The Influence of Human Endotoxemia on CD95-Induced Apoptosis

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Background: The responses of monocyte and neutrophil tumor necrosis factor receptor type 1 (TNFR-1) and TNFR-2 during systemic inflammation have been described previously. Several other members of the TNFR superfamily also appear to have regulatory roles in immunocyte function, including apoptosis. However, the response of these other receptor members, such as CD95, to systemic inflammation is unclear.

Objectives: To compare the response of CD95 with that of TNFR during systemic inflammation and to assess the influence of the inflammatory milieu on CD95 function.

Setting: Adult clinical research center of a university hospital.

Subjects and Methods: Five healthy male subjects were administered intravenous endotoxin (2 ng/kg), and systemic response was measured by cytokine analysis and receptor expression assays during a 48-hour period. CD95 function during systemic inflammation was assessed using a Jurkat cell bioassay for degree of apoptosis.

Results: Monocyte and neutrophil CD95 expression exhibited changes parallel to that of TNFR following endotoxin injection. In contrast to soluble TNFR, which was transiently elevated during endotoxemia, soluble CD95 levels remained unchanged from baseline. Jurkat cells incubated in normal and post-endotoxin serum samples equally exhibited less than 10% spontaneous apoptosis. No soluble CD95 ligand was detectable in experimental human endotoxemia.

Conclusions: Cell-associated CD95 exhibited changes parallel to its receptor family member TNFR following endotoxin administration. Soluble CD95 is present in human serum samples, but the levels remained unchanged following endotoxin administration. No soluble CD95 ligand activity was detectable by enzyme-linked immunosorbent assay or by functional assay. The potential protective role of soluble CD95 in human serum samples against CD95 ligand–induced apoptosis remains to be defined.

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SUBJECTS AND METHODS

SUBJECTS

A total of 5 male subjects, with a mean ± 5E age of 27 ± 6 years, were admitted for study to the Adult Clinical Research Center of Robert Wood Johnson University Hospital, New Brunswick, NJ, after documentation of good health by medical history, physical examination, and hematologic and biochemical screening. The study was approved by the institutional review board, and written informed consent was obtained from all subjects prior to study enrollment. The subjects were admitted the evening prior to the study, fasted, and administered intravenous maintenance crystalloid fluids. On the morning of the study, a 20-gauge catheter was placed in the radial arteries of all subjects to continuously monitor heart rate and blood pressure and to obtain blood samples. At 0 hour, all subjects received a 2-µg/kg intravenous injection of lipopolysaccharide (National Reference Endotoxin, Escherichia coli 0113, lot EC-5). A rectal probe was inserted in all subjects to allow continuous measurement of core temperature. Blood samples were obtained at 0, 1, 2, 3, 6, 12, 24, and 48 hours. Other than samples for flow cytometry, all blood samples were centrifuged at 4°C for 20 minutes at 48 hours. Other than samples for flow cytometry, all blood samples were centrifuged at 4°C for 20 minutes at 4°C.

CYTOKINE AND SOLUBLE RECEPTORS

The following soluble factors were measured in plasma by enzyme-linked immunosorbent assay: TNF-α (CLB Biotechnology, Amsterdam, the Netherlands), interleukin 1β (IL-1β) (R&D Systems, Minneapolis, Minn), IL-6 (PharMingen, San Diego, Calif), IL-8 (PharMingen), IL-10 (PharMingen), soluble TNFR-1 (R&D Systems), soluble TNFR-2 (R&D Systems), soluble CD95 (MBL, Nagoya, Japan), soluble CD95 ligand (MBL). All assays have a sensitivity of 15 pg/mL with the exception of soluble CD95 and soluble CD95 ligand, which had sensitivities of 500 pg/mL and 100 pg/mL, respectively. Microplates were analyzed with the EL312e microplate reader (Bio-Tek Instruments, Winooski, Vt) with KC Junior Software (Bio-Tek Instruments).

