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GROWTH INHIBITORY ACTIONS OF PROTHROMBIN ON NORMAL HEPATOCYTES:

INFLUENCE OF MATRIX

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Abstract

Most hepatomas have a defect in prothrombin carboxylation, and can secrete under-carboxylated prothrombin or des- γ -carboxy-prothrombin (DCP), the function of which is unknown. We considered that prothrombin-DCP axis might also be involved in growth control. Hepatocytes and hepatoma cells were treated with prothrombin, and DNA synthesis and cytoskeleton were studied. Prothrombin inhibited DNA synthesis in hepatocytes on fibronectin, but not collagen matrix. Hepatoma cell lines were not inhibited. We found that hepatoma cell matrix conferred resistance to hepatocytes. Prothrombin decreased fibronectin but not collagen amounts, but only in the presence of hepatocytes and not hepatoma cells, indicating that it has a differential action on matrix proteins. It also caused changes in cell shape and actin depolymerization. In vivo, there was a decrease in plasma prothrombin activity after a partial hepatectomy (PH) concomitant with a peak of DNA synthesis by the hepatocyte at 24 h after PH. Injection of warfarin at the time of PH, further inhibited PT activity and enhanced this 24 h peak of DNA synthesis. Furthermore, repeated injection of prothrombin lowered the peak DNA synthesis after PH. The data support the hypothesis that prothrombin can act as a hepatocyte growth inhibitor, likely at the level of fibronectin loss and result in cytoskeletal changes. Hepatomas resist this action, possibly due to their different matrix proteins. This represents a novel mechanism for growth regulation and provides a possible biological significance for the tumor marker DCP.

Keywords

Prothrombin; hepatocyte growth; cell matrix

1. Introduction

Human and rat hepatocellular carcinomas (HCCs or hepatomas) have a characteristic biochemical defect, resulting in an inability to make mature prothrombin by post-translational carboxylation of immature, under-carboxylated prothrombin (des-gamma-carboxy-prothrombin, DCP; or PIVKA-II, Protein Induced by Vitamin K Absence). As a result, the underlying normal liver produces mature prothrombin, but HCCs within that liver produce under-carboxylated or immature prothrombin, DCP, one of the more useful HCC serum markers (Weitz et al., 1993; Huisse et al., 1994; Liebman et al., 1984; Nakao et al., 1991; Liebman et al., 1989; Li et al., 2002). The biological significance of this has never been clear, but it has recently been suggested that immature prothrombin or DCP has HCC growth promoting properties (Suzuki et al., 2005). This led us to consider that mature prothrombin, a major vitamin K-dependent blood coagulation factor, might also have growth regulatory properties, since K vitamins have previously been shown to have growth inhibitory activities

(Yoshida et al., 2003; Otsuka et al., 2004; Lamson and Plaza, 2003; Wang et al., 1995; Nishikawa et al., 1995; Hitomi et al., 2005; Bouzahzah et al., 1995) in addition to their well-described physiological effects as cofactors for several blood coagulation proteins (such as prothrombin and factors VII, IX and X).

We therefore evaluated the effects of prothrombin (PT) on both HCC cell growth and on mitogen-induced DNA synthesis in primary cultures of adult rat hepatocytes. Unlike DCP, PT was found to have neither growth promoting nor growth inhibitory activity on HCC cells. However, PT was found to have two previously unknown but likely linked actions on hepatocytes. It inhibited EGF-induced hepatocyte DNA synthesis when the cells were plated on a fibronectin (FN) matrix, but not when hepatocytes were plated on a collagen matrix. It also inhibited fibronectin amounts and caused cell shape changes. This suggests an additional cellular function for prothrombin, as well as a novel pathway for growth regulation, based upon alteration of cell matrix. Since DCP, in contrast to prothrombin, did not inhibit hepatocyte DNA synthesis, it suggests a role for DCP as a possible escape mechanism from prothrombin-mediated inhibition of cell growth.

