Upregulated Promitogenic Signaling via Cytokines and Growth Factors: Potential Mechanism of Robust Liver Tissue Repair in Calorie-Restricted Rats upon Toxic Challenge

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Previously we reported that moderate calorie restriction or diet restriction (DR, calories reduced by 35% for 21 days) in male Sprague-Dawley rats protects from a lethal dose of thioacetamide (TA). DR rats had 70% survival compared with 10% in rats fed ad libitum (AL) because of timely and adequate compensatory liver cell division and tissue repair in the DR rats. Further investigation of the mechanisms indicate that enhanced promitogenic signaling plays a critical role in this stimulated tissue repair. Expression of stimuliators of promitogenic signaling interleukin-6 (IL-6), inducible nitric oxide synthase (iNOS), hepatocyte growth factor (HGF), transforming growth factor-α (TGF-α), and epidermal growth factor receptor (EGFR) were studied during liver tissue repair after TA-induced liver injury. Plasma IL-6 was significantly higher in the DR rats, with 6-fold higher expression at 48 h after TA administration. Immunohistochemical localization revealed significantly higher expression of IL-6 in the hepatic sinusoidal endothelium of DR rats. Expression of TGF-α and HGF was consistently higher in the livers of DR rats from 36 to 72 h. EGFR, which serves as a receptor for TGF-α, was higher in DR rats before TA administration and remained higher till 48 h after TA intoxication. DR-induced 2-fold increase in hepatic iNOS activity is consistent with early cell division in DR rats after TA challenge. These data suggest that the reason behind the higher liver tissue repair after TA-induced hepatotoxicity in DR rats is timely and higher expression of the growth stimulatory cytokines and growth factors. It appears that the physiological effects of DR make the liver cells vigilant and prime the liver tissue promptly for liver regeneration through promitogenic signaling upon toxic challenge.

Key Words: Epidermal growth factor receptor; EGFR; hepatocyte growth factor; HGF; IL-6; TGF-α; thioacetamide; tissue repair; TNF-α.

Beneficial effects of moderate caloric or diet restriction (DR) such as decrease in cancer incidence, increase in life expectancy, and decrease in occurrence of age-associated diseases are well known (Allaben et al., 1990; Frame et al., 1998; Hass et al., 1996; Keenan et al., 1995; Masoro, 1988; Weindruch and Walford, 1992). DR also protects from acute toxicity and lethal effects of a variety of compounds (Berg et al., 1994; Duffy et al., 1995; Ramaiah et al., 2000). Moderate caloric restriction (35% less than ad libitum [AL] diet) for a period of 3 weeks protects male Sprague-Dawley rats from a normally lethal dose (600 mg/kg) of thioacetamide (TA). Upon challenge with TA, the DR rats experience only 30% lethality, as opposed to 90% lethality experienced by AL rats. Further studies revealed that the mechanisms of protection by DR are timely and adequate liver cell division and tissue repair in the DR rats (Ramaiah et al., 1998a,b). DR induces CYP2E1, the enzyme responsible for bioactivation of TA, and consequently, the DR rats suffer from 2.5-fold higher bioactivation-based liver injury compared with AL rats (Ramaiah et al., 2001). Nonetheless, 70% of the DR rats escape death because of early and robust liver tissue repair. This is evident by the early S-phase stimulation, which starts in DR rats a day before the AL rats (24 h in DR vs. 48 h in AL). This timely and exacting tissue repair restores the structure and function, paving the way for survival.

