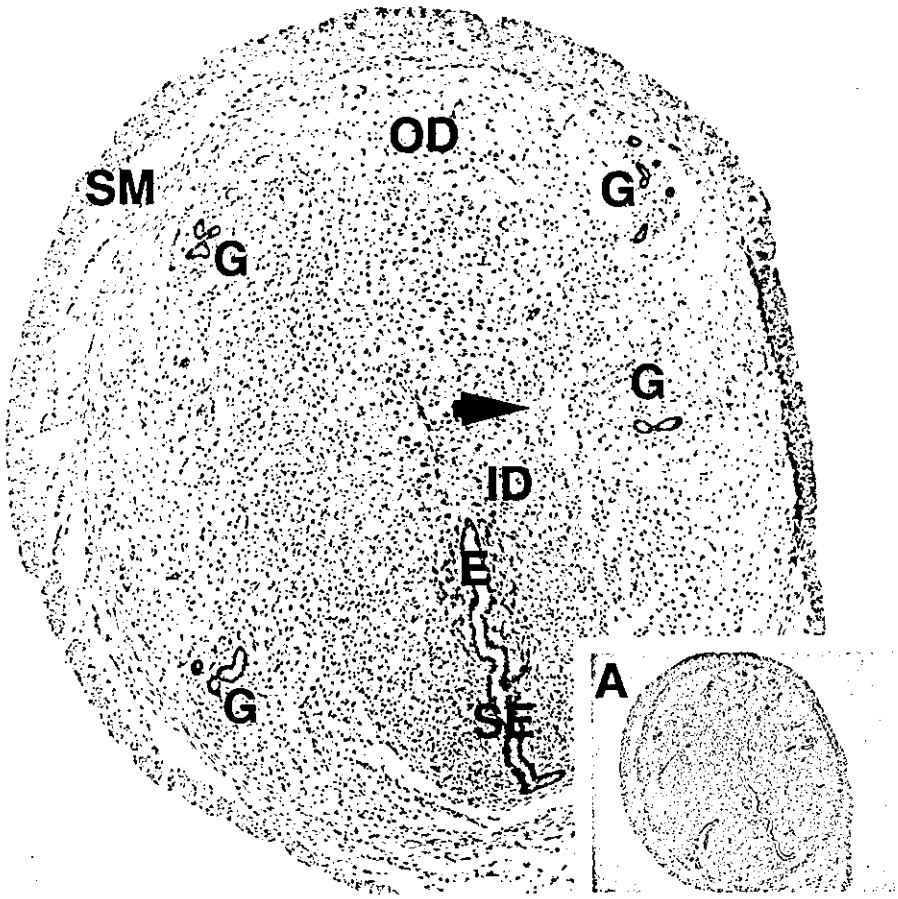

FIG. 3. Transverse section (5 μm) through gravid mouse uterus, 4.5 dpc, at the site of implantation (magnification ×4). Besides the staining of the secretory endometrial columnar epithelial cells (SE), staining of the inner decidual reaction zone (ID) and the glandular epithelial cells (G) in the outer decidual reaction zone (OD) can be seen. E, Embryonal cavity. Inset A shows negative control.

shown to play a role in influencing the growth and development of the embryo [11, 13], and the presence of α-TTP in the secretory epithelial cells suggests a major role of α-tocopherol in early pregnancy but also in the supply of the placenta with α-tocopherol throughout pregnancy.

α-TTP in the hepatocyte fulfills the role of transporting α-tocopherol after uptake from the chylomicron fraction and facilitating its secretion into plasma [4, 14, 15]. It is plausible that the secretory columnar epithelial cells can be compared with the hepatocyte regarding their expression of

