Treatment with bindarit, a blocker of MCP-1 synthesis, protects mice against acute pancreatitis

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Bhatia, Madhav, Raina Devi Ramnath, Lakshmi Chevali, and Angelo Guglielmotti. Treatment with bindarit, a blocker of MCP-1 synthesis, protects mice against acute pancreatitis. Am J Physiol Gastrointest Liver Physiol 288: G1259–G1265, 2005. First published February 3, 2005; doi:10.1152/ajpgi.00435.2004.—Chemokines are small (8–10 kDa), inducible, secreted cytokines with chemotactic and activating effects on leukocyte subsets and can be divided into major subgroups on the basis of the orientation of the first two cysteines. In CXC chemokines, the first two-cysteine residues (C) are separated by a single amino acid (X), whereas in the CC subfamily, the first two-cysteine residues are adjacent (2, 4, 6, 10, 11, 12). CXC and CC chemokines have a different pattern of tissue distribution and are believed to have different effects. The CC chemokines such as monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, and RANTES are believed to activate primarily monocytes, whereas the CXC chemokines, such as IL-8, tend to preferentially activate neutrophils. Chemokines bind to a family of seven-transmembrane-domain G protein-coupled receptors on the surface of leukocytes. Nearly 20 different types of receptors are described but perhaps only five or six are involved in the acute inflammatory response. The receptor types expressed on the surface of circulating leukocytes, together with the profile of ligand at a site of inflammation, determines the precise nature of a particular inflammatory infiltrate, which may be site specific (1). Recent work by us as well as other investigators (7, 17, 24) has, however, shown that these narrow definitions are no longer valid. We have previously shown that in knockout mice, the deletion of the CC chemokine receptor-1 (CCR1) decreased the pulmonary damage seen in severe acute pancreatitis (17). Similarly, treatment with Met-RANTES, a CCR1 antagonist, protected mice against acute pancreatitis-associated lung injury with little or no protection against local pancreatic damage (7).

We (3) have recently shown that pancreatic acinar cells produce the CC chemokine MCP-1 and that treatment with supramaximally stimulating doses of caerulein causes an up-regulation of MCP-1 production. 2-Methyl-2-[[1-(phenylmethyl)-1H-indazol-3yl]methoxy]propanoic acid (bindarit) is a novel molecule that has been shown to preferentially inhibit MCP-1 production in vitro in monocytes and in vivo without affecting the pulmonary damage seen in severe acute pancreatitis (17). We have earlier shown that pancreatic acinar cells produce the chemokine monocyte chemotactic protein (MCP)-1 in response to caerulein hyperstimulation, demonstrating that acinar-derived MCP-1 is an early mediator of inflammation in acute pancreatitis. Blocking chemokine production or action is a major target for pharmacological intervention in a variety of inflammatory diseases, such as acute pancreatitis. 2-Methyl-2-[[1-(phenylmethyl)-1H-indazol-3yl]methoxy]propanoic acid (bindarit) has been shown to preferentially inhibit MCP-1 production in vitro in monocytes and in vivo without affecting the production of the cytokines IL-1, IL-6, or the chemokines IL-8, protein macrophage inflammatory-1α, and RANTES. The present study aimed to define the role of MCP-1 in acute pancreatitis with the use of bindarit. In a model of acute pancreatitis induced by caerulein hyperstimulation, prophylactic as well as therapeutic treatment with bindarit significantly reduced MCP-1 levels in the pancreas. Also, this treatment significantly protected mice against acute pancreatitis as evident by attenuated hyperamylasemia neutrophil sequestration in the pancreas (pancreatic MPO activity), and pancreatic acinar cell injury/necrosis on histological examination of pancreas sections.

bindarit; caerulein; myeloperoxidase

Acute pancreatitis is a common clinical condition, whose incidence has been increasing over recent years (2, 4, 6, 10–12). Most cases are secondary to gallstones or excess alcohol consumption. Regardless of the cause, activation of digestive enzymes within pancreatic acinar cells is thought to be a critical initiating event. Pancreatic damage then leads to an inflammatory response. Thus inflammatory mediators play a key role in the pathogenesis of acute pancreatitis (2, 4, 6, 10–12).

