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DIFFERENTIAL CYTOKINE-MEDIATED MODULATION OF ENDOCYTOSIS IN RAT LIVER ENDOTHELIAL CELLS

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We have studied the effects of exogenous tumor necrosis factor-α, interleukin-1β, and lipopolysaccharide-induced interleukin-1β on receptor-mediated endocytosis in primary cultures of liver sinusoidal endothelial cells. Tumor necrosis factor-α and interleukin-1β enhanced endocytosis via the scavenger and mannose receptors in a dose-time dependent manner, while the function of the collagen receptor remained unaffected. The modulatory effects of the cytokines were blocked by adding specific inhibitors for both cytokines. Results from studies on binding (4°C) and internalization and degradation (37°C) of ligands indicate that the increased scavenger capacity of liver sinusoidal endothelial cells resulting from exposure to the inflammatory mediators was due to increased rate of intracellular transport of endocytosed ligands to lysosomes, rather than to increased binding to receptors. © 1995 Academic Press, Inc.

Substantial amounts of soluble connective tissue macromolecules and intracellular components are released daily from the tissues of the body during the normal catabolism of cells and matrix. For instance, a normal adult human turns over several grams of collagen and hyaluronan per day (1, 2). Recent findings suggest that only a small proportion of these substances are degraded locally. Instead, the elimination takes place distantly, in specialized non-macrophagic reticuloendothelial cells of the lymph node and the liver sinusoids (3). Thus waste macromolecules that are not eliminated by the lymph nodes, enter the general circulation and are effectively removed by receptor-mediated endocytosis in the sinusoidal endothelial cells (SEC) of the liver.

At sites of inflammation, the catabolism of cells and matrix molecules is significantly elevated, resulting in an increased flux of waste macromolecules to the circulation. Under such conditions, upregulation of the endocytic activity of SEC would be required to prevent accumulation of

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Abbreviations: TNF-α, tumor necrosis factor-α; IL-1ß, interleukin-1ß; LPS, lipopolysaccharide; SEC, hepatic sinusoidal endothelial cells; FSA, formaldehyde-treated bovine serum albumin; IL-1Ra, interleukin-1 receptor antagonist.

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harmful waste material in the circulation. The inflammatory responses are mediated by a variety of signalling proteins produced locally by several kinds of white blood cells or by complement activation. In line with this concept is the finding that the liver macrophages, or Kupffer cells produce major inflammatory mediators such as interleukin-1ß (IL-1β) and tumor necrosis factor-α (TNF-α) (4). Also SEC can be stimulated to produce IL-1β and a few other mediators (5). In the present study, we investigated the effects of exogenous TNF-α and IL-1ß, and lipopolysaccharide (LPS) induced (endogenous) IL-1ß on receptor mediated endocytosis in primary cultures of SEC. Our findings indicate that these inflammatory cytokines bring about upregulation of endocytosis via selected receptors in rat SEC.

MATERIALS AND METHODS

Chemicals – BSA and collagenase were purchased from Sigma Chem. Co., (St. Louis, MO, U. S. A.). Human serum albumin and Percoll were from CRTS (Lille, France) and Pharmacia Fine Chemicals (Uppsala, Sweden), respectively. Carrier free $Na^{125}I$ was from Institutt for Energiteknikk, (Kjeller, Norway). Cell culture RPMI 1640 and fetal calf serum was purchased from Gibco (Grand Island, NY, U.S.A.) and HyClone (Logan, UT, U.S.A.) respectively.

Inflammatory mediators and inhibitors – Human recombinant TNF-α (specific activity: 1.108 U/mg) and recombinant IL-1ß (specific activity: 5.107 U/mg) were purchased from Boehringer Mannheim (Mannheim, Germany) and contained less than 10 EU/mg LPS as determined by Limulus assay. IL-1Ra was provided by Prof. Charles A. Dinarello (New England Medical Center, Boston, MA, U. S. A.). Polyclonal anti hTNF-α was purchased from Genzyme (Boston, MA, U. S. A.)

