JOP. J Pancreas (Online) 2006; 7(2):174-184.

ORIGINAL ARTICLE

Heat Shock Factor-1 and Nuclear Factor-kappaB Are Systemically Activated in Human Acute Pancreatitis

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ABSTRACT

Context Nuclear factor-kappa B (NF-kappaB) is a transcription factor for a wide range of proinflammatory mediators while heat shock factor-1 (HSF-1) transcribes stress proteins that protect against cellular damage. Both are attractive therapeutic targets, undergoing investigation in other acute inflammatory conditions, such as sepsis.

Objective To evaluate the role of the transcription factors NF-kappaB and HSF-1 in human acute pancreatitis and their relationship to cytokine/chemokine production, disease severity and outcome.

Patients Twenty-four patients with acute pancreatitis and 12 healthy controls.

Main outcome measures Peripheral blood mononuclear cells were isolated. NF-kappaB and HSF-1 were measured by electrophoretic mobility shift assay. Soluble tumor necrosis factor (TNF) receptor II and interleukin-8 were measured by ELISA. Acute physiology scores (APS), APACHE II scores and final Atlanta designations of severity were also determined.

Results Systemic NF-kappaB activation occurs in acute pancreatitis compared to healthy controls (P=0.004). However, there was no significant difference between those with mild and severe disease (P=0.685). Systemic activation of HSF-1 was observed in acute pancreatitis compared to healthy controls although this did not reach statistical significance (P=0.053). Activation, however, was greatest in those who had a final Atlanta designation of mild pancreatitis compared to those who had a severe attack of acute pancreatitis (P=0.036). Furthermore, HSF-1 was inversely correlated with acute physiology score (APS; r=-0.49, P=0.019) and APACHE II score (r=-0.47, P=0.026).

Conclusions Both NF-kappaB and HSF-1 are systemically activated in human acute pancreatitis. HSF-1 activation may protect against severity of pancreatitis.

INTRODUCTION

The early pathophysiological events that occur during acute pancreatitis involve the premature activation and retention of proteases, within the acinar cell. These activated enzymes injure the acinar cells, which then produce cytokines and chemokines, resulting in the recruitment of inflammatory cells, such as neutrophils and macrophages. This further amplifies the inflammatory reaction and the extent of pancreatic injury [1, 2]. The degree to which these mediators escape into the circulation determines the nature of the systemic inflammatory response. The failure of therapies that sought to counteract acinar cell dysfunction [3] prompted a shift in emphasis in research in acute pancreatitis, such that control of the inflammatory process per se became the therapeutic aim [4]. A philosophy of damage limitation rather than damage prevention prevailed [5, 6, 7, 8]. Given the complexity of the inflammatory process and its myriad of mediators and cellular effectors, substances that occupy a proximal position in the inflammatory cascade represent the most logical therapeutic targets. This study investigates two such potential proximal targets; the transcription factors nuclear factor-kappaB (NF-kappaB) and heat shock factor-1 (HSF-1).

NF-kappaB designates a family of transcription factors that, upon activation, translocate from the cytosol to the cell nucleus, where they bind to their consensus sequence on the promoter-enhancer region of a spectrum of genes, which are then transcribed [9]. These genes include a variety of cytokines (e.g. TNF, IL-1), chemokines (e.g. IL-8), hemopoetic growth factors, adhesion molecules, immunoreceptors and specific enzymes [10]. Thus, NF-kappaB occupies a critical position in the inflammatory cascade. NF-kappaB activation has been demonstrated in sepsis [11, 12, 13, 14], post surgical organ dysfunction [15], experimental pancreatitis [16, 17, 18, 19, 20, 21], and human acute pancreatitis [22]. Consequently, it has attracted much attention as a potential target for anti-inflammatory therapy [23, 24].

Heat shock proteins are protective against the deleterious effects of the toxic mediators of inflammation, providing cellular protection against the damaging effects of reactive oxygen species and cytokines [25, 26]. This response involves transcriptional activation mediated by the transcription factor, HSF-1 [27]. Recent data has demonstrated that the heat-shock response inhibits nuclear translocation of NF-kappaB and the phosphorylation and subsequent degradation of its inhibitory protein, I-kappaB, thereby modulating the expression of various NF-kappaB dependent genes [28, 29, 30, 31, 32].

We sought to evaluate the role of these transcription factors in human acute pancreatitis and their relationship to cytokine/chemokine production, disease severity and outcome.

