Matrix Metalloproteinases-2 and 9 Do Not Play a Role in the Growth of Preneoplastic Liver Lesions in F344 Rats

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Matrix metalloproteinases- (MMPs) 2 and 9 (gelatinases A and B) have been implicated in tumor invasion and metastasis, and recent studies have shown increased levels of these enzymes during recovery from partial hepatectomy (PH) in rats. F344 rats are highly susceptible to the growth of glutathione *S*-transferase 7-7- (GST 7-7) positive preneoplastic liver lesions promoted using the modified resistant hepatocyte (RH) protocol. Since the RH protocol consists of 2-acetylaminofluorene (2-AAF) followed by a PH, we reasoned that MMP-2 and -9 might be critical for the growth of lesions. Using gelatin zymography, we examined the expression of these enzymes in the livers of F344 rats treated with the RH protocol and sacrificed on Days 2, 4, 7, 14, and 21 after 2-AAF/PH. We found increases in both pro- and active MMP-2 and -9 over baseline levels, with the highest levels occurring on Day 7 post-PH. Also, a 54-kDa band, likely to be proMMP-1, was elevated in a pattern similar to MMP-2 and -9. In contrast to F344 rats, identically treated Copenhagen rats that are highly resistant to promotion of liver lesion growth using the RH protocol had significantly lower levels of proMMP-1 and -2. To test the importance of these MMPs to the growth of liver lesions, F344 rats that had been initiated with diethylnitrosamine were treated using the RH protocol. They then received either the MMP inhibitor batimastat (30 mg/kg, intraperitoneally) or vehicle alone daily from Day 3 to 20 post-PH and were sacrificed on Day 21. There were no differences in the percentage of liver volume occupied by GST 7-7-positive lesions (19.1 ± 4.84 vs 19.4 ± 3.31, treated versus vehicle, mean ± SEM) or liver weight as a percentage of body weight (4.11% ± 0.15 vs 4.07% ± 0.18, treated versus vehicle, mean ± SEM) between the treated and control groups. Treatment of rats with batimastat clearly did not affect lesion growth or liver regeneration following the RH protocol. These results suggest that increases in gelatinase expression during the RH protocol are a result of the promotional stimulus rather than a mechanism by which 2-AAF/PH causes lesion growth.

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Matrix metalloproteinases (MMPs) are a family of enzymes that collectively can degrade all components of the extracellular matrix (ECM) (1, 2). MMP-2 and -9, also known as gelatinase A and B, are produced as latent pro-enzymes that must be cleaved to their active forms before they have proteolytic activity. These enzymes are involved in processes such as wound healing (1) and also in tumor invasion and metastasis (3, 4).

F344 rats are highly susceptible to the growth of preneoplastic liver lesions initiated by diethylnitrosamine (DEN) and promoted using the modified resistant hepatocyte (RH) protocol (5). This protocol that consists of 3 doses of 2-acetylaminofluorene (2-AAF) followed by a two-thirds partial hepatectomy (PH) and then a fourth dose of 2-AAF, results in the growth of glutathione *S*-transferase 7-7- (GST 7-7) positive lesions. Recent studies have shown that during recovery from PH, F344 rat livers express increased levels of MMP-2 and -9 (6, 7). Since PH is essential for the promotion of preneoplastic lesions during the RH protocol, we reasoned that MMP-2 and -9 might be critical for the growth of these lesions. To investigate the involvement of MMPs, particularly gelatinases, in promotion, we examined their expression in the livers of F344 rats treated with the RH protocol using gelatin zymography.

We have shown that in contrast to F344 rats, Copenhagen (Cop) rats are highly resistant to the promotion of GST 7-7-positive lesions using the RH protocol (8). Cop lesions stop expanding after Day 7 post-PH, whereas F344 lesions continue to grow until they occupy 50% of the liver volume by Day 21. There are no differences between Cop and F344 rats with respect to recovery from PH alone, mitoinhibition by 2-AAF, metabolism of DEN, or number of preneoplastic lesions per liver at the start of promotion (8,

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9). If MMPs are involved in the growth of lesions, Cop and F344 rats might express different levels of hepatic MMPs during the RH protocol. We tested this notion by measuring gelatinase expression in Cop rats treated with the RH protocol. Furthermore, we determined whether batimastat (BB-94), a broad-spectrum synthetic inhibitor of MMPs that has been shown to inhibit tumor growth and metastasis in rodents (10–13), inhibits lesion growth in F344 rats.

