Human recombinant Vascular Endothelial Growth Factor (hrVEGF) Reduces Necrosis and Enhances Hepatocyte Regeneration in a Mouse Model of Acetaminophen Toxicity


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Running Title page

a. Running Title: hrVEGF in acetaminophen toxicity and repair in the mouse

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d. abbreviations: APAP, acetaminophen; ALT, alanine aminotransferase; GSH, glutathione; HA, hyaluronic acid; hrVEGF, human recombinant vascular endothelial growth factor; IL-6, interleukin 6; PCNA, proliferating cell nuclear antigen; PBS, phosphate buffered saline; PECAM, platelet endothelial cell adhesion molecule; VEGF, vascular endothelial growth factor; VEGFR1, vascular endothelial growth factor receptor 1; VEGFR2, vascular endothelial growth factor receptor 2
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Abstract

We previously reported that vascular endothelial growth factor (VEGF) was increased in acetaminophen (APAP) toxicity in mice and that treatment with a VEGF receptor inhibitor reduced hepatocyte regeneration. The effect of human recombinant (hr)VEGF on APAP toxicity in the mouse was examined. In early toxicity studies, B6C3F1 mice received hrVEGF (50 ug SQ) or vehicle 30 min prior to APAP (200 mg/kg (IP) and were sacrificed at 2, 4, and 8 hr. Toxicity was comparable at 2 and 4 hr, but reduced in the APAP/hrVEGF mice at 8 hr (p<0.05) compared to the APAP/vehicle mice. Hepatic glutathione (GSH) and APAP protein adduct levels were comparable between the two groups of mice, with the exception that GSH was higher at 8 hr in the hrVEGF treated mice. Subsequently, mice received two (pre- and 10 hr) or three doses (pre-, 10, 24 hr) of hrVEGF; ALT values and necrosis were reduced at 24 and 36 hr, respectively, in the APAP/hrVEGF mice (p<0.05) compared to the APAP/vehicle mice. Proliferating cell nuclear antigen (PCNA) expression was enhanced and IL6 expression was reduced in the mice that received hrVEGF (p<0.05) compared to the APAP/vehicle mice. In addition, treatment with hrVEGF lowered plasma hyaluronic acid levels and neutrophil counts at 36 hr. Cumulatively, the data show that treatment with hrVEGF reduced toxicity and increased hepatocyte regeneration in APAP toxicity in the mouse. Attenuation of sinusoidal cell endothelial dysfunction and changes in neutrophil dynamics may be operant mechanisms in the hepatoprotection mediated by hrVEGF in APAP toxicity.
Introduction

Acetaminophen (APAP; $C_8H_9NO_2$) overdose is the most common cause of acute liver failure in the United States (Larson et al., 2005). N-acetylcysteine is the only available treatment of APAP overdose, but its efficacy is primarily limited to the initial, early stages of APAP toxicity. Currently, no other therapies exist for the management of APAP-induced liver failure, other than the management of coagulopathy and support of vital organ functions.

Previous studies have shown that numerous pro-inflammatory and anti-inflammatory cytokines and chemokines are upregulated in animal models of APAP toxicity, including interleukin 1β, interleukin 6 (IL-6), interleukin10, interleukin 13, macrophage inhibitory protein 2 (MIP-2), and monocyte chemotactic protein 1 (Jaeschke, 2005). The role of cytokines and inflammation in APAP toxicity has been reviewed (Jaeschke, 2005) and the available data suggest that cytokines may play a role in the aggravation of cellular injury, but may also limit cell injury and initiate cell and organ repair processes (Bourdi et al., 2002; James et al., 2003a; Masubuchi et al., 2003; Jaeschke, 2005; James et al., 2003b; Hogaboam et al., 1999; James et al., 2005).

Our laboratory previously demonstrated that vascular endothelial growth factor (VEGF) was significantly elevated in the late stages of APAP toxicity (Donahower et al., 2006). VEGF is a potent endothelial mitogen that is involved in angiogenesis in physiological and pathological conditions (Ferrera et al., 2003; Leung et al., 1989). In addition, VEGF has been shown to have paracrine mediated pro-mitotic effects on hepatocytes (LeCouter et al., 2003) suggesting that it could facilitate hepatocyte regeneration following liver injury. We previously reported that murine hepatic VEGF levels were significantly increased at 8 hr in the mouse, well after the onset of toxicity, and that levels of this growth factor continued to rise in the later stages of
toxicity (Donahower et al., 2006). Furthermore, treatment with a VEGF receptor inhibitor reduced hepatocyte proliferation in APAP treated mice. (Donahower et al., 2006) In addition to these data in the mouse model of APAP toxicity (Donahower et al., 2006), VEGF has been shown to be upregulated in other models of liver injury, such as partial hepatectomy, ischemia-reperfusion and carbon tetrachloride toxicity (Tsurui et al., 2005; Taniguchi et al., 2001). Collectively, these data prompted us to examine the possible role of exogenous human recombinant (hr)VEGF as a potential treatment for APAP toxicity. Treatment with hrVEGF has been previously shown to have a protective effect in a mouse model of hepatic ischemia reperfusion injury (Tsurui et al., 2005). In addition, several studies have reported beneficial effects of exogenous hrVEGF in murine models of myocardial ischemia and hindlimb ischemia (Ware et al, 1997; Banai et al., 1994). In the following study, the effect of hrVEGF on the early, intermediate and late stages of APAP toxicity was examined to test the hypothesis that hrVEGF would accelerate the liver repair process following APAP-induced hepatotoxicity in the mouse.
Methods and Materials