2. Materials and methods

2.1. Hepatocyte preparation and DNA synthesis

Hepatocytes from the livers of 2-3 month-old male Fischer F344 rats (weight 180-220 g) were isolated by a two-step *in situ* collagenase perfusion technique (Seglen, 1976). Briefly, under Metofane anesthesia, a rat liver was cannulated *via* the portal vein and was washed first with 150 ml of Hanks buffer (GIBCO BRL, Grand Island, NY) containing 20 mg/ml of EGTA for 3 min, then perfused with 300 ml of collagenase B solution (40 mg/100 ml; Boehringer Mannheim, Indianapolis, IN) for 6 min (50 ml min⁻¹). The liver was disaggregated and hepatocytes were dispersed and washed three times with L15 medium (GIBCO BRL), and resuspended in serum-free William's E medium (GIBCO BRL). Cell number was determined with a hemocytometer and viability was determined by the trypan blue exclusion method. Hepatocytes were plated at a density of 3.5×10^5 cells per 35 mm tissue-culture dish. The dishes were either fibronectin (FN)-coated or collagen-coated, at 0.5 µg per dish by covering the dish base with matrix-containing solution and allowing the solvent to evaporate over 18 hr. They were cultured at 37 °C in a 5% (v/v) CO₂ atmosphere. After a 3 hr serum-free hepatocyte attachment period, the medium was changed to 1 ml of fresh William's E medium. EGF (Boehringer Mannheim), with or without prothrombin or other test compound was added to the culture medium. To determine DNA synthesis in the cultured hepatocytes, 5 µCi of [³H] thymidine (ICN, Costa Mesa, CA) was added to each plate between 48-72 hr from the beginning of culture. At 72 hr of culture, hepatocytes were harvested, and DNA synthesis was measured using a liquid scintillation counter. Total hepatocyte protein amounts were determined by the Bradford assay (Bradford, 1976).

2.2. BrdU labeling of hepatocytes

BrdU-labeling is based on the incorporation of the pyrimidine analog BrdU instead of thymidine, into the DNA of growth-stimulated cells. Briefly, rat hepatocytes were plated in 35 mm dishes at a concentration of 3.5×10^5 per dish. After 48 hr culture with or without EGF, and with EGF plus or minus PT, BrdU (10 µmol/l) was added for another 1 hr of culture. Hepatocytes were subsequently fixed with 100% ethanol for 20 min at 20 °C and labeled with anti-BrdU antibody (Roche Diagnostics, Indianapolis, IN) for 30 min at 37 °C. After incubation with secondary antibody, hepatocytes were stained with a color-substrate peroxidase chromogen kit (Biomedica Corp, Foster City, CA) and examined in a light microscope and labeled cells were counted.

2.3. Hepatoma cell culture and growth inhibition

Human (Hep3B or HepG2) and rat (JM-1 or MH7777) hepatoma cell lines were maintained in Eagle's MEM supplemented with 10% fetal bovine serum in plastic tissue culture dishes without matrix. PT was added to the medium for the indicated times at various concentrations. Cell number was estimated by a DNA fluorometric assay using the fluorochrome Hoechst 33258 (Rago et al., 1990).

2.4. F-actin detection and quantitation

F-actin in hepatocytes was visualized by staining the actin filaments with FITC-phalloidin (Sigma Chemicals, St. Louis, MO). A stock solution of FITC-phalloidin was made in methanol at 5 mg/ml. Hepatocytes were washed with PBS and the cells were fixed for 5 min with 3.7% formalin in PBS. Cells were washed 3 times with PBS and were then permeabilized for 5 min with 0.1% Triton X-100 in PBS, and washed again 3 times with PBS. They were then stained with a 50 µg/ml solution of FITC-phalloidin in PBS for 60 min at room temperature. Excess phalloidin was removed by washing with PBS and F-actin was visualized using a fluorescence microscope.

Levels of F-actin were measured with the G-actin/F-actin in vivo assay kit (Cytoskeleton Inc., Denver, CO). Briefly, cells were lysed with lysis and F-actin stabilization buffer. Unbroken cells were removed by centrifugation at 2000 rpm for 5 min. F-actin was then pelleted by centrifugation at 100,000×g and F-actin in the pellet and G-actin in the supernatant were analyzed by western blot with anti-actin antibody (Sigma Chemicals, St. Louis, MO).

2.5. Western Blots and Immunoprecipitation

Western blotting and immunoprecipitation were performed exactly as previously described (Carr et al., 2003). Cells were plated in 100 mm tissue-culture dishes and then treated with prothrombin. After treatment, the hepatocytes were washed twice with cold phosphate-buffered saline (PBS), then lysed in 100 µl of immunoprecipitation buffer (150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 0.1% SDS, 1% Triton X-100, 1 mM orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml of leupeptin, 10 µg/ml of aprotinin). After removing cell debris, the supernatants were used for the protein concentration assay (Bradford, 1976). The lysate proteins were boiled in 40 µg of sample buffer for five minutes and the proteins were resolved by SDS-PAGE (12% polyacrylamide gel) and transferred onto a Hybond-PVDF membrane (Amersham, Arlington Heights, IL). Membranes were blocked using TBST buffer (150 mM NaCl, 0.05% (v/v) Tween 20, 10 mM Tris-HCl pH 8.) containing 1% BSA for one hour, then probed with antiphosphotyrosine antibody (Oncogene Science, Cambridge, MA) for one hour. After washing four times with TBST buffer, the membranes were probed with horseradish peroxidase-conjugated secondary antibody, to allow detection of the appropriate bands using enhanced chemiluminescence (Amersham) for Western blots. Fibronectin and collagen-I Antibodies were purchased from Santa Cruz Biotechnology, Santa Cruz, CA.