CELL SURFACE RECEPTOR MEASUREMENTS

Surface TNFR expression was determined by saturation binding of TNF-α as previously described.4 Erythrocytes from 400 µL of peripheral blood was lysed with bicarbonate-buffered, 0.826% ammonium chloride solution (pH 7.2). Leukocytes were recovered by centrifugation and washed with cold bicarbonate-buffered, 0.826% ammonium chloride solution. Specific staining was with 1-µg/mL biotinylated human TNF-α, followed by incubation for 15 minutes at 4°C. The cells were then washed with bicarbonate-buffered, 0.826% ammonium chloride solution and further stained with 0.5-µg/mL streptavidin conjugated with phycoerythrin (PE) for 15 minutes at 4°C. Nonspecific staining for background fluorescence was by the addition of 0.5-µg/mL streptavidin conjugated with PE. In the past, we have demonstrated that this concentration of streptavidin conjugated with PE provides the same background fluorescence as a 100-fold excess of unlabeled human TNF-α (data not shown). These cells were then washed and resuspended for flow-cytometric analysis. The flow cytometry photomultiplier gain was standardized using PE-conjugated beads (Calibrite, Becton-Dickinson, San Jose, Calif). Mean-channel fluorescence at greater than 570 nm of forward- and side-angle scatter-gated monocytes and PMNs was assessed. Data are presented as the difference (linear units) between mean-channel fluorescence intensities of specifically and nonspecifically stained cells.

After lysis of erythrocytes, leukocyte surface CD95 expression was determined by staining with 1-µg/mL mouse anti-human PE-conjugated CD95 antibody (Becton-Dickinson), with mouse antihuman PE-IgG1 as isotype control (Becton-Dickinson). Antibody incubation, washing, and flow-cytometric analysis were performed in similar fashion to TNFR staining using identical photomultiplier gain settings.

JURKAT CELL BIOASSAY

The influence of the inflammatory milieu on CD95 function was assessed using the Jurkat cell line, clone E6-1 (American Type Culture Collection, Rockville, Md). This assay was selected because CD95 is abundantly expressed, but TNFR expression is minimal.10 Approximately 1 × 106 cells underwent a 12-hour incubation at 37°C (5% carbon dioxide) in a 10% postendotoxin serum sample (diluted in RPMI medium with 2-mmol/L l-glutamine) obtained at 2, 12, and 24 hours after endotoxin injection. Human serum samples from 0 hours and 10% fetal bovine serum (FBS) samples served as controls. These experiments were performed in triplicate to determine if basal or inflammatory human serum samples possess CD95 signaling properties. In separate experiments, 100-ng/mL IgM CD95 agonist (clone CH11, Upstate Biotechnology, Lake Placid, NY) was added to the above cell cultures to determine if inflammatory human serum samples protect against CD95-induced apoptosis.

JURKAT CELL APOPTOSIS ASSAY

Cells were washed once in 50% FBS and Hanks balanced salt solution samples (without phenol red, Ca2+ or Mg2+) and partially resuspended in wash buffer. To fix and permeabilize the cells, 1.5 mL of cold 70% ethanol was added for 1 hour at 4°C, followed by washing with 1X bicarbonate-buffered, 0.826% ammonium chloride solution. Fixed cells were resuspended in 1 mL of propidium iodide (30 µg/mL; Sigma Chemical, St Louis, Mo) and RNAs (Sigma Chemical) and Hanks balanced salt solution for 12 to 24 hours prior to flow-cytometric analysis (EPICS profile, Coulter, Miami, Fla). Percent apoptosis is defined as the ratio of hypodiploid cells (percent <2n [normal diploid]) to viable cells times 100.

STATISTICAL ANALYSIS

All values are expressed as the mean ± SEM. Statistical differences were demonstrated by 1-way repeated measures analysis of variance and Newman-Keuls test. Significance was ascribed to a P value of less than .05 with respect to the time at 0 hour.
are less well understood. The objectives of the present study were to elucidate the response of another proapoptotic TNFR superfamily member, CD95 (Fas), following endotoxin (lipopolysaccharide) administration and to compare these responses to those of TNFR. Whereas TNFR-1 can induce either apoptosis or cell proliferation depending on accessory signals within the cell,8 the CD95 signaling pathway is presently only known to induce apoptosis.9 The present study determined if the inflammatory milieu in human endotoxemia could delay CD95-mediated programmed cell death using a Jurkat cell line bioassay.

