Effect of Warfarin on Prothrombin Synthesis and Secretion in Human Hep G2 Cells

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Prothrombin synthesis and secretion were studied in a human hepatoma cell line (Hep G2) incubated with $^{35}$S-methionine for 2 to 24 hours at 37°C. Extracellular and intracellular prothrombin were detected by immunoprecipitation with affinity-purified antiprothrombin antibody. Incorporation of $^{35}$S-methionine into prothrombin was monitored by counting specific bands excised from 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Prothrombin represented 0.3% to 0.7% of total newly synthesized protein secreted into the media.

Warfarin had no effect on total prothrombin synthesis (extracellular plus intracellular). However, warfarin inhibited secretion of newly synthesized prothrombin by 58% to 73% over a 2 to 4 hour period. This was accompanied by the intracellular accumulation of an immunoprecipitable species of prothrombin of 78 kd, 8 kd less than extracellular prothrombin. At the end of 4-hour incubation with warfarin, intracellular prothrombin increased from 44% to 82% (twofold) of total prothrombin, whereas extracellular prothrombin decreased from 56% to 19% (threefold) of total prothrombin. After 24-hour incubation with warfarin, intracellular and extracellular immunoprecipitable prothrombin approached control values. Deglycosylation of extracellular and intracellular prothrombin with hydrofluoric acid (HF) resulted in a decrease in mol wt for both species to 66 kd. Endoglycosidase-H treatment, which digests "early mannosyl" residues, resulted in a decrease in the mol wt of the intracellular species of 8 kd with no effect on the extracellular species. Thus, the lower mol wt intracellular species that accumulates following early warfarin treatment is due to the presence of incompletely processed carbohydrate chain. The data are compatible with the hypothesis that optimum glycosylation and secretion require Vitamin K-dependent carboxylation.

The role of warfarin in decreasing plasma prothrombin biologic activity has been known for 41 years. In humans, this is associated with decreased secretion of an inactive molecule, as determined by immunassay. The inactive molecule has the same approximate mol wt as the biologically active molecule but is missing the carboxylated residues on the γ carbon of ten glutamic acid residues at the N terminal end (first 33 residues) of the molecule. Vitamin K activates a microsomal carboxylase which carboxylates these ten glutamic acid residues at the γ carbon site.

We studied the effect of warfarin on human Hep G2 cells and found that intracellular prothrombin accumulates following warfarin treatment as a lower mol wt precursor. An ~6,000-kd difference between extracellular and intracellular prothrombin appears to be due to partial glycosylation of the prothrombin molecule due to a block in the removal of mannosyl residues. The details of these investigations are cited below.

MATERIALS AND METHODS

All reagents were obtained from Sigma Chemical, St Louis, unless indicated otherwise. Minimum essential medium (MEM) was obtained from GIBCO, Grand Island, NY. Warfarin was obtained from Endo Laboratories, Garden City, NY. Sodium dodecyl sulfate (SDS), polyacrylamide, and Affi-gel 15 were obtained from BioRad, Richmond, CA. HF was obtained from Fisher Scientific, Fairlawn, NJ. Endoglycosidase-H was obtained from Boehringer Mannheim, Indianapolis. $^{35}$S-Methionine (1,000 Ci/mmol) was obtained from New England Nuclear, Boston. 125I-Phosphocollagen protein A (30 mCi/mg) was obtained from Amersham, Arlington Heights, IL.

Human hepatoma G2 cells (obtained from American Type Culture Collection, Rockville, MD) were grown in MEM plus 10% fetal calf serum (FCS) until confluence. They were then washed in methionine-free MEM (GIBCO) and incubated in methionine-free MEM with or without MEM (GIBCO) + 1 to 5 μg/mL warfarin (Endo) in 25-cm² tissue culture flasks (Falcon, Becton Dickinson, Oxnard, CA) for 1 to 24 hours at 37°C with $^{35}$S-methionine (150 μCi/mL) and 10⁻⁴ mmol/L phenylmethylsulfon fluoride (PMSF) in a total volume of 3 mL containing ~1 x 10⁴ cells. Extracellular and intracellular total synthesized protein and prothrombin were determined after careful attention to experimental detail, which required development of reliable methodology for these measurements.