Reagents. APAP (paracetamol) was obtained from Sigma Chemical Co. (St. Louis, MO). Coomassie Plus Protein Assay Reagent were obtained from Pierce Chemical Co. (Rockford, IL). Gills Hematoxylin II and Permount were both acquired from Fisher Scientific, Inc. (Pittsburgh, PA). Human recombinant VEGF (hrVEGF or hrVEGF\textsuperscript{165}) was obtained through a material transfer agreement with the National Cancer Institute. The hrVEGF sequence was expressed in S\textsubscript{f}21 cells and the protein was purified by sequential chromatography to greater than 97 percent purity.\cite{Leung1989} Its biological activity has been previously examined in human umbilical vein endothelial cells (HUVECs) and it has been shown to stimulate \textsuperscript{3}H-thymidine incorporation, with an ED\textsubscript{50} of 2-6 ng/ml.\cite{Conn1990} The monoclonal anti-PCNA antibody (1:500) was obtained from DAKO Cyotomation (Carpinteria, CA).

Experimental Animals. Six-week old male B6C3F1 mice (mean weight, 24.4 grams) 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 one week prior to the planned experiments. Mice were fed \textit{ad libitum} and were housed in individual cages on a 12 hr light/dark cycle. On the day prior to experiments, mice were fasted overnight and dosing studies began at 0800 the following morning. In one experiment (Fig 1), mice were dosed with hrVEGF (50 ug SQ in PBS; Tsurui et al., 2005) 30 min prior to APAP (200 mg/kg, IP) and were sacrificed at 2, 4, and 8 hr (n = 5 per time point and treatment group). Treatment control mice received PBS vehicle 30 min prior to APAP. Control mice received PBS. The same dose of APAP and hrVEGF was used in all subsequent studies.
In the intermediate study (Fig 1B), mice received hrVEGF 30 min prior to and 10 hr after APAP and were sacrificed at 18 and 24 hr (n = 6 per time point). In a third experiment (Fig 1C), mice were dosed with hrVEGF 30 min prior to and 10 hr and 24 hr after APAP and were sacrificed at 36 hr (n = 11 per time point). In the final experiment, (Fig 1D) mice received hrVEGF at 2, 10 and 24 hr after APAP and were sacrificed at 36 hr (n=12 per treatment group).

At the indicated times, animals were anesthetized with CO₂. Blood was removed from the retro-orbital plexus, allowed to coagulate at room temperature, then centrifuged and the serum was removed for measurement of alanine aminotransferase (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° for additional analyses.

**Toxicity, histology and metabolism Assays.** Serum ALT levels were measured using a Cobas Mira Autoanalyzer (Roche Diagnostics). Hematoxylin and eosin (H&E) staining was performed for histological examination of mouse livers. The extent of necrosis in liver sections was quantified by outlining the necrotic areas with the interactive spline measuring tool in the AxioVision 4.6.3 program (Carl Zeiss Inc., Germany). Three images were obtained from each section at 10X magnification. Quantification of the extent of necrosis was expressed as a percentage of the entire histological field. For examination of neutrophil counts, H&E stained sections were examined by an investigator who was blinded to the experimental groups. In each mouse, the slide was scanned, three regions of high neutrophil accumulation were identified, and the number of neutrophils within the interstitium was counted (40X magnification; Olympus BX50). Hepatic glutathione (GSH) was measured by a colorimetric method using Ellman’s reagent as previously modified by Mitchell et al. (Mitchell et al., 1973). APAP protein adducts in
the liver were measured by assaying supernatants of liver homogenates using a high performance liquid chromatograph-electrochemical detection method developed by our laboratory (Muldrew et al., 2002).

**Cytokine, Growth Factor and Hyaluronic Acid 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. Cytokines, growth factors and growth factor receptors (hVEGF, murine VEGF, VEGFR1, VEGFR2, IL-6) were measured in the supernatants of liver homogenates using highly specific ELISA kits, as per the manufacturer’s instructions (R & D, Minneapolis, MN). Very low cross-reactivity (0.2%) for the murine VEGF ELISA and exposure to high (50,000 pg/mL) concentrations of hVEGF has been noted (R & D, Minneapolis, MD). Hyaluronic acid in plasma was measured using an ELISA kit purchased from Corgenix (Broomfield, CO).

**Immunoblotting and Immunohistochemistry.** Proliferating cell nuclear antigen (PCNA) and platelet endothelial cell adhesion molecule (PECAM) expression in liver homogenates was measured by western blot as previously described (Donahower et al., 2006). Band detection was performed using ECL Plus detection (Amersham, Piscataway, NJ).

**Quantitative Image Analysis.** To quantify the effect of hrVEGF on the proliferation of hepatic nuclei, slides of liver sections were scanned to digital file using the Aperio Scanscope T2 system (Aperio, Vista, CA). This system produces a navigable high-resolution file of each slide at magnification up to 200X. PCNA-labeled slides were analyzed over the entire slide using the Aperio Immunohistochemistry Nuclear algorithm. This algorithm is based on optical density of staining using color deconvolution to analyze only chromagen staining. Nuclei are recognized
by shape, size, and staining characteristics. Results were reported as percent positive nuclei. Algorithm settings were optimized for PCNA liver staining in our laboratory.

