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 PCNA, increased throughout the time course, with maximal expression at 48, 8, and 72 h, respectively. Prevention of endogenous VEGF is critically important to the process of hepatocyte regeneration in APAP-induced hepatotoxicity in the mouse.

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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 Tris·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 vol) 2-mercaptoethanol, 1 phenylmethylsulfonyl fluoride, 2 leupeptin, and 1 pepstatin A. Homogenates were centrifuged at 1,500

Effect of SU5416 on hepatocyte regeneration following APAP toxicity. To elucidate 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 APAP plus 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 (neoangiogenesis) (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). 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.

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DISCUSSION

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
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.

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).
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 postischemic liver injury. Fukumura 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 of proteins previously implicated in the process of angiogenesis.

Previous studies in animal models of liver injury have supported a role for VEGF in the recovery stages following liver injury (17, 45). Ishikawa et al. (17) showed that mRNA levels of VEGF and its receptors were increased in a rat model of carbon tetrachloride toxicity. Similar findings have been reported in studies of partial hepatectomy in the rat (35, 45). In vitro, VEGF induced the proliferation of endothelial cells, including hepatic sinusoidal endothelial cells (10). Increased expression of mRNA for VEGF was shown in isolated cellular fractions including hepatocytes, Kupffer cells, and stellate cells in rats treated with carbon tetrachloride (17).

Previous data suggest that VEGF receptors may have differential effects (reviewed in Ref. 43). VEGF is best known for its proangiogenic effects, most of which are mediated by VEGFR2 (31). Comparatively less is known about the specific effects of VEGFR1, whereas VEGFR3 is important in lymphangiogenesis (43). With the use of receptor-specific mutants in a coculture model of endothelial cells and hepatocytes, selective activation of VEGFR1 on endothelial cells increased mRNA levels of hepatocyte mitogens (IL-6) and hepatocyte growth factor (HGF) (31). It was thus proposed that VEGFR1 mediates “cross-talk” between parenchymal and nonparenchymal cells, causing the release of IL-6 and HGF by endothelial cells leading to prosurvival effects on hepatocytes (7, 31). Previous data have shown that endothelial cells are metabolically active, are 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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