Pancreatitis-Associated Protein 2 Modulates Inflammatory Responses in Macrophages

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Pancreatitis-Associated Protein 2 Modulates Inflammatory Responses in Macrophages

Domenico Viterbo, Martin H. Bluth, Yin-yao Lin, Cathy M. Mueller, Raj Wadgaonkar, and Michael E. Zenilman

Pancreatitis-associated proteins (PAP) are stress-induced secretory proteins that are implicated in immunoregulation. Previous studies have demonstrated that PAP is up-regulated in acute pancreatitis and that gene knockdown of PAP correlated with worsening severity of pancreatitis, suggesting a protective effect for PAP. In the present study, we investigated the effect of PAP2 in the regulation of macrophage physiology. rPAP2 administration to clonal (NR8383) and primary macrophages were followed by an assessment of cell morphology, inflammatory cytokine expression, and studies of cell-signaling pathways. NR8383 macrophages which were cultured in the presence of PAP2 aggregated and exhibited increased expression of IL-1, IL-6, TNF-α, and IL-10; no significant change was observed in IL-12, IL-15, and IL-18 when compared with controls. Chemical inhibition of the NFκB pathway abolished cytokine production and PAP facilitated nuclear translocation of NFκB and phosphorylation of IκBα inhibitory protein suggesting that PAP2 signaling involves this pathway. Cytokine responses were dose dependent. Interestingly, similar findings were observed with primary macrophages derived from lung, peritoneum, and blood but not spleen. Furthermore, PAP2 activity was inhibited by the presence of serum, inhibition which was overcome with increased PAP2. Our results demonstrate a new function for PAP2: it stimulates macrophage activity and likely modulates the inflammatory environment of pancreatitis. The Journal of Immunology, 2008, 181: 1948–1958.

Acute pancreatitis is an acute inflammatory condition of the pancreas that varies widely in presentation, course, and outcome. The majority of patients with acute pancreatitis have a mild and manageable disease process, whereas ~30% of patients suffer from a severe episode, which can include organ dysfunction secondary to a systemic inflammatory process (1). Although a great deal is known regarding etiologic causes of pancreatitis, the molecular and pathophysiological mechanisms of its disease progression are not fully understood (2). The systemic organ injury associated with pancreatitis is likely mediated by substances released from the pancreas during stress.

Pancreatitis-associated proteins (PAP) are members of the Reg gene family—14- to 17-kDa secretory proteins which have been shown to be strongly induced during acute pancreatitis (3). Although originally identified during acute pancreatitis, they have been reported in other inflamed pathologic organ systems including Crohn’s disease, inflammatory bowel disease, liver injury, neuronal, ovarian, and cardiac tissue damage (4–10). In rats, there are three homologous PAP isoforms, referred to as PAP1, PAP2, and PAP3 (11). Reported functional roles for PAP proteins include cellular apoptosis, mediators of cell regeneration and proliferation, carcinogenesis, immunity, and inflammation (12).

Emerging evidence supports the notion that PAP proteins play regulatory roles during the inflammatory process of pancreatitis. Studies by our laboratory reported that Ab neutralization of PAP (and Reg I) in rats with acute pancreatitis caused an increased inflammatory response in the pancreas (13). We then demonstrated that antisense inhibition of all three PAP isoforms correlated with worsening of pancreatitis severity (14). The immunomodulatory function of PAP was studied by Vasseur et al. (15), who showed that PAP1 inhibits activation of macrophages by TNF-α and IL-6. Furthermore, studies by Gironella et al. (6) found that PAP is anti-inflammatory in patients with inflammatory bowel disease. Although some have shown that PAP1 protects against the lung injury during pancreatitis (16), others have shown that high doses of PAP1 can induce lung inflammation (17). PAP3 has been shown to be a strong macrophage chemokine (18), and the mouse homolog to PAP3 (referred to as Reg3γ) has been shown to be involved in innate immunity (19). We have demonstrated that various Reg/PAP isoforms appear to increase in pancreatitis (20, 21), suggesting an immunomodulatory relationship within the Reg family.

Although PAP1 and PAP3 have been characterized, the physiologic inflammatory relevance of the PAP2 isoform has not been elucidated. Interestingly, we have found that of the three PAP isoforms, PAP2 is consistently induced the most after pancreatitis (11, 14), and we chose to study its effects further.

In this study, we report that the PAP2 isoform modulates macrophage inflammatory responses. Our results suggest that PAP2 is a potential mediator of early inflammation, acts specifically to orchestrate the macrophage inflammatory response, and may do so by working in concert with other PAP isoforms in acute pancreatitis.
Materials and Methods

Cell culture and NR8383 macrophage PAP assays

The rat macrophage cell line, NR8383 (American Type Culture Collection), was cultured in F12-K medium supplemented with 15% FCS at 5% CO₂ and 37°C. Before experimentation, the macrophages were plated and grown to confluence. Cells were treated with various concentrations of rPAP2 (0–15 μg/ml) for the specified time period (0–72 h), and cell medium was analyzed for secreted inflammatory proteins. Unless stated, the dosage of 5 μg/ml for PAP2 was used in all experiments. This dosage was selected because this concentration corresponded to the lower half of the log phase of preliminary dose-response experiments performed on NR8383 cells. Two versions of rPAP2 (GST-PAP and PAPHis) were used in these studies. Comparison of the activity of both recombinant proteins demonstrated that treatment of NR8383 macrophages with 5 μg/ml PAP2-GST and PAP2-GSTM showed equal activity as exhibited by cytokine analysis. Controls consisted of cells treated with rGST protein or with vehicle control (0.8 M urea, 300 mM NaCl, 20 mM Tris (pH 7.5)), neither of which had any discernible effects on the cells.