The primary objective of this study was to investigate the critical role played by proinflammatory cytokine- and growth factor-mediated signaling in stimulation of liver tissue repair in DR rats upon TA challenge. The molecular mechanisms of liver regeneration have been studied extensively both in partial hepatectomy models and to some extent after chemical hepatectomy (Dalu et al., 1995; Diehl, 2000; Fausto et al., 1995). Extensive research has shown that this process is highly regulated by cytokines and growth factors expressed by the remaining liver cells in temporally and spatially controlled fashion. Various studies have highlighted the role of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and growth factors such as transforming growth factor-α (TGF-α) and hepatocyte growth factor...
(HGF) (Diehl, 2000; Fausto et al. 1995; Michalopoulos and DeFrances, 1997). It was hypothesized that increased expression of cytokines and growth factors in DR rats stimulates the enhanced tissue repair response in these rats after TA administration. We studied five signaling pathways known to be involved in liver regeneration. TNF-α pathway and Janus-activated kinase signal transducer (JAK-STAT) pathway are the proinflammatory cytokine pathways. Mitogen-activated protein kinases (MAPK) and HGF/c-met are the growth factor pathways, and the nitric oxide (NO) pathway involves free radical signaling. Plasma TNF-α and IL-6 and hepatic expression of TGF-α and HGF were assessed in livers of AL and DR rats after TA administration over a time course. Epidermal growth factor receptor (EGFR) protein levels were assessed in livers of DR and AL rats after TA administration. Inducible NO synthase (iNOS), the primary enzyme responsible for production of NO, plays a significant role in liver regeneration (Rai et al., 1998). The role of iNOS and NO was evaluated by estimation of iNOS activity in livers from TA-intoxicated AL and DR rats over a time course. We report here that upon toxic challenge, all pathways studied except TNF-α are inhibited in AL rats, whereas DR rats exhibit upregulation of JAK-STAT (IL-6), MAPK, HGF/c-met, and iNOS pathways. This upregulated promitogenic signaling leads to timely and robust liver cell division, tissue repair, and survival.

**MATERIALS AND METHODS**

**Chemicals.** All the chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise mentioned and were of highest analytical grade. Thirty percent acrylamide:bis acrylamide solution, glycine, Tris, Tween-20, and other materials related to Western blotting such as grade. Thirty percent acrylamide:bis acrylamide solution, glycine, Tris, (St. Louis, MO) unless otherwise mentioned and were of highest analytical.

**Animals and treatment.** Male Sprague-Dawley rats (250–290 g) were subjected to moderate DR as described previously (Ramaiah et al. 1998b). Briefly, DR rats were allowed to eat 65% of the AL food consumption (Harlen Teklad Rat chow No. 7001, Madison, WI: protein 25%, fat 4.25%, fiber 4.67%, vitamins and minerals supplemented, calories 3.94 Kcal/g) and were allowed free access to water for 21 days. AL rats had free access to food and water at all times. On day 22, rats in both groups received a single normally lethal dose of TA (600 mg/kg ip in 6 ml saline/kg). For lethality studies, rats were observed for 14 days twice daily, and observations were made regarding signs of toxicity and deaths. For the time course studies, separate groups of AL and DR rats were treated with the lethal dose and sacrificed at various time points. Blood was collected from the dorsal aorta in heparinized tubes and plasma was centrifuged and used for the estimation of alanine aminotransferase activity (ALT; EC 2.6.1.2.) as marker of liver injury using Sigma kit No. 59 UV (ALT) (Sigma Chemical Co., St. Louis, MO). Liver cell division was estimated by incorporation of ³H-thymidine (³H-T) in hepatocellular DNA, as previously described (Chang and Looney, 1965). Rats were treated with 50 μCi ³H-T/rat (ip) 2 h prior to sacrifice at each time point. Total DNA content was measured by diphenylamine reaction.

**Assessment of apoptotic cells.** Apoptic cells were visualized and counted by TUNEL assay using ApoTag kit ( Oncor, Gaithersburg, MD) according to the manufacturer’s protocol. Paraffinized liver sections of samples collected from AL and DR rats over the 0–72 h time course after TA administration were used. Three slides per group per time point were stained and apoptotic cells identified by dark brown staining were counted under a microscope.

**TNF-α and IL-6 ELISA.** TNF-α levels were estimated in the plasma of AL and DR rats treated with TA using a rat-specific TNF-α sandwich ELISA (Amersham Life Sciences, Piscataway, NJ) according to manufacturer’s protocol. Briefly, heparinized plasma samples (n = 4 per time point) were incubated with anti–TNF-α capture antibody for 1 h followed by biotinylated detection antibody. Visualization was carried out by peroxidase reaction, and color intensity was measured at 450 nm in a BioRad Model 550 microplate reader (BioRad, Hercules, CA). IL-6 was measured in plasma samples collected at different time points using a rat-specific IL-6 ELISA kit (R&D Systems, Minneapolis, MN) according to manufacturer’s protocol similar to TNF-α ELISA.

