Clin. exp. Immunol. (1991) **83**, 448–491 ADONIS 0009910491000897

Acute-phase protein synthesis in human hepatoma cells: differential regulation of serum amyloid A (SAA) and haptoglobin by interleukin-1 and interleukin-6

J. G. RAYNES, S. EAGLING & K. P. W. J. McADAM *Department of Clinical Sciences, London School of Hygiene and Tropical Medicine, London, England*

(Accepted for publication 9 October 1990)

SUMMARY

Interleukin-6 (IL-6, BSF-2 or IFN-β2) is thought to be the major regulator of the acute-phase protein response that follows tissue injury and inflammation, with interleukin-1 (IL-1), tumour necrosis factor and more recently, LIF or HSF III, slightly stimulatory on only certain acute phase proteins. The synthesis of the major acute-phase protein SAA, originally described as being synthesized in response to IL-1, has been claimed recently to be mainly under IL-6 regulation. Our results show that in the human hepatoma cell line HuH-7, IL-1 is the major stimulating cytokine increasing SAA synthesis by a factor in excess of 100-fold. We also show that under most conditions interleukin-6 and tumour necrosis factor stimulate additively in combination with IL-1. Isoelectric focusing has demonstrated that SAA1 and SAA2α are expressed but not SAA2β. The HuH-7 cell line is IL-6 responsive since haptoglobin is stimulated mainly by IL-6.

Keywords interleukin-1 serum amyloid A haptoglobin acute-phase protein interleukin-6

INTRODUCTION

Acute-phase proteins are synthesized by the liver as a non-specific response to inflammation with the primary function of modulating the inflammatory response to tissue damage or infection. The interplay of four cytokines, interleukin-1 (IL-1), IL-6, tumour necrosis factor (TNF) and leukaemia-inhibitory factor (LIF) and other hormones for which there are receptors on hepatocytes, together with the nerve supply to the liver, provides the signals that account for the specificity of the hepatic acute-phase response (Gauldie *et al.*, 1987; Baumman & Wong, 1989; Heinrich, Castell & Andus, 1989; Sehgal, 1990). Serum amyloid A (SAA) is a high-density lipoprotein (HDL) associated apolipoprotein and is the most impressive of the acute-phase proteins, increasing within 24 h by up to 1000-fold (Pepys & Baltz, 1983). IL-1 was originally described as the stimulator of SAA in primary cultures (Selinger *et al.*, 1980) but the more recent discovery of IL-6 as the major stimulator of most acute-phase proteins relegated IL-1 to a supportive role (Heinrich *et al.*, 1989). Studies on regulation of acute-phase proteins by cytokines have used either primary cell culture or hepatoma cell lines; both these have potential problems. Primary cell cultures express high background acute-phase protein levels after the trauma of cell isolation. Hepatoma cell lines often have altered patterns of acute-phase protein responses; of the many tumour cell lines examined so far, the human hepatomas Hep 3B and PLC/PRF/5 produce only small amounts of SAA (Kushner, Ganapathi & Schultz, 1989).

The HuH-7 cell line was developed as a well differentiated tumour cell line that produced many plasma proteins (Nakabayashi *et al.*, 1982), and its identification, reported here, as the first cell line to synthesize SAA in amounts quantifiable by ELISA has allowed us to investigate SAA regulation systematically.

Correspondence: Dr J. G. Raynes, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, UK.

MATERIALS AND METHODS

Cell culture

HuH-7 cells (Nakabayashi *et al.*, 1982) were grown in 24-well plates to confluence in RPMI 1640 medium (Flow Labs, Irvine, UK) containing 10% fetal calf serum, 1 mM pyruvate, 2 mM glutamine, penicillin (100 μg/ml) and streptomycin (100 μg/ml). Wells were then stimulated with various cytokines in medium containing 10^{-7} M dexamethasone and 10^{-8} M insulin. In time-course experiments cytokines were added on the first day and supernatants were taken every 24 h thereafter. Supernatants were assayed for SAA, haptoglobin and albumin. Cells were counted after trypsinization. Recombinant human IL-1β and TNF were kindly provided by Dr C. Dinarello (Tuft's University, Boston) and recombinant human IL-1α was a gift from Dr Lomedico (Hoffman LaRoche, Nutley, NJ). Recombinant human IL-6 was provided by Dr S. Clark (Genetics Institute, Cambridge, MA).