Ligands – Mannan was from Sigma Chem. Co. (St. Louis, USA). Cod skin collagen was prepared as described (6). Formaldehyde-treated bovine serum albumin (FSA) were prepared as described (7).

Labeling techniques – The three types of ligands were labeled with [^{125}I] by a direct reaction employing Iodobeads (Pierce, Rockford, IL, U. S. A.), according to instructions provided by the producer. The labeled proteins were purified free of unlabeled [^{125}I] on a PD-10 column (prepacked Sephadex G-25 from Pharmacia Fine Chemical, Uppsala, Sweden) eluted with PBS. This procedure resulted in a specific radioactivity of $3x10^6$ cpm/µg. Radioactivity was measured using a gamma-counter (Auto Gamma Scintillator Spectrometer; Packard Instruments, Warrenville, IL, U. S. A.).

Isolation of liver endothelial cells – Preparation of pure cultures of functionally intact LEC from rat liver has been described elsewhere (8). After collagenase perfusion of the liver, and isopycnic centrifugation of the resulting dispersed cells through a two-step density gradient of Percoll, pure monolayer cultures of LEC were established by selective attachment on substrates of fibronectin. Liver parenchymal cells were separated from sinusoidal cells by sedimentation through 50% Percoll.

Stimulation procedure – 90-95% pure monolayer cultures of SEC were established in 24-well dishes (approx. $5x10^5$ cells attached and spread per well). After 15 h of incubation in RPMI+10% fetal calf serum, the cells were washed and treated with the inflammatory mediators in RPMI 1640 with 1% human serum albumin for 6 h. Endocytosis experiments were carried out as explained below.

Endocytosis studies – SEC cultures were washed and supplied with fresh medium containing 1% human serum albumin and trace amounts of radiolabeled protein (20.000 cpm per culture) in a total incubation volume of 200 µl per well. Incubations carried out for 1.5 h were terminated by transferring the media, along with one wash (0.5 ml) of PBS, to tubes containing 0.75 ml of 20% trichloroacetic acid. This procedure precipitates only undegraded protein of high molecular weight. The extent of degradation was determined by measuring the radioactivities in pellet and supernatant

after centrifugation. Cell-associated ligand was quantified by counting radioactivity in washed cells following solubilisation in 1% sodium dodecyl sulphate. Total endocytosis was obtained by adding cell-associated radioactivity and acid-soluble radioactivity in the medium.

Endocytosis pulse-chase studies – Monolayers of SEC established in 2 cm^2 dishes were allowed to bind radioiodinated ligands at 4°C for 2 h. After washing the cultures to remove unbound ligand, the incubation temperature was raised to 37°C and after various time periods of incubation, cultures were solubilized in 1% sodium dodecyl sulphate. Binding, uptake and degradation were measured as described above.

Assay for detection of IL-1β – IL-1β activity was determined by a two-stage bioassay (9, 10), involving the mouse thymocyte EL-4 NOB-1 cell line, which produces high amount of IL-2 in response to IL-1β and HT-2 cells, IL-2 dependent mouse T-cell line. The MTT assay was used to test cell viability.

RESULTS

A dose-time study was carried out to establish optimal conditions for effects of IL-1ß, TNF-α, or LPS on scavenger-receptor mediated endocytosis in SEC. Maximum stimulation was reached after 6-12 h exposure to 100 U/ml IL-1β, 100 U/ml TNF-α and 100 ng/ml LPS (Fig. 1). At higher concentrations of IL-1β and TNF-α the stimulatory effect on endocytosis was lower, and when the concentration was 500 U/ml or above no stimulation was observed (results not shown).

