Vol. 157, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1988 Pages 271-277

ROLE OF INTERLEUKIN-6 IN REGULATING SYNTHESIS OF C-REACTIVE PROTEIN AND SERUM AMYLOID A IN HUMAN HEPATOMA CELL LINES

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Received September 29, 1988

Previous studies have demonstrated that synthesis of the two major human acute phase proteins, C-reactive protein (CRP) and serum amyloid A (SAA), by human hepatoma cell lines was not affected by preparations of interleukin-1 (IL-1) or tumor necrosis factor α but was increased after exposure to conditioned medium from lipopolysaccharide-activated monocytes. We report that neutralizing antibodies raised against E. coli-derived recombinant human interleukin-6 (IL-6) were capable of inhibiting induction of CRP and SAA by monocyte conditioned medium in both NPLC/PRF/5 and Hep 3B cell lines. Partially purified IL-6 from lipopolysaccharide-stimulated human fibroblasts and recombinant derived IL-6 induced both CRP and SAA synthesis in a concentration dependent manner in the NPLC/PRF/5 cell line. In contrast, in Hep 3B cells, IL-6 alone had no discernable effect on the induction of either CRP or SAA, but was capable of causing increased synthesis of α_1-protease inhibitor and fibrinogen and reduced synthesis of albumin. The addition of IL-1 to IL-6 led to induction of both CRP and SAA in Hep 3B cells, but did not augment the response of either CRP or SAA in NPLC/PRF/5 cells. These studies indicate that IL-6 plays a central role in induction of both CRP and SAA synthesis in the two human hepatoma cell lines. The different observations in the two hepatoma lines we studied likely reflect differences between these transformed cell lines in genetic regulatory mechanisms or other intracellular mechanisms by which extracellular signals affect expression of the CRP and SAA genes. Furthermore, our findings suggest that the mechanisms by which IL-6 regulates synthesis of CRP and SAA in Hep 3B cells differ from those involved in induction of α_1-protease inhibitor and fibrinogen and in inhibition of albumin synthesis.

Tissue injury and infection are followed, in vertebrates, by reorchestration of the pattern of gene expression of plasma proteins in

Abbreviations: CRP, C-reactive protein; SAA, serum amyloid A; IL-1, interleukin-1; IL-6, interleukin-6; BSF-2, B-cell stimulatory factor-2, IFN B2, Interferon B2; SDS, sodium dodecyl sulfate.

0006-291X/88 $1.50

hepatocytes; the resulting changes in plasma concentrations are collectively referred to as the acute phase response (1-3). In humans, these changes are characterized by decreased synthesis of albumin and several other "negative" acute phase proteins, modest increases in synthesis of a number of positive acute phase proteins and most strikingly, by dramatic increases in synthesis (up to 1000 fold or more) of the two major acute phase proteins, C-reactive protein (CRP) and serum amyloid A (SAA). These changes can be induced in a variety of different cell culture systems by unfractionated conditioned medium from activated monocytes (4-9). Two defined cytokines, interleukin-1 (IL-1) and tumor necrosis factorα (TNFα), have been previously shown capable of inducing synthesis of a number of acute phase proteins (4,6,8-13). It has recently become clear (6) that another cytokine capable of acute phase protein induction, monocyte-derived hepatocyte stimulating factor, is a product of the same gene which codes for proteins variously described as interferon B2 (IFN B2), B-cell stimulatory factor-2 (BSF-2), hybridoma plasmacytoma growth factor, 26 kDa protein and interleukin-6 (IL-6) (14,15). This cytokine has emerged as a major mediator of the acute phase response (6,7,16-19).

We and others have shown that recombinant derived preparations of IL-1 and TNFα, although capable of inducing some acute phase protein changes in human hepatoma cell lines, do not induce CRP (4,5,9,11) or SAA (9,11) in such systems. Recent reports of the possible role of IL-6 in induction of CRP and SAA describe contradictory findings. Two studies employing human primary hepatocyte cultures suggest that IL-6 enhances synthesis of both CRP and SAA (19,20). In contrast, studies in the human hepatoma cell line Hep 3B revealed that IL-6 had a minimal effect in enhancing transcription of a recombinant plasmid carrying the CRP 5' flanking region fused to a reporter gene (21). SAA was not examined in the latter study. The present study was undertaken to elucidate the role played by IL-6 in induction of CRP, SAA and certain other acute phase proteins, employing two human hepatoma cell lines as model systems. We report marked differences between two different cell lines in the responses of the CRP and SAA genes.