Recombinant human LIF was kindly provided by Dr G. Wong (Genetics Institute) and neutralizing monoclonal antibody to IL-1α (M3) was a gift from Dr S. Gillis (Immunex Corporation, Seattle, WA).

Polyclonal rabbit antibody to human IL-6 was kindly provided by Dr L.A. Aarden (Netherlands Red Cross Blood Transfusion Service, Amsterdam).

Specific protein assays

Immulon 2 plates (Dynatech) were coated with affinity-purified anti-human SAA in 0·13 M borate, pH 9·6, at 4°C overnight, washed with phosphate-buffered saline (PBS) containing 0·05% Tween 20. The antibody to SAA was raised in a goat and affinity-purified against SAA (using human SAA conjugated to Sepharose 4B) and absorbed twice against normal human serum–Sepharose 4B. The standards and samples were added to the wells with an SAA–alkaline phosphatase conjugate prepared by the one-step glutaraldehyde method. The SAA was purified as described previously (Raynes & McAdam, 1988). The ability of sample to inhibit SAA conjugate binding was compared with that of the standards. Substrate was *p*-nitrophenol in bicarbonate/carbonate buffer, pH 9·6, containing 2 mM $MgCl_2$. Standards were HDL with a known SAA concentration diluted in culture medium. The SAA was determined by the method of Godenir *et al.* (1985) in which a range of dilutions of pure SAA and acute-phase HDL are subjected to SDS–PAGE before quantification of the SAA band by staining and measurement of the absorbance of the band. Bovine HDL does not interfere in the assay.

Haptoglobin assays were performed in a similar manner using a rabbit anti-human haptoglobin antibody (The Binding Site, Birmingham, UK) coated onto Immulon 2 plates, and peroxidase-conjugated haptoglobin (prepared using the periodate method) was competed with unlabelled sample haptoglobin. Standards were purified haptoglobin diluted in culture medium. Albumin was assayed by a similar ELISA method. Rabbit anti-human albumin (Dako) and an alkaline phosphatase conjugate (single-step glutaraldehyde) of human albumin were the reagents used. All results were expressed per 10^6 cells.

SAA isotype analysis

SAA was extracted from 12 ml of supernatant on 50 μl of octyl sepharose beads. The beads were washed in PBS five times and in distilled water once before elution with 8 M urea containing 2% NP40 and 2% ampholines (pH range 5–8). The eluate was loaded directly onto a vertical slab isoelectric focusing (IEF) gel comprising 5% v/v ampholines pI 5–8, 7% acrylamide, 7 M Urea; 150 V for 30 min and 200 V for 150 min were applied to the gel with the upper buffer of 25 mM orthophosphoric acid and lower buffer of 50 mM NaOH. After the gel was semi-dry electroblotted to PVDF membranes, using 48 mM Tris 39 mM glycine, 1·3 mM SDS and 20% methanol, the membrane was first blocked in 1% milk powder, 1% bovine serum albumin (BSA). The SAA isotypes were revealed by an affinity-purified goat anti-human SAA and an alkaline-phosphatase-labelled anti-goat immunoglobulin second antibody. Substrate was 75 μl of 5-chloro-4-bromo-3-indolylphosphate (50 mg/ml in 70% dimethylformamide) and 100 μl for nitro-blue-tetrazolium (75 mg/ml in 70% dimethylformamide) in 22·5 ml of carbonate/bicarbonate buffer, pH 9·6, containing 2 mM $MgCl_2$.

