Rat hepatic stellate cells produce cytokine-induced neutrophil chemoattractant in culture and in vivo

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Maher, Jacquelyn J., John S. Lozier, and Myron K. Scott. Rat hepatic stellate cells produce cytokine-induced neutrophil chemoattractant in culture and in vivo. Am. J. Physiol. 275 (Gastrointest. Liver Physiol. 38): G847–G853, 1998.—Hepatic stellate cells are widely recognized for their contribution to liver fibrosis. This study investigated whether these cells also promote hepatic inflammation by producing neutrophil chemoattractants. Specifically, stellate cells were examined as potential sources of cytokine-induced neutrophil chemoattractant (CINC), a rat chemokine resembling human interleukin-8. Stellate cells from normal rat liver expressed little or no CINC. In culture, CINC mRNA was induced rapidly, coinciding with the phenomenon of culture activation. CINC mRNA rose 4.6-fold within 3 days and was accompanied by secretion of immunoreactive and biologically active CINC protein (4.1 ng·µg DNA$^{-1}$·day$^{-1}$). Studies in vivo demonstrated that CINC could be induced in stellate cells during liver injury. CINC mRNA rose significantly (4- to 6-fold in two models of liver disease, both of which cause stellate cell activation. In summary, the data indicate that CINC is induced during stellate cell activation in culture and in vivo. They suggest that stellate cell-derived CINC can promote hepatic inflammation in vivo.

IN MANY CHRONIC LIVER DISEASES, parenchymal inflammation coincides with hepatic fibrogenesis. Leukocytes are often present in areas of collagen deposition, giving the impression that inflammatory foci in liver progress to hepatic scars. Close associations between inflammation and fibrosis are not unique to liver (2, 16); in some organs, they have prompted the notion that a single population of cells can control both processes. For example, in kidney (12) and synovium (24), fibroblasts or fibroblast-like cells simultaneously produce collagen and compounds that induce inflammation. In the liver, the main collagen-producing cells are hepatic stellate cells (8, 19). They reside in the perisinusoidal space of Disse, adjacent to hepatocytes. Although the involvement of stellate cells in liver fibrosis is clear, their participation in hepatic inflammation is less established.

For stellate cells to promote hepatic fibrogenesis, they must first undergo a process called “activation.” Activation is a coordinated series of events that results in transformation of stellate cells from a static, pericyte-like phenotype to one resembling myofibroblasts (8). In vivo, stellate cell activation occurs in response to a variety of hepatic insults such as alcohol or toxin exposure (22), viral infection (4), or biliary obstruction (19); most if not all of these insults also cause hepatic inflammation. Thus, if stellate cells acquire proinflammatory activity in liver disease, such activity is likely to be induced during activation. A possible link between proinflammatory activity and stellate cell activation has been suggested in cell culture (13). Because stellate cells activate spontaneously in primary culture, this model has been used extensively to study cellular events involved in the activation process.

One specific means by which stellate cells could induce hepatic inflammation is through production of chemokines. Chemokines are leukocyte chemoattractants that are produced by many types of cells, including fibroblasts (26). All chemokines contain four conserved cysteine residues, but they are grouped into two major subfamilies based on amino acid sequence, chromosomal location, and target cell specificity (1). In one subfamily, the first two cysteines are directly adjacent (C-C chemokines). In the other, the C-C motif is replaced by C-X-C (C-X-C chemokines). C-C chemokines preferentially attract mononuclear cells, eosinophils, and basophils, whereas C-X-C chemokines exhibit strong chemotactic activity toward neutrophils (1).

Our laboratory has an active interest in C-X-C chemokines as mediators of neutrophilic hepatitis (20). To date we have focused on cytokine-induced neutrophil chemoattractant (CINC), a rat chemokine that is analogous to human interleukin-8 (IL-8) (35). In the current study, our aim was to determine whether stellate cells produce CINC and, if so, whether stellate cell-derived CINC could promote neutrophil chemotaxis. We examined CINC regulation in stellate cell culture and two independent models of liver disease in vivo. In both instances, CINC was induced in stellate cells during activation and exhibited the potential to cause hepatic inflammation.