2.6. Partial hepatectomy and BrdU labeling of hepatocytes in vivo

The Higgins and Anderson 2/3 partial hepatectomy (PH) technique was performed in white male Fisher F344 rats, 150-200 g (Higgins and Anderson, 1931). Hepatic DNA synthesis and nuclear labeling (BrdU) were done as previously described (Russell and Bucher, 1983). Briefly, male Fischer F344 rats (200 g) were anesthetized with isoflurane inhalation from a nose cone. The abdomen hair was shaved. A one inch incision was made and the whole liver was squeezed out of the peritoneum. The three larger liver lobes were ligated with a tight knot of suture and they were excised. The remaining 33% of the liver comprising of the smaller lobes were put back in the peritoneum and the abdominal incision was closed with suture. The outer skin was closed with metal clips.

Warfarin (Sigma Chemicals, St. Louis, MO) was dissolved in PBS and was injected intraperitoneally at a dose of 2.5 mg/kg body weight 1 hr after PH. Prothrombin was also injected intraperitoneally at a dose of 50 U/kg body weight at 1 hr, 3 hr and 20 hr after PH. BrdU, dissolved in buffered saline at a concentration of 50 mg/ml, was injected intraperitoneally with a dose of (50 mg/kg body weight), at 22 h after PH. Rats were then sacrificed at 24 hr after PH and liver sections were fixed overnight in 10% formalin. The fixed liver sections were processed for immunohistochemistry with anti-BrdU antibody (Roche Diagnostics, NJ) and anti-mouse-IgG conjugated with horseradish peroxidase.

2.7. Hepatoma cell matrix preparation

Extracellular matrix of the Hep3B hepatoma cell line was prepared by incubating a confluent culture of these cells with serum-free medium containing 1% Triton X-100 for 24 hr at 37°C. The lysed cells were removed and the dishes were then washed 3 times with serum-free medium. The extracellular matrix remaining on the culture dish was used as a substrate to plate freshly-prepared primary hepatocytes for analysis of their sensitivity to prothrombin action. An identical procedure was used to prepare an extracellular matrix from hepatocyte cultures.

2.8. Fibronectin isolation from bovine fibrinogen

Bovine fibrinogen (Fraction I, Sigma, St. Louis, MO) solution (200 ml of 1% in 50 mM Tris pH 7.5, 0.9% NaCl) was loaded onto a Sepharose 4B (Pharmacia, Sweden) column (12 cm × 3 cm) and washed with 2-column volumes TBS buffer (1 M NaCl and 50 mM Tris, pH 7.5). The flow-through fractions were collected and loaded onto a Gelatin-Sepharose 4B (Pharmacia, Sweden) column (10 cm × 3 cm). The Gelatin-Sepharose 4B column was then eluted with a buffer containing 5 M urea and 50 mM Tris (pH 7.5). Peak fibronectin-containing fractions (5 ml/fraction) were collected and pooled (24 Scott et al., 1981). Urea in the buffer was removed by dialysis in 5 M CAPS (pH 11). Protein concentration was determined by measuring the absorbance at 280 nm. The fibronectin was used to coat tissue culture dishes, which were then incubated over 18 hr in a tissue culture hood without their covers. Then the dishes were washed with PBS and used for plating cells.

2.9. Plasma prothrombin measurements and warfarin and prothrombin treatment in vivo

Laurell rocket method was employed for quantitating rat plasma prothrombin antigen. Plasma was applied to wells punched in an agarose gel containing a homogenous mixture of antibodies. The prothrombin antigens under estimation migrate on application of an electric field (20 mA constant current). An antigen antibody complex forms within the gel resulting in precipitation. The height of the precipitates is proportional to the amount of antigen, and was quantified against known pure standard prothrombin (Laurell, 1972). Warfarin (2.5 mg/kg body weight) was injected intraperitoneally 1 h after PH. PT (200 U/mg body weight) was also injected intraperitoneally 4 times at 1, 5, 12 and 18 h after PH. Hepatocyte DNA synthesis was measured in vivo by incorporation of BrdU (50 mg/kg body weight, intraperitoneal injection).