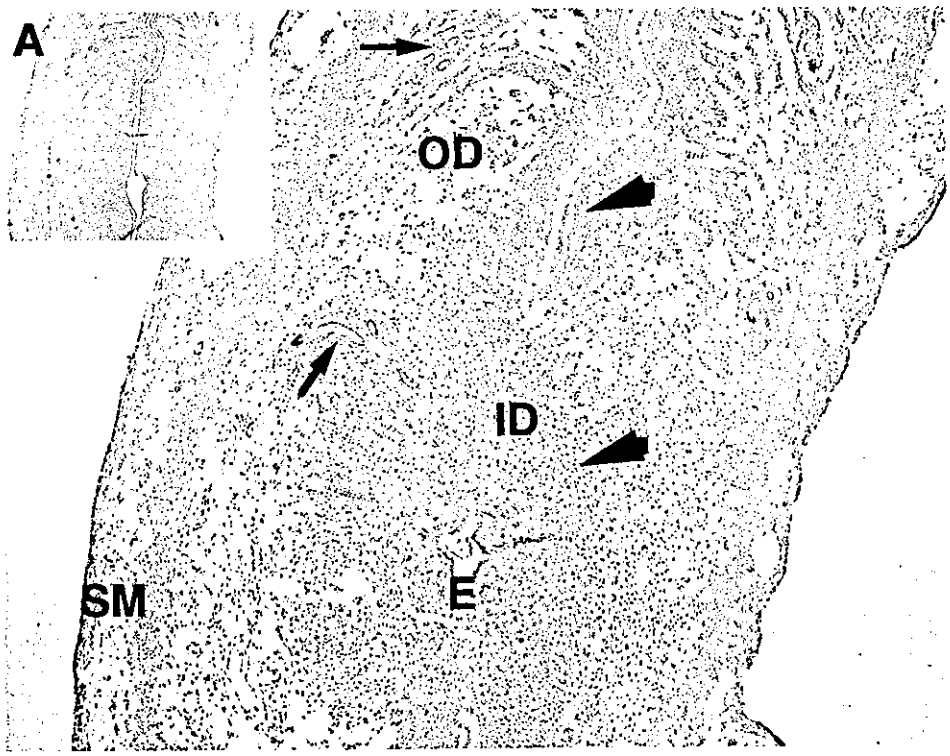

FIG. 4. Immunohistochemical localization of α-TTP-specific monoclonal antibody binding to secretory endometrial columnar epithelium (upper arrowhead) as well as to the glandular epithelial cells (lower arrow) in the inner decidual reaction zone (ID) and binding to the inner decidual reaction zone itself (lower arrowhead), pregnant uterus at 6.5 dpc (magnification ×4). Upper arrow shows nonstained glandular epithelium in the outer decidual reaction zone (OD). E, Embryonal cavity with embryonal tissue; SM, two layers of smooth muscle cells. Inset A shows negative control.

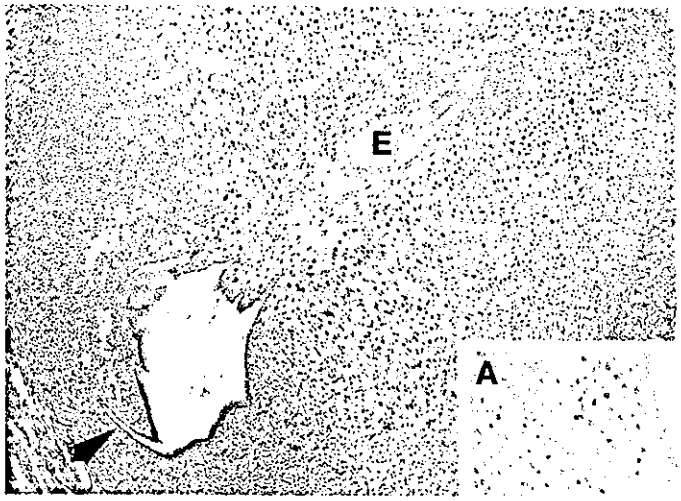

FIG. 5. Immunohistochemical localization of α-TTP-specific monoclonal antibody binding to secretory endometrial columnar epithelium (arrowhead) at 8.5 dpc. Note enlarged embryonal cavity with embryo (E) and enhanced decidualization around embryonal cavity. Inset A shows negative control of secretory endometrial columnar epithelium, magnification ×60.

α-TTP, facilitating the secretion of maternal α-tocopherol to the placenta and consecutively supplying the embryo and fetus with this antioxidative substance. The cellular localization of α-TTP is also of interest: While during implantation, α-TTP has no specific localization in the secretory columnar epithelial cell, α-TTP seems to be more abundant at the apical border of these cells facing the placenta at midpregnancy.

