The vitamin K-dependent carboxylation system in human osteosarcoma U2-OS cells

Antidotal effect of vitamin K₁ and a novel mechanism for the action of warfarin

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An osteoblast-like human osteosarcoma cell line (U2-OS) has been shown to possess a vitamin K-dependent carboxylation system which is similar to the system in human HepG2 cells and in liver and lung from the rat. In an *in vitro* system prepared from these cells, vitamin K₁ was shown to overcome warfarin inhibition of γ -carboxylation carried out by the vitamin K-dependent carboxylase. The data suggest that osteoblasts, the cells involved in synthesis of vitamin K-dependent proteins in bone, can use vitamin K₁ as an antidote to warfarin poisoning if enough vitamin K₁ can accumulate in the tissue. Five precursors of vitamin K-dependent proteins were identified in osteosarcoma and HepG2 cells respectively. In microsomes (microsomal fractions) from the osteosarcoma cells these precursors revealed apparent molecular masses of 85, 78, 56, 35 and 31 kDa. When osteosarcoma cells were cultured in the presence of warfarin, vitamin K-dependent ¹⁴C-labelling of the 78 kDa precursor was enhanced. Selective ¹⁴C-labelling of one precursor was also demonstrated in microsomes from HepG2 cells and from rat lung after warfarin treatment. In HepG2 cells this precursor was identified as the precursor of (clotting) Factor X. This unique ¹⁴C-labelling pattern of precursors of vitamin K-dependent proteins in microsomes from different cells and tissues reflects a new mechanism underlying the action of warfarin.

INTRODUCTION

Vitamin K is essential for post-ribosomal processing of certain secretory glycoproteins by the vitamin K-dependent carboxylase [1,2]. The carboxylase converts specific glutamic acid residues to γ -carboxyglutamic acid residues in a reaction which requires CO₂, O₂ and reduced vitamin K, giving the proteins Ca²⁺-binding properties [1].

Tissues and cells receive vitamin K from the environment as the quinone form of the vitamin [1]. In the cell the vitamin is reduced to the hydroquinone form, the active cofactor used by the carboxylase [3]. On completion of the γ -carboxylation reaction, vitamin K hydroquinone is converted into vitamin K 2,3-epoxide [1]. The epoxide can be reduced back to the hydroquinone, thus establishing a cyclic interconnection of the reduced/oxidized forms of vitamin K known as the 'vitamin K cycle' [1,4]. From liver studies it has been shown that vitamin K is reduced to the active cofactor by enzymes belonging to two independent reduction pathways [3,5]. These are: (1) pathway I, a pathway catalysed by vitamin K epoxide reductase, a microsomal enzyme which can reduce both the epoxide and the quinone form of the vitamin; this enzyme is highly sensitive to inhibition by coumarin anticoagulant drugs and is currently believed to mediate the anticoagulant effect of these drugs [1]; (2) pathway II, a pathway catalysed by nicotinamide-nucleotide-dependent enzymes which are less sensitive to inhibition by these drugs [5,6]. Pathway II mediates the antidotal effect of vitamin K in overcoming coumarin poisoning [3].

Recently, Price & Kaneda [8] have presented results which question the ability of vitamin K to overcome warfarin inhibition of synthesis of vitamin K-dependent proteins by bone. They showed that the serum concentration of the vitamin K-dependent

bone protein osteocalcin was not enhanced when large doses of vitamin K were administered to warfarin-treated rats [8]. This has raised the question of whether or not pathway II is present in osteoblasts, the cells responsible for osteocalcin synthesis. Using an osteoblast-like cell line from rat, Ulrich *et al.* [9] obtained data which suggest that pathway II is not present in these cells, thus providing a tentative explanation of the observations by Price & Kaneda [8].

Our laboratory has developed an *in vitro* system which mimics the vitamin K-dependent carboxylation system in rat and human tissues and measures the activities of pathways I and II [3]. We have used this system to study vitamin K-dependent carboxylation in the human osteosarcoma cell line U2-OS, which has been shown to have characteristics similar to osteoblasts [10]. These cells have a vitamin K-dependent carboxylation system which includes pathway II. The cells respond to warfarin by a mechanism that appears to be similar to that in human HepG2 cells and also in liver and lung from rats.