**Immunohistochemistry.** TGF-α, HGF, and IL-6 protein were evaluated by immunohistochemical method using paraffinized sections of liver samples collected over 0–96 h time course. Briefly, antigen retrieval was carried out by boiling the liver sections with 0.05% saponin for 30 min followed by treatment with TGF-α specific antibody (Ab-1, Oncogene, Cambridge, MA) at a concentration of 1:500 for 18 h at 4°C. Antigen retrieval for HGF was performed by citrate buffer treatment; no antigen retrieval for IL-6 was necessary. The concentration of primary antibody was 1:50 for both HGF and IL-6 immunohistochemistry. After treatment with secondary antibody (concentration 1:10,000) and streptavidine conjugate, visualization was achieved by peroxidase reaction in all cases. To get a quantitative estimate of the growth factor production from the staining intensity, a scoring scheme was devised using a scale of 0–4 staining intensity. This type of scoring scheme for staining intensity has been used by other investigators to get a quantitative estimate of immunohistochemical analysis (Roberts et al., 1991; Williams et al., 1996). Four slides were stained and coded per time point. Slides were observed under a light microscope, 10–15 central veins were observed per slide, and staining intensity was graded in single-blinded manner at a scale of 0–4 (0, no staining; 1, low staining intensity; 2, medium staining intensity; 3, high staining intensity; and 4, very high staining intensity).

**iNOS assay.** iNOS activity was determined according to the method of Bredt and Snyder (1994). Briefly, the reaction mixture composed of the iNOS assay medium (400 μl) containing 100 mM HEPES, pH 7.2; 2 mM NADPH; ['H]-arginine (0.2 μCi/ml), and 400 μg of liver cytosolic protein was incubated for 30 min at 37°C. The reaction was stopped by addition of stop buffer containing 20 mM HEPES and 10 mM EGTA at pH 5.5. The entire reaction mixture was passed through Dowex AG 50 W-X8 resin (Na⁺ form) to elute the fraction containing ['H]-citrulline, which was counted in a Beckman 6000 liquid scintillation counter.

**Preparation of cell lysate and Western blot analysis.** Liver cell lysate was prepared in lysis buffer (1% Triton-X-100, 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, 2 mM Na vanadate, 0.2 mM phenylmethylsulfonylfluoride, 1 mM HEPES, 1 μg/ml leupeptin, 1 μg/ml aprotinin), and protein concentration was estimated using a BioRad protein assay kit (BioRad, Hercules, CA). Western blot analysis of EGFR was conducted according to Dalton et al. (2000). Briefly, 50 μg of cell lysates was resolved by electrophoresis on a 7.5% sodium dodecyl sulfate (SDS) polyacrylamide gel (100 V, 1.5 h) in a running gel buffer containing 25 mM Tris, pH 8.3, 162 mM glycine, and 0.1% SDS. The samples were transferred to nitrocellulose membrane for 3 h at 500 mA using 25 mM Tris, 192 mM glycine buffer with methanol reduced to 10% and 0.02% SDS included to facilitate transfer of large molecular weight proteins. The membranes were blocked with 5% nonfat dry milk in TTBS (1
Liver Injury and Tissue Repair after TA Administration

Liver injury was assessed by plasma ALT levels over a time course after TA administration in AL and DR rats (Fig. 1A). ALT levels increased significantly in AL rats within 12 h and remained elevated throughout the time course. In DR rats, liver injury increased slowly, with a peak during 48 to 60 h where the ALT values were more than 2-fold higher than those in AL rats (Fig. 1A). Decrease in the liver injury was observed in DR rats, with ALT values returning to control levels by 120 h. All the deaths (90% deaths in AL vs. 30% in DR) were noted after 96 h. Evaluation of aspartate aminotransferase and sorbitol dehydrogenase yielded results similar to those published elsewhere (Ramaiah et al., 1998b). Liver cell division was measured by pulse-labeling experiments with $^3$H-T incorporation into hepatonuclear DNA. S-phase stimulation in AL rats was observed almost 1 day after it appeared in DR rats at 48 h after TA administration (Fig. 1B). S-phase stimulation remained suppressed in AL rats until 48 h, and the increase observed at 72 h is the data obtained from few surviving rats. It was clear that those rats survived because of the high compensatory tissue repair observed at 72 h. DR rats had an early stimulation of S-phase DNA synthesis, which increased between 24 and 36 h after TA administration. The compensatory cell division in DR rats started early and continued for a longer period of time, which restored the lost liver tissue and restored the function of the liver. The findings of $^3$H-T pulse-labeling studies were corroborated by PCNA immunohistochemical staining on AL and DR liver sections after TA administration (Ramaiah et al., 1998a).