MATERIALS AND METHODS

Monocyte conditioned medium, used as a positive control for all experiments, was prepared as previously described (9). Recombinant BSF-2/IL-6 and antibodies against BSF-2/IL-6, obtained from Drs. T. Kishimoto and T. Hirano, Osaka University, Osaka, Japan, were employed in some experiments, as indicated in the figure legends. In other experiments, we used partially purified IFN B2/IL-6 from human foreskin fibroblasts (FS-4), E. coli derived recombinant human IFN B2/IL-6 and rabbit antiserum containing antibodies against IL-6, prepared as reported earlier (18,22). Induction of acute phase protein changes observed with the two different recombinant IL-6 preparations were comparable. Recombinant IL-1α and IL-1β were from Genzyme, Boston, MA.

The human hepatoma cell lines NPLC/PRF/5 and Hep 3B were generous gifts of Dr. Barbara Knowles, Wistar Institute, Philadelphia, PA and Dr. Gretchen

Darlington, Baylor College of Medicine, Houston, TX, respectively. The cells were maintained and induced with cytokines as described earlier (9) with minor modifications; induction was carried out in 35 mm dishes and cells were labelled with 0.1 mCi Tran [^{35}S]-label (L-Methionine [^{35}S]:L-cysteine [^{35}S]) (ICN, Irvine, CA). Total volume of the incubation mixture and of the subsequent labelling medium was 1.0 ml. For experiments involving SAA determinations in the NPLC/PRF/5 cell line, inductions were carried out in 60 mm tissue culture dishes employing conditions described previously (9).

Radiolabelled CRP, α_1-protease inhibitor and albumin were analyzed by immunoprecipitation and sodium dodecyl sulfate (SDS) polyacrylamide-gel electrophoresis (9). Aliquots of 0.8, 0.05 and 0.1 ml of the [^{35}S]-methionine labelled medium were used for immunoprecipitation of CRP, α_1-protease inhibitor and albumin, respectively. For immunoprecipitation of SAA, [^{35}S]-methionine labelled medium (0.1 ml for Hep 3B and 0.3 ml for NPLC/PRF/5 cells) was diluted 1:1 with 20 mM Tris pH 7.4, 0.15 M NaCl, 2% Triton X-100, 2% sodium deoxycholate and 5 mg/ml bovine serum albumin, and incubated overnight at 4°C in the presence of 3 µl of rabbit anti-human SAA (Calbiochem, La Jolla, CA). The antigen-antibody complex was isolated according to the procedure of Auerbach et al. (23), with the exception that 13 mM Tris pH 7.4, 0.1 M NaCl was substituted for PBS, and subjected to SDS polyacrylamide-gel electrophoresis.

RESULTS

Monocyte conditioned medium enhanced CRP and SAA synthesis in both NPLC/PRF/5 and Hep 3B cells (Fig. 1). We examined the ability of antibodies directed against human recombinant derived IL-6 to inhibit the enhancement of CRP and SAA synthesis by conditioned medium in these two cell lines. Induction of CRP and SAA by conditioned medium was completely inhibited by antibodies to rIL-6 in both lines (Fig. 1, lanes 1-3). Induction of α_1-protease inhibitor (Fig. 2, lanes 1-3) and fibrinogen and decrease in synthesis of albumin (data not shown) by conditioned medium were also completely inhibited by anti-IL-6 in Hep 3B cells.