MATERIALS AND METHODS

Diagnosis and Study Subjects

The diagnostic criteria for acute pancreatitis were based upon the classification system adopted at the Atlanta symposium in 1992 [33]. The diagnosis was based upon clinical manifestations consistent with the disease and a serum amylase greater than three times the upper limit of normal. In accordance with this classification system, an attack was classified as mild if associated with minimal organ dysfunction and an uneventful recovery. An attack was classified as severe if associated with organ failure and/or local complications.

Twenty four patients with acute pancreatitis, who had been admitted to the Department of Surgery, Derriford Hospital, Plymouth, UK underwent measurement of NF-kappaB, HSF-1, soluble tumor necrosis factor receptor II (sTNFRII) and interleukin-8 (IL-8), if admitted within twenty four hours of symptom onset. Acute physiology scores (APS), acute physiological and chronic health evaluation (APACHE II) scores and organ failure scores (OFS) were also calculated to further characterize the disease episode [34]. Of these, 16 were male and 8 were female, with a median age of 59.5 years (range 24-90 years). Aetiology was attributed to gallstones in 14 patients and alcohol in 10 patients. Twenty patients had mild pancreatitis while 4 had severe pancreatitis, according to the Atlanta classification. Of the 4 patients with acute severe pancreatitis, 1 had acute respiratory failure, acute renal failure and disseminated intravascular coagulopathy, 1 had acute respiratory failure and hypocalcaemia, 1 had acute respiratory failure only and 1 had acute upper gastrointestinal haemorrhage. One of the 4 patients with acute severe pancreatitis died of multi-organ dysfunction syndrome within the first week of admission.

Twelve healthy control subjects, recruited from medical and research personnel, also underwent measurement of NF-kappaB, HSF-1, sTNFRII and IL-8.

Peripheral Blood Mononuclear Cell (PBMC) Extraction

PBMCs are a mixed cell population, composed largely of monocytes and lymphocytes. As these cell types participate in intercellular signaling, which can modify cell function, we chose to analyze this whole mononuclear cell population rather than isolated monocytes as this method more accurately reflects cellular action *in vivo*.

Blood was collected in tubes containing EDTA and diluted with an equal volume of phosphate-buffered saline (PBS). Diluted blood was layered over Lymphoprep (Nycomed, Oslo, Norway). Following centrifugation, the band at the sample/ medium interface, containing peripheral blood mononuclear cells (PBMCs), was removed with a Pasteur pipette. The harvested fraction was diluted in PBS and the cells pelleted by centrifugation.

Preparation of Nuclear Extracts

Nuclear extracts were prepared, with modification, as previously described [35]. Briefly, pelleted cells were resuspended in Buffer A, containing 10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2% NP-40, 100 mM 4-(2-aminoethyl)benzene-sulfonylfluoride (AEBSF), 18.4 mg/mL Na_3VO_4, 42 mg/mL NaF and 2.2 mg/mL aprotonin and incubated on ice for 15 minutes. Lysates were then centrifuged at 13,000 rpm for 15 minutes. The supernatant, containing cytoplasmic protein was removed and stored for future use. Nuclear pellets were then resuspended in Buffer C containing 20 mM HEPES, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.2 mM EDTA, with the same concentrations of protease inhibitors as Buffer A, incubated on ice for 15 minutes and centrifuged at 13,000 rpm for 15 minutes. The resulting supernatant contained the nuclear proteins. Protein concentrations for nuclear fractions were determined using a Bradford assay (BioRad, Hertfordshire, UK).

Electrophoretic Mobility Shift Assay

Oligonucleotides for NF-kappaB, 5'-AGTTGAGGGGACTTTCCCAGGC-3'. (Promega Life Sciences, Southampton, UK) and HSF-1, 5'-GCCTCGAATGTTCGCGAAGTT-3' (MWG, Ebersberg, Germany) were labeled with gamma-^{32}P-ATP using T4 kinase. The labeled probe was incubated with nuclear protein (10 µg) at room temperature for 20 minutes in a binding buffer containing poly (di-dC) oligonucleotide. Protein-DNA complexes were resolved on a 5% native polyacrylamide gel. Competition experiments were performed with a molar excess of unlabelled NF-kappaB or HSF oligonucleotide. All gels were transferred to Whatman 3M paper, (Whatman Inc., Maidstone, UK) dried and exposed to photographic film overnight at -80°C with an intensifying screen. Bands were quantified using a phosphorImager (BioRad, Hertfordshire, UK) with MultiAnalyst (BioRad, Hertfordshire, United Kingdom) software. Representative radiographs are illustrated in Figures 1 to 6.