Pathway inhibition studies

To evaluate potential second messenger signaling pathways, NR8383 macrophages were cultured with either the p38 inhibitor SB203580 (10 μM), JNK inhibitor SP600125 (25 μM), or ERK inhibitor PD98059 (20 μM), or NF-κB inhibitor BAY11 (25 μM) (all purchased from EMD/Calbiochem), for 1 h, followed by the addition of 5 μg/ml rPAP2 for 24 h. Preliminary data from our laboratory has demonstrated that second messenger signaling can be evaluated at this early time period. Total RNA was harvested and subjected to real-time PCR analysis of IL-1α, IL-1β, IL-6, TNF-α, and IL-18. Cell viability was always >90% as determined by trypan blue analysis.

Isolation of primary macrophages

Following nembutal anesthesia, primary macrophages were isolated from the indicated organ systems of rats. In all purifications, cell viability was >95% as determined by trypan blue staining. Macrophage cell morphology was examined by light microscopy. Assessment of macrophage function, as determined by NO production, was determined after LPS stimulation.

Peritoneal macrophages. Rat peritoneal macrophages were obtained by i.p. injection of 15 ml of cold Hank’s buffer 4 days after i.p. injection of 10 ml of 4% thioglycolate. Macrophages were centrifuged at 1500 rpm for 15 min and the cell pellet was resuspended in F-12K medium and plated in 12-well tissue-culture plates. After 1 h of incubation, nonadherent cells were removed by washing three times with PBS. Adherent cells, consisting of 95% macrophages, were supplemented with fresh F-12K medium and incubated at 37°C for 2 h before experimentation.

Alveolar macrophages. Rat alveolar macrophages were isolated from lung tissue by bronchoalveolar lavage. Lungs were lavaged three times via a tracheal cannula with 10 ml of cold HBSS. The lavage solution was centrifuged at 1500 rpm for 15 min and the cell pellet was resuspended in F-12K medium and plated in 12-well culture plates. Adherent cells, consisting of >90% macrophages, were subjected to real-time PCR analysis of IL-1α, IL-1β, IL-6, TNF-α, and IL-18. Cell viability was always >90% as determined by trypan blue analysis.

Preparation of rPAP2 proteins

Recombinant PAP2GST and PAPHis were generated from bacteria as follows.

PAP2-GST. Full-length PAP2 was PCR amplified and digested with Xho I. EcoRI restriction enzymes. Digested PAP2 PCR amplicons were inserted in-frame into the PGEX-5x-1 (Amersham Pharmacia Biotech) bacterial expression vector and sequenced. Escherichia coli BL21 cells were transformed with positive constructs, grown to a density of 2.0 OD in 1 liter of Terrific Broth and induced by the addition of 0.1 mM isopropyl-β-thiogalactoside for 2 h at 4°C. The bacterial pellet was resuspended in resuspension buffer (300 mM NaCl, 20 mM Tris (pH 7.5), 0.5% Triton X-100, 2 mM DTT) to contain protease inhibitors and sonicated on ice. Lysates were centrifuged and PAP-containing supernatants were batch incubated with glutathione sepharose beads (Amersham) for 3 h. After binding, sepharose beads were extensively washed with wash buffer (300 mM NaCl, 20 mM Tris (pH 7.5), 0.5% Triton X-100, 2 mM DTT) to contain protease inhibitors and sonicated on ice. Soluble bacterial proteins were collected after centrifugation and placed over a G50 size exclusion chromatography column and 2-ml fractions were collected and analyzed by SDS-PAGE. Samples containing abundant PAP2 protein were combined and batch purified with a 1.5-ml bed volume of cation exchange chromatography and 2-ml fractions were collected and analyzed by SDS-PAGE. Samples containing abundant PAP2 protein were combined and batch purified with a 1.5-ml bed volume of cation exchange beads (SP Sepharose Fast Flow; Sigma-Aldrich) for 2 h. Beads were washed and batch eluted with elution buffer 1 (2 M NaCl, 6 M urea) and dialyzed in dialysis buffer (6 M urea, 500 mM NaCl, 20 mM Tris (pH 7.5), 30 mM imidazole) for 6 h. The dialyzed PAP2 was then dialyzed twice in dialysis buffer (300 mM NaCl, 20 mM Tris (pH 7.5) 10 h). PAPHis. PAP2 amplicons were inserted in-frame into the PET24a bacterial expression vector (Novagen). Positive clones were transformed into BL21 (DE3) E. coli, grown to a density of 2.0 OD in 500 ml of TB broth and induced for 3 h at 37°C with 2 mM isopropyl-β-thiogalactoside. Bacteria was subsequently centrifuged and resuspended in resuspension buffer (300 mM NaCl, 20 mM Tris (pH 7.5), 1.5% Triton X-100, 2 mM DTT) to contain protease inhibitors and sonicated on ice. Soluble bacterial proteins were collected after centrifugation and placed over a G50 size exclusion chromatography column and 2-ml fractions were collected and analyzed by SDS-PAGE. Samples containing abundant PAP2 protein were combined and batch purified with a 1.5-ml bed volume of cation exchange beads (SP Sepharose Fast Flow; Sigma-Aldrich) for 2 h. Beads were washed and batch eluted with elution buffer 1 (2 M NaCl, 6 M urea) and dialyzed in dialysis buffer (6 M urea, 500 mM NaCl, 20 mM Tris (pH 7.5), 30 mM imidazole) for 6 h. The dialyzed PAP2 was then bound to a 1-ml bed volume of nickel beads for 2 h and eluted with elution buffer 2 (6 M urea, 500 mM NaCl, 20 mM Tris (pH 7.5), 250 mM imidazole, 10 mM