2.10. Statistical significance calculation

Statistical significance was calculated by "t-test".

3. Results

3.1. Inhibition of hepatocyte DNA synthesis by prothrombin

Freshly-prepared primary cultures of normal adult rat hepatocytes, were plated on a matrix of fibronectin 0.5 µg/dish, and incubated in the continuous presence of EGF (10 ng/ml), with fresh daily change of medium and EGF, with or without prothrombin. Addition of various concentrations (0, 0.125, 0.25, 0.5, and 1 U/ml) of prothrombin led to a dose dependent decrease

in EGF-induced DNA synthesis (Fig. 1A). Stimulation of DNA synthesis by HGF was also inhibited (data not shown). When hepatocytes were incubated with prothrombin for various times (0, 1, 6, 24, 48, 72 h), a time-dependent inhibition of DNA synthesis was also found (Fig. 1B), suggesting that some cellular event might be required for prothrombin inhibitory actions. Cultured hepatocytes treated with prothrombin were labeled with BrdU (10 μ mol/l for 1 h) in order to confirm the inhibitory effects of prothrombin on EGF-mediated DNA synthesis. Fig. 1C shows photomicrographs of prothrombin-treated and-untreated cells stained with BrdU. 30% of hepatocytes, which had been incubated only EGF only, stained positively, but only 9% of hepatocytes stained positively after treatment with both EGF and prothrombin. Another K vitamin dependent coagulation factors IX also showed this growth-inhibitory action. However, prothrombin that had been stripped of its carboxyl groups, resulting in DCP, no longer had this growth inhibitory (nor any growth stimulatory) action (Fig. 1D).

3.2. Matrix and cytoskeletal changes induced by prothrombin

Unlike tumor cell lines, normal hepatocytes do not attach to tissue culture dishes unless the dishes have previously been coated with a matrix. Fibronectin, collagen type I, IV or serum are commonly used. We determined the effects of prothrombin on normal hepatocytes, which were plated on either fibronectin or collagen type I coated dishes and found that although the hepatocytes responded similarly to EGF-stimulation, whether they were plated on fibronectin or collagen type I matrix, the EGF-induced DNA synthesis was inhibited by prothrombin, in a dose dependent manner (from 0.25 to 1 U/ml), only in cells plated on fibronectin but not on collagen type I (Figure 2A). The cells also underwent a change in cell shape and become elongate (Figure 2B). In order to examine possible mechanisms of cell detachment from fibronectin, but not from collagen matrix, we examined whether there might be a decrease of fibronectin amount when it was incubated with PT (1 U/ml) for various time periods (0, 6, 12, 24, and 48 h), either in the presence or absence of hepatocytes. We found that incubation of pure fibronectin in the presence of prothrombin and hepatocytes resulted in a time dependent loss of fibronectin amounts (Fig. 3A lane 2). Staining of the gels with Coomassie blue revealed the presence of lower molecular weight bands on the gels, suggesting the appearance of fibronectin degradation fragments over time. This effect was not observed in the absence of hepatocytes (Fig 3A, lane 1). Since we had found changes in cell shape (Fig. 2B), we stained the hepatocyte cultures for the presence of fibrillar actin (F-actin) and found that there was a loss of this polymerized form of the actin cytoskeleton (Fig. 3B). Western blots of protein lysates from hepatocytes that had been cultured in presence of prothrombin, showed a time-dependent (0 to 48 h) decrease in F-actin with concomitant increase in G-actin (Fig. 3C). Thus, prothrombin action resulted in a loss of fibronectin, the depolymerization of actin and change in cell shape.

3.3. Hepatoma growth is not inhibited by prothrombin, likely due to altered matrix

We next examined whether there might be a differential sensitivity to growth inhibition on the part of HCC cell lines compared with normal hepatocytes. Two human (Hep3B, HepG2) and two rat (MH7777, JM-1) hepatoma cell lines were grown in serum-free conditions (to exclude exogenous fibronectin) on plastic tissue culture dishes and examined for their sensitivity to growth inhibition by prothrombin (0 to 2 U/ml) action. None of the cell lines had any significant growth inhibition after 3 d at doses of prothrombin (1 and 2 U/ml) that completely inhibited EGF-induced hepatocyte DNA synthesis (Fig. 4A). Primary cultures of adult rat hepatocytes require a matrix for optimum viability and response to mitogens. However, continuous cultures of a variety of hepatoma cell lines grow well when plated directly in plastic tissue culture dishes, without the need for any exogenous matrix. We considered the possibility that the HCC cell lines might secrete their own matrix, which could optimize their cell growth and this might be responsible for their resistance to the growth inhibitory actions of prothrombin, since we found that hepatocytes are also resistant to the growth effects of prothrombin, depending on

