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The Involvement of the Apoptosis-Modulating Proteins ERK 1/2, Bcl-xL and Bax in the Resolution of Acute Inflammation in Vivo

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Inflammatory cell recruitment, activation, and apoptosis are highly regulated processes involving several checkpoints controlling the resolution of inflammation. We investigated the role of the mitogen-activated protein kinase (MAPK) signaling pathway (ie, ERK1/2) and apoptosis-regulating Bcl-2 family members (ie, Bcl-xL and Bax) in the resolution of a rat carrageenan-induced pleurisy model. The specific ERK1/2 inhibitor PD98059 enhanced the resolution of inflammation, whereas the MEK1/2 inhibitor U0126 had no effect and the flavonoid apigenin, a nonspecific inhibitor of ERK1/2 and COX-2, augmented inflammation. Specifically, PD98059 significantly decreased the total number of macrophages and neutrophils in the pleural cavity, mainly by increasing the rate of neutrophil apoptosis, as measured by Annexin V labeling and morphological analysis. Conversely, a specific inhibitor of proapoptotic Bax (V5) increased inflammation, indicating that by preventing apoptosis in vivo, resolution of inflammation is delayed. This was associated with a decrease in neutrophil apoptosis and an increase in macrophage and neutrophil numbers perpetuating the inflammatory response. In conclusion, this study shows that ERK1/2, Bax, and Bcl-xL play important functional roles in the resolution phase of the acute inflammatory response in vivo by influencing apoptosis. Importantly, these data may provide novel therapeutic targets for the treatment of inflammatory diseases.

ney for inflammatory diseases such as asthma, rheumatoid arthritis, and inflammatory bowel disease.

Recently, we have shown the cellular profile of apoptosis in an acute pleurisy model of inflammation in vivo. One of the key events that initiate the resolution of inflammation (24 to 36 hours) is the induction of neutrophil apoptosis, which allows for safe clearance by phagocytes, such as macrophages. During resolution the majority of macrophages migrate to the local lymph node, however we have shown that some macrophages undergo apoptosis in situ at 72 hours, when inflammation has almost fully resolved. The signaling molecules and mechanisms that govern inflammatory cell survival and apoptosis have not yet been fully elucidated and more importantly, whether modulating these molecules can prevent or promote the resolution of the inflammatory response in an in vivo scenario has not been evaluated. There are numerous signal transduction molecules that play a role in determining the fate of the cell, which are triggered by extracellular signals before committing to caspase-dependent or -independent cell death. The Bcl-2 and mitogen-activated protein kinase (MAPK) pathways have been well characterized in terms of cell apoptosis and cell survival regulation in vitro, however their effects on the resolution of inflammation in vivo is still unclear. Bax and Bcl-xL, proapoptotic and anti-apoptotic Bcl-2 family members, respectively, play key roles in the regulation of apoptosis. For example, it has been shown that eosinophils isolated from children with acute asthma had an increased expression of Bcl-2, which was inversely correlated with the expiratory flow rate. In addition, Bax expression is attenuated and Bcl-xL expression is increased in T cells isolated from the lamina propria of patients with Crohn’s disease. This shows that inflammatory cell survival, by means of prosurvival and anti-apoptotic signaling mechanisms are important in the pathogenesis of inflammatory diseases. It has yet to be determined if modifying proteins from the Bcl-2 family can modulate inflammation per se.

The three main cascades by which MAPK homologues are released via MAPK kinase (MEK 1-7) are the extracellular signal-regulated kinase (ERK) (p42, p44, MAPK), c-Jun amino-terminal kinases (JNKs), and p38 MAPK pathways. The endogenous homologues of MAPK all play roles in cell differentiation, apoptosis, stress responses, and inflammation. In inflammatory diseases, such as asthma and rheumatoid arthritis, MAPK expression is increased. ERK1/2 can be activated for a relatively prolonged period of time under certain inflammatory conditions, in which it has been shown that macrophage inhibitory factor (MIF) induces ERK1/2 via cPLA2-induced arachidonic acid generation for a period of 24 hours in culture, which may partially account for the mechanism of ERK-induced cellular proliferation. In addition, macrophage ingestion of apoptotic cells liberate mediators that can influence inflammatory cell survival via MAPK pathways.