Total synthesized protein. Total synthesized protein secreted into the medium and present in the cell lysate was determined by applying an aliquot of either material (50 μL of supernatant, 50 μL of Triton X-100 lysate) to 10% SDS polyacrylamide gel electrophoresis (SDS-PAGE) and counting radioactivity in regions of the gel corresponding to proteins of 10 to 150 kd. This methodology was necessary to obtain reproducible quantitative data. Early experiments in which proteins were analyzed for radioactivity after precipitation with trichloroacetic acid (TCA) proved unreliable because of nonspecific adsorption of radioactivity. Cell lysate was prepared by addition of 3 mL 0.5% Triton X-100 to the tissue culture flask, which had been aspirated of media and washed three times in 3 mL fresh media. The suspension was boiled in 2% SDS for 2 minutes, and the 100,000 g supernatant was used for assays.

Prothrombin synthesis. Prothrombin synthesis was determined by immunoprecipitation of medium or cell lysate (boiled in 2% SDS) with affinity-purified rabbit anti-prothrombin antibody. The complex was isolated by addition of 200 μL media or cell lysate to 600 μL buffer A plus 40 μL affinity-purified antiprothrombin antibody (0.2 mg/mL) and was incubated overnight at 4°C. Buffer A contained 0.5% Triton X-100, 150 mmol/L NaCl, 5 mmol/L EDTA, 0.02% sodium azide, and 50 mmol/L Tris-HCl, pH 7.4. Eighty microliters of a 50% suspension of insoluble staphylococcal cell wall protein A (Sigma) in buffer A was then added, and the mixture was incubated at room temperature for 2 hours. The suspension was centrifuged at 13,000 g, washed six times in buffer A, and resuspended in 50 μL of

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buffer A. An equal volume of a solution containing 2% SDS, 10% glycerol, 5% mercaptoethanol, 10 μg prothrombin, 0.06 mmol/L Tris-HCl, pH 6.8, was then added, and the suspension was heated at 95°C for 5 minutes. The material was then centrifuged as above, and the supernatant was applied to 10% SDS-PAGE for excision of radioactive bands following staining with Coomassie brilliant blue, as well as for autoradiography (Fig 1). Excision of the specific radioactive prothrombin bands eliminated the error of nonspecific adsorption of contaminating radioactive bands to the immune precipitate. This methodology was necessary to obtain reproducible quantitative data.

Preparation of affinity-purified rabbit anti-human prothrombin antibody. Human prothrombin purified by the method of Bajaj and Mann showed one band on 10% SDS-PAGE prior to immunization. Rabbit antiprothrombin IgG was purified by chromatography on diethylaminoethyl (DEAE)-52 after precipitation from serum with 50% saturated ammonium sulfate. The precipitate was dissolved in 0.1 molar phosphate buffer, pH 7.6, dialyzed against the same buffer and applied to a DEAE-52 column equilibrated in the same buffer. The flowthrough material contained purified IgG. The purified IgG was applied to an Affi-gel 15 column to which 5 mg/mL purified human prothrombin had been coupled. The adsorbed antibody was eluted with 0.1 mmol/L glycine, pH 2.5, and neutralized with 1 mmol/L Tris buffer to a pH of 7.0. This antibody, when used in a Western Blot of human plasma, developed with Coomassie brilliant blue, gave a single band of ~84 kDa on autoradiography.

Deglycosylation. Deglycosylation was performed by treatment of aliquots of cellular or media samples with HF by the method of Keutmann et al. Prothrombin-containing samples were immunoprecipitated as described above, and the immune complexes were collected on immobilized protein A (Sigma). The prothrombin (along with IgG) was removed from the agarose beads by heating in 1% SDS at 90°C for 10 minutes, followed by centrifugation. The sample was then reduced with 1 mmol/L 2-mercaptoethanol and alkylated with 1.1 mmol/L iodoacetamide. After dialysis against water, the samples were lyophilized in Teflon-coated flasks and then suspended in 1 mL anisole. After evaporation, samples were cooled in liquid nitrogen, and 10 mL of HF (condensed by cooling in liquid nitrogen) was added. After vigorous stirring at 0°C for 1.5 hours, the HF was removed under vacuum. Residual anisole was removed by washing with cold anhydrous ether, and the deglycosylated proteins were dialyzed against phosphate-buffered saline (PBS) prior to SDS-PAGE.