METHODS

Cell isolation and culture. Stellate cells were isolated from the livers of adult male Sprague-Dawley rats (500–600 g) by in situ perfusion with Pronase and collagenase (9). Crude cell suspensions were purified to >95% homogeneity by centrifugation over a discontinuous gradient of Accudenz (Accurate Scientific, Westbury, NY) (21). In some experiments, purified cell isolates were suspended immediately in guanidine isothiocyanate for RNA extraction (see below). In others, the cells were suspended in DMEM with 10% FCS (GIBCO BRL, Grand Island, NY) and plated on uncoated tissue culture plastic at a density of 1.5 x 10⁴ cells/cm². Cells were maintained in serum-containing medium for 3–7 days.

Collection of stellate cell-conditioned medium. At 3 or 7 days of culture, depending on the experiment, stellate cell monolayers were washed thoroughly with PBS and replenished with serum-free DMEM containing 0.5% BSA. Some cultures were treated with recombinant human interleukin-1 (IL-1, 20 pg/ml; R&D Systems, Minneapolis, MN) or recombinant murine tumor necrosis factor (TNF, 20 ng/ml; GIBCO
BRL, Gaithersburg, MD). Conditioned medium was collected at 24 h after incubation. Cellular debris was removed by centrifugation; clarified medium was stored at 20°C for CINC assay.

Measurement of CINC. CINC was measured in stellate cell culture medium by a sandwich ELISA developed by Wittwer et al. (36). The assay employs a goat anti-CINC antibody (provided by Dr. John Zagorski, National Institute of Dental Research, Bethesda, MD) and a separate rabbit anti-CINC purchased from Peptides International (Louisville, KY). With purified CINC as a standard (Peptides International, Louisville, KY), the assay is linear from 0.1 to 2.0 ng/ml. Samples of serum-free culture medium were diluted as necessary with PBS containing 1% BSA to fall within the standard curve.

Measurement of CINC mRNA. Total RNA was extracted from fresh or cultured stellate cells by the method of Chomczynski and Sacchi (5). CINC mRNA was measured by RNase protection, using a 32P-labeled cRNA (α-32P]CTP, >800 Ci/mmol; Amersham, Arlington, IL) transcribed from a 210-bp cDNA encoding mature CINC (14). Autoradiographic signals were quantitated by scanning densitometry (Hoefer Scientific Instruments, San Francisco, CA). CINC mRNA signals were normalized to a control signal encoding the ribosomal protein S14 (30).

Neutrophil isolation. Whole blood (5 ml) was collected from human volunteers and anticoagulated with heparin. Neutrophils were separated from mononuclear leukocytes by centrifugation through Percoll (1.080 g/ml; Pharmacia, Uppsala, Sweden). Neutrophils and erythrocytes were harvested together from the Percoll gradient; erythrocytes were then removed by lysis with ammonium chloride. Purified neutrophils were suspended in DMEM containing 0.5% BSA at a concentration of 2.5 × 10⁶ cells/ml. Viability was >95% as measured by trypan blue exclusion.

Chemotaxis assay. Conditioned medium from cultured stellate cells was assayed for chemotactic activity in a blind-well Boyden chamber fitted with a 5-µm-pore polycarbonate filter (Osmonics Laboratory, Livermore, CA). Some aliquots of conditioned medium were pretreated for 1 h before assay with goat anti-CINC antibody or normal rabbit IgG (1:500 vol/vol). Treated and untreated samples of conditioned medium were analyzed in parallel for chemotactic activity and were compared with DMEM-0.5% BSA as a control. Neutrophils (0.5 × 10⁶) were added to the upper half of each chemotactic chamber in a volume of 200 µl DMEM-0.5%BSA. The filled chambers were incubated for 30 min at 37°C in a 5% CO₂ atmosphere. At the end of the experiment, the filters were stained with LeukoStat (Fisher Scientific, Pittsburgh, PA) and mounted upside down on glass microscope slides. Neutrophils that had migrated to the bottom of each filter were visualized microscopically and counted.