MATERIALS AND METHODS

Cell culture

U2-OS osteosarcoma cells and human HepG2 cells were obtained from the American Type Culture Collection. The U2-OS cell line was grown in McCoy's medium containing 5% (v/v) fetal-bovine serum and the HepG2 cell line in minimal essential medium (MEM) supplemented with 10% (v/v) fetal-calf serum. Upon confluency, (25–30) × 10⁶ cells were incubated for 24 h with various concentrations of warfarin present in the medium. The concentrations used are stated in the text. Controls had saline (0.9% NaCl) instead of warfarin added to the medium.

Abbreviations used: DTT, dithiothreitol; warfarin, 3-(α -acetylbenzyl)-4-hydroxycoumarin; vitamin K₁H₂, fully reduced vitamin K₁; iPr₂P-F, diisopropyl fluorophosphate; MEM, minimal essential medium.

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Preparation of cell microsomes (microsomal fractions)

Cells were harvested by low-speed centrifugation and washed three times in 25 mM-imidazole/250 mM-sucrose, pH 7.2, containing 5 mM-benzamidine and 1 mM-di-isopropyl fluorophosphate (iPr₂P-F) (buffer A). The final cell pellet was suspended in 1 ml of buffer A and sonicated on ice for three 10 s intervals at 50 % duty cycle using a Branson Sonifier 250. Sonicated cells were centrifuged at 10000 *g* for 10 min. Microsomal particles from the sonicated material were obtained as a pellet after centrifugation of the 10000 *g* supernatant at 100000 *g* for 60 min. Tissue microsomes from rat lung and liver were obtained as described in [11].

Enzyme activities

Vitamin K-dependent carboxylase activity was measured as ¹⁴CO₂ incorporation into the synthetic pentapeptide Phe-Leu-Glu-Glu-Leu as described in [3]. All incubations contained 2 mM-pentapeptide and 5 mM-DTT. Incubations with chemically reduced vitamin K₁ (vitamin K₁H₂) contained 100 µg of the reduced vitamin/ml. Incubations with the quinone form of the vitamin (vitamin K₁) contained 100 µg of the commercially available vitamin K₁ preparation Aquamephyton/ml. Carboxylase activity supported by pathways I and II was determined in incubations containing vitamin K₁+DTT and vitamin K₁+NADH+warfarin respectively. The concentrations of NADH and warfarin were 2 mM and 50 µM respectively [3]. Incubations were carried out for 30 min at 25 °C.

Vitamin K epoxide reductase activity was measured in cell microsomes resuspended in buffer A. Vitamin K₁ 2,3-epoxide (10 µM) and DTT (5 mM) were added to 0.5 ml of the suspensions and incubated at 25 °C for 30 min. Enzymic conversion of vitamin K₁ 2,3-epoxide into vitamin K₁ was measured after separation and quantification of the two components on a C₁₈ reversed-phase h.p.l.c. column as described in detail elsewhere [7].

DT-diaphorase (EC 1.6.99.2) activity was measured as described by Dallner [12], with dichlorophenol-indophenol as electron acceptor and NADH as donor. The activity which could be neutralized by 50 µM-dicumarol was determined as enzyme activity [11].