To check if the cytokines modulated endocytosis in a direct manner we investigated the ability of specific cytokine inhibitors to neutralize the effect. Cultures were incubated with IL-1 receptor antagonist (IL-Ra) or polyclonal anti-TNF antibodies 30 min prior to exposure to the inflammatory mediators. IL-Ra neutralized stimulation with IL-1ß and LPS. In contrast, stimulation with TNF-α could only be neutralized by anti-TNF-antibodies (Fig. 1). Measurements of biologically active IL-1ß released by cultured SEC showed 2- and 3-fold enhanced production of this cytokine after stimulation with LPS and TNF-α, respectively (Fig. 2). This explains the observation that stimulation with LPS could be inhibited by IL-Ra. However, the fact that the effect of TNF-α could not be blocked by the IL-1Ra, indicates that TNF-α, in addition to inducing the production of other mediators, such as IL-1β, has a direct effect on the endocytic activity in SEC.

Next, we wanted to study the effect on endocytosis via two additional receptors, namely the mannose- and collagen α-chain-receptors. To this end we incubated cultured SEC with radioiodinated collagen and mannan after exposure to IL-1ß and TNF-α. The treatment resulted in enhanced endocytosis of mannan, as was already observed with FSA. In contrast, endocytosis of collagen was unaffected. (Fig. 3).

To study if the cytokine-mediated increased endocytosis of FSA was due to increased number or affinity of the scavenger receptor, or stimulation at some later stage of the endocytic process, we performed pulse- (binding at 4°C) chase (washing and transfer to 37°C) experiments with or without prior exposure of SEC to TNF-α and IL-1ß at 37°C. Results in Fig. 4 show that degradation, but not binding was upregulated in stimulated cells.