RESULTS

HuH-7 cells were grown to confluence and stimulated with a variety of cytokines and hormones. Initially the cytokines IL-1α and IL-1β, and IL-6 were used in dose–response experiments which showed that IL-1 was the major stimulator. Recombinant IL-1β (Fig. 1a) or IL-1α (Fig. 1b) in the presence of dexamethasone stimulated SAA with an effective concentration of 10–1000 pg/ml. The half maximal concentration was about 100 pg/ml, in agreement with concentrations required for stimulation of other acute-phase proteins in other hepatoma cell lines (Baumann, Richards & Gauldie, 1987). The effect of IL-6 and TNF alone and in combination with IL-1 can be seen in Fig. 2. The effect of IL-6 in combination with IL-1α or IL-1β was approximately addititive, with no evidence for synergy. When added alone, IL-6 was effective in stimulating a two- to five-fold increase and TNF also stimulated but to a lesser extent (Fig. 2). LIF at 1, 10 or 100 U/ml induced less than 100%. In some experiments, the response to IL-1 showed an increase of over 100-fold. After treatment with IL-1, the albumin levels decreased (results not shown) compared with controls, as previously shown for other hepatomas and hepatocyte cultures (Baumann *et al.*, 1987; Gauldie *et al.*, 1987; Kushner *et al.*, 1989). Albumin was not

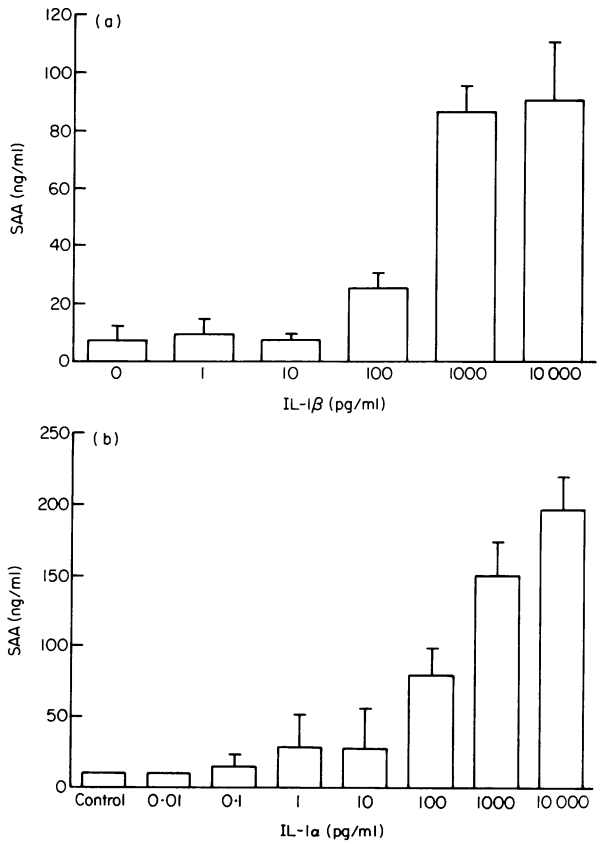

Fig. 1. Effect of increasing (a) IL-1β or (b) IL-1α concentration on serum amyloid A (SAA) synthesis by HuH-7 cells. HuH-7 cells were grown to confluence in 24-well plates and stimulated with the same media containing various concentrations of rIL-1β or IL-1α and 10^{-7} M dexamethasone plus 10^{-8} M insulin for 48 h and supernatants were then assayed for SAA using an ELISA. SAA concentrations are expressed as ng/ml per 48 h supernatant from 10^6 cells. Mean ± s.e.m. for four replicates.