Studies have also shown that inhibitors of p38 MAPK, such as RWJ-67657, SB203580, and SB242235, are effective anti-inflammatory agents in suppressing cytokine release in human clinical trials ex vivo and also in the rat adjuvant-induced arthritis and in murine collagen-induced arthritis models in vivo. Inhibitors of ERK 1/2, such as PD98059, have also been shown to inhibit eosinophilic proinflammatory cytokine release, differentiation, and degranulation in vitro. Interestingly, flavonoids, such as apigenin, also possess anti-inflammatory properties in vitro through antioxidant mechanisms; inhibition of cell adhesion molecule expression, such as ICAM-1, and by attenuating the release of inflammatory mediators such as IL-1β-induced PGE2 via the inhibition of MAPK. In vivo studies have shown that flavonoids attenuate the onset of acute inflammation in murine models of carrageenan-induced paw edema and peritonitis, which is attributable to PGE2 and LTB4 inhibition. Although flavonoids, such as apigenin, are effective at preventing the onset of inflammation, it is unclear whether flavonoids exert anti-inflammatory effects once inflammation is established and whether they can promote the resolution of inflammation. We therefore hypothesize that inhibiting prosurvival factors, such as ERK 1/2 and Bcl-xL, or inducing proapoptotic proteins, such as Bax, may lead to the safe clearance of inflammatory cells and aid the resolution of acute inflammation. In this study, we examined the expression profile of apoptosis-modulating proteins during the inflammatory response and investigated whether inhibition of these specific proteins can result in the advancement or delay of the resolution of inflammation.

Materials and Methods

All materials were purchased from Sigma-Aldrich Ltd. (Poole, UK) unless otherwise stated. Bax-inhibiting peptide (V5), PD98059, and U0126 were purchased from Calbiochem (Nottingham, UK). Viscarin (λ-carrageenan) was a kind gift from Marine Colloids Inc. (Philadelphia, PA). Antibodies to Bax, Bcl-xL, and pERK 1/2 were purchased from Autogen Bioclear (Wiltshire, UK).

Carrageenan-Induced Pleurisy

Male Lewis rats (250 to 300 g) (Tuck and Sons, Battlebridge, UK) were housed in the University of Edinburgh animal facilities in accordance with local guidelines. Animals were fed on a normal diet with tap water ad libitum and were housed in a 12-hour light/dark cycle, at 20 to 21°C and with 50% humidity levels. The first described model of carrageenan-induced inflammation was by Winter and co-workers in the paw edema model. Since then, the carrageenan-induced pleurisy model has been further developed and widely used as an acute resolving model of inflammation. Briefly, the animals were anesthetized with halothane, a small incision was made to expose the musculature and 0.15 ml of 1% viscamin (λ-carrageenan) was injected intrapleurally with a blunted 21-gauge needle. Animals were killed by a rising concentration of CO2 at various time points after carrageenan administration, from the onset (6 hours), the peak (24 hours), and during the resolution of inflammation (36, 48, and 72 hours). Pleural cavities were washed with 1 ml...
of 3.15% (w/v) sodium citrate in physiological saline. Total inflammatory cell pellets were harvested for analysis by Western blotting.

In Vivo Treatment with 2′-Amino-3′-Methoxyflavone (PD98059), 1,4-Diamino-2,3-Dicyano-1,4-Bis(O-Aminophenylmercapto) Butadiene (U0126), or Bax-Inhibiting Peptide V5