Table I. Primer sets for cytokine gene expression

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<th>Quantitative PCR</th>
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<td>IL-1α</td>
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<td>Forward</td>
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<td>Reverse</td>
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<td>Reverse</td>
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 Administration of rPAP2 protein causes morphologic changes in macrophages. A, NR8383 macrophages were cultured with 5 μg/ml of the indicated protein for 24 h. GST treatment (top row) did not have any affect on macrophage morphology. Treatment with PAP2-GST caused a time-dependent increase in macrophage aggregation. B, Macrophages migrated to beads that were coated with PAP2-GST protein. Treatment with PAP2-GST caused a time-dependent increase in macrophage aggregation (upper picture). C, Aggregation of NR8383 after PAP2-GST administration were quantitated by microscopic analysis. Clusters consisted of at least 20 cells. Results are the mean of clusters counted in six different microscope fields at ×10 magnification following treatment with 5 μg/ml PAP2-GST for 24 h. D, Treatment with PAP2-GST did not affect macrophage viability. Similar responses were observed with PAP2His protein (data not shown). Experiments were repeated three times with similar findings; *, p < 0.05.

Table II. Primer and probe sets for cytokine gene expression

<table>
<thead>
<tr>
<th>Real-Time PCR</th>
<th>Primer and Probe Sets</th>
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</thead>
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<td>IL-1α</td>
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<td>IL-β</td>
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<tr>
<td>IL-6</td>
<td>Forward: GCCGCTTCTTCTGACTCTTGGAATGA; Reverse: ATCTGCTTGTGCTTCTGACG; Probe: CCTGCTGAGTTCGCTTCTA</td>
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</table>

Flow cytometry analysis of PAP2 binding to NR8383 macrophages

Following treatment with 10 μg/ml PAP2 for 3 h, 1.0 × 10⁶ NR8383 macrophages were washed and incubated in FACS blocking buffer (Sigma-Aldrich) for 30 min. To determine PAP2 binding to NR8383, macrophages were incubated with 10 μg/ml PAP2 for 3 h, 1.0 × 10⁶ NR8383 macrophages were cultured with 5 μg/ml rPAP2GST for 3, 12, and 24 h. Total RNA was extracted from cultures with TRizol (Invitrogen Life Technologies), quantitated by spectrophotometric measurement of absorbance at 260 nm and analyzed on a 1% agarose gel for quality. Purified RNA was treated with DNase free (Ambion) for 1 h followed by cDNA synthesis using the RETROscript reverse transcriptase kit (Ambion) according to the manufacturer’s recommendations. Expression of cytokines, including IL-1, IL-6, IL-10, IL-12, IL-15, IL-18, and TNF-α was analyzed by standard PCR, using 1 μl of cDNA per reaction.

Immunofluorescence

NR8383 macrophages were grown on cover slips to 70% confluence and incubated with 10 μg/ml rPAP2His or vehicle for 2 h at 5% carbon dioxide and 37°C. Cells were then fixed in 3.7% formaldehyde for 5 min and blocked with 2% BSA for 30 min. Cells were subsequently incubated with rabbit polyclonal anti-PAP2 Ab (1/100 dilution) for 1 h and washed three times with PBS. Macrophages were then incubated with a 1/500 dilution of Alexa Fluor 555 goat anti-rabbit IgG secondary Ab for 1 h (Invitrogen Life Technologies) and washed three times with PBS. Labeled cells were analyzed using a confocal laser-scanning module attached to a Zeiss microscope equipped with an argon-krypton ion laser.

Semiquantitative cytokine expression analysis

NR8383 macrophages were grown near confluence in 12-well plates and cultured with 5 μg/ml rPAP2GST for 3, 12, and 24 h. Total RNA was extracted from cultures with TRizol (Invitrogen Life Technologies), quantitated by spectrophotometric measurement of absorbance at 260 nm and analyzed on a 1% agarose gel for quality. Purified RNA was treated with DNase free (Ambion) for 1 h followed by cDNA synthesis using the RETROscript reverse transcriptase kit (Ambion) according to the manufacturer’s recommendations. Expression of cytokines, including IL-1, IL-6, IL-10, IL-12, IL-15, IL-18, and TNF-α was analyzed by standard PCR, using 1 μl of cDNA per reaction.
reaction. PCR consisted of 50-µl reactions that included 2.5 U of Taq DNA polymerase, 1x Taq buffer, 1 mM MgCl₂, 1 mM dNTPs and 1 µM of each specific primer (Table I). PCR products were analyzed on a 2% agarose gel that was stained with ethidium bromide (500 ng/ml) and photographed. Band intensities were quantitated with Quantity One analysis software (Bio-Rad).

Quantitative real-time PCR

Total RNA was purified from NR8383 macrophages by TRIzol extraction. A total of 100 ng of RNA was used for each real-time PCR. Amplification (40 cycles) was conducted in a 25-µl reaction, containing 2× PCR master mix (Applied Biosystems) and 100 ng of RNA. Primer and probes used to analyze for IL-1, IL-6, TNF-α, and β-actin expression are summarized in Table II. Gene expression was quantitated relative to β-actin; relative expression of the target gene was calculated as $2^{-\Delta\Delta Ct}$, where $\Delta Ct$ is the difference between the threshold cycle ($Ct$) for the gene of interest and the $Ct$ for β-actin. In each experiment, the value of the relative expression of the control sample (untreated) was given a value of 1 and the expression of other treatments was plotted relative to the control.