RESULTS

INFLAMMATORY CYTOKINE RELEASE

Soluble TNF-α, IL-6, IL-8, and IL-10 were all detectable within 6 hours following endotoxin administration (peaks [mean ± SEM], 1030 ± 312 pg/mL, 1795 ± 369 pg/mL, 235 ± 100 pg/mL, 198 ± 100 pg/mL, respectively), and the temporal sequence of appearances was as previously reported,11-13 with TNF-α first, followed by IL-6, IL-8, and IL-10. At all points assayed, IL-1β was undetectable.

MONOCYTE AND PMN RECEPTOR EXPRESSION

Monocyte TNFR expression was significantly reduced (P<.03) at 2 hours after endotoxin administration and recovered to supranormal levels at 24 hours (Figure 1, A). Monocyte CD95 expression was also reduced (P<.05) in parallel with TNFR expression at 2 hours and also recovered to supranormal levels beyond 24 hours. To a lesser magnitude, a reduction in PMN-associated TNFR expression was also observed (P<.05) at 2 hours and recovered to normal levels by 24 hours. The expression of PMN-associated CD95 also reached a nadir at 2 hours (P<.05) but recovered to normal levels by 12 hours without subsequent overexpression (Figure 1, B).

SOLUBLE RECEPTOR RELEASE

Soluble TNFR-1 was transiently released into the circulation after endotoxin administration, which peaked at 2 hours (98 ± 15 pg/mL, P<.05) and returned below detection limits by 48 hours. Soluble TNFR-2, which was detectable prior to endotoxin administration (70 ± 12), exceeded baseline (358 ± 29 pg/mL, P<.01) at 2.5 hours and returned to normal levels by 48 hours.

While both soluble TNFRs were transiently elevated during experimental human endotoxemia, soluble CD95 levels did not change significantly throughout the study period (range, 1800-2100 pg/mL).

HUMAN SERUM SAMPLES POSSESS LOW SOLUBLE CD95 LIGAND ACTIVITY

Soluble CD95 ligand was undetectable in normal and postendotoxin serum samples. To confirm the lack of CD95 ligand activity, Jurkat cells were incubated for 12 hours in a 10% serum sample obtained at 2, 12, and 24 hours after endotoxin injection. These cells did not exhibit any increase in apoptosis (<10%) compared with 0 hour human serum samples or FBS samples (Figure 2).

HUMAN SERUM SAMPLES PREVENT CD95 LIGAND–INDUCED APOPTOSIS

When 100-ng/mL CH11, the CD95 agonist, was incubated with Jurkat cells in 10% serum sample obtained from 0, 2, 12, and 24 hours after endotoxin injection, there was a significant increase in apoptosis (15%-20%; P<.05). However, a 2.5-fold increase in apoptosis of cells incubated in normal FBS samples compared with cells in human serum samples was observed when 100-ng/mL CH11 was added to the medium (Figure 2). Again, there was no notable difference in apoptosis between cells incubated in normal serum samples (0 hour) and posten-
death-inducing signaling complex. Formation of the proteins, FADD and caspase-8, that collectively form the leading to the recruitment of 2 critical signaling pro-
cascade of protease activities and mitochondrial dis-
A new one. It has been shown that membrane-bound distinct intracellular signaling pathways with sequelae not limited to cell death, the only known function of CD95 activation is to induce cell death. CD95 induces apoptosis through activation of intracellular death domains, leading to the recruitment of 2 critical signaling proteins, FADD and caspase-8, that collectively form the death-inducing signaling complex. Formation of the death-inducing signaling complex leads to a complex cascade of protease activities and mitochondrial disruption, eventuating in endonuclease activation, DNA fragmentation, cell membrane blebbing, formation of smaller membrane-bound apoptotic bodies, and orderly disposal of apoptotic remnants without release of noxious mediators often characteristic of necrosis.