TNF-α and IL-6 ELISA

Plasma collected from AL and DR rats at different time points after TA administration was used to evaluate levels of TNF-α. Plasma TNF-α levels increased significantly in AL rats within 1 h of TA administration and remained elevated throughout the time course (Fig. 2). DR itself did not change the plasma TNF-α levels, but within 1 h of TA administration significantly higher plasma TNF-α was observed in DR rats. The elevated levels of plasma TNF-α remained high throughout the time course similar to AL rats. Thus, both AL and DR rats had similar plasma levels of TNF-α after TA administration at all time points except at 72 h where levels in DR rats were significantly higher (Fig. 2).

Plasma levels of IL-6 did increase in AL rats after TA administration but were significantly higher than the level at
0 h in AL rats only at 24 h, before declining to control levels. DR rats had significantly higher plasma IL-6 even before TA administration, which increased further at 12 h after TA administration and peaked at 48 h (Fig. 3). DR rats had significantly higher plasma IL-6 at 0, 12 and 48 h; at 48 h, IL-6 was 6-fold higher than in AL rats.

**Immunohistochemical Analysis of TGF-α, HGF, and IL-6**

Liver sections from AL and DR rats obtained after TA administration were probed using an anti-TGF-α antibody. TGF-α expression was found primarily in the hepatocytes. The hepatocytes immediately next to the necrotic cells stained positive for TGF-α. TA produces necrosis in the centrilobular region and these necrotic cells were surrounded by healthy liver cells that stained positive for TGF-α. During initial time points the expression was limited to a 2–3 cell layers as evident at 24 h (Fig. 4A) from the central vein, while in the later time points such as 60 h, most of the surviving hepatocytes stained positive for TGF-α in DR rats (Fig. 4B). As previously reported (Ramaiah et al., 1998b), PCNA analysis in this model showed that many of the TGF-α-positive cells were in S-phase. Four slides per time point per group were scored using the staining intensity scoring system described in the Materials and Methods section (Fig. 5A). TGF-α expression increased significantly at 12 h after TA administration in AL rats and peaked at 36 h. However, after 36 h, a steady decline in TGF-α expression was observed that decreased and diminished even below the control levels between 60 and 96 h. Diet restriction itself did not change the expression of TGF-α. After TA administration, TGF-α increased in DR rats significantly at 24 h and remained elevated until 60 h. Thereafter, it further increased and peaked at 96 h. Until 36 h after TA treatment, both AL and DR rats had similar expression of TGF-α, except at 24 h (Fig. 4A). From 48 to 96 h, DR rats exhibited consistently higher expression, whereas TGF-α levels were diminished in the AL rats during these later time points.

HGF expression was found in both hepatocytes and inflammatory cells, especially in the monocytes infiltrated in the necrotic areas. Similar to TGF-α, HGF expression was found in the cells that lie immediately next to the necrotic foci (Fig. 6). The staining intensity scoring revealed that significantly higher HGF expression was observed at 24 h after TA administration in AL rats, after which it decreased and returned to control levels by 72 h. DR rats had significantly higher HGF at 0 h, which further increased significantly at 12 h after TA administration, where it was five to six cell layers from the central vein (Fig. 6A). HGF further increased and peaked at 48 h and remained consistently high at 60 and 72 h (Figs. 5B and 6B) in the DR rats. After 48 h, most of the surviving hepatocytes and infiltrated cells stained positive for HGF, as observed in Figure 6B. DR rats exhibited significantly higher HGF during early time points (0 and 12 h after TA administration) and later during 36 to 72 h, which coincides with increased S-phase stimulation.
IL-6 immunohistochemistry was conducted to determine which cells in the liver secrete IL-6 during the regeneration process after TA administration. Mainly sinusoidal endothelial cells stained positive, indicating those to be the main source of IL-6 in the liver. In addition, the infiltrated monocytes, polymorphonuclear cells, and Kupffer cells stained positive for IL-6, especially at the time points that had significant infiltration. Significantly higher IL-6 staining was observed in DR rats 12 h after TA administration (Fig. 7A) and remained higher till 72 h. Immunohistochemical analysis further corroborated the plasma IL-6 results.