Cytokine ELISA

IL-1, IL-6, TNF-α, and IL-10 were measured in tissue-culture medium by their respective ELISA kits (R&D Systems) in accordance with the manufacturer’s recommendations. The ELISAs for these cytokines were sensitive to 50 pg/ml of the respective recombinant cytokine. Unless stated, all cytokine assays were performed after treatment with 5 µg/ml rPAP2 for 24 h.

**NF-κB analysis**

**Nuclear translocation.** NF-κB nuclear translocation was quantified in NR8383 cells by visual fluorescent microscopy. Cells were plated on to cover slips to 70% confluence and cultured with 5 µg/ml PAP2His for 3 h. Cells were washed in PBS and fixed with 0.1% Triton X-100. Slides were blocked with 2% BSA for 30 min followed by incubation with 1/300 anti-NF-κB for 1 h and Alexa Fluor 555-conjugated secondary Ab (Molecular Probes). Preliminary data from our laboratory has demonstrated that second messenger signaling can be evaluated at this early time period. Fluorescence was assessed by a confocal laser microscope.

**Immunoblot assay.** NR8383 cells were stimulated with 5 µg/ml PAP2His for 5, 15, 30, 60, 120, 180 min. After the indicated times, cells were washed once with PBS, scraped, pelleted, and resuspended in protein extraction buffer (radioimmunoprecipitation assay buffer: 150 mM NaCl, 10 mM Tris pH 7.0, 0.1% SDS, 1% Triton X-100, 5 mM EDTA) and incubated on ice for 15 min. Lysates were centrifuged at 15,000 × g for 20 min at 4°C; supernatants were collected for immunoblots. Protein concentrations were determined using the modified Lowry assay. Samples were placed on a 10% SDS-PAGE followed by transfer onto nitrocellulose paper. The membrane was blocked with 2% BSA and probed with polyclonal Ab against P-IκBα, and total IκB. Primary Abs were detected with a HRP-conjugated anti-rabbit polyclonal Ab, which was visualized using the Supersignal West Pico Chemiluminescent Substrate kit (Pierce) and film.

**FIGURE 2.** PAP2 binding to macrophages. A, NR8383 macrophages were cultured with 10 µg/ml PAP2 for 2 h. Cells were fixed with 3.7% formaldehyde and cell surface immunofluorescence staining for PAP2 binding was analyzed with a polyclonal anti-PAP2 and Alexa 555 fluorescent secondary. Control untreated macrophages which were similarly analyzed were negative. B, Histogram of flow cytometry analysis of cell surface PAP2 macrophage interaction after treatment with 10 µg/ml PAP2 reveals 45–60% of cells bind PAP2 after a 2-h incubation. Experiment were repeated three times with similar findings.
Statistical analysis

All data represent a minimum of three independent experiments and are expressed as the value ± the SD. Statistical analysis was performed using a two-tailed Student t test. A p value of <0.05 was considered statistically significant.

Results

Morphologic effects of rPAP2 protein on macrophages

Because macrophages frequently coordinate the inflammatory response, we postulated that PAP2 may directly affect macrophage activity. To this end, the macrophage cell line NR8383 was cultured with increasing concentrations of PAP2-GST. As shown in Fig. 1A, cells cultured with PAP2-GST demonstrated an aggregated phenotype which was absent in control (GST) treated macrophages. Additionally, incubation of macrophages with glutathione sepharose beads bound with PAP2-GST resulted in the migration, binding, and agglutination of macrophages toward the beads, a phenomenon which was absent in glutathione sepharose beads bound with GST protein alone (Fig. 1, B and C). Cellular viability was always >95% after incubation with PAP2 protein (Fig. 1D).

PAP2 binding to NR8383 macrophages

The observation that PAP2 mediates a change in macrophage morphology suggests a protein-protein interaction between PAP2 and the macrophage cell surface. To further investigate this finding, NR8383 cells were cultured with PAP2 protein and cell surface binding was determined by immunofluorescence and flow cytometry. As demonstrated in Fig. 2A, localization of PAP2 on the surface of nonpermeabilized macrophages can be appreciated by fluorescent microscopy. Confirmatory flow cytometric analysis of macrophages cultured in the presence of PAP2 shows that ~45% of macrophages bind PAP2 (Fig. 2B), suggesting that macrophages express a potential PAP2 receptor.

rPAP2 induces the production of select macrophage inflammatory cytokines

We postulated that with the strong and rapid induction of PAP in the inflammatory setting and the effects of aggregation on macrophages, PAP may induce mediators of inflammation in macrophages. We assessed the expression of macrophage-derived inflammatory cytokines after PAP2 administration. NR8383 macrophages were incubated with 5 µg/ml PAP2 and screened for a change in expression of inflammatory cytokines by semiquantitative PCR at 3, 12, and 24 h. Treatment with rPAP2 resulted in increased expression of IL-1α, IL-1β, IL-6, TNF-α, and IL-10, which began at 6 h and later increased at 24 h (see Fig. 3). In contrast, no significant differences in expression of IL-12, IL-15, or IL-18 were observed at any time points.

