Vascular endothelial growth factor and hepatocyte regeneration in acetaminophen toxicity

Brian Donahower, Sandra S. McCullough, Richard Kurten, Laura W. Lamps, Pippa Simpson, Jack A. Hinson, and Laura P. James. Vascular endothelial growth factor and hepatocyte regeneration in acetaminophen toxicity. Am J Physiol Gastrointest Liver Physiol 291: G102–G109, 2006. First published March 24, 2006; doi:10.1152/ajpgi.00575.2005.—VEGF or VEGF-A is a major regulator of angiogenesis and has been recently shown to be important in organ repair. The potential role of VEGF in acetaminophen (APAP)-induced hepatotoxicity and recovery was investigated in B6C3F1 male mice. Mice were treated with APAP (300 mg/kg ip) and killed at various time points that reflect both the acute and recovery stages of toxicity. VEGF-A protein levels were increased 7-fold at 8 h and followed the development of hepatotoxicity. VEGF receptor 1, 2, and 3 (VEGFR1, VEGFR2, and VEGFR3, respectively) expression increased throughout the time course, with maximal expression at 48, 8, and 72 h, respectively. Treatment with the VEGF receptor inhibitor SU5416 (25 mg/kg ip at 3 h) had no effect on toxicity at 6 or 24 h. In further studies, the role of SU5416 on the late stages of toxicity was examined. Treatment of mice with APAP and SU5416 (25 mg/kg ip at 3 h) resulted in decreased expression of PCNA, a marker of cellular proliferation. Expression of platelet endothelial cell adhesion molecule, a measure of small vessel density, and endothelial nitric oxide synthase (NOS), a downstream target of endothelial cell adhesion molecule, a measure of small vessel density, were obtained from Sigma (St. Louis, MO). Universal DAKO LSAB + (labeled streptavidin-biotin) peroxidase kit and DAKO protein block (serum free) were purchased from DAKO (Carpinteria, CA). Immunoperoxidase suppressor and Coomassie plus protein assay reagent were obtained from Pierce Chemical (Rockford, IL). Gills hematoxylin II and Permound were both acquired from Fisher Scientific (Pittsburgh, PA). The VEGF inhibitor SU5416 was obtained from CalBiochem (Spring Valley, CA).

Experimental animals. Six-week-old male B6C3F1 mice (mean weight 24.4 g) were obtained from Harlan Sprague Dawley (Indianapolis, IN). All animal experimentation was in accordance with the criteria of the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences. Protocols for animal experimentation were approved by University of Arkansas for Medical Sciences Animal Care and Use Committee. Mice were acclimatized 1 wk before the planned experiments. Mice were fed ad libitum and were housed in individual cages on a 12:12-h light-dark cycle. On the day before experiments, mice were fasted overnight and dosing studies began at 0800 the following morning. In initial experiments, mice were dosed with APAP (300 mg/kg ip in saline) and killed at 1, 2, 4, and 8 h after APAP (n = 5–6 mice per time point). In another experiment, mice were dosed with APAP (300 mg/kg ip in saline) and killed at 24, 48, 72, and 96 h after APAP (n = 5–6 mice per time point). Control mice received saline only. In other experiments, animals received SU5416 (25 mg/kg sc) 3 h after APAP and were killed at 6, 24, 48, or 72 h after APAP (n = 5–6 per treatment group). The SU5416 was administered in 200 μl of a solution containing CMC (0.5%), NaCl (0.9%), polysorbate 80 (0.4%), and benzyl alcohol (0.9%) in deionized water as previously described (25, 30). Control mice in this experiment received APAP plus vehicle. At the indicated time, animals were anesthetized with CO2. Blood was removed from the retroorbital plexus and allowed to coagulate at room temperature. Blood was centrifuged, and the serum was removed for measurement of alanine aminotransferase.

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ACETAMINOPHEN (APAP) overdose is the most prevalent cause of drug-induced acute liver failure. Recent studies have reported the upregulation of a number of cytokines and chemokines in APAP toxicity (14–16, 21, 22, 24, 29). Several cytokines have been shown to have hepatoprotective properties, such as promoting hepatocyte regeneration or controlling pro-inflammatory cytokine expression. For instance, our laboratory previously reported that IL-6 knockout (KO) mice had delayed hepatocyte regeneration following the administration of toxic doses of APAP (22). In addition, a number of chemokines, such as macrophage inhibitory protein 2 (MIP-2) and IFN-γ inducible protein (IP-10), have also been shown to be important in hepatocyte regeneration following APAP toxicity (3, 16, 21).