After an initial pancreatic acinar cell injury, trafficking of leukocytes from the circulation into pancreatic acinar cells takes place. This trafficking requires communication between the circulating leukocytes and the vascular endothelial cell barrier (10). As part of the migration process, the leukocytes and the inflammatory cells must first adhere to the endothelium via expression of adhesion molecules. This adhesion subsequently leads to leukocyte transendothelial migration (diapedesis) toward damaged pancreatic acinar cells. This migration of leukocytes is dependent on several adhesive interactions and leukocyte-specific chemotactic molecules, and these series of events are collectively called “chemotaxis” (10). Chemotaxis is the fundamental process by which the leukocytes are directed to the sites of tissue damage under the influence of a concentration gradient of the soluble chemotactic molecules or chemoattractants. It is a well orchestrated process that involves a number of proteins, including proinflammatory cytokines, adhesion molecules, matrix metalloproteinases, and the large cytokine subfamily of chemotactic cytokines, the chemokines (10).

Chemokines are a family of small (8–10 kDa), inducible, secreted cytokines with chemotactic and activating effects on leukocyte subsets and can be divided into major subgroups on the basis of the orientation of the first two cysteines. In CXC chemokines, the first two-cysteine residues (C) are separated by a single amino acid (X), whereas in the CC subfamily, the first two-cysteine residues are adjacent (2, 4, 6, 10, 11, 12). CXC and CC chemokines have a different pattern of tissue distribution and are believed to have different effects. The CC chemokines such as monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, and RANTES are believed to activate primarily monocytes, whereas the CXC chemokines, such as IL-8, tend to preferentially activate neutrophils. Chemokines bind to a family of seven-transmembrane-domain G protein-coupled receptors on the surface of leukocytes. Nearly 20 different types of receptors are described but perhaps only five or six are involved in the acute inflammatory response. The receptor types expressed on the surface of circulating leukocytes, together with the profile of ligand at a site of inflammation, determines the precise nature of a particular inflammatory infiltrate, which may be site specific (1). Recent work by us as well as other investigators (7, 17, 24) has, however, shown that these narrow definitions are no longer valid. We have previously shown that in knockout mice, the deletion of the CC chemokine receptor-1 (CCR1) decreased the pulmonary damage seen in severe acute pancreatitis (17). Similarly, treatment with Met-RANTES, a CCR1 antagonist, protected mice against acute pancreatitis-associated lung injury with little or no protection against local pancreatic damage (7).

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In this paper, we report the effect of bindarit on MCP-1 production from pancreatic acinar cells. Furthermore, we have studied the role of MCP-1 in acute pancreatitis by using bindarit, a blocker of MCP-1 production.

MATERIALS AND METHODS

Preparation of pancreatic acini and in vitro treatment with caerulein and bindarit. Pancreatic acini were obtained from mouse pancreas by collagenase treatment as described previously (3). Briefly, pancreas from Swiss mice (20–25 g) were removed, infused with buffer A (in mM: 140 NaCl, 4.7 KCl, 1.13 MgCl2, 1 CaCl2, 10 glucose, 10 HEPES, pH 7.2) containing 200 IU/ml collagenase and 0.5 mg/ml soybean trypsin inhibitor and incubated in a shaking water bath for 10 min at 37°C. The digested tissue was passed through 50 μm BSA and washed twice with buffer A before further experiments. Caerulein was obtained from Bachem (Bubendorf, Switzerland). Experiments were performed to examine the effects of caerulein and bindarit (ACRAF; Aprilia, Italy) treatment, on MCP-1 production by mouse pancreatic acini. Briefly, acini were preincubated with/without bindarit (460 μM) for 30 min, and further incubated with caerulein (10−12−10−7 M) for 30 min. The dose of bindarit was chosen based on earlier reports, in which it was shown to inhibit MCP-1 synthesis (20, 30). MCP-1 and amylase assays were performed as described in MCP-1 assay and Amylase estimation.