In acute systemic inflammation, the down-regulation of cell-associated TNFR expression is due, in part, to receptor shedding of both TNFR-1 and TNFR-2. The systemic release of soluble TNFR has also been correlated to the outcome in critically ill patients with sepsis and may be an endogenous host defense mechanism by which circulating TNF-α is neutralized. Soluble CD95 in this model of experimental human endotoxemia remained unchanged from baseline. The range of soluble CD95 levels in normal human subjects and experimental human endotoxemia reported herein are in consonance with other reports measuring soluble CD95 levels in certain autoimmune diseases and low-grade nonhematologic malignancies. The observed down-regulation of cell-associated CD95 thus suggests receptor internalization during endotoxemia rather than receptor shedding. It should be noted that soluble CD95 can shed from activated peripheral blood monocytes and transfected cell lines following in vitro high-dose stimulation with mitogens.

Monocytes are sensitive to CD95-mediated apoptosis, but functionally, it remains to be determined if CD95 down-regulation in human endotoxemia correlates with resistance to CD95 ligand–induced apoptosis.

The appearance of TNF-α during acute endotoxemia is rapid and early, typically rising above 1000 pg/mL by 1.5 hours and returning to undetectable levels by 3 hours. Soluble CD95 ligand, however, was not detectable in either normal or endotoxemic serum samples. This lack of soluble agonist release is also suggested by the lack of enhanced apoptosis in Jurkat cells exposed to post-endotoxin serum samples. CD95 ligand is a type 2 membrane protein that belongs to the TNF family, and cleavage of the extramembrane portion by matrix metalloproteinase activity forms the 26-kd soluble glycoprotein, soluble CD95 ligand. Cell-associated CD95 ligand is predominantly expressed on activated T cells, but the soluble form has, thus far, only been detectable in patients with certain hematologic malignancies, such as leukemia of natural killer or T-cell–type large granular lymphocytes, or natural killer lymphoma. The systemic tissue damage seen in most patients with such malignancies has been attributed, in part, to soluble CD95 ligand release.

Functional studies using human recombinant soluble CD95 ligand found that it induces acute liver failure and rapid lethality in mice but only after large doses were administered. Subsequent studies demonstrated that membrane-bound CD95 ligand possessed far greater cytotoxic potency than soluble CD95 ligand. Function mediated by CD95 is only activated on receptor trimerization, which would more likely occur with membrane-bound CD95 ligand induction than with the oligomeric soluble CD95 ligand. The concept of differential targeting between membrane-bound and soluble ligands is not a new one. It has been shown that membrane-bound TNF-α is far more efficient than soluble TNF-α in activating the p75 (type 2) TNFR.

HUMAN SOLUBLE CD95 AS ENDOGENOUS CD95 LIGAND ANTAGONIST

Soluble CD95 was readily detectable by enzyme-linked immunosorbent assay in normal human serum samples obtained from subjects (1800 ± 500 pg/mL), whereas none was detectable in FBS samples.

This study reports for the first time that both monocyte-associated CD95 and PMN-associated CD95 expression exhibit transient down-regulation with supranormal recovery after endotoxin administration. This is similar to the responses previously described for TNFR. Previously, changes in monocyte-associated TNFR expression were determined to be a sensitive marker for experimental and clinical responses to endotoxin. Monocyte-associated CD95 expression also appears to be such a marker in experimental endotoxemia. Efforts are being undertaken to determine the sensitivity of monocyte-associated CD95 expression as a marker in clinical sepsis or injury.