**EGFR**

AL rats had low initial EGFR protein, which increased about 2-fold at 24 h after TA challenge, but declined to undetectable levels at 48 h. EGFR protein was overexpressed in DR rats, which increased slightly at 24 h and remained at that level at 48 h after TA administration (Fig. 8). There was no significant increase observed in EGFR in DR rats compared with the 0 h DR control after TA administration, but it was significantly higher than in AL rats at 0 and 48 h time points.

**iNOS Activity**

The most notable effect observed in iNOS occurred as a direct result of DR, as evident at 0 h time point (Fig. 9). DR rats exhibited 2-fold higher iNOS activity compared with AL rats. After TA administration, iNOS activity decreased in AL rats and reached statistical significance only at 48 h. It increased to control levels at 60 h and remained at control level thereafter (Fig. 9). This indicates an overall inhibition of iNOS expression or activity in AL rats receiving TA. After TA administration, DR-induced iNOS decreased significantly at 12 h and further at 36 h. A second surge of iNOS was observed 48 h after TA administration, which elevated iNOS activity from the low point of 36 h, but the high levels observed at 0 h were never achieved in the DR rats. AL and DR rats followed a

**FIG. 4.** Immunohistochemical analysis of TGF-α on liver sections of AL and DR rats after TA administration (600 mg/kg). Paraffin-embedded liver sections were stained with TGF-α antibody as described in Materials and Methods section. Brown indicates TGF-α positive staining. H, TGF-α positive hepatocytes; N, necrosis; C, central vein. Earlier, higher, and sustained expression of TGF-α was evident in DR rat livers compared with AL rats.

AL 24 h

DR 24 h

AL 60 h

DR 60 h
similar pattern of decrease in iNOS activity after TA administration, but the DR rats had significantly higher iNOS activity than AL rats at 0 h and later at 48 h. The 2-fold higher iNOS activity in DR rats before they received TA administration appears to put these rats at an advantage in priming the hepatocytes for cell division.

Apoptosis in AL and DR Rats after TA Treatment

Apoptotic cells were detected in liver sections by TUNEL assay and counted under a light microscope (Fig. 7B). The increase in apoptotic cells in AL rats was not statistically significant, and 36 h after TA treatment it decreased to control levels. DR rats did not have higher apoptosis before TA administration, but a significant increase in the number of apoptotic cells was detected in the perivenous region in DR rats after TA treatment. The number of apoptotic cells increased significantly by 12 h in DR rats to 5% and further increased to 25% at 48 h, after which it decreased and diminished to control levels by 72 h (Fig. 10). DR rats had significantly higher apoptosis than AL rats between 12 and 60 h after TA administration. Although a significant increase in apoptosis was found in DR rats after TA treatment, the primary type of cell death was necrosis, as was evident by massive number of necrotic cells in the centrilobular region.