Growth factors are small molecular weight proteins that are closely related to cytokines. Growth factors have received little study in drug-induced liver toxicity. Previous studies have shown that VEGF-A is upregulated in several models of hepatic injury, such as partial hepatectomy, carbon tetrachloride toxicity, and ischemia-reperfusion injury (17, 35). VEGF is a potent mitogen for endothelial cells and is well-known for its effects on angiogenesis in physiological and pathological conditions. The following study was conducted to examine the role of VEGF in APAP-induced hepatotoxicity and to examine the effect of VEGF neutralization on toxicity and hepatocyte regeneration in our model.

MATERIALS AND METHODS

Reagents. APAP (paracetamol) and carboxymethylcellulose (CMC) were obtained from Sigma (St. Louis, MO). Universal DAKO LSAB + (labeled streptavidin-biotin) peroxidase kit and DAKO protein block (serum free) were purchased from DAKO (Carpinteria, CA). Immunoperoxidase suppressor and Coomassie plus protein assay reagent were obtained from Pierce Chemical (Rockford, IL). Gills hematoxylin II and Permound were both acquired from Fisher Scientific (Pittsburgh, PA). The VEGF inhibitor SU5416 was obtained from CalBiochem (Spring Valley, CA).
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(ALT). Mice were then euthanized with CO2, and the livers were removed. The livers were weighed, and a portion was preserved in formalin for histological sections. The remaining livers were snap frozen in liquid nitrogen and stored at −80°C for additional analyses.

Toxicity assays. Serum ALT was determined with a spectrophotometric diagnostic kit (Teco Diagnostics, Anaheim, CA).

Growth factor protein receptor assays. Snap-frozen liver samples were thawed, weighed, and homogenized in solutions containing 1 ml of protease inhibitor cocktail (Complete; Boehringer-Mannheim, Indianapolis, IN). The resulting supernatants were analyzed in duplicate and standardized to the weight of the homogenized liver sample. VEGF-A and other growth factors were measured in the supernatants of liver homogenates by a Luminex 100 analyzer (Applied Cytometry Systems, Dinnington, UK) using a mouse growth factor four-plex antibody bead kit (BioSource International, Camarillo, CA) as per the manufacturer’s instructions. The limits of detection for growth factors were as follows: VEGF-A (15 pg/ml), fibroblast growth factor (30 pg/ml), platelet-derived growth factor (15 pg/ml), and granulocyte colony-stimulating factor (15 pg/ml).

Western blot analysis. VEGF receptor 1 (VEGFR1 or Flt-1), VEGF receptor 2 (VEGFR2 or Flk-1), VEGF receptor 3 (VEGFR3 or FLT4), platelet endothelial cell adhesion molecule (PECAM), and endothelial nitric oxide synthase (eNOS) protein levels were measured by Western blot analysis. Liver tissue was homogenized on ice in 25 mM HCl buffer containing (in mM) 1 EDTA, 1 EGTA, 0.1% (vol/vol) 2-mercaptoethanol, 1 phenylmethylsulfonyl fluoride, 2 leupeptin, and 1 pepstatin A. Homogenates were centrifuged at 1,500 g for 10 min to remove cell debris. Liver homogenates (50 μg) were separated by SDS-PAGE and then transferred to nitrocellulose membranes by electroblocting. Membranes were blocked overnight at 4°C in blocking buffer (5% nonfat dry milk) followed by a 2-h incubation at room temperature with a monoclonal anti-Flt-1 antibody (1:400), a monoclonal anti-Flk-1 antibody (1:400), a polyclonal anti-flt-4 antibody (1:250), a polyclonal anti-PECAM antibody (1:200), or a monoclonal anti-eNOS antibody (1:750). All antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). After the membranes were treated with the primary antibodies, a peroxidase-conjugated goat anti-mouse IgG secondary antibody was used at a 1:2,000 dilution for 1 h at room temperature. Band detection was performed using ECL plus detection (Amersham, Piscataway, NJ).