Induction of liver injury in vivo. Two independent procedures were used to induce liver injury in rats. One group of animals underwent laparotomy with complete ligation of the common bile duct. A second group received carbon tetrachloride (0.5 ml/kg) by gavage twice weekly for 4 wk. Stellate cells were isolated from bile duct-ligated rats on day 7 or 14 postoperatively. Stellate cells were isolated from carbon tetrachloride-treated rats at 5 wk, 7 days after the last dose of toxicant.
RESULTS

Initial studies examined CINC gene expression and regulation in stellate cells from normal rat liver. Freshly isolated stellate cells contained little or no CINC mRNA (Fig. 1, top); however, when the cells were plated in primary culture, CINC mRNA was rapidly induced. CINC mRNA rose 4.6-fold within 3 days of plating and remained elevated for up to 18 days (Figs. 1 and 2). Some experiments, such as the one illustrated in Fig. 1 (top), demonstrated an early rise in CINC gene expression in stellate cell culture with a subsequent decline. The combined data from several experiments indicate that this downward trend in late culture is minor (Fig. 1, bottom).

Stellate cells are known to acquire a myofibroblastic phenotype when plated in primary culture. This transition occurs over ~7 days and is characterized in part by induction of type I collagen gene expression and collagen synthesis. Figure 2 illustrates the time course of CINC gene regulation in stellate cell culture compared with type I collagen. CINC mRNA rises in stellate cells before type I collagen mRNA; CINC mRNA also reaches a plateau in stellate cell culture, whereas type I collagen mRNA rises progressively over time. This suggests that the CINC and type I collagen genes in stellate cells are under independent regulation.

CINC mRNA induction in cultured stellate cells was accompanied by secretion of immunoreactive CINC protein. On day 3 of culture, stellate cells secreted 4.1 ng CINC/µg DNA into the culture medium constitutively over 24 h (Fig. 3); secretion remained at this level through day 7 of culture. Basal production of CINC by cultured stellate cells was high, but the cells could be stimulated to produce even more CINC if treated with cytokines. IL-1 (20 pg/ml) promoted a 30–60% increase in CINC secretion over 24 h. TNF, at a higher dose (20 ng/ml), induced a 60–70% increase in CINC secretion.

Stellate cell CINC secretion and response to cytokines. Graph depicts amount of CINC secreted by stellate cells (A) and Kupffer cells (B) over 24 h of primary culture, in presence or absence of interleukin-1 (IL-1, 20 pg/ml) or tumor necrosis factor (TNF, 20 ng/ml). CINC was measured in culture medium by sandwich ELISA (see METHODS). Stellate cells secreted more CINC constitutively than did Kupffer cells, but Kupffer cells exhibited a greater response to cytokine stimulation. Values represent means ± SE for n = 4, *P < 0.05 vs. control by Student’s t-test.

Fig. 3. Stellate cell CINC secretion and response to cytokines. Graph depicts amount of CINC secreted by stellate cells (A) and Kupffer cells (B) over 24 h of primary culture, in presence or absence of interleukin-1 (IL-1, 20 pg/ml) or tumor necrosis factor (TNF, 20 ng/ml). CINC was measured in culture medium by sandwich ELISA (see METHODS). Stellate cells secreted more CINC constitutively than did Kupffer cells, but Kupffer cells exhibited a greater response to cytokine stimulation. Values represent means ± SE for n = 4, *P < 0.05 vs. control by Student’s t-test.