**Statistical and Pharmacokinetic Analysis.** Results are expressed as means ± SE. Comparisons between multiple groups were by one-way analysis of variance followed by the Tukey HSD post-hoc test. SPSS Version 10.0 (SPSS Inc, Chicago, IL) was used for statistical analyses. hrVEGF concentration data from the initial experiment was standardized to baseline and vehicle values.
Results

Demonstration of hrVEGF Uptake in Mouse Liver and Its Effect on Receptor Expression

In the early toxicity experiment (Fig 1A), mice were dosed with APAP (200 mg/kg, IP) and vehicle (PBS, SQ) or APAP and hrVEGF (50 ug SQ in PBS). Control mice received PBS only. Mice were sacrificed at 2, 4 and 8 hr after APAP. To verify that hrVEGF entered the liver, hVEGF levels were measured in the supernatants of liver homogenates. In the mice that received hrVEGF, hVEGF levels peaked at 2 hr and remained significantly elevated at 4 hr, compared to vehicle/APAP treated mice (Fig 2A). Administration of hrVEGF to mice had no significant effects on murine VEGF levels (data not shown).

In the intermediate toxicity experiment (Fig 1B), mice were dosed with hrVEGF as above, followed by a second dose of hrVEGF 10 hr following APAP, and were sacrificed at 18 or 24 hr after APAP. hVEGF was higher in the mice that received hrVEGF at both 18 and 24 hr, and this finding was statistically significant at 18 hr (Fig 2B). A late toxicity experiment (Fig 1C) was also performed in which mice received 3 doses of hrVEGF or vehicle (veh) at 30 minutes prior, 10 hr and 24 hr after APAP and were subsequently sacrificed at 36 hr. hVEGF levels were increased by 30% in the hrVEGF treated mice in this experiment (354.3 ± 153.9) compared to the APAP/veh mice (164.7 ± 93.9) but this change was not at a level of statistical significance.

In addition, the effect of hrVEGF on the expression of VEGF receptor 1 (VEGFR1) and VEGF receptor 2 (VEGFR2) was examined using ELISA. Modest increases in VEGFR1 and VEGFR2 expression were observed in the mice that received hrVEGF, compared to the APAP/vehicle mice. These relative changes in receptor expression were significant at the 36 hr time point for VEGFR1 (Fig 3A; Fig 3B).
Effect of hrVEGF on APAP Toxicity and Metabolism

The effect of hrVEGF on APAP toxicity was examined by measurement of serum ALT and histological analysis of liver sections. ALT levels were lower in the APAP/hrVEGF mice compared to the APAP/vehicle mice at 8 hr (p<0.05; Fig 4A). Area necrosis measures of H&E stained sections (Fig 4B) were in agreement with the ALT data. Representative liver sections from the 8 hr animals are shown in Fig 4C. Hepatic glutathione (GSH) was measured to assess the effect of hrVEGF on hepatic metabolic function. GSH levels were significantly reduced to comparable levels in both APAP groups at 2 hr. By 8 hr, GSH had recovered to baseline in the APAP/veh mice, while the APAP/hrVEGF treated mice had GSH levels that were approximately 40 percent higher than mice receiving APAP/veh (Fig 4D). To further examine the effect of hrVEGF on the metabolism of APAP, APAP-cysteine adducts were measured in the supernatants of liver homogenates. Adducts were increased in all APAP-treated mice and no differences were present between the APAP/veh and the APAP/hrVEGF groups at any time point (Fig 4E). These data indicated that hrVEGF did not alter the metabolism of APAP in the early stages of APAP toxicity, as reflected by the comparable values for hepatic GSH and hepatic protein adducts at the 2 and 4 hr time points.

In the intermediate experiment (Fig 1B), mice that received hrVEGF had ALT levels that were 25 and 43 percent lower at 18 and 24 hr, respectively, compared to APAP/veh mice. This difference was statistically significant at 24 hr (Fig 5A). In the late experiment (Fig 1C), mice received 3 doses of hrVEGF or vehicle control and were sacrificed at 36 hr after APAP. Mice treated with hrVEGF had significantly lower ALT levels compared to the APAP/veh mice at 36 hr (Fig 5B). Histological necrosis measurements of the H&E stained sections in these two
experiments were in agreement with the ALT data (Fig 5C). Thus, in summary, the toxicity data showed that mice pre-treated with hrVEGF had reduced hepatic injury at 8, 24, and 36 hr.

No differences in hepatic GSH levels were detected between the treatment groups in the intermediate and late toxicity experiments, in contrast to the 8 hr data (Fig 4C).

VEGF, initially known as vascular permeability factor, can increase vascular extravasation (Zhang et al., 2000) and microvascular permeability (Senger et al., 1983). To examine for any potential adverse events related to these known mechanisms, hemoglobin and hematocrit values of the mice were examined in the 18 and 24 hr experiments. No differences were found at these time points for these parameters between the APAP/hrVEGF, the APAP/veh, and the vehicle treated mice (data not shown).

**Effect of hrVEGF on Hepatocyte Regeneration**

In a previous study, we showed that treatment with an inhibitor of VEGF-mediated signaling reduced hepatocyte proliferation in APAP toxicity. Therefore, the effect of administration of hrVEGF on PCNA expression was examined. As demonstrated in Figure 6, mice dosed with hrVEGF and sacrificed at 18 or 24 hr had increased upregulation of PCNA compared to APAPveh treated mice (Fig 6A). Similarly, mice that received three doses of hrVEGF had noticeable upregulation of PCNA compared to vehicle treated mice (Fig 6B). Densitometric analysis indicated that PCNA expression was significantly higher at 24 and 36 hr in the hrVEGF treated mice (Fig 6C; p<0.05), compared to the APAP/veh treated mice. To further examine PCNA expression between the two groups, quantitative image analysis of PCNA immunostained slides was performed and showed an increased percentage of nuclear staining for PCNA in the APAP/hrVEGF mice compared to the APAP/veh mice (Fig 6D).
Effect of hrVEGF on IL-6 Production

Our laboratory previously showed that IL-6 was unregulated in the early stages of APAP toxicity and that IL-6 was important to hepatocyte regeneration in APAP toxicity in the mouse. Specifically, we found that IL-6 knock out mice had reduced PCNA expression in the late stages of APAP toxicity compared to comparable wild type mice. To determine if administration of hrVEGF altered the endogenous production of IL-6 in APAP toxicity, IL-6 levels were examined in the early, intermediate, and late stages of toxicity. As demonstrated in Figure 7, significant differences in hepatic IL-6 levels between the treatment groups were detected at the 8, 24, and 36 hr time points. While lower levels of IL-6 were found in the mice receiving hrVEGF at other time points, these values were not significantly different.