We next determined whether IL-6 alone directly affected synthesis of CRP and SAA. Both partially purified IL-6 and rIL-6 by themselves increased synthesis of CRP and SAA in the NPLC/PRF/5 cell line (Fig. 1, lanes 4 and 5). The induction of these two APP was inhibited by antibodies to IL-6. Induction of both CRP and SAA by IL-6 in the NPLC/PRF/5 cell line was concentration dependent. In contrast, none of the three preparations of IL-6 by themselves at similar concentrations led to an increase in synthesis of CRP or SAA in the Hep 3B cell line (Fig. 1, lanes 4 and 5). Recombinant IL-6 alone was, however, capable of inducing other acute phase protein changes in Hep 3B cells; this cytokine induced synthesis of α_1-protease inhibitor (Fig. 2) and fibrinogen and decreased albumin synthesis in these cells. In each instance the effect of IL-6 was inhibited by anti-rIL-6.

Studies of the effect of IL-1 alone or in combination with IL-6 on induction of CRP and SAA revealed that IL-1α or IL-1β by themselves did not induce either CRP or SAA in either cell line (Fig. 3, lanes 3 and 4). In the NPLC/PRF/5 cell line, addition of either IL-1α or IL-1β together with IL-6 did

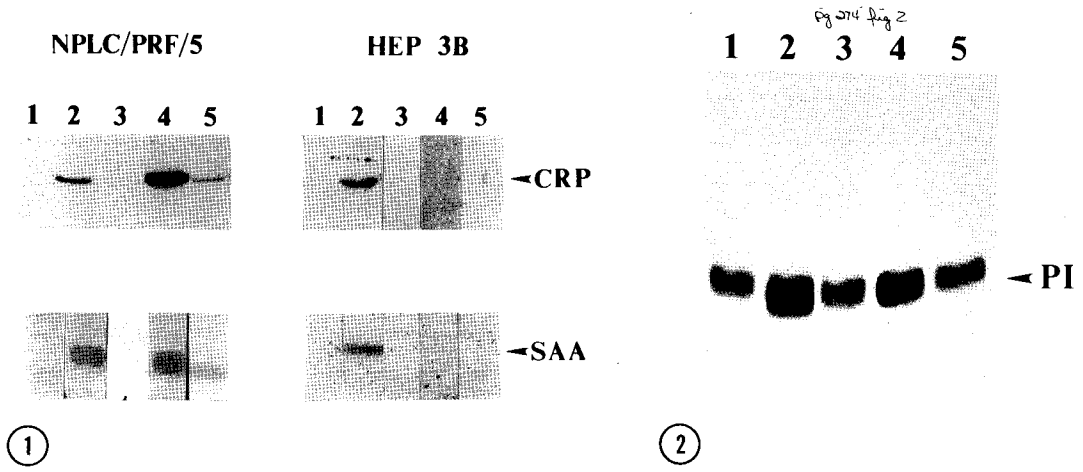

Figure 1. Response of CRP and SAA to monocyte-conditioned medium treated with antibodies to E. coli derived IL-6 and to partially purified and recombinant-derived preparations of IL-6 in human hepatoma cell lines. The NPLC/PRF/5 and Hep 3B cell lines were incubated for 18-24h in the absence (lane 1) or presence of 20% conditioned medium (lane 2), 20% conditioned medium treated with antibodies to recombinant-derived IL-6 diluted 1:40 (lane 3), 50 ng/ml partially purified IL-6 from human foreskin fibroblasts (lane 4) and rIL-6 (lane 5). Following the initial incubation period, medium was removed and cells labelled with Tran [^{35}S]-label for 4h as desribed under "Materials and Methods." Radiolabelled CRP and SAA secreted into culture medium were analyzed by immunoprecipitation and SDS polyacrylamide gel electrophoresis as described under "Materials and Methods." For CRP analysis in NPLC/PRF/5 cells, recombinant-derived IL-6 from Drs. T. Kishimoto and T. Hirano was employed at a final concentration of 5 ng/ml (lane 5). In the rest of the experiments recombinant-derived IL-6 was employed at a concentration of 200 ng/ml.