¹⁴C-labelling of microsomal carboxylase substrates

Cell microsomes were suspended in 25 mM-imidazole/0.5 % CHAPS, pH 7.2 containing 1 mM-iPr₂P-F and 5 mM-benzamidine (buffer A) using a Dounce homogenizer. The suspension was incubated for 30 min at 25 °C in the presence of 5 mM-DTT/H¹⁴CO₃⁻ (40 µCi/ml)/1 mM-MnCl₂/chemically reduced vitamin K₁H₂ (100 µg/ml). Controls were identical incubations carried out in the absence of reduced vitamin. ¹⁴C-labelling of vitamin K-dependent proteins in liver and lung microsomes were carried out as described in [13]. In some experiments the microsomal membrane fragments that were present in incubations after ¹⁴C-labelling of carboxylase substrates were separated from soluble protein by centrifugation at 100000 *g* for 60 min. The isolated membrane fragments were washed by resuspending them in buffer A followed by centrifugation at 100000 *g* for 45 min. Complete incubation mixtures or separated membrane fragments and soluble protein from such mixtures were dialysed against 10 mM-NH₄HCO₃/5 mM-benzamidine and freeze-dried before labelled proteins were subjected to SDS/PAGE.

Quantitative immunoabsorption

Microsomal membrane fragments were solubilized in 25 mM-imidazole / 1.5 % Triton X-100 / 0.5 M-KCl / 250 mM-sucrose, pH 7.2, containing 5 mM-benzamidine and 1 mM-iPr₂P-F (buffer

B). In preliminary experiments, various concentrations of anti-serum were added to the solubilized membrane fragments in order to estimate the amount of antiserum required to remove quantitatively the antigen from the solution. Usually 25–50 µl of antiserum was needed to remove the antigen from a 1 ml solution containing 10 mg of protein. Removal of antigen was confirmed by Immunodot blotting as described below. Immunocomplexes were removed from the solution with *Staphylococcus aureus* Protein A particles (Pansorbin, Calbiochem, La Jolla, CA, U.S.A.) and prepared for SDS/PAGE and fluorography as described in [2].

Immunodot blotting

For quantitative estimation of prothrombin and Factor X antigen in microsomal membrane fragments, aliquots of 1–2 µl of membranes solubilized in buffer B were spotted on nitrocellulose membranes and allowed to react with antisera against human prothrombin and human Factor X respectively as described in [2]. The purified clotting factors were used as standards. The nitrocellulose membrane was then allowed to react with 0.1 µCi of ¹²⁵I-Protein A/ml and prepared for scintillation counting as described in [2]. Antisera were obtained as described in [2].

SDS/PAGE and fluorography

SDS/PAGE was performed as described by Laemmli [14] in reducing 10 %-polyacrylamide gels. Standard proteins used for molecular-mass estimations were [¹⁴C]methylated standard proteins purchased from Amersham Corp. Fluorography of SDS/PAGE gels was carried out as described in [2] after treating the gel with En³Hance (du Pont–New England Nuclear). Scanning of fluorograms was carried out with a Hoefer Scientific Instruments scanning densitometer.

Materials

Vitamin K₁ and warfarin were from Sigma, St. Louis, MO, U.S.A. The vitamin was reduced to vitamin K₁H₂ with dithionite as described by Sadowski *et al.* [15]. The pentapeptide Phe-Leu-Glu-Glu-Leu was from Vega Fox Biochemicals Division (Tucson, AZ, U.S.A.). NaH¹⁴CO₃ (60 mCi/mol) was from ICN, Costa Mesa, CA, U.S.A. ¹²⁵I-Protein A (88 µCi/µg) was from NEN Research Products, Boston, MA, U.S.A. Aquamephyton was from Merck Sharp and Dohme. All other chemicals were reagent grade or better. Protein was measured with the Bio-Rad (Richmond, CA, U.S.A.) protein assay.

RESULTS

Human HepG2 cells are known to produce vitamin K-dependent clotting factors [16] and were therefore used as a positive control to study the vitamin K-dependent carboxylation system in human osteosarcoma U2-OS cells. When chemically reduced vitamin K₁H₂ was added to the carboxylase test system, microsomes from the HepG2 cells, and also the osteosarcoma cells, revealed significant carboxylase activity (see Table 1; vitamin K₁H₂). The data in Table 1 also demonstrate that both cell lines have the enzymes that are necessary to provide the carboxylase with reduced vitamin K₁ cofactor in the presence of warfarin. Indeed when vitamin K₁ reduction was carried out by pathway II (vitamin K₁+NADH), both cell lines revealed significant carboxylase activity with 50 µM-warfarin present in the test system (see Table 1). Vitamin K₁ reduction by the warfarin-sensitive pathway I could also be demonstrated in both cell lines (Table 1; vitamin K₁+DTT). Carboxylase activity triggered with pathway I was, however, never as high as this activity triggered with pathway II (Table 1).