Whereas activation of the TNFRs propagate distinct intracellular signaling pathways with sequelae not limited to cell death, the only known function of CD95 activation is to induce cell death. CD95 induces apoptosis through activation of intracellular death domains, leading to the recruitment of 2 critical signaling proteins, FADD and caspase-8, that collectively form the death-inducing signaling complex. Formation of the death-inducing signaling complex leads to a complex cascade of protease activities and mitochondrial disruption, eventuating in endonuclease activation, DNA fragmentation, cell membrane blebbing, forma-

![Figure 2. Effects of different serum samples on CD95-mediated apoptosis using Jurkat cell bioassay. The serum concentration was 10%, and the incubation time was 12 hours. FBS indicates fetal bovine serum; NHS, normal human serum; CH11 (−), the control group; CH11 (+), received 100-ng/mL CH11; asterisk, P = .004; and dagger, P < .001, when compared with all NHS and human serum samples from different points.](image-url)
Persistent activation and excessive inflammatory cytokine release by monocytes and neutrophils during injury or severe infections may contribute to multiple organ failure and late mortality in critically ill patients. Clinical studies aimed at neutralizing excessive circulating inflammatory mediators have yielded only modest success. Knowledge of the kinetics of CD95 expression and function in immunocytes during systemic inflammation may be the first steps in identifying potential therapeutic adjuvants whereby activated immunocytes responsible for proinflammatory mediator production and organ injury can be selectively deleted.

The bioassay system using Jurkat cells has been extensively described as a functional CD95-mediated cytotoxicity assay because these T lymphoma–derived cells are almost exclusively rich in CD95 expression but poor in TNF expression. To test our hypothesis of whether the inflammatory milieu can delay or protect against CD95-mediated apoptosis, we added the CD95 agonist CH11 to cells incubated in FBS and human serum samples obtained at 0, 2, 12, and 24 hours postendotoxemia. The dose and type of CD95-agonist used in our experiments have been standardized by others in Jurkat cell bioassays and is also calculated to approximate the molar concentration of soluble CD95 in human serum samples. Figure 2 demonstrates that CH11 induces apoptosis (15%-20%) in Jurkat cells cultured in normal and postendotoxemic human serum samples. However, the inflammatory serum samples from 2, 12, and 24 hours after endotoxin administration neither enhanced nor inhibited CH11-induced apoptosis when compared with 0 hour (ie, normal) human serum samples. These data suggest that the inflammatory milieu induced by mild human endotoxemia is not sufficient to alter CD95-mediated apoptosis.

An area of particular interest relates to the role of CD95 in organ injury during systemic inflammation. While the sequelae of severe sepsis have been known to precipitate hepatic dysfunction, it is increasingly recognized that TNFR-1 (p55) and CD95 are both potent and independent mediators of this process. Indeed, resident Kupffer cells and sinusoidal endothelial cells cultured with lipopolysaccharide expressed high levels of CD95 ligand messenger RNA. It has been described that endogenous soluble CD95 can protect against CD95 ligand–induced apoptosis, and corroborating evidence suggests that soluble CD95 produced by hepatocytes can neutralize CD95 ligand.

In summary, we have described the kinetics of monocyte-associated CD95 and PMN-associated CD95 expression during acute endotoxemia, which parallels that of its superfamily member, TNFR. The inflammatory mediators characteristic of experimental human endotoxemia do not appear to alter CD95-mediated apoptosis. However, this does not preclude the influence of inflammation on CD95 function in severe illness, such as septic shock and multiple organ failure. In addition, any potential effects of inflammatory mediators on apoptosis may also occur at the level of postreceptor signal transduction. The regulatory role of soluble CD95 in systemic inflammation, sepsis, and the fate of activated immunocytes requires further investigation.

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REFERENCES

DISCUSSION

Alfred Ayala, PhD, Providence, RI: This paper represents another in the series of studies that have come from Drs Lowry and Calvano’s laboratory looking at endotoxemia as a septic challenge in humans, the obviously most relevant animal subject you could utilize. Here the authors have looked at members of the nerve growth family, specifically TNFR and Fas antigen receptor, as well as their associated ligands, and have attempted to assess their possible contribution to the suppression of phagocyte apoptosis. That said, I have several questions.