Quantitative analysis of proinflammatory cytokines

To validate our PCR-based screening of PAP-induced cytokine changes, we analyzed PAP2-treated macrophages using quantitative real-time PCR. As shown in Fig. 4A, analysis of IL-1α, IL-1β, IL-6, and TNF-α revealed a 26-, 33-, 58-, and 21-fold increase in gene expression when compared with GST-treated cells. Furthermore, boiling PAP2 before macrophage incubation significantly decreased cytokine gene expression for IL-1α, IL-1β, IL-6, and TNF-α (Fig. 4A). To confirm the RNA data, protein levels of IL-1α, IL-1β, IL-6, and TNF-α in the culture supernatant were measured by ELISA and revealed a 48-, 42-, 16-, and 104-fold increase over GST-control-treated macrophages, respectively (Fig. 4B). Similarly, denaturing PAP2 (boiling) before treating macrophages with it caused a significant drop in activity as exhibited by a 86, 94, 93, and 93%, reduction in protein levels of IL-1α, IL-1β, IL-6, and TNF-α, respectively (Fig. 4B). Similar data were observed with PAP2-His protein (data not shown).

Dose-related response

Stimulation of macrophages with increasing concentrations of PAP2His resulted in dose-dependent increases in the expression of IL-1α, IL-1β, IL-6, and TNF-α (Fig. 5). However, the up-regulation of inflammatory cytokines plateaus at 10 µg/ml PAP2 and subsequently decreases after treatment with 15 µg/ml PAP2 (Fig. 5A). This may represent a saturation point for the biologic effect of PAP2 or a desensitization of the putative PAP2 receptor, or its signaling pathway. Analysis of protein levels of these inflammatory cytokines within the culture medium demonstrated a similar pattern with the exception that a reduction in secretion was observed at higher doses of PAP2 (Fig. 5B); decreases in IL-1β and IL-6 were observed after treatment with 15 µg/ml PAP2 whereas TNF-α secretion decreased after 17.5 µg/ml. In all cases, the viability of the macrophages was not affected by increasing concentrations of PAP2 proteins (data not shown). Similar results were observed with PAP-GST protein (data not shown).

Effect of PAP2 on cytokine expression in primary macrophages

PAP is increased in serum in acute pancreatitis and may modulate the multiorgan dysfunction which occurs in this disease. We therefore sought to examine the effects of rPAP2 on resident primary macrophage populations from various organ systems. We isolated rat alveolar, peritoneal, splenic, and blood monocytes from their respective organs. We initially studied the effect of PAP2 on primary alveolar macrophages and compared its activity to splenic macrophages. As shown in Fig. 6A, primary alveolar macrophages cultured in the presence of PAP2 had increased expression of IL-1α, IL-1β, IL-6, and TNF-α as demonstrated by a 26-, 67-, 39-, and 149-fold change, similar to

![Figure 3](http://www.jimmunol.org/Downloaded_from.http://www.jimmunol.org/Downloaded_from.http://www.jimmunol.org/Downloaded_from.http://www.jimmunol.org/Downloaded_from.http://www.jimmunol.org/)

**FIGURE 3.** Screening for a change in expression of macrophage-derived inflammatory cytokines after culturing with 5 µg/ml PAP2 for 3, 12, and 24 h. RT-PCR of total RNA isolated from untreated NR8383 macrophages and PAP2-treated macrophages were generated. Expression of each cytokine was analyzed by PCR using specific primers (Table I). Respective PCR fragment were analyzed on a 1% agarose gel. There was no difference in the expression levels of IL-12, IL-15, and IL-18. An increase in IL-1α, IL-1β, IL-6, IL-10, and TNF-α was observed following PAP2 treatment, starting at 12 h and increasing at 24 h. Data represent one of three experiments; all showed similar results.


<table>
<thead>
<tr>
<th>Cytokine</th>
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<th>24 Hour</th>
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those observed with NR8383 cells. In contrast, there was no significant PAP2 mediated up-regulation of proinflammatory cytokines in splenic macrophages after incubation for 24 h. To further assess and confirm select cytokine protein levels from various macrophage populations, we again isolated and treated primary alveolar macrophages, as well as peritoneal macrophages and

FIGURE 4. Quantification of inflammatory cytokines after culturing with PAP2. A, Using specific primers for real-time PCR (Table II), the expression of IL-1α, IL-1β, IL-6, and TNF-α in GST, PAP2-GST, and boiled PAP2-GST-treated NR8383 macrophages was analyzed. Total RNA was extracted from macrophages and subjected to one step real-time PCR analysis. PAP2-GST-treated macrophages revealed a significant increase in the listed cytokines which was abrogated after heat denaturation. Control GST-treated macrophages did not alter cytokine expression. One representative of three experiments is shown. B, Analysis of cytokine production in GST, PAP2-GST, boiled PAP2-GST NR8383 macrophages was performed from cell culture medium using ELISAs that are specific for each cytokine. Secretion of the listed cytokines is significantly induced by PAP2 and decreases to nearly basal levels after heat inactivation. Culturing with control GST did not have any effect; p < 0.05 for all values compared with PAP/GST.

FIGURE 5. Dose-related response analysis of PAP2-mediated cytokine expression. A, NR8383 macrophages were cultured with increasing concentrations of PAP2His ranging between 2 and 15 μg/ml for 24 h. Macrophages were harvested at 24 h and total RNA was subjected to real-time PCR analysis. Saturation of cytokine transcription was achieved at 10 μg/ml. Desensitization of PAP2-mediated cytokine production is demonstrated by a decreased response at 15 μg/ml. B, Similarly, as described above NR8383 macrophages were cultured with increasing doses of PAP2, ranging from 2 to 25 μg/ml. Culture medium were collected for each group and cytokine levels were analyzed by ELISA at 24 h. Macrophage viability remained above 90% for all PAP2-treated groups as demonstrated by trypan blue at 24 h (data not shown). Data represent the mean ± SD of a minimum of three experiments.
blood monocytes, with PAP2 protein over a 24-h period. Confirmatory ELISA protein analysis of the culture supernatant, respectively, revealed a 34-, 25-, and 42-fold increase in secreted TNF-α (Fig. 6B).