Immunohistochemistry. Fresh liver tissues, previously trimmed to ~2-mm thickness, were placed in plastic cassettes and immersed in neutral buffered formalin for 24 h. Paraffin-embedded tissue sections were deparaffinized with xylene (2 × 5 min, 25°C), then rehydrated in a series of graded ethanol washes and deionized water. The sections were then placed in Pierce Immunopore peroxidase suppressor for 30 min to quench endogenous peroxidase activity. Next, DAKO protein block (serum-free) was added to each tissue section for 30 min to block nonspecific binding. After being washed in PBS, the sections were incubated with a mouse monoclonal VEGF-A (1:50) antibody for 60 min at room temperature. For color development, the protocol described in the DAKO LSAB kit was used. The slides were counterstained with Gill’s hematoxylin II for 2 min, and after being rinsed in deionized water, were immersed in ammonia blue for 2 min. The slides were dehydrated and mounted with Permount. Sections of pancreatic parenchyma served as the positive control for VEGF immunohistochemistry (50). Normal mouse liver tissue with the primary antibody replaced by mouse serum was used as a negative control. Immunohistochemical analysis with PCNA was performed using the DAKO ARK peroxidase kit (DAKO) at an antibody concentration of 1:75. PCNA is a commonly used assay of cellular proliferation that correlates with DNA synthesis and liver thymidine kinase activity (47).

Statistical analysis. Results are expressed as means ± SE. Comparisons between multiple groups were by one-way analysis of variance followed by the Tukey’s Honestly Significantly Different post hoc test; P ≤ 0.05 was considered statistically significant. Comparison of differences in PCNA immunohistochemistry was performed using a Mann-Whitney nonparametric test. SPSS version 10.0 (SPSS, Chicago, IL) was used for statistical analyses.

RESULTS

VEGF upregulation in APAP toxicity. To determine whether VEGF was upregulated in APAP-induced hepatic necrosis, mice were treated with APAP (300 mg/kg ip). The mice were killed at 1, 2, 4, and 8 h, and in a separate experiment, the mice were killed at 24, 48, 72, and 96 h. Consistent with previous data from our laboratory (24), significant hepatotoxicity [elevation of serum ALT and histological necrosis (data not shown)] was apparent at 4 h (Fig. 1A), peaked at 8–24 h, remained high for 48 h, and resolution occurred between 48 and 96 h (Fig. 1B). As shown in Fig. 1C, a significant increase in VEGF-A protein was detected in liver homogenates at 8 h. This increase followed the onset of toxicity (Fig. 1A). By 24 h, VEGF-A levels were extremely high (a 34-fold elevation over controls), and levels peaked at this time point and subsequently declined (Fig. 1D).

The upregulation of VEGF-A in APAP-treated livers was also examined by immunohistochemical analysis of tissue sections. These data showed that VEGF-A staining was present in the hepatocytes of the portal and mizonal areas of the APAP-treated liver (Fig. 2B). The immunohistochemical expression of VEGF-A in hepatocytes is consistent with data reported in other models of liver injury (4, 17).

Levels of other growth factors (fibroblast growth factor, platelet-derived growth factor, granulocyte colony-stimulating factor) were not significantly elevated in the APAP-treated mice (data not shown).

VEGF receptor upregulation in APAP toxicity. The protein expression of the three VEGF receptors [VEGFR1, VEGFR2, and VEGFR3 (42)] was examined by immunoblots (Fig. 3). Minimal expression of all three receptors was present in the saline-treated mice. Increased expression of VEGFR1, VEGFR2, and VEGFR3 protein (Fig. 3, A–C) was apparent throughout the time course, with peak expression for VEGFR1 at 48 h, for VEGFR2 at 8 h, and for VEGFR3 at 72 h.

Effect of SU5416 on APAP toxicity. To examine the role of VEGF in the early stages of APAP toxicity, mice were treated with APAP followed by SU5416, a synthetic VEGF inhibitor that is highly specific for VEGF receptor signaling (11, 32). The mice received SU5416 3 h after APAP, a time point beyond the metabolic phase of toxicity (23, 34, 38). The mice were killed at 6 or 24 h after APAP. Both biochemical evidence of toxicity (ALT values; Fig. 4A) and histological examination of hematoxylin and eosin-stained slides (Fig. 4B) showed comparable degrees of toxicity in the two groups of mice at both time points. Thus treatment with SU5416 did not alter APAP toxicity.