The literature is now relatively clear that the form of Fas ligand that induces apoptosis is primarily the cell-associated type and not the soluble ligand. Thus, it is not so surprising that there isn’t much of a change or difference in your circulating levels. Have you assessed Fas ligand expression on these cells in your model? Down these lines, while you have looked at phagocytes here, have you any data on the nonphagocytic cells, in other words, the lymphocytes in this population, for their expression of these antigens as well as the ligand? Also, while you have utilized Jurkat T cells as a responder, and I think that is a good model for a responder to Fas, my question becomes whether or not you actually looked for evidence of apoptosis in the actual model itself? In other words, did neutrophils show apoptosis following the injection of endotoxin in this model?

You used bolus IV endotoxemia as a model of the septic state, but it is clear from the literature that endotoxin exposure in healthy volunteers, as well as similar models of endotoxemia in animals, may not always adequately emulate either sepsis or shock states. Have you examined similar serum samples from patients in this setting?

Work by Conrad Liles at the University of Washington, Seattle, suggests that both human PMNs as well as monocytes maintain preformed pools of Fasl, which appear to be involved in inducing endothelial as well as epithelial damage in paracrine fashion. How would you reconcile your findings with their observations?

And finally, 2 brief technical questions with respect to the TNFR expression. Can you provide us with any insight as to which subpopulation of the TNFR on these cells has been altered or shed? Also, you have presented flow-cytometric data as mean-channel fluorescence. It would be also interesting to know what the changes in the actual percentage of cells are that are expressing these antigens.

Dr Lin: Thank you, Dr Ayala, for your several questions. I am going to lump your first few regarding Fas ligand and Fas receptor expression into 1 dissertation here.

It has been demonstrated that membrane-associated Fas ligand is approximately 10-fold more potent than soluble Fas ligand, but soluble Fas ligand is also functional at very high doses. We have not measured any Fas ligand expression in monocytes and neutrophils. We have attempted to measure it in lymphocytes, and the work is still in progress. Technically, it is a challenging process.

When you referred to the work by Dr Liles from the University of Washington, I believe you are referring to articles in the Journal of Experimental Medicine and Journal of Immunotherapy in the last couple of years. Their group has shown that monocytes contain preformed Fasl ligand in intracellular compartments, and on stimulation in an in vitro system with pHA or superantigens, these molecules can be released into the extracellular environment. While their observations are reasonable, we have not seen that in our in vivo model. Perhaps under different conditions, we will be able to make the same observations.

This leads me to your other point. Have we measured Fas ligand in patients who are septic or in septic shock or multiple organ failure? The answer to that is yes. The manuscript is in preparation right now.

Regarding the differences between T lymphocyte Fasl ligand and Fas receptor expression, and how they compare to monocytes and neutrophils, I can’t give you complete data on Fasl ligand, but soluble Fas ligand is also functional at very high doses. We have not measured any Fas ligand expression in monocytes and neutrophils. We have attempted to measure it in lymphocytes, and the work is still in progress. Technically, it is a challenging process.

In response to your technical questions, TNF receptors, both types 1 and 2, are down-regulated after endotoxin administration. For that I can refer you to a paper from our laboratory in the Journal of Immunotherapy in 1997. We do have data on the total number of leukocytes present even though I only showed you data for the cell receptor expression. On the flow cytometer, we set regions specifically for populations of monocytes and neutrophils. Indeed, the number of these cells expressing Fas is 100%. This enables us to measure receptor density as mean channel fluorescence.

Dr Gordon: I just want to verify that these are indeed circulating cells that you are examining.

Dr Lin: Yes.

Dr Gordon: I was wondering whether you had considered the possibility that these cells were rapidly cleared and whether within the spleen there was an increased rate of apoptosis of your cells or any difference of apoptosis?

Dr Lin: That would be difficult to do. We report systemic responses measured by peripheral blood and serum. In terms of tissue-fixed or organ-fixed immunoocyte function and their longevity, I can’t give you specific answers except to say that, in animal models, they are subject to complex autocrine and paracrine influences.