Fig. 4. Chemotactic activity of stellate cell-conditioned medium. Graph depicts chemotactic activity of stellate cell-conditioned medium toward neutrophils either alone (CM) or after pretreatment with rabbit anti-CINC (CM + anti-CINC) or nonimmune rabbit IgG (CM + IgG). DMEM-0.5% BSA was used as a control. Values represent means ± SE for n = 4, *P < 0.05 vs. control by Student’s t-test.
These changes were significant but modest in magnitude. For comparison, IL-1 and TNF were added in the same doses to cultured rat Kupffer cells. The two cytokines caused a 130 and 560% increase in Kupffer cell CINC secretion, respectively. Thus culture-activated stellate cells produce large amounts of CINC constitutively but are less responsive to cytokines than cultured hepatic macrophages.

To determine whether stellate cell-derived CINC attracts neutrophils, conditioned medium from unstimulated stellate cells (culture day 7) was assayed for chemotactic activity in a Boyden chamber. The conditioned medium, which contained 9 ng/ml CINC by ELISA, caused significant directed migration of neutrophils over a 30-min interval (Fig. 4). Neutralization of CINC by preincubation of the conditioned medium with anti-CINC resulted in an 82% reduction in directed neutrophil migration.

To determine whether CINC is upregulated in stellate cells in vivo under conditions that promote activation, CINC mRNA was quantitated in stellate cells from normal rats and rats with liver injury. Cells were isolated from rats with two different forms of liver disease, caused by bile duct ligation or repeated doses of carbon tetrachloride. Both insults are known to cause stellate cell activation and liver fibrosis. Bile duct ligation causes portal fibrosis within 7 days (15); carbon tetrachloride promotes centrilobular fibrosis over 4–8 wk (28). Figure 5 illustrates the histological changes induced by bile duct ligation and carbon tetrachloride treatment and demonstrates that the degree of liver injury caused by carbon tetrachloride varies among individual rats. Bile duct ligation and carbon tetrachloride treatment both induced CINC mRNA in stellate cells (Fig. 6). CINC mRNA was barely detectable in cells from control rats but was present in all rats with
and in vivo; moreover, stellate cell-derived CINC is a C-X-C chemokine and potent neutrophil chemoattractant. CINC is inducible in stellate cells in culture and in vivo; moreover, stellate cell-derived CINC is biologically active as a neutrophil chemoattractant. Thus stellate cells are likely to play an important role in recruiting neutrophils to the hepatic parenchyma.

Our experiments indicate that CINC production is closely linked to stellate cell activation. In culture, CINC is induced rapidly in stellate cells; its upregulation parallels that of many other genes involved in myofibroblastic transformation, such as smooth muscle α-actin (29), transforming growth factor-β (8), and type I collagen (19). Moreover, CINC is induced in cultured stellate cells in the absence of an exogenous stimulus, suggesting that its regulation is part of the general phenomenon of culture activation. The time course of CINC regulation in stellate cells differs somewhat from that of other transformation-related genes. CINC is maximally induced early in culture (3 days), whereas smooth muscle α-actin and type I collagen typically increase later (between 3 and 7 days). The early appearance of CINC in activating stellate cells fits logically into the context of many liver diseases. For example, in viral, toxic, and alcoholic liver injury, inflammation often precedes fibrosis. Thus, if stellate cells produce CINC in the early stages of liver injury, they could be important contributors to this inflammatory process. As liver disease progresses in vivo, inflammation is ultimately replaced by fibrosis. This transition was also observed experimentally during the later stages of stellate cell activation. CINC expression remained stable or declined in late stellate cell culture, whereas type I collagen gene expression rose significantly.