**FIGURE 6.** Effect of PAP2 on primary macrophages. A, Splenic and alveolar macrophages were cultured with 5 μg/ml PAP2His for 24 h. Control macrophages were cultured with vehicle. Total RNA was extracted and subjected to one-step real-time PCR analysis. PAP2 treatment of splenic macrophages did not significantly increase cytokine expression. A significant increase in cytokine production in PAP2-treated alveolar macrophages was observed. B, Monocytes, peritoneal, and alveolar macrophages were treated with 5 μg/ml PAP2His for 24 h. Macrophages treated with vehicle served as controls. Secreted TNF-α was measured in culture medium using cytokine-specific ELISA. Similar findings were observed for IL-1 and IL-6 (data not shown). Data represent the mean ± SD of a minimum of three experiments; *, p < 0.05.

**FIGURE 7.** Effects of MAPK and NF-κB-signaling pathway inhibitors on PAP2-mediated cytokine production. Cells were pretreated with BAY11 (25 μM; NF-κB inhibitor), PD98059 (20 μM; ERK inhibitor), SB203580 (5 μM; p38 inhibitor), or SP600125 (25 μM; JNK inhibitor) for 1 h followed by incubation with 5 μg/ml PAP2. After 24 h, total RNA was purified and cytokine expression was measured by real-time PCR. Data represent mean (±SD) obtained from triplicate samples and are representative of two experiments.

**PAP2-mediated cytokine up-regulation signals through the NF-κB pathway**

Previous studies have reported that the anti-inflammatory effects of PAP1 are mediated through the inhibition of the NF-κB pathway (6, 23, 24). Because our previous findings demonstrated a pro-inflammatory function for the PAP2 isoform, we considered the possibility that this isoform may elicit its effects through mechanisms distinct from PAP1. Thus, we assessed PAP2 signaling by analyzing, in vitro, four pathways known to be involved in inflammation: NF-κB, p38, JNK, and ERK (25–28). NR8383 cells were incubated with rPAP2His plus inhibitors that block each of these specific pathways. Interestingly, inhibition of the NF-κB pathway completely suppressed PAP2-induced cytokine production, indicating that this pathway is important for the activity of PAP2.
FIGURE 8. PAP2-mediated activation of NF-κB transcription factors. NR8383 macrophages were cultured ± 5 μg/ml PAP2 for 3 h and fixed with formaldehyde. Cells were subsequently stained with anti-NF-κB Ab and Alexa-labeled secondary Ab. Cells were visualized by confocal fluorescence microscopy. A. Untreated cells exhibit the localization of NF-κB in the cytoplasm. B. PAP2-treated macrophages display a significant increase in the translocation of NF-κB into the nucleus. C. Nuclear NF-κB was quantitated by visual fluorescent microscopy. Quantification was reported as the average of five fields representing the number of positive cells per 100 cells. D. Western blot analysis reveals a time-dependent increase of IκBα phosphorylation status (P-IκB) after treatment with 5 μg/ml PAP2 for the indicated time. Total IκB analysis shows a modest decrease at 180 min. β-actin analysis served as the loading control. Data represent one of three experiments with similar results; * , p < 0.05.

(Fig. 7). Inhibition of the ERK and JNK pathway with their respective inhibitors caused a partial blockade of IL-1α, IL-1β, and TNF-α production and a significant decrease in IL-6. In contrast, inhibition of the p38 pathway did not have any effect on PAP2-mediated cytokine expression. These data suggest that the PAP2 isoform up-regulates the expression of IL-1α, IL-1β, IL-6, and TNF-α in macrophages via the NF-κB pathway.

Building on this, we then investigated the ability of PAP2 to increase the DNA-binding activity of NF-κB. We first addressed this by analyzing for the degradation of the NF-κB inhibitor, IκBα, which is one of the first steps to occur before NF-κB activation and translocation into the nucleus. We assessed this process by screening for S32 phosphorylation of IκB at specific time points after stimulation with PAP2 (Fig. 8D). Phosphorylation of IκB was detectable at 15 min, reaching maximal phosphorylation at 60 min which was maintained through 180 min. Similar activation of the NF-κB pathway at these early time points have been reported (24). Because IκB degradation alone does not fully denote for the functional activity of NF-κB, we assessed for the translocation of this transcription factor into the nucleus by immunofluorescence (Fig. 8B). After a 2-h incubation of macrophages with PAP2, the number of cells with NF-κB nuclear staining reached over 75% (Fig. 8C). To ensure equal protein loading, samples were immunoblotted with an Ab against β-actin. In all cases, viability of these cells was not compromised (data not shown).