Materials and Methods

Chemicals. DEN (Eastman Kodak Co., Rochester, NY) was 98% pure by gas chromatography. Normal swine serum and swine anti-rabbit biotinylated antibody were obtained from DAKO (Mississauga, Ontario, Canada). 2-AAF, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal), gelatin, and p-aminophenylmercuric acetate (APMA) were from Sigma Chemical Co. (St. Louis, MO). The rabbit anti-rat GST 7-7 antibody was a gift from Dr. Tom Rushmore (14) and the streptavidin-β-galactosidase conjugate was purchased from Boehringer Mannheim (now Roche Biochemical, Dorval, Quebec, Canada). Batimastat was kindly provided by British Biotech Pharmaceuticals (Oxford, UK).

Animals. Cop and F344 rats, purchased from Harlan Sprague-Dawley (Indianapolis, IN), were allowed to acclimatize for 1 week prior to the start of experiments at which time they were 7 to 8 weeks old. Food (6% fat, Harlan Teklad, Madison, WI) and acidified water (pH 2.8) were provided *ad libitum* and a 12:12-hr light:dark cycle was maintained automatically.

Animal Treatments. In the first experiment, 25 Cop and 25 F344 rats were administered a single intraperitoneal dose of 200 mg/kg DEN dissolved in 0.9% NaCl solution. All rats were then treated using a modified RH protocol (5). Briefly, 18 days after DEN, three daily gavages of 20 mg/kg 2-AAF in DMSO and corn oil (1:29, v/v) were given followed by a PH. A fourth 20 mg/kg dose of 2-AAF was given the day after PH. Groups of five rats from each strain were sacrificed on Days 2 and 4 post-PH, six rats from each strain on Day 7, and four Cop and four F344 rats on Day 14 post-PH. Representative liver samples were fixed in 5% acetic acid in methanol or snap frozen in liquid nitrogen. Using this regime we found that a number of rats of both strains became moribund around Day 11 post-PH such that there were too few rats to make comparisons at our intended Day 21 time point. Therefore, to obtain data at 21 days, a group of five Cop and eight F344 rats were treated as above, except the fourth dose of 2-AAF was given 4 days after PH and was lowered to 5 mg/kg. This is a commonly used regime that has a promoting effect equivalent to the protocol we used for the rats sacrificed on Days 2 through 14 (5).

In the batimastat experiment, 15 F344 rats were initiated with DEN and were promoted with the RH protocol as described above using the 5 mg/kg dose of 2-AAF 4 days after PH. Starting on Day 3 after PH, nine of the rats received daily intraperitoneal doses of the MMP inhibitor batimastat (30 mg/kg) suspended by sonication at a concentration of 2.5 mg/ml in sterile PBS with 0.01% Tween-20. Previous studies have demonstrated that 1 hr following this dose in rats, serum levels of at least 10× the IC_{50} for MMP-1, -2, and -9 were reached and were maintained over 48 hr (13). The other six control rats received the equivalent volume of vehicle alone. All the rats were sacrificed on Day 21 after PH.

Zymography. Protein was isolated from snap-frozen livers by homogenization in PBS on ice, and debris was removed by centrifugation at 15,000*g* for 20 min at 4°C. Gelatin zymography (15) was performed as follows: 30-μg samples of protein per lane diluted in Laemmli sample buffer were separated on 8% SDS-PAGE gels containing 1 mg/ml gelatin. As a positive control, a 20-μL aliquot of medium from a rat mammary tumor cell line was also run on each gel. This control also facilitated comparisons between gels. The gels were then rinsed in water and washed twice in 2.5% Triton X-100 in water for 15 min each to remove SDS. The gels were incubated in 50 mM Tris buffer (pH 8.0) with 5 mM $CaCl_2$ at 37°C for 24 hr. Following incubation, the gels were rinsed in water, stained with Coomassie blue, and destained until pale bands of gelatinase activity could be seen on a dark blue background. Bands were identified based on their size and by comparison to the positive control. In order to identify pro and active forms of MMPs, 10 mM APMA was added to Day 7 samples and incubated for 2 hr at 37°C (16). These activated samples were then run on gels and stained as above. To confirm that bands were MMPs, a gel containing Day 7 samples was incubated in the same Tris buffer, but without $CaCl_2$ and with 20 mM EDTA to act as a chelator to inhibit all MMP activity.