the matrix on which they are plated (Fig. 2A). We tested this possibility by growing Hep3B cells on plastic tissue culture dishes and then lysed the cells in Triton X-100, which dissolved and removed the cellular structure. These same dish on which the Hep3B cells had grown but were now removed, were washed 3 times and were then used to plate freshly isolated primary adult rat hepatocytes, using the matrix that had been only deposited by the growing Hep3B cells. We found that when the effects of prothrombin (1 U/ml) were tested in an identical manner to that used in Fig. 1, the hepatocytes were now entirely resistant to the inhibitory effects of prothrombin on EGF-induced DNA synthesis (Fig. 4B). Matrix-coated dishes were also made in an identical manner from primary cultures of normal hepatocytes. However, after these hepatocytes were lysed, and fresh hepatocytes were then plated on the hepatocyte matrix, prothrombin (1 U/ml) inhibited EGF-induced DNA synthesis, just as on fibronectin matrix (Fig. 4B). This result suggested that the HCC cell lines had deposited a matrix on the dish, which likely was different than that from normal hepatocytes, and caused subsequently-plated hepatocytes to be resistant to the growth inhibitory effects of prothrombin.

3.4. Prothrombin role in liver regeneration

The regenerative response of rat liver after a 2/3 partial hepatectomy (PH) is a well studied model of epithelial cell growth in adult mammals and the first wave of hepatocyte DNA synthesis in vivo after a PH has been used for the study of relatively synchronized normal epithelial cell growth in vivo (Michalopoulos and DeFrances, 1997). It has previously been shown that after a PH in both rats and humans (hepatic lobectomy or trisegmentectomy), there is an increase in the plasma prothrombin time, reflecting a transient decrease in prothrombin amounts and action, likely secondary to loss of 2/3 of the liver mass that is the source of prothrombin. We found that there was a similar decrease in prothrombin amounts in the plasma, starting approximately 6 hr, reaching a minimum at 24 h and returning to near-normal levels by 72 hr post PH (Fig. 5A).

In order to examine whether prothrombin could alter DNA synthesis in vivo, as it did in vitro, we examined the effect of the prothrombin inhibitor warfarin in vivo on DNA synthesis after a PH. Warfarin is used in humans as an anticoagulant in the treatment and prevention thrombosis. It is thought to work by inhibiting the vitamin K cycle and leads to a controllable and reversible increase in the prothrombin time, which in turn is used clinically to adjust the doses of warfarin (Fasco et al., 1982; Wallin and Martin, 1987). We found that a single dose of warfarin (2.5 mg/kg body weight) could enhance (2-fold) the peak level of DNA synthesis at 24 hr post PH, consistent with the idea that levels of prothrombin in vivo might have an effect in modulating liver cell growth (Fig. 5B).

We next examined the effects of prothrombin injections after PH. A single intraperitoneal dose of prothrombin (200 U/kg body weight) did not alter the peak of DNA synthesis. However, 4 injections caused a partial antagonism of this peak (Fig. 5B). The reasons why prothrombin could not completely abrogate the DNA synthesis response to a PH are not yet clear. However, we were also not able to completely restore the plasma prothrombin levels to baseline by administration of exogenous prothrombin during PH. In addition, there are several other K-vitamin dependent blood coagulation factors, which could also be involved in limiting the regenerative response and their levels would not have been enhanced by exogenous prothrombin, as well as TGF- β , which may also play a role in the negative control of hepatocyte DNA synthesis in vivo.