Levels of PAP in serum and its biologic effects

Previous studies in our laboratory (11, 29, 30) and by others (3, 31) have shown that experimental acute pancreatitis in rats leads to a significant increase in serum PAP levels. Clinical studies have similarly elevated levels in patients with acute pancreatitis (32–34). Preliminary studies in our laboratory have demonstrated that serum obtained from acute pancreatitis animals induces morphologic changes in macrophages, and that, in certain cases, detection of PAP requires serum-based components (31, 35). Therefore, we set out to study whether the biologic activity of purified PAP2 would be affected by incubation with normal rat serum. PAP2 protein was incubated with 5% normal rat serum for 2 h before culturing with NR8383 cells. As demonstrated in Fig. 9A, the presence of 5% normal rat serum suppresses PAP-induced TNF-α gene expression. Similar findings were observed for IL-1 and IL-6 (data not shown). These results indicate the presence of a potential PAP-binding protein, within serum, which inhibits its activity. Increasing protein concentrations of PAP2 were incubated with a constant amount of normal rat serum after which cytokine expression levels were determined. As shown in Fig. 9B, saturation of the putative regulatory serum protein was achievable, where higher concentrations of PAP2-mediated up-regulation of macrophage cytokines. A. A total of 5 μg/ml PAP2 was incubated with 5% normal rat serum for 1 h followed by culturing NR8383 macrophages with this mixture for 24 h. This was compared with macrophages cultured with PAP2 at the same concentration. Controls included culturing macrophages with 5% normal serum. Secretion of TNF-α into the culture medium was determined by ELISA. In the presence of normal serum, PAP2 was unable to induce TNF-α secretion, indicating the presence of a serum regulatory protein. B. Increasing concentrations of PAP2 were incubated with 5% normal rat serum for 1 h before macrophage assay. PAP2 plus serum dose response analysis was assessed for TNF secretion. Saturation of the serum protein was noticeable at 10 μg/ml and significantly increased levels of TNF-α was noticeable at 20 μg/ml.
PAP2 resulted in a significant increase in secreted TNF-α. It is interesting to note that TNF-α levels were suppressed by 65% when treated with PAP2 at a concentration of 20 μg/ml plus normal rat serum compared with PAP treatment alone.

Discussion
The key role of PAP in acute pancreatitis is not fully understood. In rats, three PAP isoforms (PAP1, PAP2, PAP3) are highly induced during pancreatitis. Furthermore, the PAP2 isoform is consistently expressed at a higher level than PAP1 and 3. Over the past few years, PAP proteins have been reported to be new effector molecules of macrophage function. PAP1 has been shown to inhibit macrophage activation by down-regulating the synthesis of TNF-α and IL-6, thereby promoting an anti-inflammatory state (15). PAP3 has been shown to be a macrophage chemokine, recruiting macrophages to areas of neural tissue damage (18). Here, we demonstrate that PAP2 mediates the up-regulation of inflammatory cytokines and stimulates macrophage activity thus identifying a new function for PAP2: it is a modulator of the inflammatory response. We postulate that the relationship of PAP isoform expression, secretion, or regulation relative to each other may define the course of the inflammatory response which occurs in pancreatitis.

The importance of PAP proteins in animals has been evaluated by RNA inhibition (14, 15, 36), Ab neutralization (15, 21), and PAP up-regulation studies (11, 37). Our laboratory has previously demonstrated that the inhibition of all three PAP isoforms in rats with experimental acute pancreatitis caused a significant increase in pancreatic tissue damage (14). We also demonstrated that the induction of all three isoforms before the induction of acute pancreatitis decreased pancreatic damage (11). Others noted that decreased expression of PAP in a P8 transcription knockout mouse was associated with increased acute pancreatitis severity (15). We have also noted that PAP affects leukocytes and that the areas within the parenchyma which express PAP2 protein 1) do not appear inflamed and 2) appear to be infiltrated by leukocytes. In light of these findings, it is possible that PAP2 serves to recruit macrophages and other leukocytes to areas devoid of tissue damage as a means to protect these areas from subsequent damage. Others have shown that chemical stimulation of macrophages, in the context of experimental acute pancreatitis, results in improved animal survival rates (38, 39). In contrast, other studies have shown that increased macrophage activation plays a role in the pathogenesis of pancreatic injury (40, 41). The discrepancies in the data of the role of macrophages and other leukocytes in acute pancreatitis is likely due to differences in animal models, activating agents, free radical species, and the time and type of macrophages/leukocytes studied (42), or simply differences in PAP1 vs PAP2 levels.

To investigate PAP2-mediated macrophage physiology, we generated two different rPAP2 proteins from E. coli expression systems, both of which behave in a similar fashion in vitro. Purified soluble protein was used to perform assays on the rat macrophage cell line NR8383. Following incubation of macrophages with rPAP2, we observed a noticeable shift in macrophage phenotype: macrophages aggregated in a way that increased in intensity over time, without compromising cell viability. In addition, immunofluorescence studies demonstrated that PAP2 protein binds macrophages suggesting a direct effect on macrophage responses.

The observations that elevated levels of TNF-α, IL-1β, and IL-6 are involved with pancreatitis and that they come from macrophages (43–45) suggests that activated macrophages mediate the pathologic process of pancreatitis. Recent reports suggest that the release of inflammatory components from extrapancreatic macrophages is secondary to a pancreatic-derived agent (2, 46, 47). Here, we postulate that in acute pancreatitis, the overproduction and secretion of PAP1 and PAP2 leads to the modulation of macrophages, which may directly impact the local and systemic inflammatory responses. We showed that PAP2 induced significant increases in IL-1, IL-6, IL-10, and TNF-α, but not IL-12a, IL-12b, IL-15, and IL-18, which was initially observed at 12 h and increased at 24 h. The fact that PAP2 induced expression of select cytokines but did not induce a pan-cytokine response, as evidenced by the lack of difference in expression of IL-12 and IL-15, further suggests that macrophages may be orchestrating the inflammatory response in acute pancreatitis.