The intensity of bands on photographs of zymograms was measured by using AlphaEase software (version 5.5, Alpha Innotech Corporation, San Leandro, CA). All bands were measured relative to the proMMP-2 band in the positive control lane, facilitating comparisons within and between gels. Results were analyzed using a one-way ANOVA and a Tukey post-test was used to compare Cop and F344 groups at each time point.

Immunohistochemical Staining. After fixing for 3 to 4 hr, liver samples from the batimastat experiment were embedded in paraffin wax and 2-μm sections were prepared on microscope slides. Immunohistochemical staining was carried out for GST 7-7 using X-Gal as a chromogen, as described by Stinchcombe *et al.* (17). Briefly, following deparaffinization and rehydration, sections were blocked with 10% normal swine serum for 10 min and then rabbit anti-GST 7-7 antibody was applied (1:2000) overnight at 4°C. The following were then applied to the sections at room temperature, washing with PBS between steps: biotinylated swine anti-rabbit antibody at 1:500 for 2 hr, streptavidin-β-galactosidase complex at 1:200 for 2 hr, and X-Gal substrate for 1 hr. All dilutions were made in 1% normal

swine serum in PBS. Sections were finally counterstained with H+E and were then mounted.

Analysis of Stained Sections. The areas of GST 7-7-positive lesions in liver sections were measured as described previously (9) using morphometry (Bioquant IV, Zeiss Instruments, Wetzlar, Germany) and were converted to the percentage of liver volume occupied by lesions and the number of lesions per liver using the method of Enzmann *et al.* (18). Only lesions with radii greater than 35 μm were included in the analysis (19, 20).

Results

To investigate a possible role for MMP-2 and -9 in promoting preneoplastic liver lesion growth, we measured the hepatic levels of these enzymes in F344 and Cop rats following initiation with DEN and promotion using the RH protocol. Figure 1 shows gelatin zymograms of liver protein from rats sacrificed at various times during promotion. To provide evidence that the bands seen on the zymograms are indeed MMPs, gels identical to Day 7 of Figure 1 were incubated without $CaCl_2$ and with EDTA as a chelator. Under these conditions, the zymograms displayed no gelatinase activity, showing that the enzymes are inhibited by EDTA and are, therefore, likely to be MMPs (Fig. 2a). To

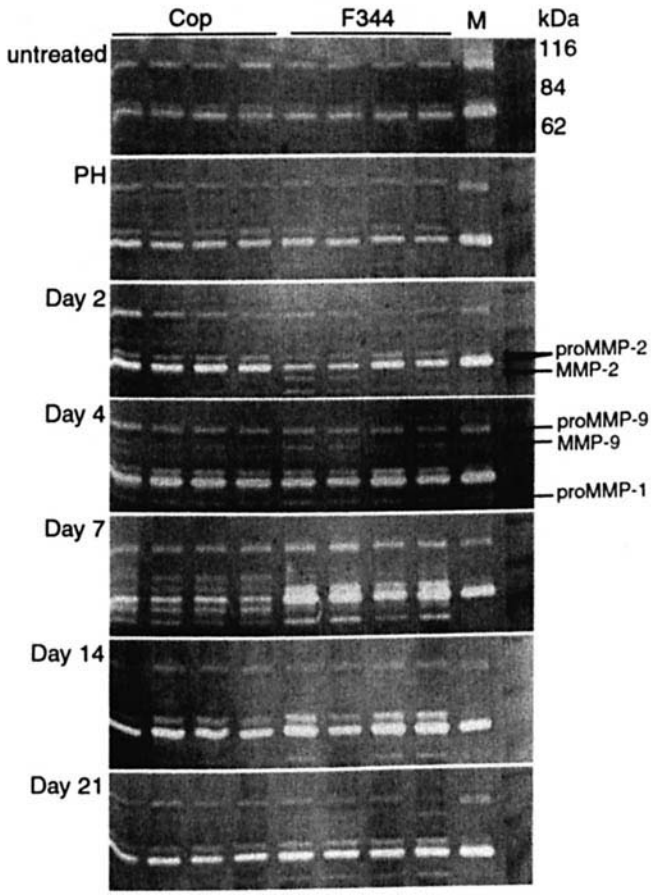

Figure 1. Gelatin zymograms of livers from four Cop and four F344 rats at each time point. M, positive control media. Rats were either untreated or initiated with DEN and promoted using the RH protocol and livers were taken at various times following PH.