4. Discussions

Two serum markers for HCC have long been recognized. The best known is alpha-fetoprotein, which we have found to be elevated in about 50% of our HCC patients (Virji et al., 1992). By contrast, the more recently recognized marker DCP (des- γ carboxy prothrombin) has been

found to be increased in over 70% of patients with HCC, although it is less specific than AFP, since it is also increased in biliary diseases and by the anticoagulant action of warfarin (Weitz et al., 1993; Huisse et al., 1994; Liebman et al., 1984; Nakao et al., 1991; Liebman et al., 1989; Li et al., 2002; Lefrere et al., 1987; Shah et al., 1987; Tsai et al., 1990). The action of warfarin has been identified to be on the vitamin K cycle, in which oxidized vitamin K cannot be cycled back to the reduced and physiologically active form (Fasco et al., 1982), when warfarin was present. As a consequence of warfarin action, vitamin K cannot act as a carboxylase cofactor, leading to a decrease in mature prothrombin production (and an increase in DCP), and thus to anticoagulation. The exact biochemical defect in HCCs, in which immature prothrombin or DCP is secreted by the HCC (but not by the underlying liver) instead of mature prothrombin, has never been clearly identified, although it is thought to result from an inability of the carboxylase to function normally, possibly as a result of a vitamin K transport defect (Li et al., 2002). We have previously shown that this is partly dependent on the vitamin K dose available, since in human hepatoma cell lines in culture, we were able to correct this defect by addition of exogenous vitamin K (Wang et al. 1995). The same correction was found after administration of pharmacological doses of vitamin K1 to HCC patients (Lefrere et al., 1987; Carr BI, 1996). We also found that vitamin K1 could weakly inhibit cell growth both in vitro and possibly in human HCC patients (Carr BI, 1996). Others have also found that K vitamins have some inhibitory action on cell growth in vitro (Yoshida et al., 2003; Lamson and Plaza, 2003; Yoshiji et al., 2005; Yokoyama et al., 2005; Oztopcu et al., 2004; Shibayama et al., 2003; Miyazawa et al., 2001; Sun et al., 2000; Taper et al., 2004). The mechanism(s) for the weak growth inhibitory actions of natural K vitamins have not been identified. Since the major physiological function of K vitamins is to act as a cofactor for carboxylation of prothrombin (factor II) and other K vitamin dependent coagulation factors (VII, IX and X) as well as proteins C and S, we considered the possibility that prothrombin itself might have growth regulatory activity. In addition, it was recently shown that DCP has some mild growth stimulatory activity for hepatoma cells in vitro (Suzuki et al, 2005), and therefore we wished to examine whether the fully carboxylated form of prothrombin might also have growth regulatory effects.

The current experiments show that prothrombin is an inhibitor of EGF-induced DNA synthesis in attached primary cultures of normal adult rat hepatocytes in tissue culture on a fibronectin matrix. This is both a time dependent and a dose dependent phenomenon. Prothrombin does not seem to exert its inhibitory activity if it is only present for 6 hr in cell culture, suggesting that some other biochemical event, which requires time, might be necessary for its action inside the cell, such as conversion to thrombin. Hepatocytes that were plated on collagen, did not have any inhibition of their EGF-induced DNA synthesis after exposure to prothrombin. This suggested a possible mechanism, namely that a primary effect of the prothrombin might be on the fibronectin matrix. This was examined by measuring the amounts of fibronectin in the presence of prothrombin and hepatocytes. Prothrombin caused a time dependent decrease in fibronectin amounts, but only in the presence of hepatocytes. This was thought likely to be caused by fibronectin degradation, as additional lower molecular weight proteins were seen on gels from prothrombin-treated hepatocytes.

Since cell shape also changed on treatment with prothrombin, inferring cytoskeletal changes, the cells were stained for polymerized F-actin. They were found to have lost actin fibers after prothrombin treatment and thus had a major change in their cytoskeleton. A Western blot for F-actin amounts was performed on lysates of cells exposed to prothrombin. This showed a loss of the polymerized F-actin and a concomitant increase in the unpolymerized G-actin. Prothrombin thus caused a loss of fibronectin, depolymerization of the cytoskeletal actin and change in cell shape. The mechanisms by which prothrombin did this are not yet examined, although we speculate that protease action is likely involved, either by prothrombin itself, or after its conversion to the protease thrombin. The integrins have been shown to be involved in cell signaling, and are in turn influenced by and tethered to the cell matrix. Their alteration is

therefore likely to disrupt the mitogenic (EGF, HGF) signaling pathways. Since actin is tethered to integrins, any integrin changes would probably disrupt growth factor receptor-linked signaling pathways. These will need to be examined in future work, as well as the mechanisms of fibronectin alterations.