Determination of sTNFRII and IL-8 Concentrations in Plasma

Plasma was separated from blood by centrifugation during the process of PBMC extraction. Assays for sTNFRII (Quantikine kit, R&D Systems, Abingdon, UK) and IL-8 (OptEIA kit, Pharmingen, San Diego, USA) were performed using a quantitative sandwich enzyme immunoassay technique. Optical density of each well was determined using a microplate reader set to 570 nm (Immuno-Assay System, Dynatech, Billinghurst, United Kingdom). Concentrations were determined by subtracting the mean zero standard

Figure 1. Representative radiographs of HSF-1 competition experiments in PBMCs of patients with acute pancreatitis and healthy controls. Lane 1: positive control; lane 2: 1x specific inhibitor; lane 3: 10x specific inhibitor; lane 4: non-specific inhibitor. The middle two lanes are blank, as should be expected.

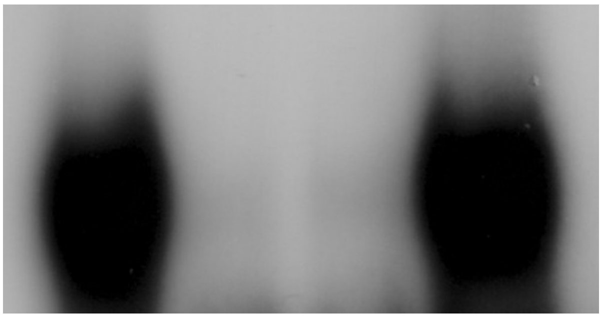

Figure 2. Representative radiographs of NF-kappaB competition experiments in PBMCs of patients with acute pancreatitis and healthy controls. Lane 1: positive control; lane 2: 1x specific inhibitor; lane 3: 10x specific inhibitor; lane 4 non-specific inhibitor. The middle two lanes are blank, as should be expected.

Figure 3. Representative radiographs of HSF-1 activation in PBMCs of healthy controls. Lanes 1-5.

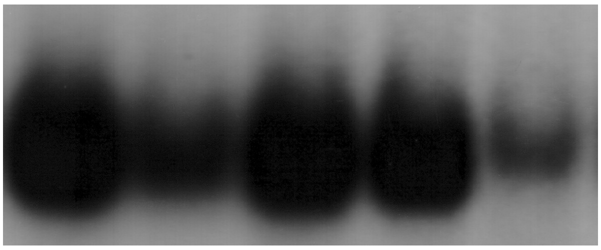

Figure 4. Representative radiographs of HSF-1 activation in PBMCs of patients with acute pancreatitis. Lanes 1-5.

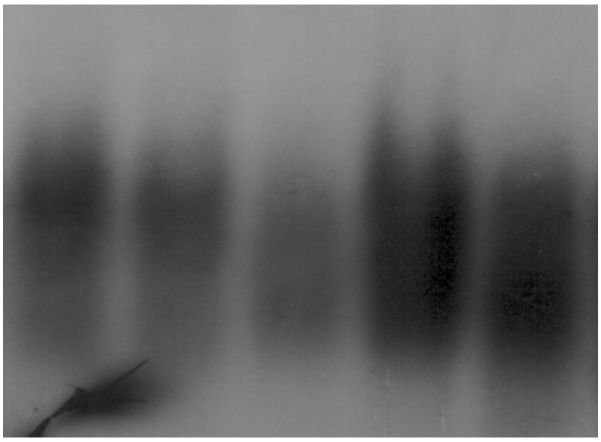

Figure 5. Representative radiographs of NF-kappaB activation in PBMCs of healthy controls. Lanes 1-5.