Endoglycosidase-H digestion. Endoglycosidase-H digestion was performed with aliquots of cellular or media samples to determine the presence or absence of "high" mannose oligosaccharide chains by a modification of the method of Tarentino et al. Prothrombin–antibody complexes were collected on immobilized protein A as described above. The beads were then washed in 0.01 mmol/L PBS, pH 7.4, suspended in PBS containing 2 mmol/L PMSF and incubated with 20 μU/mL of endoglycosidase-H overnight at 30°C. Residual prothrombin was eluted with 1% SDS and heated at 90°C as above, and the supernatant was applied to SDS-PAGE.

RESULTS

Synthesis of a lower molecular weight intracellular prothrombin species and effect of cycloheximide and warfarin on newly synthesized extracellular and intracellular prothrombin. Figure 2 shows an autoradiogram of prothrombin synthesis at 2 and 4 hours of incubation without and with cycloheximide (10^{-5} mmol/L) or warfarin (1 μg/mL). The extracellular apparent 84-kd band (~2.3, SD, nine experiments) immunoprecipitated by antiprothrombin antibody in lanes 1 and 4, representing newly synthesized prothrombin, and the apparent 78-kd band (~4.5 SD, nine experiments) in lanes 7 and 9, representing newly synthesized intracellular prothrombin. Cycloheximide inhibited extracellular (lanes 2 and 4).

![Fig 1. Coomassie brilliant blue stain of 10% SDS-PAGE of an aliquot of media obtained from a 4-hour incubation with 1 × 10^6 Hep G2 cells. Varying concentrations of purified human prothrombin were added after immunoprecipitation with affinity-purified rabbit anti-human prothrombin antibody. Lanes 1 through 4 refer to addition of prothrombin at 25, 12.5, 6.25, and 0 μg. The gel was dried on Whatman 3 MM paper, and the region corresponding to the prothrombin band was excised for radioactive measurements of prothrombin synthesis in other studies. The position of standard mol wt markers is shown (far right lane) in kilodaltons.](fig1)

![Fig 2. Autoradiogram after 10% SDS-PAGE of immunoprecipitates of aliquots of media and Hep G2 cells incubated with cycloheximide or warfarin for 2 and 4 hours at 37°C in MEM plus 29-3S-methionine. Lanes 1 through 3 represent aliquots of media from control, cycloheximide (2 × 10^{-5} mmol/L) or warfarin (1 μg/mL) incubations at 2 hours. Lanes 4 through 6 represent the same aliquots at 4 hours. Lanes 7 through 9 represent aliquots of cell lysates from control, cycloheximide, or warfarin-treated cells at 4 hours. Outer lanes refer to positions of radioactive standard mol wt markers.](fig2)
and 5) and intracellular (lane 8) prothrombin synthesis. Warfarin inhibited the secretion of newly synthesized prothrombin (lanes 3 and 6) but resulted in the accumulation of newly synthesized intracellular prothrombin at 4 hours (lane 9). Confirmation that the radioactive bands at 84 and 78 kd were prothrombin was obtained by demonstrating inhibition of immunoprecipitation with purified human prothrombin at 1 mg/mL (Fig 3) with lack of inhibition at 100 µg/mL prothrombin (data not shown).

Effect of deglycosylation on extracellular and intracellular prothrombin species. Figure 4 shows the effect of deglycosylation on the mol wt of intracellular and extracellular prothrombin without and with warfarin. The resultant identical apparent mol-wt species is ~66 kd.

Effect of endoglycosidase-H on extracellular and intracellular prothrombin species. Figure 5 shows the effect of endoglycosidase-H on the molecular weight of intracellular and extracellular prothrombin without and with warfarin. The decrease in apparent mol wt of the intracellular species at 2 and 4 hours from 79 (lanes 6 and 8, respectively) to 71 kd (lanes 5 and 7, respectively), without an effect on the extracellular mol-wt species (lanes 1 through 4), indicates the presence of mannosyl residues on the intracellular species.

Cycloheximide inhibition of total secreted protein synthesis. Figure 6 shows a kinetic analysis of secreted newly synthesized total protein without and with cycloheximide, at 2 and 4 hours. Cycloheximide inhibited secreted total protein synthesis by ~75%.
Effect of warfarin on newly synthesized secreted prothrombin. Newly synthesized secreted prothrombin varied between 0.27% and 0.67% of total secreted protein synthesis during a 2- to 24-hour period of incubation (Table 1). Warfarin inhibited secretion of newly synthesized immunologically detectable prothrombin by 58% to 73% during a 2-to 4-hour period. However, this inhibition was no longer detectable after 24 hours of incubation (Fig 7), media lane 2 (4 hours) as compared with lane 4 (24 hours). This could be accounted for by a decrease in warfarin-induced accumulation of intracellular prothrombin at 24 hours as compared with accumulation at 4 hours (Fig 7, cells in lane 4 (24 hours) as compared with lane 2 (4 hours)). A kinetic analysis is cited below.