DISCUSSION

Moderate caloric restriction or DR in animals including nonhuman primates and humans has a variety of beneficial effects. These include prolongation of life span with a relatively disease-free life, decrease in cancer incidences, increased sensitivity of the peripheral tissues to insulin, and protection from chemical-induced toxicity (Allaben et al., 1990; Berg et al., 1994; Duffy et al., 1995; Hass et al., 1996; Lane et al., 1999; Ramaiah et al., 2000). We have reported that moderate DR protects male SD rats from lethal challenge of TA because of timely and robust tissue repair response (Ramaiah et al., 1998a,b). The process of liver regeneration has been explored in great detail after partial hepatectomy and to some extent after chemical-induced injury (Dalu et al., 1995; Diehl, 2000; Fausto et al., 1995; Lindroos et al., 1991; Soni et al., 1999). The priming action of proinflammatory cytokines such as TNF-α and IL-6 is essential to fully activate the mitogenic stimulus of growth factors such as TGF-α and HGF, along with their receptors, and by NO produced via induction of iNOS (Galun et al., 2000; Lindroos et al., 1991; Rai et al., 1998; Scotte et al., 1997; Webber et al., 1998). The objective of the present work was to test the hypothesis that upregulated signal transduction via cytokines and growth factors stimulates enhanced liver regeneration in DR rats upon toxic challenge. Our observations indicate that the reason behind delayed repair and lethality of AL rats is failure to stimulate prompt mitogenic signaling after toxic challenge.

FIG. 5. Staining intensity scores of (A) TGF-α and (B) HGF immunohistochemistry. Four slides were stained for either TGF-α or HGF from both AL and DR groups at various time points after thioacetamide (600 mg/kg) administration. Staining intensity scores were assigned in a blindfold fashion using a scoring scheme of 0–4. Ten to fifteen central vein areas were viewed per time point for each group. 0, no staining; 1, low staining; 2, medium staining; 3, high staining; 4, very high staining. Values are expressed as mean ± SE (n = 4). *Significantly different from AL group at the corresponding time point. !Significantly different from 0-h DR group. #Significantly different from 0-h AL group.
This supports the hypothesis that a high dose of a chemical is capable of inhibiting tissue repair by decreased expression of growth-stimulatory molecules and signal transduction. In the DR rats, in spite of the high injury, signaling mechanisms were intact, which led to a prompt tissue repair response. Keenan et al. (1995) have demonstrated that both male and female Sprague-Dawley rats fed a special diet (Purina Certified Rodent Chow 200-9), which restricted the calories to 65% of AL, had higher hepatocyte division after 106 weeks of diet restriction. Such increase in hepatocyte division was not observed in our model, mainly because of the short time of diet restriction, but hepatocyte division increased promptly after toxic challenge in the DR rats. Cuenca et al. (2001) have recently reported that calorie restriction improves liver regeneration after partial hepatectomy because of early mobilization of hepatocytes into S-phase (Cuenca et al., 2001). These data support our observation that DR rats have higher compensatory regeneration after TA challenge.

TNF-α has been implicated as a priming factor during liver regeneration, stimulating progression of quiescent hepatocytes from G₀ gap phase to G₁ (Webber et al., 1998; Yamada and Fausto, 1998). TNF-α levels in plasma increased after TA administration over the time course and remained higher until 96 h in both AL and DR groups but there was no significant difference between the two groups. Hepatic expression of TNF-α mRNA measured by semiquantitative RT-PCR indicated that DR rats have significantly higher mRNA at 48 and 72 h after TA administration (data not shown). These data indicate that TNF-α–mediated signaling may play a role in the stimulation of tissue repair induced by TA injury, but it does not explain the enhanced compensatory liver tissue repair in DR rats over and above the AL rats. TNF-α is also known to stimulate production of TGF-α, which further stimulates hepatocytes to move from G₁ to M phases (Gallucci et al., 2000; Webber et al., 1998). Our data support this notion. Increase in TNF-α levels precedes an increase in TGF-α after TA administration in both the groups.

Gene knockout experiments have revealed the critical role played by IL-6 during liver regeneration. IL-6−/− mice have delayed regeneration, and other experiments including human studies with post-liver transplantation regeneration have highlighted crucial involvement of IL-6 during liver tissue repair (Galun et al., 2000; Rai et al., 1998; Streetz et al., 2000). IL-6 expression was suppressed in the AL rats after treatment with

![Image](FIG. 6. Immunohistochemical analysis of HGF on liver sections of AL and DR rats after TA administration (600 mg/kg). Paraffin-embedded liver sections were stained with HGF antibody as described in Materials and Methods section. Brown indicates HGF positive staining. H, HGF positive hepatocytes; N, necrosis; C, central vein.)
a high dose of TA. DR rats had significantly higher IL-6 than AL rats as early as 12 h after TA administration that further increased, reaching a peak at 48 h where the IL-6 levels were 6-fold higher in DR rats. The increase in plasma levels of IL-6 was further corroborated by immunohistochemical analysis of IL-6 in livers of AL and DR rats after TA administration. Sinusoidal endothelial cells were identified as the primary source of IL-6 in the liver. The increase in IL-6 coincides with increase in S-phase stimulation in the DR rats, indicating potential involvement of IL-6 in liver tissue repair in DR rats after toxic insult.