The fact that α-TTP is expressed in the uterine secretory epithelium throughout pregnancy stresses the fact that the fetus is not able to accumulate large amounts of α-tocopherol during pregnancy and is therefore dependent on a continuous supply by the mother [16]. Fetal tissue concentrations are extremely low and α-TTP in fetal rat liver is practically not detectable [17]. This is of great clinical importance with respect to the nutritional requirements in premature infants and their supplementation with vitamin E [18, 19]. Premature infants without major clinical symptoms are born with adequate vitamin E levels with respect to their gestational ages [19] but deplete very quickly if a sufficient supply with vitamin E is not feasible. In utero, the continuous supply of vitamin E, namely α-tocopherol, supplied via α-TTP in the uterine secretory epithelial cells to the placenta and fetus, is maintained. By premature birth, this supply is cut off and deficiency states can occur in sick, premature infants.

It has been reported that a 15-kDa transport protein for tocopherols exists in many tissues, including the human placenta [20, 21]. So far, the presence of the 30-kDa α-TTP could not be verified in the mouse placenta, but the necessity of this α-TTP and its transport of α-tocopherol for the normal development of the labyrinthine portion of the mouse placenta could be documented in our α-TTP-disrupted mouse model [9]. Due to the fact that the labyrinth of the mouse and the chorionic villi in the human placenta are homologous but not identical structures [22] and that the interhemal barrier in the mouse is hemotrichorial versus hemomonochorial in humans, the necessity of α-TTP for the development of the human placenta can be postulated. Furthermore, it could also be documented that the embryos in the α-TTP-disrupted mouse model showed developmental failure from 10.5 dpc, showing mainly neural tube malformations [9]. This, together with the observations made in premature infants, suggests that α-tocopherol also plays an essential role in normal fetal development in midterm and later pregnancy.

It is well known that the feto-placental unit is exposed to oxidative stress and that the placental membrane is very susceptible to peroxidation [2]. Therefore, the efficiency of enzymatic and nonenzymatic reactive oxygen scavengers must be ensured throughout pregnancy for normal fetal growth and development, not only in early but also in late pregnancy. The transport of vitamin E, mainly α-tocopherol

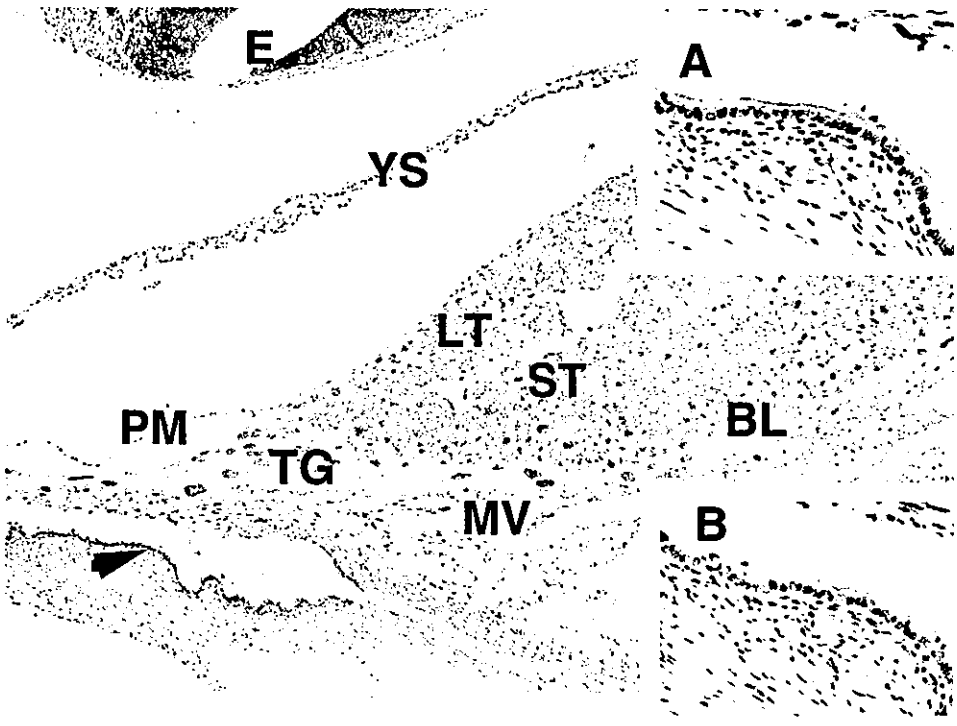

FIG. 6. α-TTP-specific monoclonal antibody binding to secretory endometrial columnar epithelium (arrowhead) of the pregnant uterus at midterm, 10.5 dpc, magnification ×4. Secretory endometrial columnar epithelium shows weaker binding than at prior gestational stages. E, Embryonal tissue; YS, visceral yolk sac; PM, placental membrane; LT, labyrinthine trophoblast; ST, spongiotrophoblast; TG, trophoblast giant cells; BL, basal layer of placenta; MV, dilated maternal blood vessels. Inset A shows magnification ×60 of secretory endometrial columnar epithelium marked by arrowhead; inset B is negative control.