Rats were treated with 0.15 ml of saline control, 0.15 ml of 0.1% or 0.8% dimethyl sulfoxide vehicle control, or with 300 μmol/L PD98059, 300 μmol/L U0126, or 200 μmol/L Bax-inhibiting peptide (V5) intrapleurally 36 hours after injection with 1% carrageenan. Animals were culled by a rising concentration of CO2 48 hours after injection with carrageenan (12 hours after drug treatment). Pleural cavities were washed with 1 ml of 3.15% (w/v) sodium citrate in physiological saline. Edema formation was measured by weighing the total pleural exudates, and total cell counts were measured with a Coulter Counter (model DN; Coulter Electronics, Luton, UK). An aliquot of 1 × 10⁶ inflammatory cells were washed in PBS three times and labeled with fluorescein isothiocyanate-conjugated Annexin V according to the manufacturer’s instructions. Total cell apoptosis was measured by Annexin V labeling according to the manufacturer’s instructions and analyzed by the BD FacsCalibur (Becton-Dickinson, Mountain View, CA). Lymphocyte, polymorphonuclear leukocyte (PMNs), and monocyte/macrophage total numbers were counted by light microscopy from hematoxylin and eosin (H&E)-stained cytospin preparations and confirmed by flow cytometry as described. Total neutrophil apoptosis was also measured by morphological analysis by light microscopy from H&E-stained cytospin preparations of pleural inflammatory cells with data presented as the percentage of apoptotic neutrophils from the total number of neutrophils present.

Western Blot Analysis

Inflammatory cell pellets were lysed in the presence of protease inhibitory buffer containing 1 μmol/L phenylmethyl sulfonyl fluoride, 1.5 μmol/L pepstatin A, and 0.2 μmol/L leupeptin. Protein concentrations were determined by the Bradford assay, and equal protein loading was confirmed by β-actin blotting (data not shown). Samples were boiled for 5 minutes with equal volumes of Laemmli gel loading buffer (5 mmol/L Tris, 10% sodium dodecyl sulfate, 10% glycerol, 10% β-mercaptoethanol, and 2 mg/ml bromophenol blue). Total protein equivalents of 10 μg for each sample were run on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were transferred to nitrocellulose paper (Amersham, UK) using the Bio-Rad minigel system (Bio-Rad, Hemel Hempstead, UK). Nonspecific antibody binding was blocked with 5% dried milk protein, and blots were incubated with either polyclonal goat or rabbit antibodies against pERK 1/2 (1:1000 dilution), Bax (1:500 dilution), or Bcl-xL (1:1000 dilution).

Expression Profiles of Signaling Molecules Involved in Apoptosis

Inflammatory cells from the onset of inflammation in a rat carrageenan-induced pleurisy model (6 to 36 hours after carrageenan) expressed the prosurvival molecules pERK 1/2 and Bcl-xL with very low levels of Bax expression found (Figure 1). Conversely during the resolution of inflammation (48 hours after carrageenan), Bax protein expression was increased with negligible levels of pERK 1/2 and Bcl-xL expression.

Modulation of Proapoptotic or Prosurvival Proteins Can Enhance or Prevent the Resolution of Inflammation in Vivo

Functional studies were conducted to assess whether inhibition of the prosurvival ERK1/2 could alter the resolution of inflammation in the carrageenan-induced pleurisy model. Once inflammation was established in the pleural cavity 36 hours after carrageenan, the flavonoid apigenin, a nonspecific ERK1/2 inhibitor, was given locally by intrapleural injection. Although apigenin did not affect edema, it was found to exacerbate inflammation with an increased total amount of pleural cell numbers of 112.6 ± 6.4 cells compared with vehicle-treated control of 71.7 ± 6.7 cells (P < 0.05; Figure 2).

The specific ERK1/2 inhibitor PD98059 augmented the resolution of inflammation by reducing cell numbers in

Statistics

All experiments were performed with six to eight rats per group, with experiments repeated to verify the original findings. Statistical analysis was performed by a one-way analysis of variance with Bonferroni multiple comparison post hoc test with a 95% confidence interval or by a Student’s t-test as appropriate. Data are expressed as mean ± SEM.

Results

Expression Profiles of Signaling Molecules Involved in Apoptosis

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The specific ERK1/2 inhibitor PD98059 augmented the resolution of inflammation by reducing cell numbers in
the pleural cavity to $50 \times 10^6 \pm 5.2 \times 10^6$ cells ($n = 8$; Figure 3B) compared with vehicle control of $81 \times 10^6 \pm 5.6 \times 10^6$ cells ($^{**}P < 0.01$) when given 36 hours after carrageenan. PD98059 pretreatment halved the total cell number of PMNs ($^{*}P < 0.05$) and also monocytes/macrophages ($^{***}P < 0.001$) compared with vehicle-treated control (Figure 5A). Edema formation decreased with PD98059 treatment by 30% when compared with saline control; however, PD98059 treatment was not significantly different from vehicle-treated control (Figure 3A).