In vivo, CINC was inducible in stellate cells in two independent models of liver injury (bile duct ligation and carbon tetrachloride administration). Both models promote stellate cell activation, and thus the findings in vivo are consistent with those in culture that CINC appears during activation. Of note, however, is that only one of the two models of liver injury employed in our study is believed to cause significant hepatic inflammation. Carbon tetrachloride promotes neutrophilic and mononuclear inflammation of the liver before ultimately causing fibrosis. Bile duct ligation, on the other hand, causes hepatic fibrosis without apparent antecedent inflammation. Induction of CINC in this second “noninflammatory” model of liver disease is difficult to reconcile. To address this issue, we have recently reexamined bile duct-ligated livers in an effort to identify neutrophilic inflammation. We find that neutrophils are present in these livers and that they localize to areas of bile duct proliferation and fibrogenesis (18). Thus induction of CINC in stellate cells in vivo does coincide with hepatic inflammation in both experimental models of liver disease.

Although our experiments identify stellate cells as a source of CINC in diseased liver, they do not address the importance of these cells in relation to other liver cells. Hepatocytes and Kupffer cells as well as biliary cells (J. Maher and J. Saito, unpublished observations) also produce CINC (17, 31). In the diseased liver in vivo, CINC is likely to be produced by several cell populations. The precise mix of contributing cells prob-
ably depends on factors such as the local cytokine milieu. Our experiments indicate that stellate cells, which are activated in culture, produce large amounts of CINC even in the absence of exogenous cytokines or endotoxin. This may be an important distinction, since it suggests CINC is inducible in stellate cells by factors that do not affect hepatocytes or Kupffer cells. The precise nature of the stimulus that induces CINC in stellate cell culture is under investigation.

CINC appears to be the dominant neutrophil chemoattractant produced by stellate cells. In our experiments, it accounted for over 80% of the neutrophil chemotactic activity in conditioned medium from culture-activated stellate cells. This is in general agreement with a recent study showing that a related C-X-C chemokine, macrophage inhibitory protein 2 (MIP-2), is not produced constitutively by culture-activated stellate cells (32). MIP-2 is inducible in stellate cells by TNF or endotoxin, but the amount of MIP-2 produced by these cells is small in comparison to the amount of CINC measured in our cultures. Thus it appears that CINC outweighs MIP-2 as a stellate cell chemokine. CINC and MIP-2 are structurally and functionally very similar. Why the two chemokines would be regulated differently in stellate cell culture is unknown. We considered that CINC might be induced spuriously in our experiments by inadvertent contamination of cell cultures with cytokines or endotoxin, but this is highly unlikely, since parallel cultures of hepatocytes and Kupffer cells produce much less CINC (Fig. 3; Ref. 17). Few studies exist comparing the regulation of different C-X-C chemokines in a single cell type. One report indicates that CINC is more prominent than other C-X-C chemokines in cultured fibroblasts (25). This supports our observation that CINC is the dominant C-X-C chemokine in stellate cells.

Although chemokines are classically viewed as leukocyte chemoattractants, they can also influence other processes, such as tumor growth and angiogenesis (3, 33, 34). One recent study indicated that MCP-1 stimulates lung fibroblasts to produce collagen and transforming growth factor-β (11). In view of the latter findings, it is intriguing to speculate that CINC is induced in stellate cells not only to attract neutrophils but also to provoke or perpetuate the myofibroblastic phenotype. We have begun to investigate possible autocrine effects of CINC on stellate cells. To date, we find no influence of CINC on stellate cell proliferation (J. Saito and J. Maher, unpublished observations). Studies of CINC as a mediator of stellate cell collagen synthesis are ongoing.

In summary, the current study demonstrates that stellate cells produce CINC, a compound that attracts and activates neutrophils. CINC is induced in stellate cells both in culture and in vivo, since they transform from a quiescent to a myofibroblastic phenotype. CINC is induced early in the process of stellate cell activation; this coincides with clinical and histological observations that hepatic inflammation often precedes fibrosis. The current study strongly supports the notion that stellate cells promote neutrophilic inflammation in liver. Further study should clarify whether CINC has independent autocrine effects on stellate cells that stimulate or enhance hepatic fibrogenesis.

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