Effect of hrVEGF on platelet endothelial cell adhesion molecule (PECAM) Expression

In a previous study, we showed that inhibition of VEGFR2 signaling reduced PECAM expression. PECAM is a commonly used marker of neo-vascularization. PECAM expression in whole liver homogenates was increased in both groups of APAP-treated mice, compared to the saline group, but no differences were noted between the treatment groups (data not shown).

Effect of hrVEGF given after APAP Administration

In the final experiment, the effect of hrVEGF given after APAP administration was examined (Fig 1D). Mice were dosed with APAP as described above and received hrVEGF or vehicle at 2, 10, and 24 hr. The mice were sacrificed at 36 hr. As with the late toxicity experiment above, ALT values were significantly lower (Fig 8A) and the extent of necrosis (Fig 8B) was reduced in the mice that received hrVEGF compared to the treatment control mice.
addition, PCNA expression by western blot analysis (Fig 8C) and by image analysis of PCNA staining of hepatocyte nuclei (Fig 8D) was greater in the hrVEGF treated mice than the PBS treated mice. Thus, hrVEGF delivered after APAP had hepatoprotective effects, including reduced toxicity and the augmentation of hepatocyte regeneration.

**Effect of hrVEGF on Hyaluronic Acid Expression**

To understand the potential mechanisms of effect of hrVEGF as a hepatoprotectant in APAP toxicity, plasma levels of hyaluronic acid were examined. We postulated that hrVEGF may play a role in improving sinusoidal endothelial cell dysfunction as VEGF has mitotic effects on endothelial cells, the cells that line the hepatic sinusoid. In addition, VEGF alters vascular permeability (Senger et al., 1983) which could have effects on the hepatic microcirculation. Previous data have shown that hepatic microvascular injury, including sinusoidal cell injury, occurs early in APAP toxicity (Walker et al., 1985; McCuskey et al., 2006; McCuskey et al., 2008). Since hyaluronic acid (HA) is cleared by normal endothelial cells, increased levels of plasma HA represent a functional marker of sinusoidal cell injury. **Figure 9A** demonstrates the time course of plasma HA levels in mice treated with APAP (200 mg/kg IP) and sacrificed at 1, 2, 4, 6, 8, 24, 48, 72, and 96 hr after APAP. HA levels in plasma gradually increased from baseline and were significantly increased at 4, 8, 24, and 48 hr after APAP and thereafter abruptly returned to baseline levels (Fig 9A). In addition, elevation of plasma HA followed the onset of ALT elevation in the APAP treated mice. To assess the role of treatment with hrVEGF on sinusoidal endothelial cell dysfunction, plasma levels of HA were determined in the mice treated with hrVEGF from Experiments 3 and 4 (Figure 1). HA levels were markedly increased in the APAP/veh treated mice (Fig 9B; Experiment 3) in comparison to saline treated mice (p<0.05). In comparison, mice that received hrVEGF prior to APAP had significantly reduced
plasma HA levels that were comparable to the saline treated mice. Furthermore, mice that received hrVEGF 2 hr (Fig 9B; Experiment 4) after APAP had reduced HA levels as well. Thus, the data suggest that one mechanism for the hepatoprotective effects of hrVEGF on APAP toxicity in the mouse is alleviation of sinusoidal endothelial cell dysfunction.

**Effect of hrVEGF on Neutrophil influx in APAP Toxicity**

Neutrophil influx to the liver in APAP toxicity has been reported by a number of investigators (Lawson et al., 2000). Liu et al. postulated that neutrophils play a role in the propagation and severity of liver injury (Liu et al., 2004, Liu et al., 2006). The summary data (mean ± SE) for the neutrophil counts is presented in Table 1. The data showed that neutrophils were increased above control mice in the APAP/veh mice and the APAP/hrVEGF mice at 8 hr. Modest changes in neutrophil counts between the two treatment groups were noted at some of the time points but these were not statistically significant. However, at the 36 hr time point, mice that received hrVEGF had lower neutrophil counts than the APAP/veh treated mice. This finding was observed in the mice that were pre-treated with hrVEGF (Experiment 3) and in those that received hrVEGF at 2 hours (Experiment 4). The data thus suggest that hrVEGF altered the dynamics of neutrophil accumulation in APAP toxicity.
Discussion

The cytokine VEGF is the only known mitogen to act specifically on endothelial cells. It has pro-angiogenic effects in the developing vasculature and has been implicated in the pathogenesis of diabetes, tumors and retinopathy and numerous other conditions (Ray et al., 2004; Schoeffner et al., 2005). Our laboratory previously reported that endogenous murine VEGF levels were markedly increased in the late stages of APAP toxicity and that inhibition of VEGF signaling significantly reduced hepatocyte proliferation (PCNA expression) in the late stages of toxicity (Donahower et al., 2006). In light of these previous data, we examined the effects of administration of exogenous VEGF on various phases of APAP toxicity in the mouse (Fig 1).