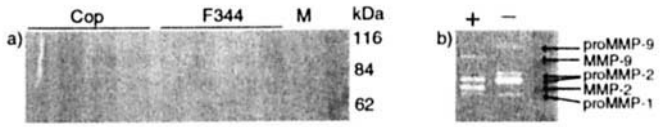

Figure 2. (a) Gelatin zymogram identical to Figure 1 Day 7, but incubated with EDTA added to the incubation buffer and no $CaCl_2$. No gelatinase activity is present. (b) Gelatin zymogram of a sample from an F344 rat at Day 7 following 2-AAF/PH. +, sample was incubated with APMA to convert pro forms of MMPs to active forms; −, no APMA.

provide further evidence for the identity of the pro and active bands, APMA, an activator of MMPs, was incubated with samples that were then run together with untreated samples on a zymogram (Fig. 2b). Pro forms of MMP-2 and -9 were cleaved to their smaller, active forms, confirming their identity. A 54-kDa band was present, though very faint, at the time of PH and was most prominent on Day 7. This band has previously been identified immunologically as proMMP-1 (interstitial collagenase) (21) and gelatin zymograms are commonly used to measure proMMP-1 levels (22–24). Although MMP-3 (stromelysin-1) is of a similar size, casein zymograms that detect stromelysin activity revealed no bands even after a week of incubation (data not shown).

ProMMP-2 and -9 are clearly present in the livers of untreated rats as well as rats treated with 2-AAF (Fig. 1, untreated and PH, respectively). 2-AAF treatment had no significant effect on MMP expression, except for a modest increase in proMMP-2 (data not shown). Treatment with 2-AAF/PH resulted in an increase in all pro and active MMPs measured in F344 rats (Figs. 1 and 3). In order to examine the significance of these changes, we compared F344 livers with livers from Cop rats, a strain that is highly resistant to promotion using the RH protocol. As shown in Figures 1 and 3, Cop rats did not have the same pattern of gelatinase expression as F344s. ProMMP-2 and proMMP-1 levels did not increase significantly in Cop rats following 2-AAF/PH, and F344 rats had significantly higher levels of proMMP-2 on Days 7, 14, and 21 post-PH, and proMMP-1 on Days 7 and 14 post-PH compared with Cop rats. Pro and active MMP-9 and active MMP-2 levels were not significantly different between the strains at any time point.

Our results show that gelatinase levels are lower overall in resistant Cop rats than in susceptible F344 rats during promotion with the RH protocol. To test whether this difference plays an important role in controlling the growth of preneoplastic lesions, we treated F344 rats with batimastat, an inhibitor of MMP activity, from Day 3 post-PH until they were sacrificed on Day 21. All the rats survived until Day 21 and there were no signs of morbidity in either the batimastat or the vehicle-treated control group. Liver weights as a percentage of body weights were not different between groups (4.11% ± 0.15% vs 4.07% ± 0.18%, treated versus vehicle, mean ± SEM). The percentage of liver volume occupied by GST 7-7-positive lesions was not different in batimastat and vehicle-treated control rats (19.1% ± 4.84%