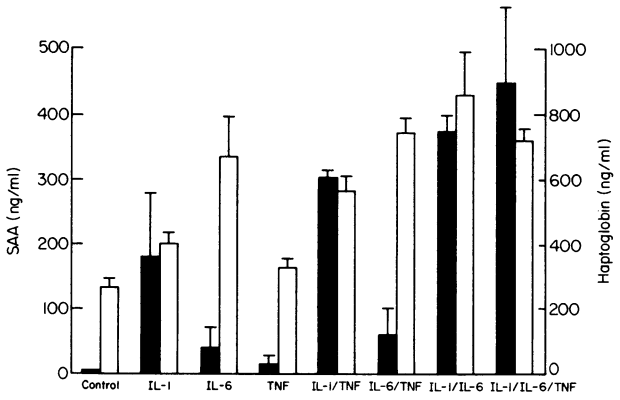

Fig. 2. Effects of IL-1, TNF and IL-6 on SAA (■) and haptoglobin (□) synthesis by HuH-7 cells. HuH-7 cells were grown to confluence in 24-well plates and stimulated with rIL-6 (200 U/ml), rIL-1β (10 ng/ml) and TNF (10 ng/ml). SAA and haptoglobin were measured by ELISA and expressed as ng/ml per 48 h per 10^6 cells. Mean ± s.d. for six replicates.

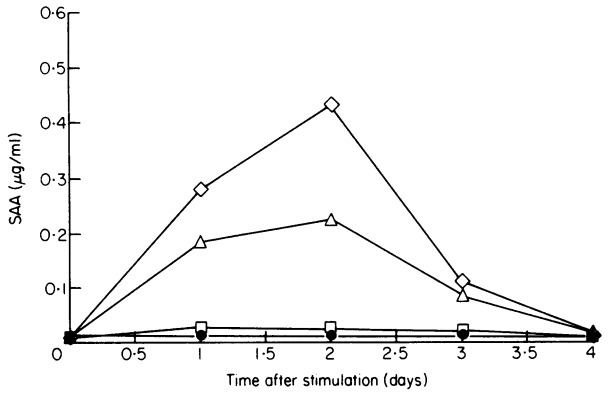

Fig. 3. Time course of synthesis of SAA by HuH-7 cells after cytokine stimulation. Medium was changed every 24 h and assayed for SAA as previously described (SAA expressed as μg/ml per 24 h per 10^6 cells). Stimulation on the first day only was with IL-1β (10 ng/ml) and IL-6 (10 U/ml) (◇), IL-1β alone (△), IL-6 alone (□) or control unstimulated (●). Mean ± s.d. of six replicates.

affected by IL-6, as shown previously (Morrone *et al.*, 1988). As shown previously (Baumann *et al.*, 1987), SAA synthesis was increased in the presence of dexamethasone (results not shown) which was included at 0·1 μM in all cultures in this report. The lack of stimulation by IL-6 prompted us to examine the response of another acute-phase protein which was found to be under IL-6 regulation in other human cell lines (Heinrich *et al.*, 1989). As shown in Fig. 2, it was apparent that the cells were fully responsive to IL-6 since haptoglobin was induced approximately three-fold by IL-6 but only 50% by IL-1.

To determine whether the time taken for the cells to respond was similar to that observed after *in vivo* stimulation, we looked at SAA concentrations at daily intervals (Fig. 3). Synthesis of SAA was similar on day 1 and 2 suggesting a peak between 24 h and 48 h after stimulation. It was apparent that the rate at which the SAA synthesis returned to normal was consistent with the

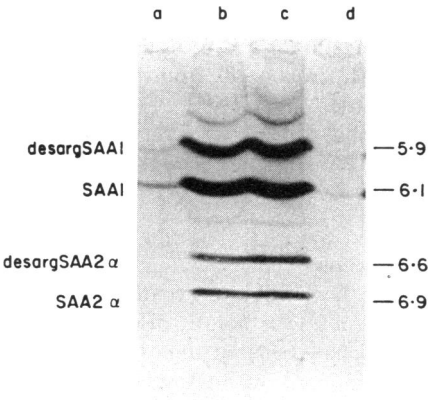

Fig. 4. Isoelectric focusing of SAA extracted from HuH-7 supernatants. HuH-7 cells were stimulated with optimal concentrations of cytokines and SAA was extracted as described in Materials and Methods. Lane d shows an unstimulated supernatant, lane a was stimulated with IL-6 (20 U/ml), lane b with IL-1β (10 ng/ml), lane c with IL-1α (10 ng/ml).

rapid return to normal levels seen *in vivo* following an inflammatory stimulus such as after surgery. The half-life of SAA in plasma has been estimated at less than 24 h (Bausserman *et al.*, 1984).

