C5a receptor and thymocyte apoptosis in sepsis[1]

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SPECIFIC AIMS

Thymocyte- and lymphocyte-induced apoptosis during experimental sepsis is well documented and thought to be part of the immunosuppressive outcome of sepsis. Since we recently showed that blockade of C5a in rats with sepsis (after cecal ligation/puncture) greatly reduces thymocyte apoptosis but were unable to show direct induction of in vitro apoptosis in thymocytes by C5a, the aim of the current study was to investigate the presence and regulation of the C5a receptor (C5aR) on thymocytes during sepsis and the ability of C5a to directly induce apoptosis under conditions in which C5aR is up-regulated during sepsis.

PRINCIPAL FINDINGS

1. ^{125}I-labeled recombinant rat C5a (rrC5a) binds specifically to rat thymocytes

We used binding experiments with ^{125}I-rrC5a to detect C5aR in rat thymocytes from normal rats. Saturation was reached at ~500 pM C5a. With increasing amounts of unlabeled rrC5a, binding of ^{125}I-rrC5a to thymocytes could be competed against. Between 10^{-14} and 10^{-11} M unlabeled rrC5a, the binding signal achieved with 200 pmol ^{125}I-rrC5a could be reduced by ~50%. Therefore, binding of ^{125}I-rrC5a appeared to be highly specific and of high affinity.

2. Binding of ^{125}I-rrC5a to rat thymocytes from CLP rats and from rats treated with lipopolysaccharide (LPS) or interleukin 6 (IL-6) is increased

To determine whether enhanced binding of C5a occurred in rat thymocytes during the onset of sepsis, thymocytes were isolated 3 h after CLP and compared to normal thymocytes. Significantly increased binding of ^{125}I-rrC5a to CLP thymocytes was seen. To further investigate mediators possibly responsible for these results, thymocytes from normal rats were isolated and stimulated in vitro with LPS and IL-6 at various concentrations for 3 h at 37°C. Each mediator was capable of significantly increasing ^{125}I-rrC5a binding to rat thymocytes in a dose-dependent manner. At concentrations of 100 ng/ml, IL-6 and 500 ng/ml LPS, binding appeared to be maximally increased.

3. mRNA expression for C5aR is increased in rat thymocytes during sepsis and after stimulation in vitro with LPS and IL-6

To further confirm findings of the binding experiments, we conducted RT-PCR experiments to detect mRNA for C5aR in rat thymocytes. In vitro stimulation of rat thymocytes with LPS or IL-6 increased mRNA expression for C5aR in a dose-dependent manner (**Fig. 1*A***). At 3, 6, and 12 h after CLP, mRNA expression was clearly enhanced compared with that from normal

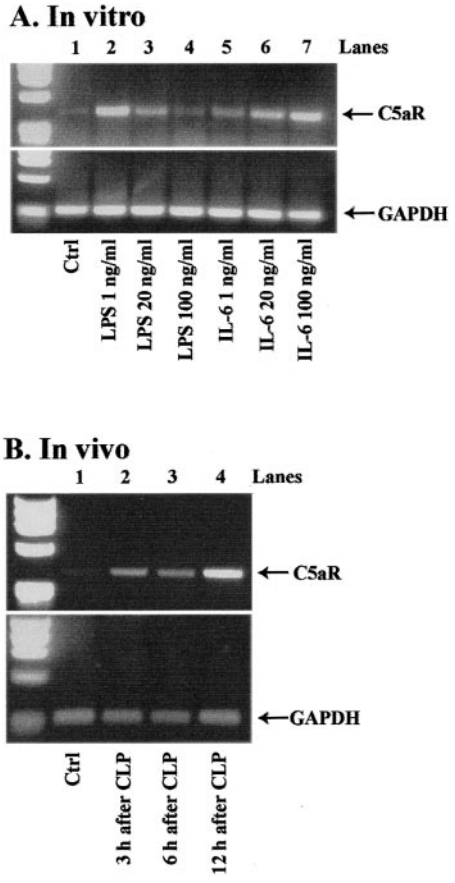

Figure 1. Semiquantitative RT-PCR analysis for C5aR mRNA in rat thymocytes. Thymocytes from normal rats were stimulated with LPS and IL-6 in vitro at different concentrations (*A*). Induction of C5aR mRNA in vivo as early as 3 h and as late as 6 and 12 h after CLP (*B*). Equal loading conditions were demonstrated with bands for GAPDH-mRNA production (lower panels in both frames). Data are representative of results from 2 or 3 independent experiments.

1 To read the full text of this article, go to http://www.fasebj.org/cgi/doi/10.1096/fj.02–0033fje; to cite this article, use *FASEB J.* (April 23, 2002) 10.1096/fj.02–0033fje.