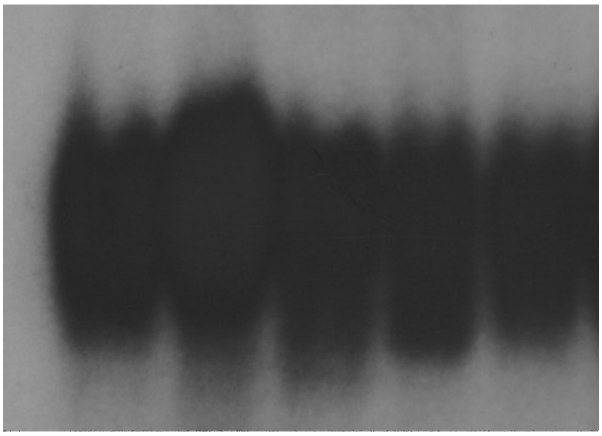

Figure 6. Representative radiographs of NF-kappaB activation in PBMCs of patients with acute pancreatitis. Lanes 1-5.

absorbance from the mean absorbance for each set of duplicate standards, controls and samples, followed by multiplication by the dilution factor.

ETHICS

Local Research Ethics Committee approval was obtained. Informed consent was obtained from each patient and the study protocol conforms to the ethical guidelines of the "World Medical Association Declaration of Helsinki - Ethical Principles for Medical Research involving Human Subjects" adopted at the 18th WMA General Assembly, Helsinki, Finland, June 1964 as revised in Tokyo, 2004" as reflected in a priori approval by the local research ethics committee.

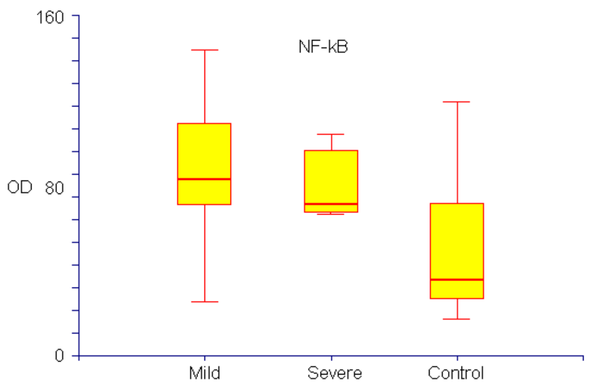

Figure 7. Box-and-whisker plot of NF-kappaB activity showing the distribution of patients according to disease severity compared with healthy controls. Systemic NF-kappaB activation occurs in acute pancreatitis (mild and severe pooled together) compared to healthy controls (P=0.004). However, there was no significant difference between those with mild and severe pancreatitis, (P=0.685). Boxes represent the interquartile range (IQR: i.e., the middle 50% between the 2nd and 3rd quartiles); whiskers represent the minimum and the maximum value in the absence of outliers or extreme values. No outliers or extreme values were detected.

STATISTICS

Values are expressed as medians and interquartile ranges (IQRs). The Mann-Whitney U test was employed in order to compare the various groups. Simple linear regression was used to examine relations between transcription factors and cytokine/chemokine production and severity scores of acute pancreatitis, and the Pearson correlation coefficient was calculated. All statistical tests were two sided and significance was assumed when P value was less than 0.05. The SPSS 8.0 for Windows was used to analyse the data.

RESULTS

NF-kappaB is Activated in Acute Pancreatitis

Systemic NF-kappaB activation occurs in acute pancreatitis (median optical density (OD): 77.0) compared to healthy controls (median OD: 35.5; P=0.004). However, there was no significant difference between those with mild (median OD: 72.8) and severe disease (median OD: 73.3; P=0.685) (Figure 7).

HSF-1 Activation Occurs in Acute Pancreatitis but Is Diminished in Severe Pancreatitis

Systemic activation of HSF-1 was observed in acute pancreatitis (median OD: 29.8) compared to healthy controls (median OD: 22.2), although this did not reach statistical significance (P=0.053). Activation, however, is greatest in those who have final Atlanta designations of mild pancreatitis (median OD: 40.6) compared to those who have a severe attack of acute pancreatitis (median OD: 11.3; P=0.036) (Figure 8).

sTNFRII Concentration is Elevated in Severe Acute Pancreatitis

sTNFRII concentration was elevated in acute pancreatitis (median concentration: 2,619 pg/mL) compared to healthy controls (median concentration: 1,099 pg/mL; P=0.002). The concentration was greater in those with severe