The role of TGF-α and HGF has been particularly well studied during regeneration after toxic insult (Jiang and Hiscox, 1997; Lindroos et al., 1991; Michalopoulos and DeFrances, 1997; Scott et al., 1997). TGF-α expression has been extensively studied in hepatocarcinomas in human subjects and in partial and chemical hepatectomy in animal models (Dalu et al., 1995; Harada et al., 1999; Tomiya et al., 1998). TGF-α and c-myc double knockout mice lose their ability to regenerate liver after partial hepatectomy (Factor et al., 1997). TGF-α signals via EGFR with downstream activation of MAPK (Boylan and Gruppuso, 1994; Stromblad et al., 1993). Immunohistochemical localization of TGF-α indicated hepatocytes as the primary source of TGF-α, with the cells immediately surrounding the necrotic areas staining positive. Both AL and DR rats had similar expression of TGF-α during the initial time points (0–36 h), but in AL rats, TGF-α expression decreased and diminished during the later time points. In DR rats, it remained consistently higher during later time points (48–96 h). These data support the observations of other investigators that TGF-α expression in hepatocytes increases during tissue repair after toxicant administration (Burr et al., 1993; Dalu et al., 1995; Kobayashi et al., 2000). Western blot analysis of EGFR indicated AL rats had lower initial expression, which increased comparable to that in DR rats at 24 h, but diminished to undetectable levels at 48 h after TA treatment. Higher EGFR protein was observed initially in DR rats, which further increased slightly at 24 h and remained higher 48 h after TA administration. Both the ligand (TGF-α) and its receptor (EGFR) are upregulated in DR rats, and their temporal corre-
lation with increased cell division points toward an important role of these factors in stimulation of liver tissue repair after toxic insult in DR rats.

HGF is a potent mitogen for a number of cell types including hepatocytes and was thought to be produced in extrahepatic tissues such as salivary glands and Brunner’s gland in the intestine and transported to liver (Wolf et al., 1991). However, recent investigations suggest that under certain conditions such as embryonic and fetal development, endotoxin challenge, and cirrhosis, the liver parenchymal cells can also express HGF. It is known that cytokines such as TNF-α and IL-6 are capable of stimulating expression of HGF (Masson et al., 2001). In our study, hepatocytes, Kupffer cells, and infiltrated macrophages stained positive for HGF. HGF is produced in an inactive monomer form and is processed to a dimer in hepatocytes and stimulates the cells in autocrine and paracrine fashion using the transcription factor AP-1 (Gao et al., 1999; Pediatdiktakis et al., 2001). The antibody used in the present study stains the un-processed form of HGF. Immunohistochemical localization indicated that in AL rats HGF expression increased after TA challenge and attained similar levels as in DR rats at 24 h, but decreased by 48 h and was diminished at 72 h after TA administration. DR rats had slightly higher HGF initially, which quickly increased further after TA treatment and remained higher until 72 h. Western blot analysis of c-met, the receptor for HGF, indicates higher c-met protein at 48 h in DR rats after TA administration (data not shown). The early increase during the first 12 h and persistent expression of growth factors and their receptors during late time points (36–72 h) coinciding with cell division indicate that HGF-mediated signaling may have an important role in enhanced cell division in DR rats.