To confirm the specificity of the response, monoclonal antibody to IL-1α (M3) was pre-incubated with the cytokine for 1 h and added to the cultures. The response to 1 ng/ml of IL-1α could be inhibited 85% with mouse monoclonal antibodies to IL-1α (25 μg/ml). Rabbit polyclonal antibody to IL-6 did not reduce the response to IL-1.

Using isoelectric focusing of culture supernatants extracted with octyl sepharose (Raynes & McAdam, 1988) it is shown that both SAA1 and SAA2α were induced in response to IL-1α or IL-1β and weakly in response to IL-6 (Fig. 4). The isotype SAA2β was not synthesized by the HuH-7 cell line.

DISCUSSION

The HuH-7 human hepatoma cell line is comparatively well differentiated in comparison with, e.g. PLC/PRF/5 (Nakabayashi *et al.*, 1982); we have been able to show in this cell line that SAA protein is regulated by IL-1. The obvious conflict between our results and those with the human primary liver culture (Castell *et al.*, 1989) and hepatoma cell lines which suggested that IL-6 was the major stimulator of SAA (Kushner *et al.*, 1989) led us to examine the induction of haptoglobin, a known IL-6-responsive gene in other human hepatoma cell lines. However, the ability of the HuH-7 cell line to express IL-6 receptors and respond to IL-6 was shown by the fact that haptoglobin was induced about three-fold by IL-6 (Fig. 2). It has been suggested that IL-1 exerts its activity through stimulating IL-6 synthesis in hepatoma cell lines, but the observations that IL-6 alone is a poor inducer of SAA even at 48 h, while it produces a maximal haptoglobin response, and that antibody to IL-6 does not inhibit IL-1 induced SAA synthesis, suggest that IL-6 is of secondary importance to IL-1 in the stimulation of acute-phase SAA synthesis in this cell line. The demonstration of IL-1 activity in stimulating SAA synthesis is in agreement with the 50-fold stimulation of SAA mRNA in transfected L

cells observed by Woo *et al.* (1988) and their more recent experiments with a human SAA–CAT construct transfected into Hep G-2 cells which showed PMA stimulation of CAT acting through a nuclear factor kB-like factor (Edbrooke *et al.*, 1989). It is not clear how these data compare with the primary hepatocyte results which suggest that IL-1 is without effect (Castell *et al.*, 1988, 1989; Kushner *et al.*,1989). However, it may be that the hepatocyte isolation procedure was sufficient to stimulate the cells by the IL-1-dependent mechanism, a possibility reinforced by consideration of the high levels of SAA seen in unstimulated primary cultures. In the experiments with human primary cells the albumin was reduced by IL-6, a result not seen in the hepatoma cells. Evidence for *in vivo* activity of IL-1 comes from the observation that IL-1 caused SAA synthesis much faster than endotoxin (Sipe & Ramadori, 1986).

It is apparent that the high rate of synthesis of SAA combined with the inherent advantages of a homogeneous cell population will make this an important model for studying transcriptional and post-transcriptional regulation of the acute-phase protein response. We have described how in this cell line, SAA is regulated by IL-1 with lesser contributions from IL-6 and TNF. For the reasons stated previously, these data support the notion that IL-1 may be the major *in vivo* stimulatory cytokine for SAA.

ACKNOWLEDGMENTS

We thank the Arthritis Research Council and Wellcome Trust for supporting the research covered in this report, and all those who generously provided reagents.

REFERENCES

BAUMANN, H. & WONG, G.G. (1989) Hepatocyte-stimulating factor III shares structural and functional identity with leukemia-inhibitory factor. *J. Immunol.* **143,** 1163.

BAUMANN, H., RICHARDS, C. & GAULDIE, J. (1987) Interaction among hepatocyte stimulating factors, interleukin 1 and glucocorticoids for regulation of acute phase plasma proteins in human hepatoma (Hep G2) cells. *J. Immunol.* **139,** 4122.

BAUSSERMAN, L., HERBERT, P.N., RODGER, R. & NICOLOSI, R.J. (1984) Rapid clearance of serum amyloid A from high density lipoproteins. *Biochim. biophys. Acta*, **792,** 186.