Effect of warfarin on extravascular and intracellular newly synthesized prothrombin at 4 and 24 hours. In the experiment to determine the effect of warfarin on extravascular and intracellular newly synthesized prothrombin at 4 and 24 hours, prothrombin synthesis at 4 hours represented 0.39% ± 0.04% (SEM) of total protein synthesis; 56% was extravascular and 44% intracellular. Warfarin (1 μg/mL) had no effect on total prothrombin synthesis, (442 ± 43 (SEM) vs 511 ± 56 cpm, P > .2, with and without warfarin, respectively, eight experiments). However, extravascular secretion decreased to 19% (3-fold, P < .001), and intracellular accumulation increased to 82% (1.9-fold, P < .001, Fig 8).

Prothrombin synthesis at 24 hours represented 0.31% ± 0.08% of total protein synthesis: 73% was extracellular and 27% intracellular. However, warfarin no longer had its marked effect on intracellular prothrombin accumulation (Figs 7 and 8). Intracellular accumulation decreased from 82% to 42% (twofold, P < .001) whereas extravascular secretion increased from 19% to 59% (threefold, P < .001). This was confirmed by performing a Western blot analysis of extravascular and intracellular prothrombin without and with warfarin, obtained from samples incubated in the absence of 35S-methionine (data not shown). Similar results were obtained with as well as without 10% FCS. This effect was

| Table 1. Effect of Warfarin on Secreted Newly Synthesized Prothrombin |
|-----------------------------|-----------------|-----------------|-----------------|-----------------|
| Experiment | 2 h | Change (%) | 4 h | Change (%) | 24 h | Change (%) | P |
| 1. Control (4) | 0.672 ± 0.164 | — | 0.518 ± 0.126 | — | — | — | — |
| 2. Control (4) | 0.274 ± 0.029 | — | 0.303 ± 0.063 | — | — | — | — |
| Warfarin (4) | 0.085 ± 0.030 | —69 | 0.083 ± 0.009 | —73 | — | — | — |
| 3. Control (4) | — | — | 0.382 ± 0.043 | — | 0.376 ± 0.102 | — | <.004, <.007 |
| Warfarin (4) | — | — | 0.161 ± 0.027 | —58 | 0.301 ± 0.045 | —20 | <.02, >.10 |

Data are expressed as percentage of prothrombin synthesized ± SEM. Secreted newly synthesized total protein was calculated from radioactivity of excised radioactive bands on SDS-PAGE from 10 to 150 kd. Secreted newly synthesized prothrombin was calculated from radioactivity of the excised prothrombin band on SDS-PAGE of immunoprecipitated prothrombin, localized with added prothrombin marker. P value refers to differences between control and warfarin for 2 and 4 hours, respectively (experiment 2), or 4 and 24 hours, respectively (experiment 3), obtained by Student’s t test, matched samples.
EFFECT OF WARFARIN ON PROTHROMBIN

Fig 8. Quantitation of effect of warfarin on newly synthesized extracellular and intracellular prothrombin at 4 hours (panel A) and 24 hours (panel B). Percentage of extracellular and intracellular prothrombin was calculated from radioactive measurements obtained from excised extracellular and intracellular prothrombin bands after electrophoresis of prothrombin immunoprecipitates on 10% polyacrylamide SDS gels. C and W refer to control and warfarin incubations, respectively. SEM is given.

not due to inactivation of warfarin, since similar results were obtained with 5 μg/mL warfarin; and warfarin in the media from the 24-hour warfarin incubation was still active when added to fresh Hep G2 cells for 4 hours (ie, accumulation of intracellular prothrombin and decrease in secretion of extracellular prothrombin, data not shown).