We examined the effects of prothrombin on the growth of 2 rat and 2 human HCC cell lines, which grow well in plastic tissue culture dishes without the need for matrix supplementation, and found that none of them had significant inhibition of their growth by prothrombin. Since it has been previously reported that HCCs make different matrix components from normal liver (Torbensoen et al., 2002; Gulubova et al., 1997; Zhao et al., 1996; Koukoulis et al., 1995; Torimura et al., 1994; Grigioni et al., 1991; Jagirdar et al., 1985; Szendroi et al., 1985; Sell and Ruoslahti, 1982; Theret et al., 2001; Giannelli et al., 2001; Lebail et al., 1997; Kin et al., 1994; Tabarin et al., 1987), we examined the possibility that this relative resistance of HCC cell lines to prothrombin, could be attributable to an HCC matrix that was different from normal hepatocytes. We tested this idea by growing Hep3B human hepatoma cells to partial confluence and the HCC cells were then lysed in their tissue culture dishes. The tissue culture dishes were then washed and these same dishes with the secreted matrix proteins from the growth of the HCC cells, were then used as matrix for addition of freshly isolated rat hepatocytes, which were then plated on these 'used' dishes. We found that these normal rat hepatocytes were completely resistant to the inhibitory effect of prothrombin on EGF-induced DNA synthesis, providing support for the idea that the effects of prothrombin might be on the hepatocyte interaction with its matrix. Dishes containing matrix secreted by normal hepatocytes were also used for subsequent fresh hepatocyte attachment. These cells underwent EGF-induced DNA synthesis and prothrombin-mediated inhibition, just as found using a fibronectin matrix. This would provide a novel mechanism for growth regulation, based on control of cell matrix components. Previous reports from other labs have indicated that the composition of the cell matrix can influence intracellular signaling (Sawada et al., 1986; Bissell and Choun, 1988; Mooney et al., 1992; Nagaki et al., 2000; Hong et al., 2000; Bhadriraju and Hansen, 2002; Kim et al., 2003; Rahaman et al., 2005; Dipersio et al., 1991). The experiments reported here suggest two different mechanisms by which HCCs might evade normal growth regulation by prothrombin. In the first, their secretion of different matrix proteins would confer on them the ability to resist prothrombin effects on cell growth. In the second, their well-described secretion of DCP instead of mature prothrombin, could be viewed as an adaptive mechanism for escaping the negative growth controls of prothrombin in the tumor microenvironment.

We considered the possibility that prothrombin might also have a negative regulatory control on growth in vivo, since it has previously been shown that with major hepatic lobectomies in human liver surgery, prothrombin time has been found to increase (reflecting a decrease in prothrombin activity) both in humans and rats (Furnival et al., 1976; Poulsen et al., 1985; Pelton et al., 1998; Antivic et al., 1993; Yildirim et al., 1981; Farinati et al., 2005; Oguro et al., 1993; Warner, 1939; Ettore et al., 2001). Although this was thought to be simply a loss of protein amounts due to loss of liver mass, we reasoned that this might also be a reflection of an in vivo growth regulatory mechanism based upon prothrombin actions in preventing liver over-growth. If true, then the late increase in prothrombin levels after 48 hr post PH could be viewed as an in vivo mechanism for limiting the degree of the regenerative response of the liver. We tested this idea both directly and indirectly. To test it indirectly, we treated rats after PH with warfarin, to further increase the plasma prothrombin time (resulting from a decrease in prothrombin activity) and we found an increase in the peak of DNA synthesis at 24 hr in the warfarin-treated PH rats, compared to untreated control PH rats. We also tested this idea positively, by injecting rats post PH with prothrombin protein, and found that we could then decrease the peak of DNA synthesis in response to PH, but could not completely suppress it. However, we also could not completely restore the normal levels of prothrombin in the hours after PH by administration of exogenous prothrombin. Another possible explanation for the

failure of prothrombin to completely suppress the peak of DNA synthesis post PH, might be that other vitamin K dependent proteins might also be involved in normal negative regulation (such as factors IX, shown in Fig. 1) as well as other non-K vitamin dependent in vivo growth regulators such as TGF- β 1.

It is unclear whether prothrombin protein is the effector molecule for the previously-published mild growth-inhibiting actions of vitamin K for the following reasons. Prothrombin is a much more potent growth inhibitor than either vitamins K1 or K2. However, this could simply reflect the difficulty of cellular uptake of the lipid soluble K vitamins. Interestingly however, the K vitamins shared with prothrombin a preferential growth inhibitory action on normal hepatocytes, compared with HCC cell lines. Others have also shown a growth-inhibiting action for prothrombin components (74-76 Rhim et al., 1998; Lee et al., 1998; Kim et al., 2002). Taken together, these experiments show that prothrombin can inhibit hepatocyte DNA synthesis, induce cell shape change and loss of fibronectin in hepatocytes plated on fibronectin, but not on collagen matrix. Hepatoma cells are resistant to its action in all these respects. In addition, the changes in prothrombin activity in vivo are consistent with the hypothesis that prothrombin could also act as a negative growth regulator to limit the liver regenerative response in vivo. The data suggests a novel action for this major blood coagulation protein and a new mechanism for cell growth control, based on selective synthesis of an important matrix component. Details of the mechanisms for fibronectin loss still need to be established.