Table 1. Carboxylase activity in cell microsomes

γ-Carboxylation of the pentapeptide Phe-Leu-Glu-Glu-Leu was measured in microsomes isolated from human osteosarcoma U2-OS and human HepG2 cells as described in the Materials and methods section. Carboxylase activity was triggered with chemically reduced vitamin K₁H₂ (vitamin K₁H₂) and reduced vitamin produced by pathway I (vitamin K₁+DTT) and pathway II (vitamin K₁+NADH+warfarin) respectively. The results shown are the average activities measured in three parallel incubations differing by < 10%. Activities measured in the absence of vitamin K₁ were 85–100 c.p.m./mg of protein.

Additions	Cell type...	Carboxylase activity (c.p.m./mg of microsomal protein)	
		Osteosarcoma	HepG2
Vitamin K ₁ H ₂		13 648	23 994
Vitamin K ₁ +DTT		640	1 047
Vitamin K ₁ +NADH+warfarin		1 700	8 147

Table 2. DT-diaphorase and vitamin K epoxide reductase activities in osteosarcoma U2-OS and HepG2 cells

DT-diaphorase activity was measured as dicumarol-sensitive NADH reduction of 1,2-dichlorophenol-indophenol in suspensions of sonicated cells. Vitamin K epoxide reductase activity was measured as conversion of vitamin K₁ 2,3-epoxide into vitamin K₁ quinone in cell microsomes (see the Materials and methods section). Activities are averages of three independent measurements differing by < 10% and < 5% for vitamin K epoxide reductase and DT-diaphorase respectively.

Cell type	DT diaphorase activity (nmol/min per mg of protein)	Vitamin K epoxide reductase activity (pmol/mg of protein)	
		– Warfarin	+ Warfarin*
Osteosarcoma	77.2	152	11
HepG2	84.2	177	19

* Measured activity with 10 μM-warfarin present in test system.

In liver and lung one of the enzymes participating in vitamin K₁ reduction by pathway II has been identified as DT-diaphorase [11,17]. We measured the activity of this enzyme in homogenates of the osteosarcoma and the HepG2 cells and found the specific enzyme activity to be similar in the two cell lines (Table 2). DT-diaphorase activity was measured as dicumarol-sensitive diaphorase activity (see the Materials and methods section). Of total diaphorase activity measured in sonicated cells, 93 and 87% of the activity was inhibited with 50 μM-dicumarol present in assay mixtures with HepG2 and osteosarcoma cells respectively. Warfarin-sensitive vitamin K epoxide reductase activity was also measured in microsomes from both cell lines, thus providing additional evidence for the presence of pathway I (Table 2).

Precursors of vitamin K-dependent proteins synthesized by the osteosarcoma-cell line are shown in Fig. 1(a). Before SDS/PAGE these proteins had been ¹⁴C-labelled in a vitamin K-dependent carboxylation reaction and thus represent the endogenous carboxylase substrates. Five protein bands were easily distinguished on the fluorogram, and these bands appeared to have molecular masses of 85, 78, 56, 35 and 31 kDa (Fig. 1a, lane +K). The appearance of these ¹⁴C-labelled precursors on fluorograms was also investigated in samples from osteosarcoma cells that had

Fig. 1. ¹⁴C-labelled precursors of vitamin K-dependent proteins in human osteosarcoma U2-OS cells

Microsomes were isolated from control cells and cells that had been cultured for 24 h in the presence of 50 μM-warfarin. Endogenous precursors of vitamin K-dependent proteins were ¹⁴C-labelled and subjected to SDS/PAGE and fluorography as described in the Materials and methods section. A fluorogram of electrophoresed proteins from control cells is shown in (a). Lanes '–K' and '+K' have proteins from incubations carried out in the absence (–K) or presence (+K) of chemically reduced vitamin K₁H₂. (b) Shows a fluorogram of ¹⁴C-labelled proteins from control cells (lane 'cont.') and warfarin treated cells (lane 'warf.'). A 30 μg portion of microsomal protein was loaded on to each of the lanes in (b). Lane 'St.' contains standard protein markers.