Treatment of APAP treated mice with hrVEGF resulted in significant uptake of hrVEGF into the liver (Figs 2A, 2B). While calculation of the elimination half life for hrVEGF in the mice was not performed due to the limited number of samples obtained after the administration of hrVEGF, the relatively higher values of hrVEGF obtained at 24 hr (4556 ± 3128) in the intermediate toxicity experiment compared to the values at 8 hr in the intermediate toxicity experiment (2510 ± 379) suggest that clearance of hrVEGF may have been slower in the later stage of toxicity. It is possible that this observed change in clearance was secondary to hepatic congestion and the relative increase in hepatic plasma volume that has been reported to occur in APAP toxicity (Walker et al., 1985).

Administration of hrVEGF also affected the expression of VEGF receptors, particularly VEGFR1 expression (Fig 3B) which was statistically increased at 36 hr. VEGFR1 and VEGFR2 have distinct functions and unique binding affinities for VEGF under in vitro conditions. VEGFR1 has a binding affinity for VEGF that is 10 fold greater than that of VEGFR2 (Shibuya
et al., 2006). However, the tyrosine kinase activity of VEGFR1 is approximately 10 fold less than that of VEGFR2 (Shibuya et al., 2006). Thus, the increased expression of VEGFR1 at 36 hr may have been a reflection of enhanced binding affinity of VEGFR1 \textit{in vivo}. Of interest, LeCouter et al. (LeCouter et al., 2003) previously showed that treatment of liver sinusoidal endothelial cells with a selective VEGFR1 mutant increased the expression of hepatocyte pro-mitotic factors (IL-6 and hepatocyte growth factor) \textit{in vitro}. Also, \textit{in vivo} studies showed that the VEGFR1 mutant was protective in carbon tetrachloride toxicity in the mouse (LeCouter et al., 2003). The exact role of VEGF receptor 1 is controversial (Ferrara et al., 2003), and some laboratories have reported that it may serve as a decoy, making VEGF unavailable to VEGFR2, which is known to mediate the angiogenic effects of VEGF (Ferrara et al., 2003). We did not note significant changes in VEGFR2 or PECAM expression in whole liver homogenates in this study, endpoints that would suggest enhancement of the angiogenic effects of VEGF.

The primary goals of the present study were to examine the effect of hrVEGF on APAP toxicity and hepatocyte regeneration. Pre-treatment of APAP treated mice with hrVEGF resulted in a significant decrease of serum ALT values and a corresponding reduction in necrosis that was statistically significant at 8 hr (Figure 4). Of interest, Tsurui et al. (Tsurui et al., 2005) examined the effect of hrVEGR in a mouse model of hepatic ischemia reperfusion and observed a hepatoprotective response that was similar in magnitude to the data of the present study. In previous time course studies of APAP toxicity in the mouse (James et al., 2003b), we noted the elevation of serum ALT at 2 and 4 hr (Figure 9; James et al., 2003). In addition, hepatic GSH depletion occurs by 1 hr with restoration of hepatic GSH at 8 hr. The data from the present study demonstrated that pre-treatment with hrVEGF did not affect the early metabolism stages of toxicity as reflected by comparable values for serum ALT, histology, hepatic protein adduct
formation, and hepatic GSH at 2 and 4 hr. However, ALT values and histology were improved at 8 hr, suggesting that the mechanisms for hepatoprotection involved events downstream or independent of metabolism. The significance of higher values of hepatic GSH at the 8 hr time point in the present study is unclear but may indicate that there was an improvement in the metabolic capacity of the cells. In an in vitro study, VEGF treatment of endothelial cells was shown to increase intracellular levels of GSH and to serve an anti-oxidant role in a cell-based model (Kuzuya et al., 2001).

Similarly, hrVEGF treatment also reduced ALT values and the extent of necrosis at later time points in the toxicity (18, 24 and 36 hr; Figs 3 and 4). These effects were associated with enhanced regeneration of hepatocytes in the late stages of toxicity. As described above, previous studies in our laboratory have supported a role for endogenous VEGF in hepatocyte regeneration (Donahower et al., 2006) and similar data were recently reported in a rat model of APAP toxicity (Papastefanou et al., 2007). In the present study, hrVEGF enhanced PCNA expression by western blot assays performed on whole liver homogenates. At 18 hr, this effect was slight, and by 24 and 36 hr, more pronounced expression of PCNA was apparent in the hrVEGF treated mice (Fig 5), compared to the vehicle/APAP mice. Quantitative image analysis of immunohistochemical assays for PCNA expression in hepatocyte nuclei supported the findings of the western blot assays. Thus, cumulatively, the data support the hypothesis that hrVEGF enhances hepatocyte regeneration in APAP toxicity in the mouse. To further test this effect, studies were performed in which hrVEGF was administered 2 hr after APAP. As demonstrated in Figure 8, hrVEGF given at 2, 10, and 24 hr resulted in lower ALT values, reduced histologic necrosis and enhanced expression of PCNA. Thus, hrVEGF administered to mice after APAP
was hepatoprotective and the data suggest that future study of hepatoregenerative therapies are warranted in the pre-clinical model of APAP toxicity.

Treatment with hrVEGF also reduced the expression of IL-6 (Fig 6). IL-6 is known to be an important factor in hepatocyte regeneration in several models of liver injury, including partial heptatectomy and carbon tetrachloride mediated hepatotoxicity (Cressman et al., 1996). Our laboratory and others have shown that IL-6 expression is increased in APAP toxicity and that IL-6 KO mice have increased toxicity compared to wild type mice (James et al., 2003a; Masubuchi et al., 2003). We also found that IL-6 KO mice had reduced PCNA expression compared to wild type mice (James et al., 2003a). However, treatment with murine IL-6 did not alter toxicity or hepatocyte regeneration following APAP toxicity (Bajt et al., 2003; James et al., 2003a).