CASTELL, J.V., GOMEZ-LECHON, M.J., DAVID, M., ANDUS, T., GEIGER, T., TRULLENQUE, R., FABRA, R. & HEINRICH, P.C. (1989) Interleukin-6 is the major regulator of acute phase protein synthesis in adult human hepatocytes. *FEBS Lett.* **242,** 237.

CASTELL, J.V., GOMEZ-LECHON, M.J., DAVID, M., HIRANO, T., KISHIMOTO, T. & HEINRICH, P.C. (1988) Recombinant human interleukin 6 (IL-6/BSF-2/HSF) regulates the synthesis of acute phase proteins in human hepatocytes. *FEBS Lett.* **232,** 347.

EDBROOKE, M.R., BURT, D.W., CHESHIRE, J.K. & WOO, P. (1989) Identification of cis-acting sequences responsible for phorbol ester induction of human serum amyloid A gene expression via a nuclear factor kB-like transcription factor. *Mol. Cell. Biol.* **9,** 1908.

GAULDIE, J., RICHARDS, C., HARNISH, D., LANSDORF, P. & BAUMANN, H. (1987) Interferon β-2/B-cell stimulatory factor type 2 shares identity with monocyte derived hepatocyte stimulating factor and regulates the major acute phase response in liver cells. *Proc. natl Acad. Sci. USA*, **84,** 7251.

GODENIR, N.L., JEENAH, M.S., GOETZEE, G.A., VAN DER WESTERHUYZEN, D.R., STRACHAN, A.F. & DE BEER, F.C. (1985) Standardisation of the quantitation of serum amyloid A protein in human serum. *J. immunol. Methods*, **83,** 217–225.

HEINRICH, P.C., CASTELL, J.V. & ANDUS, T. (1989) Interleukin-6 and the acute phase response. *Biochem. J.* **265,** 621.

KUSHNER, I., GANAPATHI, M. & SCHULTZ, D. (1989) The acute phase response is mediated by heterogeneous mechanisms. *Ann. NY Acad. Sci.* **557,** 19.

MORRONE, G., CILIBERTO, G., OLIVIERO, S., ARCONE, R., DENTE, L., CONTENT, J. & CORTESE, R. (1988) Recombinant IL-6 regulates the transcriptional activation of a set a human acute phase genes. *J. biol. Chem.* **263,** 12554.

NAKABAYASHI, H., TAKETA, K., MIYANO, K., YAMNE, T. & SATO, J. (1982) Growth of human hepatoma cell lines with differentiated functions in chemically defined medium. *J. Cancer Res.* **42,** 3858.

PEPYS, M.B. & BALTZ, M.L. (1983) Acute phase proteins with special reference to C-reactive protein and related proteins (pentraxins) and serum amyloid A. *Adv. Immunol.* **34,** 141.

RAYNES, J.G. & MCADAM, K.P.W.J. (1988) Purification of serum amyloid A and other high density apolipoproteins by hydrophobic interaction chromatography. *Anal. Biochem.* **157,** 167.

SEHGAL, P.B. (1990) Interleukin-6: a regulator of plasma protein gene expression in hepatic and non-hepatic tissues. *Mol. Biol. Med.* **7,** 117.

SELINGER, M.J., MCADAM, K.P.W.J., KAPLAN, M.M., SIPE, J.D., VOGEL, N. & ROSENSTRICH, D.L. (1980) Monokine induced synthesis of serum amyloid A protein by hepatocytes. *Nature*, **285,** 498.

SIPE, J.D. & RAMADORI, G. (1986) Sites of SAA/AA synthesis. In *Amyloidosis* (ed. by J., Marrink & M. H. van Rijswijk), p. 319. Martinus Nijhoff, Dordrecht.

WOO, P., SIPE, J., DINARELLO, C.A. & COLTEN, R.A. (1987) Structure of a human serum amyloid A gene and modulation of its expression in transfected L cells. *J. biol. Chem.* **262,** 15790.