Inflammatory cell apoptosis, measured by Annexin V binding and analyzed by flow cytometry, was significantly enhanced with PD98059 resulting in a 25% increase in binding when compared with vehicle control ($n = 8$, $^{*}P < 0.05$; Figure 3C). Specifically, there was an increase of apoptotic neutrophils with PD98059 pretreatment by $50 \pm 6\%$ ($n = 6$, $^{**}P < 0.01$; Figure 5B), which was approximately double the amount of constitutive apoptosis found in the saline and vehicle-treated control groups. Neutrophil apoptosis was further illustrated with H&E-stained inflammatory cells (Figure 5C). In contrast, the MEK1/2 inhibitor U0126 had no effect on the exudate volume, total cell number, or cell apoptosis levels (Figure 3, D–F).

We have shown that by enhancing apoptosis, via inhibition of ERK1/2, there is a significant attenuation of inflammation. On the contrary, it was also important to assess whether direct inhibition of apoptosis can alter the resolution of inflammation. A specific inhibitor of Bax prevented the safe resolution of inflammation with an increase in numbers of inflammatory cells to $121.9 \times 10^6 \pm 16.7 \times 10^6$ cells ($n = 8$; Figure 4) compared with saline alone to $78.3 \times 10^6 \pm 9.5 \times 10^6$ cells ($n = 8$) when administered locally at the peak of inflammation. Specifically, the total amount of PMNs and monocytes/macrophages increased by 42% and 33%, respectively, compared with saline control ($^{*}P < 0.05$, $^{***}P < 0.001$; Figure 5A). There was also a significant decrease (35%, $^{*}P < 0.05$, $n = 8$) in Annexin V binding compared with control, which further supports the hypothesis that the augmented inflammation was due to the prevention of apoptosis, which would normally occur during the resolution phase of inflammation. In particular, Bax inhibition caused a decrease in the number of apoptotic neutrophils, in which only 7.2% of neutrophils were apoptotic within the total number of neutrophils present (Figure 5, B and C; $^{*}P < 0.05$). Interestingly, although TGF-$\beta_1$ was prevalent during resolution (36 hours after carrageenan),
the mean of 10 animals per group (data not shown).

Discussion

The balance between prosurvival and proapoptotic signals is a highly regulated process involving many check-points that regulate cell survival and death. Several proinflammatory mediators, such as iMLP, tumor necrosis factor-α, GM-CSF, PAF, LTβ, influence apoptosis of inflammatory cells, and trigger MAPK pathways. This study has shown that during the initiation of acute inflammation, expression of prosurvival molecules, such as ERK1/2 and Bcl-xL, are increased. Furthermore, we also show that the expression of these survival factors is down-regulated during the resolution phase. Conversely, the proapoptotic signaling molecule Bax is up-regulated when the inflammation is resolving with low expression levels found during the onset of inflammation. The functional role of cell signaling proteins, such as Bax, MEK1/2, and ERK1/2, in inflammation in vivo was previously undefined.

This study demonstrates for the first time that apoptosis-modulating pharmacological inhibitors can promote or delay the resolution of acute inflammation. Importantly, the specific ERK1/2 inhibitor, PD98059, accelerates resolution of inflammation even after inflammation was established. It is difficult to predict in patients when inflammation will occur, even in chronic inflammatory diseases, and therefore, treatment during flare-ups is more beneficial than a prolonged prophylactic drug treatment regimen. Furthermore, this is an advantageous therapeutic strategy that will allow neutrophil infiltration to combat invading pathogens but will limit persistent inflammation and excessive tissue damage. Premature neutrophil apoptosis during infection is likely to be detrimental to host defense and lead to bacterial infection and sepsis. This is a known complication in cystic fibrosis patients in which the Pseudomonas aeruginosa-derived exotoxin pyocyanin induces neutrophil apoptosis, which allows inflammatory cells and bacteria to persist contributing to pulmonary damage and patient mortality.