Despite the reduction in the IL-6 endogenous response in the present study, treatment with hrVEGF enhanced recovery. One possibility is that hrVEGF accelerated recovery and as a secondary effect, the endogenous IL-6 response was suppressed; this interpretation of the data would be consistent with previous data showing the redundancy of repair networks in the regeneration response (Bajt et al., 2003; James et al., 2003a).

Administration of hrVEGF also resulted in alleviation of sinusoidal endothelial cell dysfunction as measured by reduced plasma HA levels in mice treated with hrVEGF (Fig 9B). Changes in the cellular integrity of the hepatic sinusoid have been described in APAP toxicity (Walker et al., 1985; McCuskey, 2006; McCuskey, 2008) and include the development of large pores in the sinusoidal endothelial cells and the filling of the space of Disse with red blood cells (Walker et al., 1985). HA is cleared by normal sinusoidal endothelial cells and with the onset of sinusoidal endothelial cell injury, HA levels in plasma rise. The significant reduction of plasma HA in hrVEGF treated mice suggests that hrVEGF may have hastened the recovery of cells in
the hepatic sinusoid, but further experiments are needed to examine the potential mechanisms of hrVEGF on the hepatic sinusoid in APAP toxicity. Endogenous VEGF, as well as other angiogenic growth factors, has been shown to be important in the reconstitution of the hepatic sinusoid following hepatic injury (Enomoto et al., 2004). Another related possibility is that hrVEGF altered microvascular perfusion. In a model of cerebral stroke, treatment of rats with hrVEGF led to improvements in cerebral microvascular perfusion (Zhang et al., 2000).

Treatment with hrVEGF also resulted in significant reductions in neutrophil counts at 36 hr. Neutrophils represent the greatest fraction of inflammatory cells present in livers of APAP treated mice (Liu et al, 2004), although their role in the mediation of APAP toxicity is controversial (Jaeschke et al., 2006). Lawson et al. (2000) showed that treatment with an antibody against B2 integrins did not protect against toxicity. However, other data suggest that neutrophils are mechanistically important in the propagation of APAP toxicity (Liu et al., 2004; Liu et al., 2006). Two laboratories (Liu et al, 2006; Ishida et al., 2006) independently showed that induction of neutropenia by administration of the anti-Gr-1 antibody to APAP treated mice reduced toxicity at 6 and 24 hr. To our knowledge, the role of neutrophils in the very late stages of APAP toxicity (beyond 24 hr) has not been previously examined. The mechanism for the reduction of neutrophil counts in the hrVEGF treated mice is unclear. One possibility is that treatment with hrVEGF may have dampened other endogenous repair responses, such as chemokine upregulation which has been reported to be important in the process of hepatocyte regeneration (Hogoboam et al., 1999) but also may enhance neutrophil infiltration. Alternatively, VEGF is well known to enhance vascular permeability, and it is possible that this effect may have altered neutrophil dynamics in the hrVEGF treated mice. Further studies are warranted to examine the mechanisms responsible for neutrophil reduction in the hrVEGF mice.
It has been proposed that acute liver injury occurs in two phases - Phase 1 or initiation and Phase 2 or progression (Limaye et al., 2006). Liver regeneration is thought to limit the progression of phase 2 injury (Limaye et al., 2006). Cumulatively, the data in the present study suggest that hrVEGF had dual effects – mitigation of toxicity and enhancement of hepatocyte regeneration. Differential, time dependent effects of hrVEGF in a model of brain injury have recently been reported (Zhang et al., 2000). The effect of hrVEGF on APAP toxicity was not apparent until a relatively late time point in the toxicity (ie, 8 hr) and did not alter the metabolism of APAP at earlier time points. It is possible that the hepatoprotective effects of hrVEGF were secondary to the alleviation of sinusoidal endothelial cell injury in hrVEGF treated mice and that this process is mechanistically important in both attenuating toxicity and facilitating the onset of hepatocyte regeneration. Further study should be focused on defining the potential treatment window for which reversal of sinusoidal endothelial cell dysfunction is possible and examining the direct effects of hrVEGF on the cellular architecture within the hepatic sinusoid, as well as the resulting effects on toxicity and hepatocyte regeneration. Since the clinical antidote N-acetylcysteine primarily targets the metabolic phase of toxicity, its efficacy is limited by the time it is administered relative to APAP dose. Novel therapies focused on new molecular targets that may mitigate the progression of toxicity and/or enhance the regeneration response could represent mechanistically relevant advancements to the currently available treatment approaches for humans with APAP poisoning. Based on the present data, further studies of hrVEGF as a potential treatment for APAP toxicity appear warranted.
References


Footnotes

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Legends for Figures

Figure 1. Summary of study designs. Four experimental designs were utilized in the study. hrVEGF was administered as 50 ug SQ (denoted by triangle) and mice were sacrificed at various times (denoted by X) to characterize the effect of hrVEGF on the early (A), intermediate (B), and late (C) stages of toxicity. In addition, a rescue design study was performed (D) in which hrVEGF treatment was initiated 2 hr after APAP administration.

Figure 2. Demonstration of human recombinant vascular endothelial factor (hrVEGF) in mouse liver following treatment with hrVEGF.

A. Mice were treated with acetaminophen (APAP) (200 mg/kg, IP) plus vehicle (PBS) or APAP plus hrVEGF (50 ug, SQ) and sacrificed at the indicated times. hVEGF levels were significantly elevated at 2 and 4 hr in mice treated with hrVEGF. B. Mice were treated with APAP and received 2 doses of hrVEGF or vehicle and were sacrificed at the indicated times. hVEGF levels were significantly elevated at 18 hr in mice treated with hrVEGF. hVEGF levels in PBS treated mice were 0.0 pg/g in both experiments (data not shown). *indicates significant difference from APAP/veh groups, p<0.05.