Induction of acute pancreatitis. All animal experiments were approved by the Animal Ethics Committee of National University of Singapore and carried out in accordance with established International Guiding Principles for Animal Research. Swiss mice (20–25 g) were randomly assigned to control or experimental groups using 10 or more animals for each group. Animals were given hourly intraperitoneal injections of normal saline or saline-containing caerulein (50 μg/kg) for 10 h. Bindarit was administered to mice at a dose of 200 mg/kg ip either 30 min before or 1 h after the first caerulein injection. The dose of bindarit was chosen based on earlier reports, in which bindarit was shown to inhibit MCP-1 synthesis and inflammation (16, 19, 20, 21, 30, 32, 33). One hour after the last caerulein injection, animals were killed by an intraperitoneal injection of a lethal dose of pentobarbital. Harvested heparinized blood was centrifuged and the plasma removed and stored at −80°C. Random cross sections of the head, body, and tail of the pancreas were fixed in 4% neutral phosphate-buffered formalin then embedded in paraffin wax. Samples of pancreas were stored at −80°C for subsequent measurement of tissue MPO activity. Samples of pancreas were also used for MCP-1 assay.

MCP-1 assay. Pancreatic acinar cell supernatants were assayed for MCP-1 using a sandwich ELISA, according to the manufacturer’s instructions (Duoset kit; R&D Systems, Minneapolis, MN). Briefly, anti-MCP-1 primary antibody was aliquoted onto ELISA plates and incubated at 4°C overnight. Samples and standards were incubated for 2 h, the plates were washed, and a biotinylated anti-MCP-1 antibody was added for 2 h. Plates were washed again, and streptavidin bound to horseradish peroxidase was added for 20 min. After a further wash, tetramethylbenzidine was added for color development, and the reaction was terminated with 2 M H2SO4. Absorbance was measured at 450 nm.

Amylase estimation. Amylase activity was measured by using a kinetic spectrophotometric assay. Plasma or acinar cell supernatants were incubated with the substrate, 4,6-ethylidene (G1)-p-nitrophenol (G1)-1-o-maltobiose (Sigma, St. Louis, MO) for 2 min at 37°C, and absorbance was measured every minute for the subsequent 2 min at 405 nm (5, 8, 28). The change in absorbance was used to calculate the amylase activity.

Myeloperoxidase estimation. Neutrophil sequestration in pancreas was quantified by measuring tissue MPO activity (5, 8). Tissue samples were thawed, homogenized in 20 mM phosphate buffer (pH 7.4), and centrifuged (10,000 g, 10 min, 4°C), and the resulting pellet was resuspended in 50 mM phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide (Sigma). The suspension was subjected to four cycles of freezing and thawing and was further disrupted by sonication (40 s). The sample was then centrifuged (10,000 g, 5 min, 4°C), and the supernatant used for the MPO assay. The reaction mixture consisted of the supernatant, 1.6 mM tetramethylbenzidine (Sigma), 80 mM sodium phosphate buffer (pH 5.4), and 0.3 mM hydrogen peroxide. This mixture was incubated at 37°C for 110 s, the reaction was terminated with 2 M H2SO4, and the absorbance was measured at 450 nm. This absorbance was then corrected for the DNA content of the tissue sample (25) (fold increase over control).

Morphological examination. Paraffin-embedded pancreas samples were sectioned (5 μm), stained with hematoxylin/eosin, and examined with light microscopy. Acinar-cell injury/necrosis was quantitated by morphometry as described by an experienced morphologist who was not aware of the sample identity (8). For these studies, 10 randomly chosen microscopic fields (×125) were examined for each tissue sample and the extent of acinar-cell injury/necrosis was expressed as the percentage of the total acinar tissue that was occupied by areas meeting the criteria for injury/necrosis. Those criteria were defined as either 1) the presence of acinar-cell ghosts or 2) vacuolization and swelling of acinar cells and the destruction of the histochitecture of whole or parts of the acini, both of which had been associated with an inflammatory reaction.

Statistics. Data are expressed as the means ± SE. In all figures, vertical bars denote SE, and absence of such bars indicate that the SE is too small to illustrate. The significance of changes was evaluated by using Student’s t-test when the data consisted of only two groups or by ANOVA when comparing three or more groups. If ANOVA indicated a significant difference, the data were analyzed by using Tukey’s method as a post hoc test for the difference between groups. A P value of <0.05 was considered to indicate a significant difference.