Effect of SU5416 on hepatocyte regeneration following APAP toxicity. To elucidate the role of endogenous VEGF-A in the later stages of toxicity, mice were treated with SU5416 and killed at 48 or 72 h after APAP. Another group of mice received APAP followed by vehicle only. At 48 h, ALT values were significantly higher in the mice that received APAP plus SU5416, compared with the APAP plus vehicle group (Fig. 5).
However, histological evaluation of relative necrosis indicated that there was not a significant difference between the toxicity in the APAP plus SU5416 group and the APAP plus vehicle group. To examine the effect of SU5416 on hepatocyte regeneration, immunohistochemical analysis for PCNA was performed. PCNA is a 36-kDa acidic nuclear protein expressed in the G1 and S phases of mitosis (39, 40) and is commonly used as a measure of hepatocyte regeneration in liver injury models (2, 5, 21, 22, 47). In previous studies, our laboratory reported that PCNA staining was significantly increased by 48 h in APAP toxicity in the mouse (20, 22). Minimal PCNA staining was present in the vehicle-treated mice (Fig. 6C). Prominent staining for PCNA was apparent at the junction of the midzonal and centrilobular regions of the livers of the APAP plus vehicle groups at 48 h (Fig. 6A). In contrast, minimal staining for PCNA was present in the mice treated with APAP plus SU5416 (Fig. 6B). Quantitative image analysis showed that the intensity of PCNA staining was significantly increased in the APAP-treated mice compared with the vehicle-treated mice (132.5 ± 7.5 vs. 95.8 ± 6.8; P = 0.009) and significantly reduced in the SU5416-treated mice at 48 h (104.3 ± 8.2; P = 0.041). These data suggest that VEGF receptor signaling is critical to the process of hepatocyte regeneration in APAP toxicity.

Effect of APAP treatment on PECAM and eNOS expression. A number of proteins have been reported to be induced during angiogenesis. These include PECAM and eNOS. PECAM expression is a commonly used relative measure of microvessel density (neoangeogenesis) (11, 44, 51). eNOS is a constitutive endothelial protein important in the control of portal blood pressure by regulating nitric oxide (41) and has been shown to be induced by VEGF (9). Figure 7 shows that both PECAM and eNOS were induced at 48 and 72 h (Fig. 7B). Quantitative image analysis showed that the intensity of PECAM staining was significantly decreased in the SU5416-treated mice at 48 h and 72 h (Fig. 7B).
VEGF-A is a homodimeric glycoprotein with a relative molecular mass of 45,000 Da and is the only mitogen that specifically acts on endothelial cells. It is well-known as a proangiogenic factor in the developing vascular system and is also important in the pathogenesis of tumors, diabetes, and prematurity-induced retinopathy (28). VEGF-A is secreted by hepatocytes (1), as well as many other cell types (43), and has also been shown to be important in wound

**DISCUSSION**

Fig. 3. Time course of VEGF receptors VEGFR1, VEGFR2, and VEGFR3 protein expression in mice treated with APAP. Mice were dosed with APAP (300 mg/kg ip) and killed at the indicated times. Minimal expression of VEGFR1, VEGFR2, and VEGFR3 was present in the saline-treated mice. VEGFR1, VEGFR2, and VEGFR3 expression increased throughout the time course with peak expression of VEGFR1 at 48 h (A), VEGFR2 at 8 h (B), and VEGFR3 at 72 h (C).

Fig. 4. Effect of SU5416 on toxicity. A: mice were treated with SU5416 3 h after APAP (300 mg/kg ip) and killed at 6 or 24 h. ALT values were comparable in the mice that received SU5415, compared with the mice that received APAP + vehicle; *P < 0.05, compared with mice treated with vehicle alone. B: hematoxylin and eosin-stained sections of representative mice. Top shows mouse treated with APAP + vehicle, killed at 6 h. Bottom shows mouse treated with APAP + SU5416, killed at 6 h. The mice had comparable degrees of hepatocellular necrosis.
healing in bone and skin injury (13, 46). In the present study, the time course of VEGF-A protein and receptor expression was examined in a mouse model of APAP toxicity. VEGF-A protein and receptor levels were significantly increased in mice treated with APAP (Figs. 1–3), and this upregulation followed the onset of biochemical and histological toxicity, suggesting a role for endogenous VEGF-A upregulation in the recovery stages of toxicity. In addition, immunohistochemical studies demonstrated VEGF-A in the healthy regions of the liver (Fig. 2), known to be important in the process of hepatocyte regeneration (22).