been treated with 50 μM-warfarin overnight. As shown in Fig. 1(b), lane 'warf.', ¹⁴C-labelling of the 78 kDa band was clearly enhanced in the sample prepared from warfarin-treated cells. Densitometric scanning of the fluorogram demonstrated a 12-fold increase in labelling intensity of the 78 kDa band over the control when the integrated scanned area was calculated per μg of protein applied to the gel. On the other hand, scanning of the photographic film revealed no difference in ¹⁴C-labelling of the other precursors present in the samples from control and warfarin-treated cells (Fig. 1b, lanes 'cont.' and 'warf.' respectively).

A similar observation was made with warfarin-treated HepG2 cells. Fig. 2 shows ¹⁴C-labelled precursors of vitamin K-dependent proteins produced by these cells. Of the five precursors that easily could be distinguished on the fluorogram (molecular masses 86, 70, 56, 52 and 36 kDa), only the 70 kDa protein band revealed enhanced ¹⁴C-labelling after warfarin treatment of the cells. In this cell line it was found, as described below, that the precursor of (clotting) Factor X was responsible for enhanced labelling of the 70 kDa band. In our vitamin K-dependent ¹⁴C-labelling system, where microsomes are suspended in a 0.5% concentration of the detergent CHAPS [2], we found all detectable ¹⁴C-labelled proteins to be associated with the membrane

Fig. 2. ^{14}C -labelled precursors of vitamin K-dependent proteins in human HepG2 cells

Microsomes were isolated from control cells and cells that had been treated for 24 h in the presence of $50\text{ }\mu\text{g}$ of warfarin. Endogenous precursors of vitamin K-dependent proteins were ^{14}C -labelled and subjected to SDS/PAGE and fluorography as described in the Materials and methods section. Lanes 'C' and 'W' have ^{14}C -labelled proteins from control and warfarin-treated cells respectively. Lane '-K' has proteins from control cells that had been labelled in the absence of vitamin K.

fragments in the suspension (results not shown). Similar results have recently been obtained with rat liver microsomes [2]. Thus, for identification of ^{14}C -labelled precursors, we used the membrane fragments after extensive washing of the fragments in 0.5% CHAPS as described in the Materials and methods section. These membranes were solubilized with detergent and reacted with an excess of anti-prothrombin or anti-(Factor X) serum for quantitative immunoabsorption. Fluorograms of isolated immunocomplexes from cells treated with saline and $50\text{ }\mu\text{M}$ -warfarin are shown in the inset to Fig. 3, panels (a) and (b) respectively. Lanes a and b have ^{14}C -labelled antigens isolated with the anti-prothrombin and anti-(Factor X) sera respectively. Clearly, the radioactivity associated with the immunoabsorbed anti-(Factor X) antigen was enhanced in microsomes from warfarin-treated cells (Fig. 3, inset panel b, lane b). On the other hand, when the drug was added to the microsomal suspension used for vitamin K-dependent labelling of carboxylase substrates, instead of being added to the cell culture, warfarin had no effect on labelling of the microsomal membrane proteins. Thus the enhanced labelling of the Factor X precursor pool in the membrane was indeed the result of an effect of the drug on the HepG2 cells in culture. As the inset to Fig. 3 shows, the prothrombin and the Factor X precursor revealed approximately the same molecular mass (70 kDa) and thus appeared together as one broad band on SDS/10%-PAGE (Fig. 2).