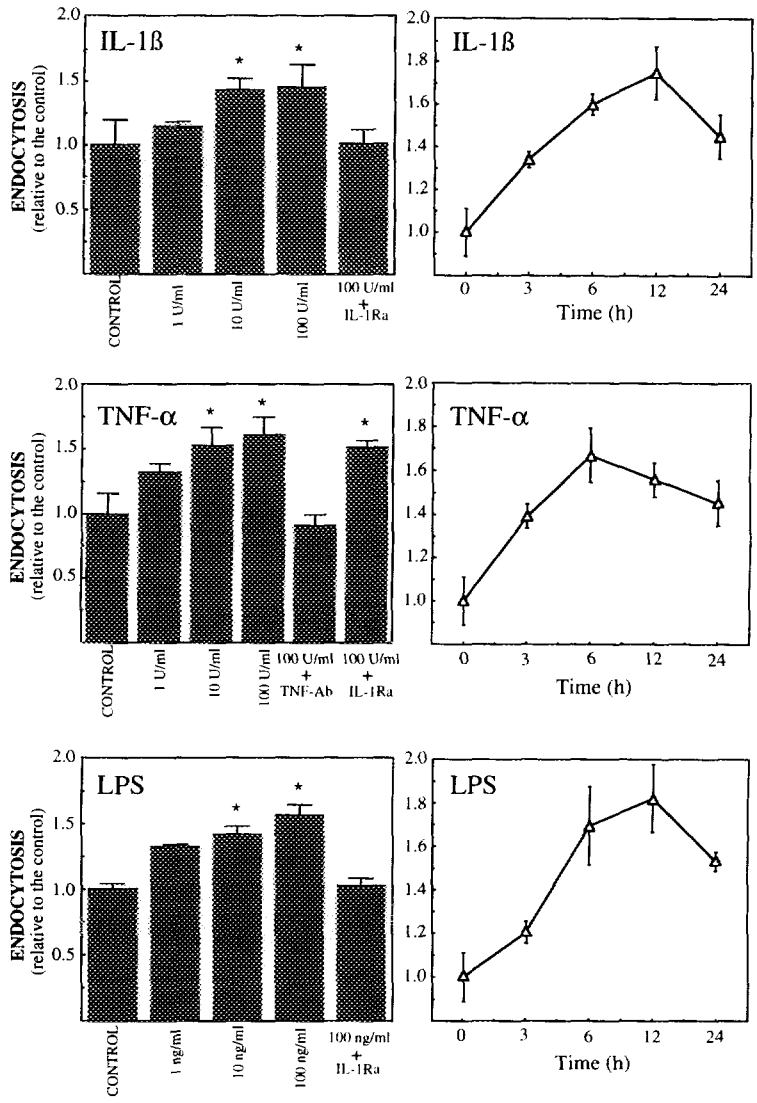

Fig. 1. Stimulatory effect of inflammatory mediators on scavenger-receptor-mediated endocytosis in SEC: dose-time relationship. Endocytosis of [^{125}I]FSA, a ligand for the scavenger receptor, was measured in cultured SEC following a 6-h exposure of the cells to increasing amounts of IL-1ß, TNF-α or LPS (bar diagrams to the left), or for various time periods, using 100 U/ml (IL-1ß or TNF-α) or 100 ng LPS/ml (line diagram to the right). Stimulation was neutralized by including IL-Ra (100 ng/ml) or anti-TNF-α-antibodies (TNF-Ab, 1:4000) in the incubation medium, along with inflammatory mediators at concentrations found to give optimal stimulation of endocytosis. The differences between endocytosis in controls and treated cultures were statistically significant (*p<0,01).

DISCUSSION

SEC constitute the most important scavenger cell system for blood clearance of the majority of physiological and foreign soluble waste macromolecules (1, 11, 12). These cells have a very high

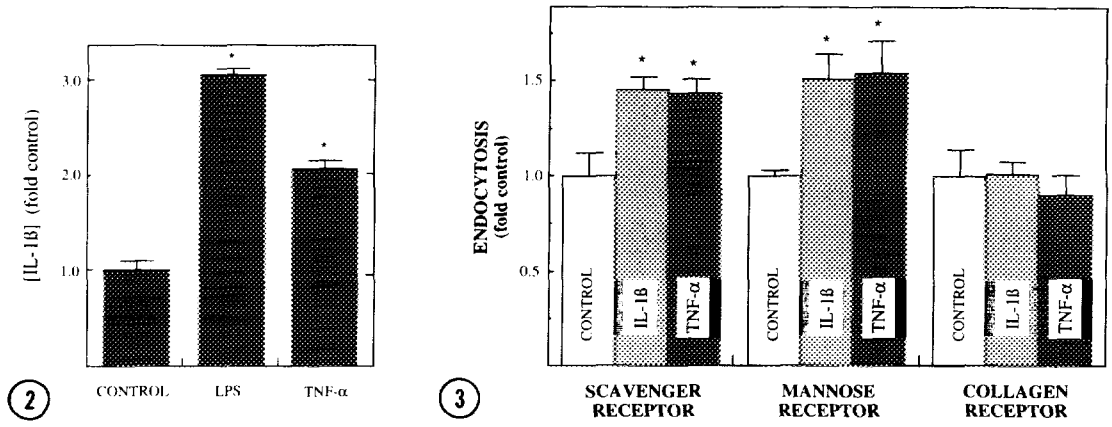

Fig. 2. Production of IL-1ß by SEC after treatment with LPS or TNF-α. Conditioned culture media were collected and analyzed for IL-1ß after incubation of SEC for 6 h with or without TNF-α (100 U/ml) or LPS (100 ng/ml) in the incubation medium. The differences in Il-1β production between treated and untreated cells were statistically significant (*p<0,001).