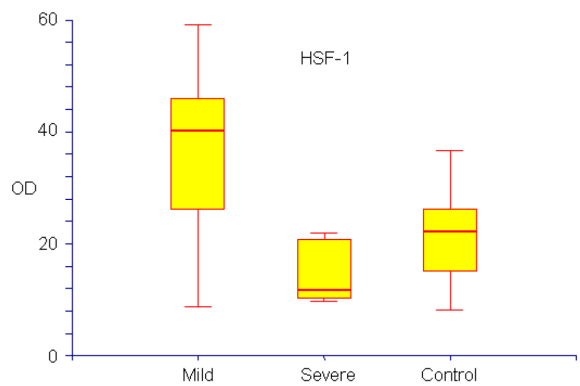

Figure 8. Box-and-whisker plot of HSF-1 activity showing the distribution of patients according to disease severity compared with healthy controls. Systemic activation of HSF-1 was observed in acute pancreatitis (mild and severe pooled together) compared to healthy controls (P=0.053). However, activation was significantly greater in those who had a final Atlanta designation of mild pancreatitis compared to those who had a severe attack of acute pancreatitis (P=0.036). Boxes represent the interquartile range (IQR: i.e., the middle 50% between the 2nd and 3rd quartiles); whiskers represent the minimum and the maximum value in the absence of outliers or extreme values. No outliers or extreme values were detected.

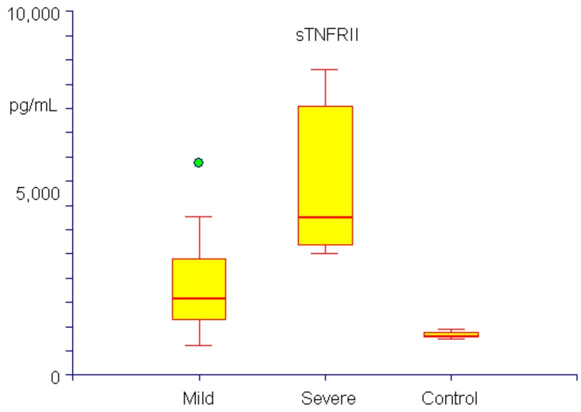

Figure 9. Box-and-whisker plot of sTNFRII concentration showing the distribution of patients according to disease severity compared with healthy controls. sTNFRII concentration was elevated in acute pancreatitis (mild and severe pooled together) compared to healthy controls (P=0.002). The concentration was greater in those with severe compared to mild acute pancreatitis although this did not reach statistical significance (P=0.059). Boxes represent the interquartile range (IQR: i.e., the middle 50% between the 2nd and 3rd quartiles); whiskers represent the minimum and the maximum value in the absence of outliers or extreme values. We have computed one outlier (green bullet) in the mild acute pancreatitis group. (Outliers were defined as values between 1.5 IQRs and 3 IQRs from the end of a box).

(median concentration 4,393 pg/mL) compared to mild acute pancreatitis (median concentration 2,612 pg/mL), although this did not reach statistical significance (P=0.059) (Figure 9).

IL-8 Concentration is Elevated in Acute Pancreatitis

IL-8 concentrations were elevated in acute pancreatitis (median concentration: 3.99 pg/mL) compared to healthy controls (median concentration 0 pg/mL), P<0.001). However, there was no significant difference in concentrations between those with mild (median concentration: 3.23 pg/mL) and severe acute pancreatitis (median concentrat-ion 4.92 pg/mL; P=0.271) (Figure 10).

HSF-1 Activation Is Inversely Correlated with APS and APACHE II Score

On simple linear regression performed in the pancreatitis patients only, HSF-1 was inversely correlated with APS (r=-0.49; P=0.019) and APACHE II score (r=-0.47; P=0.026), while sTNFRII was positively correlated with APS (r=0.49, P=0.014) and APACHE II score (r=0.53, P=0.008). NFkB and IL-8 were neither significantly correlated with APS (P=0.502, and P=0.092, respectively) nor with APACHE II score (P=0.328, and 0.135, respectively). No significant relationship was observed between all pairs of NF-kappaB, HSF-1 sTNFRII, and IL-8 (P>0.279).

DISCUSSION

Nuclear factor-kappa B (NF-kappaB) designates a family of transcription factors, composed of 5 proteins; NF-kappaB1 (p50), NF-kappaB2 (p52), p65 (RelA), c-Rel (Rel) and RelB. All share a Rel homology domain that mediates dimerization, interaction with the inhibitory protein, I-kappaB and DNA binding. In humans, a p50/p65 heterodimer