DISCUSSION

These data indicate that human hepatoma cells can secrete newly synthesized prothrombin of 83,000 daltons at a linear rate for 4 hours. Secreted prothrombin synthesis represented 0.3% to 0.7% of total secreted protein synthesis and was inhibitable by cycloheximide. Warfarin inhibited this secretion by 58% to 73%. Warfarin inhibition was accompanied by a parallel increase in the intracellular accumulation of a lower mol wt prothrombin species of 77,000 da.

The human hepatoma cell line Hep G2 has also been used by Fair and Bahnak,13 who analyzed its ability to synthesize and secrete several coagulation proteins, factor X, prothrombin, and antithrombin III. Secreted prothrombin had a mol wt of 73,000 by SDS-PAGE, 10,000 daltons lower than our findings. This discrepancy may be related to our use of the Laemmli procedure for SDS-PAGE,9 as compared with the Weber and Osborn14 technique used by Fair and Bahnak.13 Swanson and Suttie,15 using the Laemmli procedure, also recently reported a plasma mol wt of 83,000 for rat prothrombin. In addition, the same authors also noted a lower mol wt species of prothrombin in the microsomal fraction of 78,500 daltons, which accumulated with warfarin treatment. This lower intracellular mol wt species in the rat appears to be the same as the lower intracellular mol wt species that we noted in the human hepatoma cell line. Similar observations were reported in an abstract of Munns et al.16 Both intracellular species accumulate with warfarin and both are susceptible to endoglycosidase-H digestion, indicating the presence of early mannosyl residues that have been incompletely processed. These precursors are found in the endoplasmic reticulum. Endoglycosidase-H will not digest the fully mature oligosaccharide complexes that have been processed by enzymes of the Golgi apparatus.17 This is substantiated by our observation that the secreted extracellular prothrombin species was not susceptible to endoglycosidase-H, as well as the observation of Swanson and Suttie that their higher mol wt species of 83,000 daltons was also not susceptible to endoglycosidase-H digestion. In addition, our data indicate that both the intracellular and extracellular species become identical in mol wt following deglycosylation with HF.

The effect of warfarin at 4 and 24 hours is of particular interest. After 4 hours of warfarin, low-mol-wt intracellular prothrombin accumulates markedly over control values in the presence of a marked decrease in extracellular secretion with no effect on total prothrombin synthesis. Thus, secretion has become rate-limiting. However, after 24 hours of warfarin, intracellular prothrombin decreased toward control levels in the presence of an insignificant decrease in extracellular secretion, with no effect on total prothrombin synthesis. Thus, an event has occurred following 24 hours of incubation, which increases the rate of intracellular prothrombin secretion despite the presence of warfarin. Under these conditions, extracellular prothrombin did not contain the lower mol wt prothrombin species. Thus, full and proper glycosylation of the intracellular prothrombin species was necessary for prothrombin secretion. This could not be explained on the basis of inactivation of warfarin or lack of viability of the hepatoma cell line, since this was not observed. Fair and Bahnak13 also observed no effect of warfarin on intracellular accumulation of prothrombin after 5 days of cell culture. These data are compatible with the hypothesis that glycosylation is linked to Vitamin K-dependent carboxylation of γ-glutamyl residues (ie, carboxylation is required for optimal carbohydrate processing), and that proper carbohydrate processing is required for optimal secretion of the prothrombin molecule. An apparent conflict with this interpretation is the observation of Swanson and Suttie15 that glycosylation was not essential for secretion of prothrombin from the liver following treatment of rats with tunicamycin (which prevents N-glycosylation of newly synthesized proteins). However, this does not rule out the possibility that a partially glycosylated molecule with incompletely processed carbohydrate chain may be incapable of being secreted from the liver. Furthermore, Swanson and Suttie15 did note a decrease in prothrombin secretion of tunicamycin-treated rats but explained this effect on the nonspecific decrease in total protein synthesis. Preliminary studies from our laboratory with tunicamycin treatment of Hep G2 cells indicate a 45% decrease in prothrombin secretion when data are expressed as percentage of total protein synthesis.

The reason for the changeover in secretion and intracellular accumulation between 4 and 24 hours remains to be established. Further experiments will be necessary to determine whether long-term warfarin treatment induces the formation of an enzyme(s) that enhances glycosylation or stimulates transport out of the endoplasmic reticulum.
this regard, warfarin is capable of inducing at least one enzyme, the Vitamin K-dependent carboxylase of rat liver.18

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Effect of warfarin on prothrombin synthesis and secretion in human Hep G2 cells

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