Fig. 3. Differential effect of IL-1β and TNF-α on endocytosis via scavenger-, mannose-, and collagen-receptors. Endocytosis of [^{125}I]FSA (via the scavenger receptor), [^{125}I]mannan (via the mannose receptor) and [^{125}I]collagen (via the collagen alpha-chain receptor) was measured in SEC following a 6-h preincubation with or without IL-1ß (100 U/ml) or TNF-α (100 U/ml). Stimulation was significant (*$p < 0.01$) with [^{125}I]FSA and [^{125}I]mannan, but not with [^{125}I]collagen.

base level capacity to eliminate circulating waste products generated during normal catabolic processes. However, during inflammation, cells and matrix components are turned over at a greatly increased rate, and the clearance capacity of SEC may be insufficient to eliminate potentially harmful substances and intracellular enzymes from the circulation. To maintain homeostasis in such situations the organism must bring about an increased scavenger capacity of the cells that normally carry out this task. One way of doing so would be by way of the cytokine system, which is known to mediate a number of inflammatory responses. We have studied the effect of important inflammatory mediators on "scavenger endocytosis" in SEC. FSA, mannan and collagen were used as ligands for the scavenger, mannose and collagen alpha chain receptors, respectively. All these receptors are involved in the clearance of physiological waste macromolecules that are generated at an accelerated speed during inflammation: amino- terminal propeptides of types I and III procollagen (scavenger receptor, 11); lysosomal enzymes and carboxy-terminal propeptide of type I procollagen (mannose receptor, 11, 13); alpha chains of all major types and species origins of collagen (collagen alpha chain receptor, 14).

IL-1ß, TNF-α, and LPS stimulated endocytosis in cultured SEC via the scavenger and mannose receptors. This observation is compatible with the notion that inflammatory conditions upregulate the scavenger function of these cells. Surprisingly, endocytosis via the collagen alpha-chain receptor was not influenced by these mediators. It may be speculated that activated macrophages and granulocytes present at the site of inflammation produce sufficient amounts of collagenases and

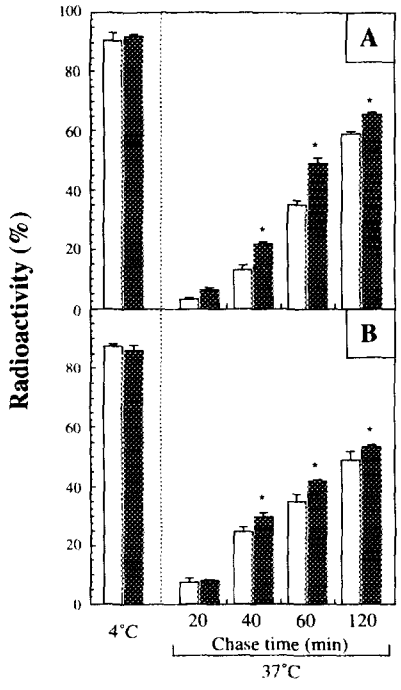

Fig. 4. Effect of cytokines on binding and degradation of FSA. Binding and degradation kinetics of endocytosed [^{125}I]FSA were studied in pulse- (binding at 4°C) chase (washing and transfer to 37°C) experiments following a 6-h incubation of cultured SEC with (shaded bars) or without (open bars) IL-1ß (100 U/ml, *A*) or TNF-a (100 U/ml, *B*). Degradation was significantly (*p<0,05) enhanced in cytokine-treated cells, upon transfer to 37°C. Columns represent percentages of bound ligand at 4°C (≈10% of unspecific binding of label to the substrate) and free iodine in the supernatant at 37°C.

other neutral proteases to break down released pieces of collagen to low molecular weight products. If this be the case, there would be no need for an upregulation of endocytosis via the collagen alpha chain receptor in SEC.

We found that both LPS and TNF-α stimulated cultured SEC to generate 3 and 2 times their base levels of IL-1ß, including these cells in the system of inflammatory regulatory cells. It has also been shown by others that these cells are able to produce IL-1ß in response to various stimuli (5). IL-Ra neutralized stimulation of endocytosis brought about by both IL-1ß and LPS, suggesting that LPS acts indirectly by stimulating the cells to produce endogenous IL-1ß, which in turn stimulates endocytosis in an autocrine manner. The finding that anti-TNF-antibodies neutralized the TNF-provoked upregulation of endocytosis, indicates that TNF-α stimulates the cells in a direct manner.