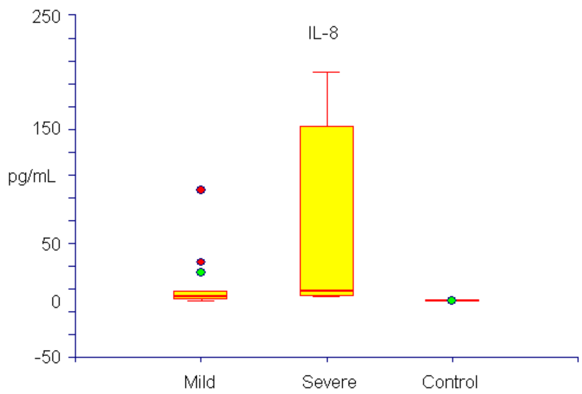

Figure 10. Box-and-whisker plot of interleukin-8 concentration showing the distribution of patients according to disease severity compared with healthy controls. Interleukin-8 concentrations were elevated in acute pancreatitis (mild and severe pooled together) compared to healthy controls (P<0.001). However, there was no significant difference in concentrations between those with mild and severe acute pancreatitis, (P=0.271). Boxes represent the interquartile range (IQR: i.e., the middle 50% between the 2nd and 3rd quartiles); whiskers represent the minimum and the maximum value in the absence of outliers or extreme values. We have computed one outlier (green bullet) and two extreme values (red bullets) in the mild acute pancreatitis group and one outlier in the control group. (Outliers were defined as values between 1.5 IQRs and 3 IQRs from the end of a box. Values more than 3 IQRs from the end of a box were defined as extreme).

complexed to I-kappaB is found in the cytoplasm of most cells. Upon activation, NF-kappaB translocates from the cytosol to the cell nucleus, where it binds to its consensus sequence on the promoter-enhancer region of a spectrum of genes, which are then transcribed. As a transcription factor for a variety of cytokines, hemopoetic growth factors, adhesion molecules, immunoreceptors and specific enzymes NF-kappaB occupies a critical position in the inflammatory cascade [9].

Our study demonstrates that systemic NF-kappaB activation occurs in acute pancreatitis, compared to healthy controls. However, there was no statistical difference between those with mild and severe disease, within 24 hours of symptom onset. This time-point was chosen, as the early prediction of severity is the foundation upon which the current management of acute pancreatitis is based [36]. NF-kappaB expression in PBMCs has been studied in the systemic inflammatory response syndrome that occurs in sepsis, trauma and post-operative organ dysfunction, with conflicting results. In sepsis, non-survivors can be distinguished from survivors by virtue of their increased NF-kappaB binding activity. [11, 12, 14]. In two separate studies, circulating levels of TNF, IL-1, IL-6 [12], and IL-6, IL-8, and sICAM-1 [14] were also elevated but unrelated to leukocyte NF-kappaB activation. Similarly, we found no correlation between cytokine/chemokine concentration and NF-kappaB activation. This may represent the fact that NF-kappaB is not the only transcriptional regulator of these inflammatory mediators, reinforcing the redundancy that exists in the immune system or alternatively, may represent the nonreflection of cellular cytokine expression by circulating concentrations. Adib-Conquy *et al*. observed a different pattern of NF-kappaB response [13]. Dysregulation of NF-kappaB expression was observed in sepsis and trauma. However, this study demonstrated global down-regulation of NF-kappaB in survivors and the presence of large amounts of an inactive homodimer in the non-survivors compared to healthy controls, indicating that although downregulation of inflammation is a normal aspect of sepsis, excessive inhibition of the process is associated with a poor prognosis. This reinforces modern concepts about the systemic inflammatory response syndrome (SIRS) as a combined pro- and anti-inflammatory state with both protective and destructive aspects [37].

NF-kappaB has attracted much attention as a potential target for anti-inflammatory therapy. Indeed, established, clinically useful substances, such as steroids [38], aspirin [39], and recombinant human activated protein C [40] seem to exert at least part of their actions through inhibiting NF-kappaB activation. More specific strategies to inhibit NF-kappaB include proteasome inhibitors, degradation resistant I-kappaB proteins and antisense DNA targeting of the NF-kappaB protein, p65 [24]. These approaches may result in a novel treatment strategy, with added specificity but reduced toxicity compared with standard immunosuppressive therapy.