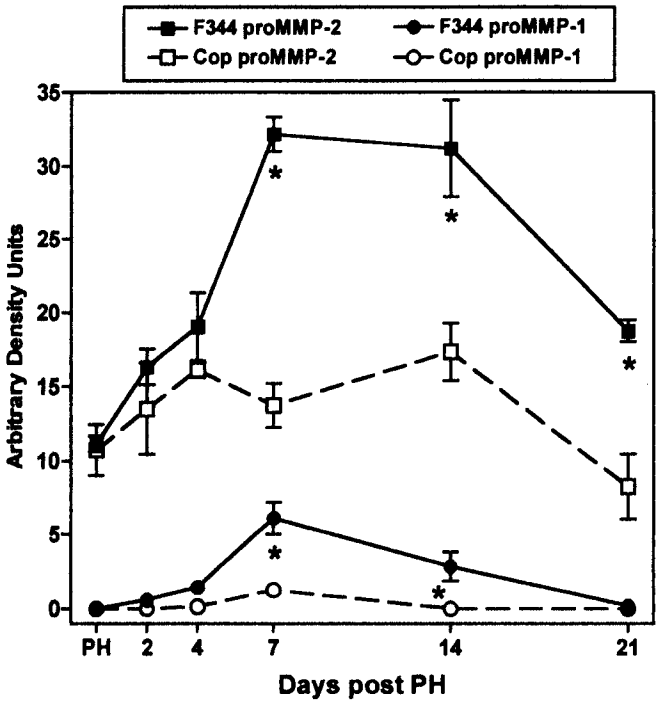

Figure 3. ProMMP-1 and 2 levels in F344 and Cop rats from Figure 1 measured by densitometry. Bands were standardized to the proMMP-2-positive control in each gel to facilitate comparisons between gels. * F344 rats had significantly higher levels of proMMP-2 on Days 7, 14, and 21 post-PH and proMMP-1 on Days 7 and 14 compared with Cop rats ($P < 0.05$, one-way ANOVA with Tukey post-tests).

vs 19.4% ± 3.31%, treated versus vehicle, mean ± SEM). The number of lesions per liver was also not different (2252 ± 236.2 vs 2959 ± 474.0, treated versus vehicle, mean ± SEM).

Discussion

Hepatic proMMP-2 and proMMP-9 have been shown to be elevated within 30 min of PH in F344 rats compared with sham-treated controls (6). Active forms were present a few hours later and the levels remained elevated until at least 72 hr after PH. In our study, F344 rats were treated with 2-AAF prior to PH, and like PH alone, active MMP-2 was present by Day 2 post-PH. However, unlike PH alone, in our study increases in pro and active MMP-2 and -9 were greatest on Day 7. The delay in MMP expression we observed might be a consequence of mitoinhibition of hepatocytes by 2-AAF. Another important difference is the presence of a 54-kDa gelatinolytic band in rats treated with 2-AAF/PH that is clearly not present with PH alone (6). This band is likely to be MMP-1 and could be critical to the promoting ability of 2-AAF since it is not present following PH alone.

Previously, we have shown that Cop rats are resistant to the promotion of GST 7-7-positive liver lesions using the RH protocol (9). The lesions of Cop rats stop expanding after Day 7 post-PH, whereas F344 lesions continue to grow until they occupy 50% of the liver volume by Day 21. In the present study, the first experiment shows that liver gelatinase levels are lower in Cop compared with F344 rats. The differences are greatest on Day 7 post-PH, just before the growth curves for GST 7-7-positive lesions in the two strains diverge (8). This suggests that the higher gelatinase levels in F344 rats might be required for the expansion of lesions into the normal surrounding liver. By the same reasoning, the inability to sufficiently degrade ECM might prevent lesions in Cop rats from expanding.

To test the importance of MMPs for lesion growth, we treated F344 rats with the broad-spectrum MMP inhibitor batimastat while their lesions were expanding. Other studies in rats have shown inhibition of mammary tumor and metastasis growth (13) and colon carcinomatosis growth and resulting hepatic metastasis (25) using the same dose of batimastat that we used here. Our results show, however, that batimastat has no effect on the growth of liver lesions promoted using the RH protocol. This suggests that the higher levels of gelatinases we observed in F344 rat livers are not an important factor in their susceptibility to hepatocarcinogenesis and may result from rather than facilitate lesion growth. Another possibility is that increases in MMPs in F344 rats may have been offset by increases in endogenous inhibitors of MMPs (3) such that overall matrix degradation might have been the same in both strains.

There is evidence that MMP-2 and -9 play an important role in recovery from PH in rats (6, 7). If these enzymes are critical for the liver to recover from PH, inhibition of MMPs by batimastat given after PH should inhibit regeneration of the liver. In our study, although we treated rats with 2-AAF prior to PH, the rats still must regenerate their livers. We saw no effect of MMP inhibition on liver regeneration despite treating the rats daily with batimastat from Day 3 after PH until Day 21. Both treated and control rats showed no morbidity and their liver weights as a percentage of body weight were almost identical. These results suggest that following 2-AAF/PH, the expression of gelatinases is not essential for liver regeneration.

In summary, we have shown that F344 rats have increased levels of hepatic gelatinases during promotion with the RH protocol. However, treatment of rats with the MMP inhibitor batimastat did not affect lesion growth or liver regeneration. The increases in gelatinase expression during the RH protocol appear to be a result of the promotional stimulus rather than a mechanism by which 2-AAF/PH causes lesion growth.

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