Receptor-mediated endocytosis may be upregulated by increasing the receptor-binding affinity, the number of receptors, and/or increasing the internalization and intracellular transport of endocytosed ligand. We measured rate of endocytosis in a pulse-chase system. Since degradation is rapid once the ligand has reached the compartment where degradation takes place, we thought it

reliable to take the release of acid soluble (low molecular weight) degradation products as a direct measure of speed of endocytosis. The finding that binding of ligand at 4°C was unaffected by cytokine treatment, suggests that endocytosis is upregulated along the intracellular transport route prior to delivery of ligand to the degradative compartment. Future studies will aim at localizing the endocytic compartment(s) that is subject to cytokine activation.

It has been shown previously that endocytosis mediated by mannose and scavenger receptors in macrophages are downregulated under the influence of proinflammatory cytokines and by agents that promote macrophage activation, such as LPS and BCG (15, 16). This is in contrast to our results that endocytosis via these receptors is enhanced in SEC. We entertain the idea that macrophages and SEC carry out complimentary tasks during an inflammatory response, macrophages by way of being activated to produce an array of powerful inflammatory mediators and enzymes, and SEC by way of increasing their scavenging capacity.

In conclusion, for the first time we show that the high base level scavenging capacity of SEC may be even further upregulated by major inflammatory mediators.

ACKNOWLEDGMENTS

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REFERENCES

1. Laurent, G. J. (1987) Am. J. Physiol. 252, C1-C9.
2. Laurent, T. C., and Fraser, J. R. E. (1991) In Regulation of Bioactive Substances: Physiology and Pathophysiology (J. H. Henriksen, Ed.), pp. 249 – 265. CRC Press, Boca Raton.
3. Smedsrød, B., Pertoft, H., Gustafson, S., and Laurent, T.C. (1990) Bioch. J. 266, 313-327.
4. Karck, U., Peters, T.and Decker, K. (1988) J. Hepatol. 27, 352-361.
5. Smedsrød, B. (1992) In Molecular and Cell Biology of Liver Fibrogenesis (A. M. Gressner, and G. Ramadori, Eds.), pp. 454 – 467. Kluwer Academic Publishers, Dordrecht, Boston, London.
6. Lewis, M. S., and Piez, K. A. (1964) J. Biol. Chem. 239, 3336-3340.
7. Mego, J. L., Bertini, F., and Mc Queen, D. J. (1967) J. Cell Biology 32, 699-707.
8. Smedsrød, B., and Pertoft, H. (1985) J. Leukocyte Biol. 38, 213-230.
9. Gearing, AJH., Bird, CR., Bristow, A., Poole, S., Thorpe, R. (1987) J. Immunol. 99, 7-11.
10. Mosmann, TR., Cherwinski, H., Bodn, MW:, Giedlin, MA:, Coffman, RL. (1986) J. Immunol. 136, 2348-57.
11. Smedsrød, B., Melkko, J., Risteli, L., and Risteli, J. (1990) Biochem. J. 271, 345-350.
12. Melkko, J., Hellevik, T., Risteli, L, Risteli, J., and Smedsrød, B. (1994) J. Exp. Med. 179, 405-412.
13. Smedsrød, B., and Tollersrud O. K. (1995) In Cells of the Hepatic Sinusoid (K. Wake, E. Wisse, and D. L. Knook, Eds.) vol.5. Kupffer Cell Foundation, Leiden, The Netherlands. In press.
14. Smedsrød, B. (1989) In Cells ofthe Hepatic Sinusoid (E. Wisse, D. L. Knook, and K. Decker, Eds.), vol.2, pp. 69-72. The Kupffer Cell Foundation, Rijswijk, The Netherlands.
15. Lenten, B., and Fogelman, A. (1992) J. Immunol. 148, 112-116.
16. Geng, Y., and Hansson, G. (1992) J. Clin. Invest. 89, 1322-1330.