The effects of endogenous or exogenous mediators depend on the kinetics of the synthesis or release or the timing of the administration of the inflammatory process. Studies have shown that in the carrageenan-induced paw edema model, apigenin had an anti-inflammatory effect when given at the onset of inflammation. This effect would be consistent with the suppression of COX-2 inhibition when given prophylactically. In the present study, the nonselective ERK1/2 inhibitor apigenin exacerbated inflammation, which in part is likely due to an inhibition of COX-2 because it has been shown that selective COX-2 inhibition by NS-398 and traditional NSAIDs, when given during the peak of inflammation, causes a prolonged inflammation. This is likely due to inhibition of the anti-inflammatory cyclopentenone prostaglandins, 15deoxyΔ12,14 PGJ2, and PGD2, which directly promote the apoptosis of neutrophils and macrophages in situ. Further evidence of the anti-inflammatory role of COX-2 was also shown by Wallace and co-workers, in which inflammation failed to resolve in COX-2 knockout mice in a model of carrageenan-induced paw inflammation.

Here, we show that pretreatment with the specific ERK1/2 inhibitor PD98059 enhanced the resolution of acute inflammation, with attenuated total cell numbers of monocytes/macrophages and PMNs in the pleural cavity. This correlated with an increase in the number of free apoptotic neutrophils found after pretreatment with PD98059. The increased amount of free apoptotic cells was probably not due to a defective phagocytosis mechanism because previous studies have shown that PD98059 does not affect phagocytosis in many different in vitro assays. In the present study, we also qualitatively show that the macrophage can still ingest apoptotic cells, as indicated in Figure 5C. The ability to visualize the free apoptotic neutrophils may be a result of a pronounced increase in apoptosis levels and a reduced number of the professional phagocytes (macrophages) in the pleural cavity. The persistence of free apoptotic cells has recently been suggested to be anti-inflammatory dependent on the environment that the macrophage is exposed to. Interestingly, autologous administration of apoptotic cells has been clinically proven to help prevent heart, lung, and renal allograft transplant rejection. This is thought to occur as a result of tolerogenic changes of immature dendritic cells after the engulfment of the apoptotic cells, which then stimulates inhibitory T-regulatory cells that suppress immune rejection. This clearly shows that the administration of apoptotic cells and their safe engulfment in hu-
mans has a role in the adaptive and the innate immune system by stimulating tolerance and promoting the resolution of inflammation. In this study, there is an overall diminished inflammatory response with PD98059 treatment in which increased apoptosis of neutrophils leads to an enhanced resolution of inflammation.

The accelerated resolution of inflammation was a specific effect for PD98059 because the MEK1/2 inhibitor
U0126 had no anti-inflammatory effect in terms of inflammatory cell numbers and apoptosis levels in vivo. U0126 has been shown to be specific for MEK1/2 and is non-competitive with ERK and ATP. This indicates that MEK1/2 may be dissociated from the ERK1/2 signaling pathway, which may provide a novel pathway for cell survival in inflammation. Alternatively U0126 could be ineffective in this model. Recently, however, administration of U0126 at the onset of inflammation in a murine model of ovalbumin-induced asthma has been shown to be effective in reducing eosinophilia, proinflammatory cytokine release, and airway hyperresponsiveness. In an in vitro study, there was a suppression of the release of eotaxin, RANTES, and GM-CSF from human airway smooth muscle cells with the MEK 1/2 inhibitor U0126; however, no effect was observed with a range of concentrations of the ERK1/2 inhibitor PD98059. The authors argue that PD98059 has poor efficacy, which is different from the findings proposed here, in which this compound augmented the resolution of inflammation. The local concentrations of the PD98059 and U0126 compounds were consistent with EC_{50} values shown in other in vitro studies.

The Bax-inhibiting peptide V5 is a cell-permeable pentapeptide that blocks the ku70 binding domain and prevents Bax conformational change and mitochondrial translocation. This peptide has been shown to inhibit staurosporine-, UV-, and anti-cancer drug-induced apoptosis in vitro. In the present study, inhibition of Bax during the resolving phase of acute inflammation exacerbated the inflammatory response in vivo. Specifically, total pleural cell numbers of monocytes/macrophages and PMNs were increased, with a corresponding decrease in the amount of neutrophil apoptosis, after pretreatment with the specific Bax inhibitor. Other studies by Maiuri and co-workers show that induction of Bax via inhibition of nuclear factor-κB in a carrageenan sponge model of chronic inflammation is associated with the apoptosis of neutrophils.