NO is a versatile molecule playing an important role in a variety of physiological processes including liver regeneration. In liver, the primary source of NO is iNOS, which is present at minimal levels in normal liver. During liver regeneration, induction of iNOS via cytokines leads to higher release of NO, further stimulating a variety of genes involved in regeneration and priming of hepatocytes for division. NO plays a dual role during liver regeneration; it stimulates cell division along with inhibition of apoptosis induced by factors such as TNF-α (Diez-Fernandez et al., 1997; Rai et al., 1998). Estimation of iNOS activity in AL and DR rats revealed an overall inhibition of iNOS induction in the AL rats treated with a high dose of thioacetamide (600 mg/kg) as described in Materials and Methods section. Values are expressed as mean ± SE (n = 4). *Significantly different from AL group at the corresponding time point. !Significantly different from 0-h DR group. #Significantly different from 0-h AL.
TA. Diet restriction induced iNOS activity 2-fold, which plays a significant role in early priming of cells to divide. iNOS activity in DR rats decreased after TA administration, with a small surge again at 48 h. This may be important for stimulation of additional cells during later time points. The DR-mediated induction of iNOS activity might be related to inhibition of apoptosis in DR rats. During diet restriction, liver cells undergo apoptosis to cope with changes in the energy budget (Frame et al., 1998; Klaunig and Kamendulis, 1999). iNOS induction may be controlling the extent of apoptosis and thus play a significant role in establishing homeostasis after caloric restriction. This is consistent with our observation that apoptotic cells increased in DR rats after TA administration from 12 to 48 h, during which a decrease in iNOS activity was observed. At 48 h, iNOS activity increased and the number of apoptotic cells decreased at 60 h. In AL rats, iNOS was suppressed and no increase in apoptotic cells was observed, indicating a deficiency in signaling. TUNEL analysis of AL and DR liver sections after TA administration revealed no significant increase in apoptotic cell in AL rats, which is consistent with our previous observation. DR rats have significantly higher apoptotic cell death after TA administration. Apoptosis has been recognized as a controlled, programmed cell death leading to minimal damage to the surrounding tissue (Barros et al., 2001). Increased apoptosis in DR rats after TA treatment increased the efficiency of tissue repair by removing the weak and damaged cells that would otherwise undergo oncosis and contribute to progression of injury. Therefore, regulated increase in apoptosis in tandem with stimulated robust cell division in DR rats after toxic challenge with TA appears to facilitate efficient tissue repair, leading to recovery from markedly higher liver injury.

These data support our observation that the high dose of TA inhibits tissue repair in the AL rats because of inhibited signaling (Fig. 11). This is clear from the decreased expression of cytokines, growth factors, and EGFR after TA administration, leading to a delayed repair response. The mechanisms by which the high dose inhibits signal transduction in AL rats are not entirely clear. On the other hand, elimination of this delay and disrepair in DR rats upon TA challenge is due to a combination of several molecular mechanisms. These involve upregulation of cytokines, especially IL-6, and growth factors, both TGF-α and HGF, along with EGFR and iNOS. This sequential orchestration, evident with increase in TNF-α, IL-6 and iNOS during the early periods after TA treatment, was followed by increases of TGF-α, HGF and IL-6 during later time points. The interaction of these molecules orchestrates the complex process of liver cell division and tissue repair in DR rats. The increase in apoptosis after TA treatment in DR rats further enhances the regeneration process. Our studies provide substantial evidence that upregulated promitogenic signaling via cytokines and growth factors is a potential mechanism of stimulated repair in DR rats that disallows progression of

FIG. 10. Apoptotic cells in AL and DR livers after thioacetamide (600 mg/kg) stained by TUNEL assay as described in Materials and Methods section. Thousand cells per slide were counted and percentage apoptotic cells were expressed as an index of apoptosis. Values are expressed as mean ± SE (n = 4). *Indicates values significantly different from AL group at the corresponding time point. ! Indicates values significantly different from 0-h DR group. # Indicates values significantly different from 0-h AL.

FIG. 11. Proposed mechanism of stimulated tissue repair in DR rats after toxic challenge. In AL rats high dose inhibits promitogenic signaling, leading to inhibited repair, whereas in DR rats signaling is intact, which leads to prompt tissue repair and survival after a lethal dose of TA.
injury on one hand and allows for restoration of liver structure and function on the other. Other possible mechanisms, such as involvement of fat mobilization and increased fatty acid metabolism and its relation to cell division (Chanda and Mehendale, 1996; Yamashita et al., 2000), that may modulate cell division in DR rats remain to be investigated.

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