Analysis of PAP2 treatment on macrophages revealed dose-dependent enhancement of secreted cytokines IL-1α, IL-1β, IL-6, and TNF-α; macrophage viability was not compromised throughout any of these experiments. Combined with our observation that PAP2 binds to macrophages, this suggested the presence of a PAP2 receptor on macrophages. It is likely that these potential PAP receptors are different for each isoform and may differentially regulate cell surface expression in pancreatitis and affect disease outcome.

Because a differential organ response to acute pancreatitis may very well be dependent on its own resident macrophage population, we purified resident (primary) macrophages from lung, peritoneum, spleen, and blood (monocytes). As we observed in the NR8383 cells, PAP2 induced IL-1, IL-6, and TNF-α expression in blood-derived alveolar and peritoneal macrophages, but not in splenic macrophages. This ex vivo finding reinforces our hypothesis and demonstrates that pancreas-derived PAP2 is capable of activating macrophages in various organ systems.

Previous studies have demonstrated that PAP1 mediates its anti-inflammatory effects by inhibiting the NF-κB pathway (6, 15, 24). Additionally, PAP1 was also shown to stimulate a neuronal survival pathway in motor neurons through the NF-κB pathway (23). Here, we found that PAP2 cell signaling was also through the NF-κB pathway. Assays performed on macrophages revealed that PAP2 can mediate the translocation of NF-κB into the nucleus and leads to the degradation of the inhibitory IκBα protein. This finding is very intriguing in that PAP1 and PAP2 appear to mediate their biologic affects in macrophages through the NF-κB pathway but have opposing physiologic outcomes, suggesting that PAP proteins are regulators of the NF-κB pathway signaling and that the inflammatory outcome is likely contingent on either the dominantly expressed isoform or the stoichiometric relationship among the isoforms. Whether PAP2 and/or other PAP isoforms use members of the stress kinase pathway including MAPKs, ERKs, JNKs, and p38—as have been demonstrated in other aspects of pancreatic disease (28)—remains to be determined.

We postulate that PAP proteins mediate the systemic inflammation response of pancreatitis. To affect distant macrophage populations, PAP2 must traverse via sera. We demonstrated that incubation of PAP2 with normal rat serum before culturing with macrophages caused a complete inhibition of its activity, as measured by the absence of cytokine production. This suggests that a regulatory serum protein binds to PAP2 and can obviate its function. Thus, one can envision that saturation of this regulatory protein would be detrimental to the overall physiology of an organism, perhaps increasing susceptibility to a systemic inflammatory reaction. Interestingly, analysis of serum levels of PAP proteins in patients with acute pancreatitis revealed a correlation: patients with severe pancreatitis had dramatically greater concentrations of PAP (>600 μg/ml) when compared with patients with mild pancreatitis (<200 μg/ml) (33, 34, 48, 49). When compared with patients with active celiac disease, Crohn’s disease, and ulcerative colitis, PAP serum levels were found to be much lower (24–130 μg/ml) (6, 50, 51). It is likely that the relationship of PAP isoforms...
with their potential receptors is integral to these inflammatory responses.

This observation can help explain the extrapancreatic complications associated with severe acute pancreatitis. Of the above-mentioned clinical studies, it is possible that in acute pancreatitis, the oversaturation of the serum component yields free, uninhibited PAP2 in serum. Unbound circulating PAP2, which is presumably active and in higher concentrations than PAP1, now has the ability to access and activate extrapancreatic macrophages. The activation of resident macrophages can result in a cytokine storm setting in motion the basis for the formation of a systemic inflammatory syndrome. For example, the activation of alveolar macrophages might precipitate respiratory distress in some patients. Elucidation of what PAP2 binds to and its relationship to PAP2 may facilitate a better understanding of the inflammatory response of acute pancreatitis.

Although a great deal of emphasis has been placed on determining the role of PAP proteins in inflammatory diseases, studies have only analyzed the PAP1 isoform. Because all rat PAP isoforms exhibit functional effects on macrophages, future functional studies in rats with acute pancreatitis must take into account the expression of all three isoforms. Moreover, because PAP proteins have been reported to self activate (24) and form homodimers as well as heterodimers with each other (52, 53), the physiologic outcome may very well be dependent on the stoichiometry of these proteins. Additionally, these proteins are shown to have different biochemical properties within the pancreas environment as demonstrated by trypsin cleavage studies on all PAP isoforms. Conceivably, as different members of the PAP family appear to have different biologic functions, macrophage physiology may be dependent on the dominating isoform, and the resulting biologic effect may depend on the levels of all three isoforms, as well as on the presence of PAP-binding proteins. Additionally, the biological function of PAP proteins may depend on several diverse factors including protein concentration, cell type, and tissue environment. Indeed, it could be that PAP2 provides a protective response in pancreatitis in that it stimulates and recruits macrophages to the site of organ damage; similar protective responses have been described for heme-activated macrophages (39).

In conclusion, our findings are the first to demonstrate a direct role of PAP2 on macrophages, and the likelihood that PAP2 binds to a receptor on these cells as well as to a binding protein in serum. These data suggest that during acute pancreatitis, the regulation of PAP2 may mediate the local vs systemic inflammatory reaction. This newly proposed mechanism for the extrapancreatic complications found in acute pancreatitis has broad implications for the clinical outcome of patients with severe acute pancreatitis as well as other inflammatory conditions.

Disclosures

The authors have no financial conflict of interest.

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