A decrease in total cell apoptosis, which could be attributed mainly to neutrophil apoptosis, was observed with the Bax peptide inhibitor. In contrast, an increase in neutrophil apoptosis contributed to an enhanced resolution with the ERK1/2 inhibitor PD98059. The percent total apoptosis of pleural cells in control and drug-treated animals were relatively small. This likely reflects the difficulty in visualizing and measuring apoptosis due to efficient clearance by phagocytosis. There is also a very limited window of opportunity to visualize free apoptotic cells in vivo, in which the estimated duration of detectable apoptosis due to rapid recognition and efficient engulfment by phagocytes is only 1 to 2 hours. However, even the levels of apoptosis that we have observed may have significant biological effects because studies have shown that small changes in apoptosis levels can drastically affect the overall total cell number. For example, in the rat liver an increase of 2% apoptosis by histological examination results in an overall 25% reduction in cell number. Furthermore, our previous work shows that an approximate 3% induction of apoptosis contributes to an 80% reduction in the number of neutrophils in the carrageenan-induced pleurisy model of inflammation in vivo.

Results of previous studies suggest that phagocytosis of apoptotic cells results in release of anti-inflammatory cytokines, such as TGF-β and IL-10. Furthermore, the released TGF-β_1 is capable of inhibiting the release of proinflammatory cytokines modulated via p38 MAPK whereby the inhibitor, SB 203580, prevented TGF-β_1-mediated inhibition of lipopolysaccharide-induced tumor necrosis factor-α and MIP-2 release. In this study, however, there was no significant difference in TGF-β or IL-10 levels found using the Bax inhibitor, PD98059, or the U0126-treated groups (data not shown). However, silent phagocytosis of early apoptotic cells can occur without affecting cytokine levels. The levels of proinflammatory cytokines, such as tumor necrosis factor-α and IL-1β, are insignificant during the resolution of inflammation; therefore, we were unable to establish whether inducing or preventing apoptosis in vivo affects these cytokines in particular.

Although we have clearly shown in this study that manipulation of pathways that influence apoptosis (namely ERK1/2 and Bax) can accelerate the resolution of an acute model of inflammation, the question remains whether our observations could be extended to a more chronic inflammatory scenario. Many of the pathways that we have investigated here can affect cell recruitment and activation, in addition to influencing cell survival and apoptosis. Furthermore, it is now becoming clear that MAPK signaling pathways and proteins involved in the regulation of apoptosis are dysregulated in a number of pathological inflammatory conditions, such as asthma. The challenge now is to investigate whether such manipulation of these pathways influences the resolution phase of inflammation in animal models of chronic inflammation or, more importantly, whether chronic inflammatory diseases in humans can be beneficially influenced. The clinical trials currently underway with compounds that directly influence apoptosis (eg, the caspase inhibitors)
and compounds that have an indirect effect on cell survival (eg, JNK, ERK, and p38 kinase inhibitors) will identify whether manipulation of apoptosis would be clinically beneficial for chronic inflammatory diseases.

Overall this study shows that direct modulation of apoptosis through interfering with the signaling of important molecules (ERK 1/2, Bcl-xL, Bax) can exacerbate or attenuate cell infiltrate and edema formation. Thus evidence from in vitro data and from the evidence presented here suggest that promoting apoptosis and safe clearance of effete cells may be a valuable new therapeutic target for inflammatory diseases. There is also a greater need to elucidate effective mechanisms of inducing apoptosis of inflammatory cells in vivo and whether this affects the outcome of the disease process once inflammation is established. This study emphasizes that there is an intricate balance of survival and apoptotic signaling pathways that mediate the kinetics of the acute inflammatory response, as illustrated in Figure 6. The enhanced resolution of inflammation by the inhibition of the pro-survival mediators, such as ERK 1/2 with PD98059 may prove to be an important advancement for the development of new treatments of inflammatory diseases.

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45. Apoptotic Signaling Pathways in Inflammation