Figure 2. Effect of recombinant-derived IL-6 and monocyte conditioned medium treated with antibodies to IL-6 on the induction of α_1-protease inhibitor (PI) in Hep 3B cells. Hep 3B cells were incubated in the absence (lane 1) or presence of 20% conditioned medium (lane 2), 20% conditioned medium plus 50 ng/ml anti-IL-6 (lane 3), 5 ng/ml IL-6 (lane 4), 5 ng/ml IL-6 plus 50 ng/ml anti IL-6 (lane 5). All preparations of rIL-6 and anti IL-6 were from Drs. T. Kishimoto and T. Hirano. Cells were labelled with Tran [^{35}S]-label as described in the legend to Figure 1 and α_1-protease inhibitor (PI) was immunoprecipitated and subjected to SDS polyacrylamide gel electrophoresis as described under "Materials and Methods".

not further increase the response of CRP or SAA above that observed with IL-6 alone (Fig. 3, lanes 6 and 7). In Hep 3B cells, however, the combination of IL-1α or IL-1β with IL-6 led to a substantial induction of both CRP and SAA synthesis (Fig. 3, lanes 6 and 7).

DISCUSSION

Interleukin-6 has emerged as a major hepatocyte stimulating factor; this cytokine has been shown to alter synthesis of a large number of acute phase proteins in both human and rat systems, including that of several plasma proteins whose synthesis is not modulated by IL-1 or TNFα (6-8,19-21). In this study we demonstrate that IL-6 plays an important role in regulating synthesis and secretion of CRP and SAA in the two human hepatoma cell lines NPLC/PRF/5 and Hep 3B. Studies using antibodies to E. coli-derived

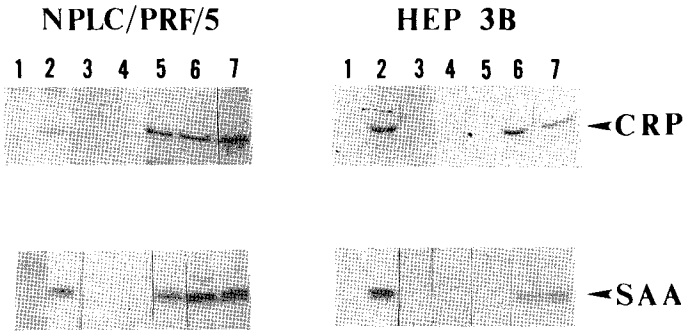

Figure 3. Effect of combination of IL-1 and IL-6 on synthesis and secretion of CRP and SAA in human hepatoma cell lines. NPLC/PRF/5 and Hep 3B cell lines were treated in the absence (lane 1) or presence of 20% conditioned medium (lane 2), 0.2 ng/ml IL-1α (lane 3), 0.2 ng/ml IL-1β (lane 4), 200 ng/ml rIL-6 (lane 5), 0.2 ng/ml IL-1α plus 200 ng/ml rIL-6 (lane 6), and 0.2 ng/ml IL-1β plus 200 ng/ml rIL-6 (lane 7), and labelled with Tran [^{35}S]-label for 4h as described under "Materials and Methods." CRP and SAA were analyzed as described in the legend to Figure 1.

recombinant IL-6 indicated that a cytokine present in monocyte conditioned medium that is required for induction of SAA and CRP in both lines is antigenically related or identical to IL-6. While IL-6 preparations alone were capable of inducing CRP and SAA in the NPLC/PRF/5 cell line, they were unable to do so in Hep 3B cells. However, the combination of IL-6 with IL-1 was capable of inducing these acute phase proteins in Hep 3B cells. These observations reveal a strong cell line-dependent influence on the ability of IL-6 to enhance gene expression of CRP and SAA.

Hep 3B cells were, however, capable of responding to IL-6 in the case of other acute phase proteins. Whereas induction of CRP and SAA in this cell line required both IL-6 and IL-1, IL-6 alone was sufficient to increase synthesis of α_1-protease inhibitor and fibrinogen and to decrease albumin synthesis in these cells. Thus, intracellular mechanisms by which IL-6 enhances CRP and SAA expression can be dissociated from those regulating gene expression of α_1-protease inhibitor, fibrinogen and albumin in Hep 3B cells.