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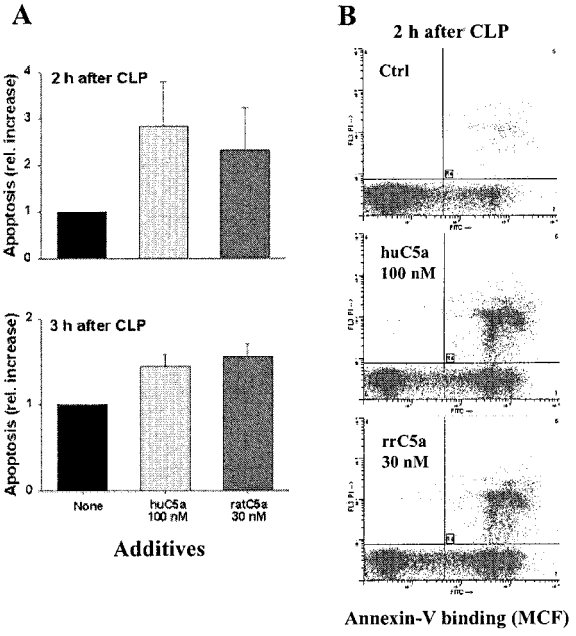

Figure 2. Flow cytometry analysis for apoptosis (annexin-V binding) in rat thymocytes. Rat thymocytes were isolated 2 and 3 h after CLP and not otherwise stimulated or incubated with huC5a (100 nM) or rrC5a (30 nM) for 30 min at 37°C. Flow cytometry was performed after staining with annexin-V for 15 min (*A*). Data are presented as increases in apoptosis relative to the corresponding control value in each experiment. Data are from 3–9 independent experiments per bar graph. Flow cytometric data from thymocytes obtained from rats 2 h after CLP and not otherwise stimulated or after exposure to hu C5a or rrC5a (30 min at 37°C) (*B*).

thymocytes (Fig. 1*B*). Therefore, enhanced binding of ^{125}I-labeled rrC5a during CLP-induced sepsis and after in vitro stimulation with LPS or IL-6 correlated with an increased expression of mRNA for C5aR.

4. C5a directly induces apoptosis in thymocytes from septic rats

In our recent study (mentioned above), we were not able to demonstrate that C5a directly induces apoptosis in normal thymocytes in vitro. Therefore, we conducted flow cytometry experiments where we stimulated thymocytes from CLP rats with C5a in vitro under conditions in which C5aR was up-regulated (as described above). Experiments showed the ability of rrC5a and human C5a to induce thymocyte apoptosis (as measured by annexin-V binding) in vitro in thymocytes isolated 2 and 3 h after induction of sepsis by CLP (**Fig. 2**). Figure 2*A* summarizes the increase of apoptosis achieved by in vitro stimulation with either human C5a or rrC5a in thymocytes 2 and 3 h after induction of CLP. Shown is the relative increase of apoptosis compared to the matching control for each experiment (no C5a addition). Figure 2*B* shows a typical flow cytometry

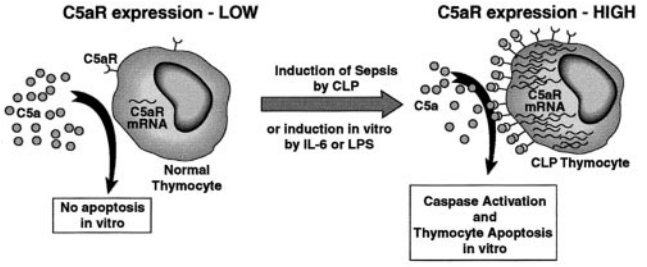

Figure 3. Schematic diagram showing the increased C5aR expression during the onset of sepsis and the correlated susceptibility to C5a-induced apoptosis.

experiment demonstrating direct induction of apoptosis by either human (100 nM) or rrC5a (30 nM) in thymocytes from rats 2 h after CLP. Such an effect could not be detected in thymocytes from normal rats (data not shown).

5. C5a-induced caspase-3, -6, and -8 activity in thymocytes from CLP rats

We conducted experiments in thymocytes obtained 3 h after CLP and measured caspase-3, -6, and -8 activity enzymatically after in vitro stimulation with human or rrC5a. Under such conditions, both mediators induced activities of the three caspases; 30 nM rrC5a appeared to be more potent than 100 nM human C5a. Under these conditions, rrC5a increased caspase-3, -6, and -8 activities between 27 and 40%.

CONCLUSIONS

Our findings suggest that C5aR is present in thymocytes from rats and up-regulated early after the onset of CLP. We demonstrated that such an increase in C5aR is accompanied by increased susceptibility of rat thymocytes to C5a-induced in vitro apoptosis. Such direct evidence for C5a-induced apoptosis in freshly isolated cells from septic animals has not yet been reported, and data showing increased C5aR expression in thymocytes during the onset of sepsis represent a new finding. C5a has been reported to have harmful effects during the onset of sepsis, but the role of C5aR expression in sepsis has not been closely studied. The evidence for a direct induction of apoptosis in thymocytes by C5a represents another step in the overall understanding of its potentially harmful effects during the onset of sepsis by suggesting a mechanism leading to rapid immunosuppression. Our findings suggest that suppressing the harmful effects of C5a during sepsis may be accomplished by interception of C5a or blockade of C5aR as a target for new drugs like antibodies or receptor antagonists. FJ