We propose two hypotheses based on our results: 1) PT is a novel inhibitor of hepatocyte DNA synthesis. This is lost when PT is undercarboxylated (DCP), which is neither an inhibitor nor a promoter of hepatocyte DNA synthesis. This happens in hepatomas, which can only synthesize DCP, suggesting an adaptive escape mechanism by hepatomas; 2) Hepatocytes are inhibited by PT in fibronectin matrix, but not on collagen type-I matrix. This could also contribute to resistance by hepatomas to PT-mediated growth inhibition, as they have a complex, but different matrix than normal hepatocytes. DCP is also a hepatoma mitogen. Hence DCP production is advantageous for hepatoma growth in 2 ways: 1) it escapes PT-mediated growth inhibition, and 2) it uses DCP as a growth factor. The inhibitory action of prothrombin on hepatocytes and loss of this effect when it is under-carboxylated (DCP) thus provides a possible biological significance for DCP secretion by HCCs.

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Abbreviations

DCP, des-gamma-carboxy prothrombin; FN, fibronectin; PH, 2/3 partial hepatectomy; HCC, hepatocellular carcinoma; WB, western blot; PT, prothrombin; BrdU, bromo-deoxyuridine; EGF, epidermal growth factor.

6. References

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Fig1.

Effects of prothrombin and K vitamin dependent proteins on EGF-induced DNA synthesis of primary hepatocytes in culture. *A*, Effects of various prothrombin concentrations on the peak of DNA synthesis at 24 h; *B*, Effects of time of exposure to prothrombin 1 U/ml on the peak of EGF induced DNA synthesis at 72 h; *C*, photograph of cells stained with BrdU after treatment with EGF (control) or after EGF plus prothrombin (1 U/ml) for 48 h; *D*, Effects on EGF induced hepatocyte DNA synthesis of prothrombin, des-gamma carboxy prothrombin (DCP), factor IX (all at 1 U/ml) and controls (EGF alone).

Fig 2. Effects of matrix on prothrombin-mediated inhibition of hepatocyte DNA synthesis. *A*, Hepatocytes were plated on fibronectin or collagen-I (0.5 μ g/dish). All cells were treated with continuous EGF (10 ng/ml) and the designated doses of prothrombin (0 to 1 U/ml) for 48 h and DNA synthesis was measured in hepatocytes following prothrombin treatment; *B*, photomicrograph of changes in cell shape of hepatocytes treated with EGF plus prothrombin (upper photo) compared to untreated control hepatocytes treated with EGF alone (lower photo)

Fig3.

Changes in matrix and cytoskeleton induced by prothrombin. *A*, effects of prothrombin (1 U/ml for 0, 6, 12, 24, and 48 h) on fibronectin amounts on culture dishes in the absence (lane 1, upper) and presence of hepatocytes (lane 2, lower); *B*, photomicrograph of hepatocytes treated with EGF alone (upper) or with EGF and prothrombin and stained with FITC-phalloidin (lower); *C*, levels of F-actin and G-actin in hepatocytes treated with prothrombin (1 U/ml) for various times (0, 6, 12, 24, and 48 h).

Fig4.

Effects of prothrombin on the growth of hepatoma cell lines. 2 human (Hep3B and HepG2) and 2 rat (JM-1, MH7777) hepatoma cell lines were grown to semi-confluence in 10% serum and then the serum was removed and replaced with serum-free medium for 1 d. *A*, prothrombin was added at the indicated concentrations (0, 0.125, 0.5, 1, and 2 U/ml) and cell growth was measured after a further 3 days; *B*, effects of prothrombin (1 U/ml) on EGF-induced DNA synthesis in hepatocytes that were plated on either fibronectin or matrix made either by Hep3B cells or hepatocytes.

A. **B.** **Fig5.**

Effects of 2/3 PH and warfarin on prothrombin plasma levels and on hepatic DNA synthesis. *A*, plasma prothrombin levels (assayed by electrophoresis) at various times after a 2/3 PH; *B*, hepatic DNA synthesis (BrdU incorporation) 24 h after sham surgery or a 2/3 PH, a PH plus warfarin, or a PH plus prothrombin injections. 4 rats were used in each group and statistical significance was calculated by "t-test".