event. Clearly there are many circumstances where IL-8 may be an important regulator of acute-phase protein synthesis.

In the present study the supernatants from MIA PaCa-2 had a much greater stimulatory effect on CRP production than an equivalent amount of recombinant IL-8. The contribution of IL-6 to the stimulatory capacity of the MIA PaCa-2 supernatants would appear to be small because the stimulatory activity of the MIA PaCa-2 supernatants was reduced by only 10% using a saturating amount of neutralizing anti-IL-6 antibody. Almost all the hepatocyte-stimulating activity of the supernatant would therefore appear to be due to IL-8. The apparent discrepancy between the stimulatory activity of recombinant IL-8 and the projected activity of the supernatant may have several explanations. One possibility is that the recombinant IL-8 may have lower biological activity than native IL-8. Alternatively, other moieties in the supernatant from the MIA PaCa-2 line may amplify the effect of IL-8 on hepatocytes without having a significant effect in the absence of IL-8. This is suggested by the almost complete abrogation of CRP production when neutralizing IL-8 antibody is present in the hepatocyte cultures containing MIA PaCa-2 supernatant.

The regulatory effect of IL-8 and IL-6 on the proteins examined in this paper appeared to be very similar. This is interesting given that the receptor complexes and signaling mechanisms for the IL-6-LIF family appear to be very different from the IL-8-chemokine family. Whereas the IL-6-LIF family of receptors comprise complexes where there is association of a low-affinity ligand-binding subunit and signal transducing components, the receptors for IL-8 contain seven transmembrane domains, as revealed by cDNA cloning (2), suggesting that they are members of the superfamily of G protein-coupled receptors. We have previously shown (18) that glucagon, in the absence of IL-6, can markedly increase CRP production from isolated human hepatocytes. The glucagon receptor is, like the IL-8 receptor, a seven transmembrane domain structure (14) and is a...
member of the superfamily of G protein-coupled receptors. It is known that this receptor can signal into the mitogen-activated protein kinase (MAPK) pathway as can the IL-6-LIF receptor family. This MAPK pathway may therefore provide a common pathway by which glucagon, IL-8, and the IL-6-LIF receptor families may influence acute-phase protein production in human hepatocytes. Further work is required to establish if these different receptor and signaling pathways may result in the critical threonine-phosphorylation of nuclear factor IL-6 through MAPK activation and thereby the activation of type 2 IL-6-responsive elements of the acute-phase protein genes.

It is interesting to note that in experiments designed to investigate the synergism between IL-8 and IL-6 there appeared to be an additive effect only with a similar stimulatory effect for equivalent amounts of the two cytokines. In this regard the role of IL-8 in influencing the acute-phase protein response in vivo is difficult to ascertain. As noted previously, IL-8 is rapidly removed from the circulation, whereas IL-6 circulates in an active form and remains capable of delivering a signal, via the cell-surface signal-transducing molecule gp130, even when bound to its soluble receptor (13). Therefore, IL-8 may only be biologically significant in vivo when produced locally in the liver or when present in excess in the circulation.

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