Fig. 3 also presents data on ^{14}C -labelling of the prothrombin and the Factor X precursors in microsomal membrane fragments from cells treated with various concentrations of warfarin. Labelling per μg of membrane protein was estimated after

Fig. 3. ^{14}C -labelling of prothrombin and Factor X precursors in microsomes from HepG2 cells

Membrane fragments from HepG2 cell microsomes with ^{14}C -labelled precursors of vitamin K-dependent proteins were used for the experiments (see the Materials and methods and also text for details). The fragments were solubilized with the detergent Triton X-100 and subjected to quantitative immunoabsorption with antisera against the human plasma coagulation factors Factor X and prothrombin. Immunocomplexes were isolated with *Staphylococcus aureus* protein A and subjected to SDS/PAGE in 10% gels. Panels (a) and (b) (inset) show fluorograms of electrophoresed immunocomplexes isolated from HepG2 cells treated with saline and $50\text{ }\mu\text{M}$ -warfarin in culture for 24 h respectively. Lanes a and b have the anti-prothrombin and anti-(Factor X) antigens respectively. The graph shows specific ^{14}C -labelling of the anti-(Factor X) (●) and the anti-prothrombin (▲) antigen pool in membranes from cells treated with various concentrations of warfarin in culture for 24 h. Specific ^{14}C -labelling (units/ μg of protein) was estimated per μg of membrane protein used for immunoabsorption. The integrated scanned area on fluorograms showing the labelled antigen was used to estimate labelling intensity.

quantitative immunoabsorption, SDS/PAGE of immunocomplexes and scanning of their fluorograms. As Fig. 3 shows, labelling of the membrane pool of the prothrombin precursor was the same whether or not the cells had been treated with warfarin. On the other hand, there was a dose-response relationship between the concentration of warfarin in the medium and labelling of the Factor X precursor pool in the membrane. As shown in Fig. 3, labelling of the factor X precursor per μg of membrane protein in membranes from cells treated with $10\text{--}50\text{ }\mu\text{M}$ -warfarin had increased 11-fold over this labelling in membranes from saline-treated cells.

Similar data were obtained when the concentration of the anti-prothrombin and anti-(Factor X precursor) antigens were estimated in the membranes. In the membranes from saline- and warfarin-treated cells the prothrombin concentration remained unchanged, with an average calculated value of $0.25\text{ }\mu\text{g}$ of precursor/mg of membrane protein. The variation in precursor concentration between the different preparations was $<10\%$. On the other hand, in the same samples, the anti-(Factor X) antigen concentration increased from 0.04 to $0.10\text{ }\mu\text{g}/\text{mg}$ of membrane protein. Thus treating the cells for 24 h with warfarin had increased significantly the membrane concentration of the Factor X precursor. Importantly, it appears that warfarin had a selective effect on the Factor X precursor concentration.

To investigate whether or not this unique ^{14}C -labelling pattern

Fig. 4. ^{14}C -labelled precursors of vitamin K-dependent proteins in rat lung and liver

Lung microsomes from control rats and microsomes from liver and lung obtained from rats treated for 24 h with 30 mg of warfarin/kg were used for vitamin K-dependent ^{14}C -labelling of endogenous proteins. The labelled proteins were subjected to SDS/PAGE in 5% gels. Lanes (b) and (c) show ^{14}C -labelled proteins in lung microsomes from control and warfarin-treated rats respectively. Each lane has 40 μg of protein. Lane (a) shows ^{14}C -labelled proteins from liver microsomes. The arrowhead points to the ^{14}C -labelled prothrombin precursor (lane a) in liver microsomes [2] which has the same apparent molecular mass as the lung protein(s) showing enhanced ^{14}C -labelling due to warfarin treatment of the rat (lane c).

reflected a common mechanism underlying the action of warfarin in cells and tissues, we included in these studies lung microsomes from normal and warfarin-treated rats. Fig. 4(c) demonstrates a similar phenomenon also in lung microsomes from warfarin-treated rats. In this experiment proteins were electrophoresed into an SDS/5% PAGE gel in order to compare more accurately the migration of proteins with molecular masses 70–80 kDa. In the lung, enhanced ^{14}C -labelling was associated with a protein of the same apparent molecular mass (78 kDa) as the rat prothrombin carboxylase substrate (Fig. 4). Scanning of the fluorogram showed a 15-fold enhancement in labelling of this protein when estimated per μg of lung protein applied to the gel. A similar estimate for the proteins appearing on the gel in the 45–55 kDa range (Fig. 4) showed < 5% variation in specific ^{14}C -labelling between proteins from normal and warfarin-treated rats.