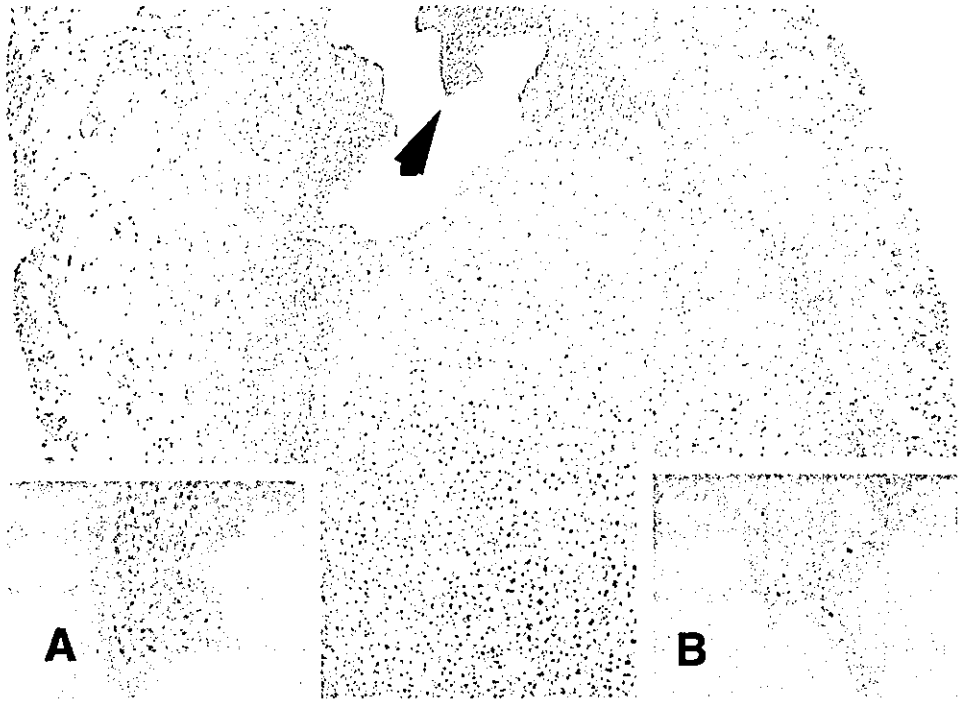

FIG. 7. α-TTP-specific monoclonal antibody staining of α-TTP knockout mouse uterus at 8.5 dpc. No staining is detectable; arrowhead marks secretory endometrial columnar epithelium. Inset **A** shows magnification ×60 of arrowhead area; inset **B** is negative control.

as a major chain-breaking antioxidant, to the fetus throughout pregnancy must be sustained and this must, at least in part, be managed by the presence of α-TTP in the uterine secretory luminal and glandular epithelial cells as well as of the inner decidual reaction zone.

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Human Placental Trophoblast Cells Express α-Tocopherol Transfer Protein

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α-Tocopherol transfer protein (α-TTP), a 30 kDa cytosolic protein first described to be present in the liver and important for α-tocopherol trafficking, plays a major role in maintaining α-tocopherol levels in plasma, while α-tocopherol is known as the major lipid-soluble antioxidant. Expression of α-TTP has not only been described in animal model liver, but also in diverse other tissues such as rat brain or pregnant mouse uterus, the latter finding stressing the importance of α-TTP for embryogenesis and foetal development. In this study, we report the identification of α-TTP in human liver by anti-human α-TTP monoclonal antibodies made in rat and the cellular localization of α-TTP in term human placenta. By immunohistochemistry, intense staining of α-TTP was seen in syncytiotrophoblast as well as in villous and invading extravillous cytotrophoblast, while basal decidual cells showed slighter, but present staining of α-TTP. Foetal vessel endothelium remained unstained. It is therefore suggested that α-TTP may play a major role in supplying α-tocopherol to the foetus prior to delivery and is likely involved in maintaining adequate α-tocopherol levels in the foetus.
Placenta (2003), 24, 439–444