Previous studies of SAA induction have yielded contradictory findings. IL-1 alone has previously been shown to induce SAA in both mouse and rabbit primary hepatocyte cultures (10,25) and in mouse fibroblasts transfected with the human SAA gene (26). However, Il-1 alone did not induce SAA in human hepatoma cell lines (9,11). Recently, induction of SAA by IL-6 in human primary heptocyte cultures has been reported (19,20). One possible explanation for these inconsistent findings is that induction of SAA by IL-1 in the former group of studies may result from induction of IL-6 in the transfected fibroblasts or the nonparenchymal cells which ordinarily contaminate primary hepatocyte cultures. Indeed, increased expression of IL-6 in response to IL-1 has been reported in fibroblasts (27-28). Our findings in

a model system consisting of two different hepatoma cell lines, in which the possibility of secondary induction of cytokines in extraneous cell types is eliminated, indicate that IL-1 alone is not capable of induction of SAA and that IL-6 plays a central role in induction of this acute phase plasma protein.

Similarly, differing conclusions about the effect of IL-6 on CRP induction have been reported. Although the CRP response to IL-6 was substantial in human primary hepatocyte cultures (19,20) and in hepatoma cells harboring plasmids containing the CRP promoter (29), only a minimal transcriptional effect of IL-6 on the CRP upstream regulatory sequences was reported in transfected Hep 3B cells (21). Possible explanations for this discrepancy are: 1) the likelihood that contaminating non-parenchymal cells may serve as a source of other cytokines and 2) the recognition that transformed cell lines are abnormal, and may differ from normal cells in some of the pathways by which extracellular signals are transmitted to the gene.

While we observed no effect of IL-6 alone on CRP synthesis and secretion by Hep 3B cells, Morrone et al (21) found a modest effect of IL-6 on expression of a transfected reporter gene fused to the CRP 5' flanking region. It may be that such an apparent difference would be expected when two such vastly different assay systems are employed. On the other hand, there are a number of instances in which different laboratories report conflicting findings in what is presumably the same cell line. For example, in one of our laboratories, IL-1 does not induce α_1-antichymotrypsin in Hep 3B cells (30), while in the other laboratory, IL-1 does induce this acute phase protein (9). It is striking that Morrone et al (21), in contrast to our results, found that IL-6 failed to influence α_1-protease inhibitor or albumin synthesis in their stock of Hep 3B cells.

In summary, our findings clearly show that IL-6 plays a critical role in induction of CRP and SAA. However, it is not clear whether IL-6 alone or IL-6 plus IL-1 is required for CRP and SAA induction *in vivo*, or indeed, whether other cytokines might not be required as well, for a maximal response. Also, these studies do not address the issue of whether some of the forms of IL-6 (17,22) are more potent than others in induction of CRP or SAA.

ACKNOWLEDGEMENTS

We thank Beverly A. Hodgdon for her excellent technical assistance and Judy Smothers for her excellent secretarial assistance.

This work was supported by U.S. Public Health Service Grants AG-02467 and AI-16262, Irma Bender Arthritis Research Fund of the Cuyahoga County Hospital Foundation, Ohio Board of Regents, Research Initiation Grant BBR WM72428, Case Western Reserve University, Cuyahoga County Hospital Foundation Grant IMED-203, Cancer Center Grant P30 CA43703, a Contract from the National Foundation for Cancer Research and an established investigatorship from the American Heart Association (P.B.S.).