RESULTS

Effect of bindarit pretreatment on MCP-1 production by mouse pancreatic acini and caerulein-induced amylase secretion. Mouse pancreatic acini were found to produce MCP-1 (Fig. 1A). Treatment of acini with supramaximal concentrations of caerulein (10−7 M) stimulated MCP-1 production by pancreatic acinar cells (Fig. 1A). Pretreatment of mouse pancreatic acini with bindarit was found to significantly inhibit MCP-1 production by mouse pancreatic acini (Fig. 1A). In accord with previously reported findings (8), biphasic stimulation/inhibition of amylase secretion by increasing concentrations of caerulein was observed when freshly prepared pancreatic acini from mice was evaluated (Fig. 1B). Similar changes were observed in acini that were pretreated with bindarit (Fig. 1B). These findings indicate that bindarit pretreatment does not alter pancreatic cell responsiveness to the secretagogue caerulein.

Effect of bindarit treatment on pancreatic MCP-1 levels in acute pancreatitis. Induction of acute pancreatitis by caerulein hyperstimulation resulted in an increase of pancreatic MCP-1 levels. Both prophylactic and therapeutic treatment with bindarit caused a significant reduction in MCP-1 levels in the pancreas (Fig. 2).

Effect of prophylactic treatment with bindarit on the severity of caerulein-induced acute pancreatitis. Evidence of pancreatic injury in acute pancreatitis induced by intraperitoneal administration of caerulein at a dose of 50 μg/kg hourly for 10 h was confirmed by an increase in plasma amylase (Fig. 3A) and pancreatic MPO as a measure of neutrophil infiltration.
Activated leukocytes, particularly neutrophils, are important in the development of organ damage in acute pancreatitis. Depletion of circulating neutrophils with antineutrophil serum (9, 22, 23) or strategies that interfere with neutrophil migration, such as the use of a blocking antibody to ICAM-1 reduce neutrophil infiltration and pancreatic damage in acute pancreatitis (27, 31).

Chemokines influence leukocyte migration into tissues in two main ways. First, binding of chemokine ligand to a specific leukocyte receptor leads to activation of cell surface integrins and allows strong adhesion to endothelium. Second, chemokines promote migration of adherent leukocytes across the endothelium and through the extracellular matrix. Chemokines are also important regulators of leukocyte activation in situ (2, 4, 6, 10 –12).

The major chemokine receptors involved in the trafficking of neutrophils have until recently been thought to be CXC dependent (CXCR1 and CXCR2). The discovery, however, that CCR1 is present on and upregulated on isolated human neutrophils after treatment with interferon-γ (13) and that subdermal injection of MIP-1α leads to neutrophil as well as monocyte influx in human volunteers (26) suggests that CC chemokines may also be involved in neutrophil trafficking in the human. Moreover, granulocyte macrophage colony-stimulat-
ing factor has recently been shown to upregulate CCR1 expression in human neutrophils (15).

Although most studies evaluating the role of chemokines in acute pancreatitis have focused on CXC chemokines, other evidence points to a critical role for CC chemokines (3, 18). We have previously shown that mice genetically deficient in the CCR1 have reduced lung damage after induction of acute pancreatitis with caerulein (7, 17). Recently, it has been de-

Fig. 3. Effects of prophylactic bindarit administration on the severity of acute pancreatitis. Mice (n = 10 in each group) were given 10 hourly injections of caerulein (Caer; 50 µg/kg ip). Bindarit was administered to mice at a dose of 200 mg/kg ip 30 min before the first caerulein injection. One hour after the last caerulein injection, mice were killed by an intraperitoneal injection of a lethal dose of pentobarbitone (Pbo), and plasma amylase activity (A), pancreatic MPO activity (B), and acinar cell injury/necrosis (C) were determined as described in MATERIALS AND METHODS. Results shown are the means ± SE. *P < 0.01 when bindarit-treated animals were compared with placebo-treated animals.