Figure 3. Effect of human recombinant vascular endothelial factor (hrVEGF) on VEGF receptor 1 (VEGFR1) and VEGF receptor 2 (VEGFR2) expression. VEGFR1 and VEGFR2 levels were examined in the mice from the early (not shown), intermediate, and late experiments. Mean (+ SE) hepatic VEGFR1 and VEGFR2 levels were increased in the hrVEGF treated mice compared to the mice that received vehicle/APAP and this finding was significant for VEGFR1 in the 36 hr experiment (*p<0.05).
Figure 4. Effect of human recombinant vascular endothelial factor (hrVEGF) on alanine transferase (ALT), histology and metabolism. Mice were treated with APAP/veh or APAP/hrVEGF and sacrificed at the indicated times. A. At 8 hr, mice receiving hrVEGF plus APAP had significantly reduced ALT levels compared to the mice that received vehicle plus APAP (*p<0.05 indicates significant difference from APAP/veh group). B. Histological examination of H&E stained slides revealed decreased necrosis in the hrVEGF treated mice at 8 hr. C. Histology of saline, APAP/veh and APAP/hrVEGF treated mice. D. At 2 and 4 hr, APAP/rhVEGF mice and APAP/veh mice had comparable reductions in glutathione (GSH) levels. At 8 hr, rhVEGF treated mice had a significantly higher GSH levels than the APAP/veh mice (*indicates significant difference from APAP/veh group, p<0.05). E. Acetaminophen protein adducts in liver homogenates of mice treated with APAP/veh or APAP/hrVEGF. Adducts were increased at 2, 4, and 8 hr in all treatment groups. No differences were present between the APAP/veh and the APAP/hrVEGF groups at any time point.

Figure 5. Effect of human recombinant vascular endothelial factor (hrVEGF) on alanine transferase (ALT) levels in the intermediate and late stages of toxicity. Mice were treated with APAP/veh or APAP/hrVEGF and sacrificed at the indicated times. A. At 18 and 24 hr, mice receiving hrVEGF had reduced ALT levels compared to the APAP/veh mice. Mean (± SE) ALT values in PBS mice were 28.9 ± 5.3 IU/L (data not shown). B. Mice were treated with hrVEGF had significantly lower ALT levels than vehicle treated mice at 36 hr. Mean ALT (± SE) values in control mice were 16.2 ± 2.1 IU/L (data not shown). *indicates significant difference from veh/APAP group, p<0.05. C. Necrosis scores of mice sacrificed at 18, 24, and 36 hr. Necrosis was reduced (*p<0.05 in the hrVEGF treated mice at 24 and 36 hr compared to the APAP/veh treated mice.
Figure 6. Effect of human recombinant (hrVEGF) on proliferating cell nuclear antigen (PCNA) expression. A. Mice were treated with APAP/veh or APAP/hrVEGF and sacrificed at 18 or 24 hr. hrVEGF treatment increased PCNA expression by western blot at 18 and at 24 hr compared to APAP/vehicle treated mice. B. Mice were treated with APAP/vehicle or APAP/hrVEGF and sacrificed at 36 hr. Mice treated with hrVEGF had increased expression of PCNA by western blot compared to vehicle treated mice. C. Densitometric analysis indicated a significant difference in PCNA expression, represented as mean (+ SE), at 24 and 36 hr in the hrVEGF treated mice (p<0.05) compared to the APAP/veh mice at the same time points. D. Image analysis of PCNA stained slides from the 36 hr experiment indicated increased PCNA labeling in the hepatic nuclei of the mice treated with hrVEGF compared to the vehicle treated mice (*p<0.05). E. Representative mouse liver section of PCNA staining in APAP/veh treated mouse. F. Representative mouse liver section of PCNA staining from APAP/hrVEGF treated mouse.

Figure 7. Effect of hrVEGF on hepatic interleukin-6 (IL-6) expression. IL-6 levels were examined in the mice from the early, intermediate, and late experiments. Mean (+ SE) hepatic IL-6 levels were reduced in mice that received hrVEGF compared to vehicle treated mice (*p<0.05).

Figure 8. Effect of treatment with human recombinant vascular endothelial factor (hrVEGF) on alanine transferase (ALT) levels, necrosis, and PCNA expression at 36 hr. Mice were treated with APAP followed by hrVEGF or vehicle at 2, 10, or 24 hr and sacrificed at 36 hr. A. At 36 hr, mice receiving hrVEGF had reduced ALT levels compared to the vehicle/APAP mice. B. Mice treated with hrVEGF had significantly reduced necrosis scores compared to the vehicle treated mice. C. PCNA expression by immunoblot. Mice treated with
hrVEGF had increased expression of PCNA by immunoblot analysis. Lanes signify representative mice and densitometry represent summary of all mice (n=12 per treatment group).

**D.** Summary of image analysis for PCNA labeled nuclei (*p<0.05 for all comparisons of APAP/veh vs. APAP/hrVEGF).

**Figure 9. Effect of hrVEGF on plasma levels of hyaluronic acid (HA).**  
**A.** Mice were treated with APAP (200 mg/kg IP) and sacrificed at the indicated times. Plasma HA levels were significantly increased at 4, 8, 24, and 48 hr (p<0.05) and the onset of plasma HA elevation occurred prior to the ALT response.  
**B.** Plasma HA levels were measured in mice that received hrVEGF prior to APAP and 2 hr after APAP. Additional doses of hrVEGF were administered at 10 and 24 hr and the mice were sacrificed at 36 hr. Plasma HA levels were reduced at 36 hr in both groups of mice treated with hrVEGF (*p<0.05).
### Table 1. Neutrophil counts.