REFERENCES

1. Kushner, I. (1982) Ann. N.Y. Acad. Sci. 389, 39-48.
2. Kushner, I., and Mackiewicz, A. (1987) Dis. Markers 5, 1-11.
3. Fey, G. H., and Fuller, G. M. (1987) Mol. Biol. Med. 4, 323-338.
4. Darlington, G. J., Wilson, D. R., and Lachman, L. B. (1986) J. Cell Biol. 103, 787-793.
5. Goldman, N. D., and Liu, T.-Y. (1987) J. Biol. Chem. 262, 2363-2368.
6. Gauldie, J., Richards, C., Harnish, D., Lansdorp, P., and Baumann, H. (1987) Proc. Natl. Acad. Sci. USA 84, 7251-7255.
7. Andus, T., Geiger, T., Hirano, T., Northoff H., Ganter U., Bauer, J., Kishimoto, T., and Heinrich, P. C. (1987) FEBS Lett. 221, 18-22.
8. Baumann, H., Richards C., and Gauldie, J. (1987) J. Immunol. 139, 4122-4128.
9. Ganapathi, M. K., Schultz, D., Mackiewicz, A., Samols, D., Hu, S.-I., Brabenec, A., Macintyre, S. S., and Kushner, I. (1988) J. Immunol. 141, 564-569.
10. Ramadori, G., Sipe, J. D., Dinarello, C. A., Mizel, S. B., and Colten, H. R. (1985) J. Exp. Med. 162, 930-942.
11. Perlmutter, D. H., Dinarello, C. A., Punsal, P. I., and Colten, H. R. (1986) J. Clin. Invest. 78, 1349-1354.
12. Geiger, T., Andus, T., Klapproth, J., Northoff H., and Heinrich, P. C. (1988) J. Biol. Chem. 263, 7141-7146.
13. Andus, T., Heinrich, P. C., Bauer J., Tran-Thi, T.-A., Decker, K., Männel, D., and Northoff, H. (1987) Eur. J. Immunol. 17, 1193-1197.
14. Sehgal, P. B., May, L. T., Tamm, I., and Vilcek, J. (1987) Science 235, 731-732.
15. Wong, G. G., and Clark, S. C. (1988) Immunol. Today 9, 137-139.
16. May, L. T., Santhanam, U., Tatter, S. B., Ghrayeb, J., and Sehgal, P.B. in Tumor necrosis factor/cachetin, lymphotoxin and related cytokines. Proc. of an International Conf. Heidelberg Sept. 14-18, 1987 (Bonarida, B., and Kirchner, H., eds) S. Karger, Basel (1988) in press.
17. May, L. T., Ghrayeb J., Santhanam, U., Tatter, B., Sthoeger, Z., Helfgott, D. C., Chiorazzi, N., Grieninger, G., and Sehgal, P. B. (1988) J. Biol. Chem. 263, 7760-7766.
18. Sehgal, P. B., Helfgott, D.C., Santhanam, U., Tatter, S.B., Clarick, R.H., Ghrayeb, J., and May, L. T. (1988) J. Exp. Med. 167, 1951-1956.
19. Castell, J. V., Gomez-Lechon, M. J., David, M., Hirano, T., Kishimoto, T., and Heinrich, P. C. (1988) FEBS Lett. 232, 347-350.
20. Moshage, H. J., Roelofs, H. M. J., van Pelt, J. F., Hazenberg, B. P. C., van Leeuwen, M. A., Limburg P. C., Aarden, L. A., and Yap, S. H. (1988) Biochem. Biophys. Res. Comm. 155, 112-117.
21. Morrone, G., Ciliberto, G., Oliviero, S., Arcone, R., Dente, L., Content, J., and Cortese, R. (1988) J. Biol. Chem. 263, 12554-12558.
22. Santhanam, U., Tatter, S. B., Helfgott D. C., Ray, A., Ghrayeb J., May, L. T., and Sehgal, P. B. (1988) in Monokines and other non-lymphocytic cytokines (Powanda, M. C., Oppenheim, J., Kluger, M. J., and Dinarello, C.A., eds.) Vol. 8, pp 29-34, Alan R. Liss, Inc., New York.
23. Auerbach, H. S., Baker, R. D., Mathews Jr., W. J., and Colten, H. R. (1984) J. Exp. Med. 159, 1750-1761.
24. Laurell, C. B. (1972) Scand. J. Clin. Lab. Invest., 29(Suppl. 124), 21-23.
25. Mackiewicz, A., Ganapathi, M. K., Schultz, D., Samols, D., Reese, J., and Kushner, I. (1988) Biochem. J., 253, 851-857.
26. Woo P., Sipe, J., Dinarello, C. R., and Colten, H.R. (1987) J. Biol. Chem., 262, 15790-15795.
27. Kohase, M., May, L. T., Tamm, I., Vilcek J., and Sehgal, P. B. (1987) Mol. Cell Biol. 7, 273-280.
28. Walther, Z., May, L. T., and Sehgal, P. B. (1988) J. Immunol. 140, 974-977.
29. Li, S. P., Liu, T.-Y., and Goldman, N. D. (1988) FASEB J., 2, A1246.
30. Helfgott, D. C., Tatter, S. B., Santhanam, U., Clarick, R. H., Bhardwaj, N., May, L. T., and Sehgal, P. B. submitted to J. Immunol.