Heat shock proteins (HSPs) are protective against the deleterious effects of the toxic mediators of inflammation. HSPs inhibit NADPH oxidase and thereby provide cellular protection against the damaging effects of reactive oxygen species [26]. HSP also protect against the adverse effects of cytokines. In particular, HSP70 interferes with TNF-mediated lipid activation by interrupting its signal transduction pathway [41]. Activation of HSF-1 is linked to the appearance of non-native proteins and the requirement for molecular chaperones (heat shock proteins) to prevent the appearance of misfolded proteins, in response to heat shock and other cellular stresses. HSF-1 exists in a control state as an inert monomer and undergoes step-wise activation to a DNA binding competent state. Induction of phosphorylation results in complete activation and subsequent transcription of heat shock genes [27]. Recent data has emphasized the relationship between the heat shock response and the activation of NF-kappaB. Activation of HSF-1 results in inhibition of the activation of NF-kappaB by different types of stimuli, leading to the simultaneous activation of cytoprotective genes and down-regulation of inflammatory genes [42, 43].

HSP production in the clinical setting of sepsis has also been examined. Enhanced HSP expression has been demonstrated in PBMCs and polymorphonuclear cells of patients with sepsis [44, 45]. However, neither study demonstrated a relationship with clinical outcome. Schroeder *et al*. have demonstrated that the *ex-vivo* endotoxin inducible expression of HSP70 in PBMCs was significantly lower in patients with severe sepsis than in non-septic patients and healthy controls. Furthermore, those who survived showed an increase in inducible HSP70 expression [46, 47]. This impaired expression of the protective HSP70 may contribute *in vivo* to immune dysfunction. Similarly, systemic activation of HSF-1 was observed in our study and was greatest in those who have final Atlanta designations of mild pancreatitis compared to those who have a severe attack of acute pancreatitis. On simple linear regression, HSF-1 was inversely correlated with APS and APACHE II score. This suggests that a failure to mount an adequate heat shock/stress response may have a detrimental effect upon an individual's ability to withstand the adverse effects of the systemic inflammatory response during acute pancreatitis.

TNF plays a pivotal role in the initiation of the cytokine network, inducing the release of the proinflammatory mediators IL-1, IL-6 and IL-8 [48, 49, 50]. It activates endothelial cells and upregulates the expression of intercellular adhesion molecules, which facilitate leukocyte-endothelial interaction. Because of its short half-life, phasic release, the masking effects of circulating inhibitors and its mainly paracrine level of function, measurement of circulating levels of TNF have not correlated well with severity of acute pancreatitis [5, 7]. Soluble TNF receptors provide a more accurate marker of TNF activity [51, 52]. This study demonstrates that the sTNFRII concentration was elevated in acute pancreatitis compared to healthy controls and correlates with APS and APACHE II score within the first 24 hours of symptom onset.

Chemokines are chemotactic cytokines that mediate the movement and activation of leukocytes in inflammation. IL-8 is a NF-kappaB responsive chemokine that is chemotactic for neutrophils and stimulates their activation [53]. In acute pancreatitis, IL-8 has been detected early in the course of the disease [54]. Levels have been detected to be higher in severe pancreatitis compared with the mild form of the disease and precede, by several hours, the rise in serum PMNE levels that indicate neutrophil activation [55]. In our study, IL-8 concentrations were elevated in acute pancreatitis compared to healthy controls however; there was no statistical difference in concentrations between those with mild and severe acute pancreatitis, at this time-point.

A specific therapy for acute pancreatitis has, thus far, proved elusive. Strategies aimed at antagonism of mediators of the systemic inflammatory response hold out hope that an effective agent may finally be within our grasp. Therapeutic optimism derives from the considerable success obtained with this approach, in the vast majority of animal experiments [6]. Furthermore, the patient with acute pancreatitis can pinpoint the onset of their disease and therefore the initiation of the cytokine cascade, enabling therapeutic intervention to take place at a time when mediator production is maximal but organ dysfunction is not yet established. The inflammatory profiles of patients with acute pancreatitis require much greater delineation. Account must also be taken of how these profiles may alter during the course of the disease. Ultimately, clinically applicable tests are required that will accurately profile the correct systemic inflammatory state of an individual patient. The propensity for inter-individual variation in the nature of the inflammatory response mounted, against injury or infection, also needs to be considered, as this appears to be genetically determined [56, 57, 58].

Received October 6th, 2005 - Accepted November 21st, 2005

Keywords Heat-Shock Proteins; Interleukin-8; NF-kappa B; Pancreatitis, Acute Necrotizing; Tumor Necrosis Factor-alpha

Abbreviations APS acute physiology scores; IQR: interquartile range; OD: optical density; OFS: organ failure scores; PBMC: peripheral blood mononuclear cell; sTNFRII: soluble tumor necrosis factor receptor II

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