Fig. 4. Morphological changes in mouse pancreas on induction of acute pancreatitis with/without prophylactic treatment with bindarit. A: control, no pancreatitis. B: caerulein-induced acute pancreatitis with placebo (prophylactic). C: caerulein-induced acute pancreatitis in mice administered bindarit (prophylactic).
onstrated that there are increased protein levels of MCP-1 (a CC chemokine) in the rat pancreas after induction of acute pancreatitis by caerulein hyperstimulation or bile salt infusion (14). Pancreatic acinar cells produce MCP-1 in vitro, and production is upregulated by hyperstimulation with caerulein (3). These observations suggest that MCP-1 may be an early inflammatory mediator in acute pancreatitis.

These observations have been substantiated in a recent clinical study (29) in which it was shown that complicated...
acute pancreatitis was associated with significantly elevated local and systemic concentrations of MCP-1, suggesting that, among the CC chemokine members, MCP-1 might play a pivotal role in the pathological mechanism of complicated acute pancreatitis (29). In this study, therefore, we have investigated the role of MCP-1 in acute pancreatitis by using bindarit, a blocker of MCP-1 synthesis (30).

Bindarit is an original indazolic derivative devoid of systemic immunosuppressive effects and of activity on arachidonic acid metabolism, which has been shown to be effective in a number of animal models involving chronic inflammation such as arthritis (16, 20–21) and lupus (19, 32, 33). Bindarit, in fact, selectively inhibits chronic inflammation and ameliorates joint damage in rat adjuvant arthritis (16, 20, 21) and is able to limit glomerular injury and prolong survival of NZB/W lupus mice (19, 33). Several studies have demonstrated that bindarit is a preferential inhibitor of MCP-1 production in vitro and in vivo and have suggested that its beneficial effects in models of joints and kidney inflammation are related to its anti-MCP-1 activity (30).

We have found an activation of MCP-1 production by pancreatic acinar cells by caerulein that could be blocked by pretreatment with bindarit. Bindarit pretreatment, however, does not alter pancreatic cell responsiveness to the secretagogue caerulein because a biphasic dose response curve for amylase secretion was observed in acini pretreated with bindarit (similar to that observed in control acini). Moreover, induction of acute pancreatitis in mice by caerulein hyperstimulation caused an increase in pancreatic MCP-1 levels. This increase in MCP-1 levels was significantly attenuated by prophylactic, as well as therapeutic, treatment with bindarit. It is interesting to note that although, in isolated pancreatic acini, bindarit treatment completely inhibited MCP-1 production, in vivo the pancreatic levels of MCP-1 were only partially reduced. The reasons for this difference are not clear but may be explained by the fact that the in vivo system is a much more complex system than isolated pancreatic acini in vitro. A possible reason for this difference is that the agent is affecting selective MCP-1 (such as acinar) pools. However, it is important to note that both in vitro as well as in vivo, bindarit treatment inhibits MCP-1 production.

In addition, our results show that treatment of animals with bindarit (either prophylactic or therapeutic) reduces the severity of pancreatitis as evidenced by a significant attenuation of hyperamylasemia and pancreatic MPO activity and by histological evidence of diminished pancreatic injury (acinar cell injury/necrosis as well as edema). The reduction in pancreatic edema was suggested by the histological examination of pancreas sections.

We believe that the mechanism by which bindarit treatment protects against acute pancreatitis is by inhibiting MCP-1 production. The drug, however, has been reported to affect other inflammatory mediators, such as TNF-α (20) but not IL-1, IL-6, or the chemokines IL-8, MIP-1α, and RANTES (30), and it is possible that this may contribute to its protective action. However, in our experiments, caerulein treatment did not cause a stimulation of TNF-α generation in mouse pancreatic acini. A nonspecific effect of bindarit on acinar cell function was, however, ruled out by the observation that bindarit treatment has no effect on amylase secretion by pancreatic acini in response to caerulein. The precise mechanism by which bindarit acts to protect against acute pancreatitis will be the subject of future studies.

In light of these results, we propose that anti-MCP-1 strategies may be of potential therapeutic value in the treatment of acute pancreatitis.

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