DISCUSSION

Here we present evidence for the existence of a warfarin-insensitive dehydrogenase enzyme(s) in osteosarcoma U2-OS cells that can reduce vitamin K_1 to provide the vitamin K-dependent carboxylase with active cofactor. In liver and lung this pathway-II enzyme(s) has been shown to mediate the antidotal effect of vitamin K_1 in overcoming warfarin inhibition of γ -carboxylation at normal tissue concentrations of the vitamin [3,11]. Our data are discordant with those reported by Ulrich *et al.* [9], who used a rat osteosarcoma-cell line for investigation of the vitamin K-dependent carboxylation system in bone. In the rat osteosarcoma-cell line these investigators were unable to measure pathway-II-supported γ -carboxylation. On the basis of similarities of this cell line with osteoblasts, those investigators proposed that osteoblasts may lack the pathway-II enzymes and thus are unable to utilize vitamin K_1 as a warfarin antidote [9]. The human osteosarcoma U2-OS cell line used here has been shown to be similar to osteoblasts. They contain calcitriol receptors, have the ability to produce alkaline phosphatase and

they responded to parathyroid hormone with cyclic AMP production [10]. As shown here, these cells also have a vitamin K-dependent carboxylation system which has an activity *in vitro* comparable with the system in human HepG2 cells. For the purposes of this Discussion, the most important characteristic of this cell line is an active pathway II. In fact in the presence of high concentrations of vitamin K_1 pathway II actually triggered a higher carboxylase activity in the test system than pathway I. This observation is the opposite of the normal activity of liver and lung cells [3,11]. Transformed cells have been shown to possess an elevated activity of the pathway-II enzyme DT-diaphorase [18]. Thus the high pathway-II activity measured in osteosarcoma and HepG2 cells was not surprising. On the basis of our data we propose that the lack of an antidotal effect of vitamin K_1 on bone, as demonstrated by Price & Kaneda [8], could result from incapability of this tissue to accumulate enough vitamin K_1 to trigger cofactor production via pathway II, which has a high K_m for the vitamin [3].

Another interesting observation in the present study is the unique ^{14}C -labelling pattern of precursors of vitamin K-dependent proteins from warfarin-treated cells and tissues. Among the several vitamin K-dependent proteins produced individually by osteosarcoma and HepG2 cells and the lung, only one protein revealed enhanced ^{14}C -labelling in response to warfarin treatment when the proteins were subjected to SDS/PAGE and fluorography. A similar phenomenon has been observed with precursors of vitamin K-dependent proteins produced in liver from warfarin-treated rats [2]. In that study the microsomal protein acquiring enhanced labelling was identified as the Factor X carboxylase substrate. It was shown that warfarin specifically induced more binding sites for the Factor X carboxylase substrate in the endoplasmic-reticulum membrane, resulting in enhanced γ -carboxylation of this precursor [2]. In the present study, similar results were obtained with human HepG2 cells. Since Factor X is not known to be synthesized by non-hepatic cells, the affected protein(s) in osteosarcoma U2-OS cells and the lung remain unknown. Two vitamin K-dependent proteins, osteocalcin and the matrix bone Gla (carboxyglutamic acid-containing) protein, are known to be produced by osteosarcoma cells [19]. The present paper provides, for the first time, evidence that osteosarcoma cells synthesize additional vitamin K-dependent proteins which may have important functions in bone metabolism.

The only known mechanism of action of warfarin has been its irreversible inhibition of vitamin K epoxide reductase, the pathway-I enzyme [20]. Thus the enhanced ^{14}C -labelling of a vitamin K-dependent protein in the microsomal fraction from different cells and tissues exposed to warfarin reflects a new mechanism underlying the action of the drug.

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