Neutrophil counts (mean ± SE) in liver in mice treated with acetaminophen (200 mg/kg IP) and human recombinant vascular endothelial growth factor (hrVEGF; 50 ug sq).

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Time point (hr)</th>
<th>APAP/veh</th>
<th>APAP/hrVEGF</th>
<th>p value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>0.66 ± 0.31</td>
<td>1.53 ± 0.79</td>
<td>0.992</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1.86 ± 0.83</td>
<td>1.0 ± 0.20</td>
<td>0.988</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>6.58 ± 1.35&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.4 ± 1.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.946</td>
</tr>
<tr>
<td>2</td>
<td>18</td>
<td>3.67 ± 2.11</td>
<td>10.48 ± 1.56&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.173</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>7.87 ± 0.66</td>
<td>8.98 ± 2.51&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.994</td>
</tr>
<tr>
<td>3</td>
<td>36</td>
<td>7.7 ± 1.96&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.88 ± 0.49</td>
<td>0.018&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>36</td>
<td>6.19 ± 1.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.83 ± 0.62</td>
<td>0.027&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Represents comparison to saline group (mean = 0.33 ± 0.24 neutrophils per high powered field; 40 X magnification); <sup>b</sup>Represents comparison between APAP/veh and APAP/hrVEGF groups.
<table>
<thead>
<tr>
<th>Experiment</th>
<th>APAP ↓</th>
<th>Sacrifice Times (X)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Early Toxicity</td>
<td>▼</td>
<td>▼ X X X</td>
</tr>
<tr>
<td>B. Intermediate Toxicity</td>
<td>▼ ▼</td>
<td>▼ X X</td>
</tr>
<tr>
<td>C. Late Toxicity</td>
<td>▼ ▼</td>
<td>▼ ▼ X</td>
</tr>
<tr>
<td>D. Post APAP</td>
<td>▼ ▼</td>
<td>▼ ▼ X</td>
</tr>
</tbody>
</table>

![Diagram](https://via.placeholder.com/150)

Fig 1

Time post APAP (hr)

▼ hrVEGF

APAP Sacrifice Times (X)

0            6          12            18            24          30          36

Table notes: The table lists the experiments and their corresponding APAP sacrifice times. The symbols ▼ represent the hrVEGF markers for each experiment.
Fig2

A

Hepatic hrVEGF (pg/gram)

Time after APAP (hr)

- vehicle/APAP; □ hrVEGF/APAP

B

Time after APAP (hr)

- vehicle/APAP; □ hrVEGF/APAP
Fig 4

A

ALT (IU/L)

Saline | 2 | 4 | 8

APAP/veh | APAP/hrVEGF

B

% Necrosis

Saline | 2 | 4 | 8

APAP/veh | APAP/hrVEGF

C

Saline | APAP/veh | APAP/hrVEGF

D

GSH (%)

Saline | 2 | 4 | 8

APAP/veh | APAP/hrVEGF

E

Adducts (mol/mol protein)

Saline | 2 | 4 | 8

APAP/veh | APAP/hrVEGF
Fig 5

A.  

ALT (IU/L)

<table>
<thead>
<tr>
<th>Hr after APAP</th>
<th>veh/APAP</th>
<th>hrVEGF/APAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B.  

ALT (IU/L)

<table>
<thead>
<tr>
<th>Time after APAP</th>
<th>Vehicle/APAP</th>
<th>hrVEGF/APAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>36 hr</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C.  

% Necrosis of 10X Field

<table>
<thead>
<tr>
<th>Time after APAP (hr)</th>
<th>Saline</th>
<th>Veh/APAP</th>
<th>hrVEGF/APAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>36</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Fig 6

A

39kDa

veh/APAP 18 hr VEGF/APAP veh/APAP VEGF/APAP

B

39 kDa

veh/APAP 36 hr hrVEGF/APAP

C

Relative intensity

18 24 36

Hr after APAP

veh/APAP hrVEGF APAP

D

% Hepatic Nuclei Labeled

0 10 20 30 40

Control APAP/veh APAP/hrVEGF

E

F

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Fig 7

![Graph showing IL-6 levels over time after APAP administration. The graph displays data for veh/APAP and hrVEGF/APAP groups with pg/g measured at various hours after APAP (2, 4, 8, 18, 24, 36).]

- **IL-6**
  - veh/APAP
  - hrVEGF/APAP

pg/g vs. Hr after APAP

- 2, 4, 8, 18, 24, 36 hours after APAP

* Indicates statistical significance.
Fig 8

A

ALT (IU/L)

0
100
200
300
400
500
600
700
800
900
1000
1100
1200
1300
1400
1500
1600

Saline  APAP vehicle  APAP hrVEGF

36 hr

B

Extent Necrosis (%)

0
10
20
30
40
50

Saline  APAP/veh  APAP/hrVEGF

36 hr

C

39 kDa

Ctrl  APAP veh  APAP hrVEGF

36 hr

D

% Hepatic nuclei labeled

0
10
20
30
40
50
60
70
80

Saline  APAP/veh  APAP/hrVEGF

36 hr

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Fig 9

A

![Graph showing ALT and Hyaluronic acid levels over time after APAP administration.]

- **ALT (IU/L)**
- **Hyaluronic acid (ng/mL)**

Time after APAP (hours):
- Saline 1, 2, 4, 6, 8, 24, 48, 72, 96

B

![Bar graph showing Hyaluronic acid levels in different conditions.]

- **Hyaluronic acid (ng/mL)**

Conditions:
- Saline, APAP, APAP Veh, APAP hrVEGF

Experiments:
- Experiment 3
- Experiment 4

* indicates significant difference.