To further examine the relationship of VEGF-A to recovery following toxicity, mice were treated with SU5416, an antiangiogenic compound that specifically inhibits VEGF-mediated signaling (32). SU5416 is a hydrophobic small molecule that has prolonged pharmacodynamic effects in mice (32) and was developed as an antitumor agent due to its inhibitory effects on angiogenesis (11). This compound is a tyrosine kinase inhibitor that inhibits VEGF receptor-mediated phosphorylation and subsequent signal transduction (11, 32, 44). SU5416 is thought to primarily have effects on VEGFR2 (11), although one study found that it may also have effects on VEGFR1-mediated signaling (19). In the present study, treatment with SU5416 after APAP had no effect on toxicity at either 6 or 24 h. In further studies, treatment with SU5416 after APAP was associated with a significant reduction in cellular proliferation as measured by immunohistochemical assays for PCNA. Thus treatment with SU5416 slowed recovery in APAP-treated mice.

![Fig. 5. Effect of SU5416 on serum ALT levels in APAP-treated mice. Mice were treated with SU5416 3 h after APAP (300 mg/kg ip). ALT levels were significantly higher at 48 h in the mice that received SU5416, compared with the mice that received APAP + carboxymethylcellulose (CMC) vehicle. *Significant difference from APAP + inhibitor group (P < 0.05). No difference was present in the 2 groups of mice killed at 72 h.](image)

![Fig. 6. Effect of SU5416 on PCNA immunohistochemistry in APAP-treated mice. PCNA staining (arrows) was present in the midzonal and centrilobular regions of the livers of mice treated with APAP and killed at 48 and 72 h (A). In contrast, PCNA was significantly reduced in the mice treated with SU5416 (B). None to minimal staining for PCNA was present in the vehicle-treated mice (C).](image)
In addition to a reduction in immunohistochemical staining for PCNA, treatment with SU5416 was associated with a reduction in PECAM and eNOS expression. Previous studies (37) have examined the relationship of VEGF and eNOS. The angiogenic effects of VEGF are mediated by eNOS in vascular endothelial (reviewed in Ref. 27) and sinusoidal endothelial cells (41). Autophosphorylation of VEGFR2 results in eNOS activation (9). Kawachi et al. (26) found that mice genetically deficient in eNOS had increased liver injury in a model of postschismic liver injury. Fuku-mura et al. (12) reported that eNOS KO mice had reduced angiogenesis. Collectively, the data from the present study suggest that endogenous VEGF is important in the recovery stages of APAP toxicity and that neutralization of VEGF slows the recovery process and is associated with a reduction in the expression of targets of APAP toxicity (8, 49), and that microcirculatory dysfunction involving endothelial cells precedes parenchymal cell injury in APAP toxicity (18). Both HGF and IL-6 have been shown to be important in hepatocyte regeneration in several models of hepatic injury, including APAP toxicity (6, 22). We previously showed that IL-6 KO mice treated with toxic doses of APAP had delayed hepatocyte regeneration (22). Thus, cumulatively, the data support a mechanism where both VEGF and IL-6 are critical mediators of hepatocyte regeneration following APAP toxicity.

One of the observations of this study was that serum ALT levels were significantly higher in the APAP plus SU5416-treated mice than the mice treated with APAP only at 48 h (Fig. 5). Examination of liver tissues from the mice revealed comparable degrees of necrosis, despite this apparent difference in ALT data between the treatment groups at 48 h (Fig. 5). Interestingly, we previously observed discrepancies between ALT values and histology in other studies of APAP toxicity (22, 33). From a pathological perspective, histological examination of liver tissue is considered to be the gold standard for the assessment of toxin-mediated liver injury. Thus other factors, possibly relating to the rate of leakage of ALT from hepatocytes into the circulation or the clearance of ALT from the circulation, may be responsible for the observed differences between ALT and histological examination of liver tissues.

In closing, we found that VEGF protein and receptor expression was markedly increased in the late stages of toxicity in APAP-treated mice. Inhibition of VEGF-mediated signaling with SU5416 significantly delayed hepatocyte regeneration, and this intervention was associated with reduced expression of PECAM and eNOS. Further study will be required to examine the possible protective effects of exogenous VEGF (36, 48) as a novel therapeutic approach for stimulating hepatocyte regeneration following APAP overdose.

**GRANTS**

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