INTRODUCTION

Vitamin E (α-tocopherol), a substance recognized as a major lipid-soluble chain-breaking antioxidant widely distributed in cellular membranes and organelles, is known as an important factor in the protection of polyunsaturated fatty acids against peroxidative damage. As a lipid-soluble vitamin, the human foetus acquires stores of vitamin E in the last 10 weeks of pregnancy, during which 90 per cent of fat deposition in the foetus occurs. While fatty acids, which make out a large proportion of the foetal fat accumulation, cross the placental microvillous and basal membranes by simple diffusion as well as by the action of membrane bound and cytosolic fatty acid binding proteins (Haggarty, 2002), the passage of vitamin E across the placenta is so far not well understood. Several clinical reports exist concerning the transport of vitamin E across the human and animal placenta: prepartum treatment with tocopheryl acetate in pregnant rabbits showed accumulation of the vitamin in the placenta, but not in amniotic fluid or foetal blood (Bortolotti, Traina, Barzago et al., 1990, and in pregnant sheep, treatment did not lead to increased serum vitamin E levels in neonatal lambs (Njeru, McDowell, Wilkinson et al., 1994, indicating inefficient placental transfer. Similarly, a recent study shows that vitamin E concentrations in piglet plasma and tissues before suckling do not increase significantly if the mother animals receive vitamin E 7 days prior to giving birth, stressing limited placental vitamin E transfer in sows (Lauridsen, Engel, Jensen et al., 2002). Studies conducted in humans (Acuff, Dunworth, Webb et al., 1998; Leger, Dumontier, Fouret et al., 1998) showed that short-term supplementation of pregnant women before delivery did not improve neonatal vitamin E status and the authors conclude that vitamin E placental transfer in humans is low. On the other hand, a study where the investigators reported a positive correlation between cord blood and maternal vitamin E levels in both a control and vitamin E supplemented group hints at a possible active transport of α-tocopherol across the human placenta (Mino and Nishino, 1973). In this study, the mothers were supplemented over an average period of three weeks in the last trimester of pregnancy, indicating that a more long-term supplementation is required for effective placental transfer of tocopherol. Due to the fact that a relevant structural diversity of the placenta among different species exists (Benirschke and Kaufmann, 2000), it is evident that animal data cannot be merely transferred to the situation in the human placenta. Human studies so far have mainly focussed on

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normal, term pregnancies, in which the foetuses can be regarded as adequately supplied with vitamin E. It is therefore of great interest to elucidate the mode of placental vitamin E transfer to the human foetus to acquire information on the possibility of effective prenatal supplementation with vitamin E in pending premature delivery, due to the fact that premature infants in critical clinical situations tend to be vitamin E deficient (Kaempf, Miki, Ogihara et al., 1994; Kaempf and Linderkamp, 1998).

α-Tocopherol transfer protein (α-TTP) with its high affinity for α-tocopherol plays a major role in maintaining adequate plasma α-tocopherol levels by excreting α-tocopherol from the hepatocyte into plasma (Arita, Nomura, Arai et al., 1997). α-TTP, which has been cloned and chromosomally localized (Arita, Sato, Miyata et al., 1995), is a 30 kDa protein identified as a product of the causative gene for ataxia with isolated vitamin E deficiency (AVED) (Gotoda, Arita, Arai et al., 1995). Patients with AVED have practically undetectable serum vitamin E levels and show severe neurological symptoms and muscular weakness. While α-TTP was primarily described as a cytosolic hepatic protein (Sato, Hagiwara, Arai et al., 1991; Sato, Arai, Miyata et al., 1993), it has meanwhile been localized in many other tissues, namely in rat brain (Hosomi, Goto, Kondo et al., 1998) and most recently in pregnant mouse uterus (Jishage, Arita, Igarashi et al., 2001; Kaempf-Rotzoll, Igarashi, Aoki et al., 2002) on the mRNA level by Northern blotting and protein level by immunohistochemical studies, while the murine placenta showed no α-TTP localization throughout day 15 of pregnancy with both methods. In this study, we examined term, human placenta with monoclonal anti-human α-TTP antibodies made in rat to elucidate the presence or absence of α-TTP in this tissue by immunohistochemical studies. We hypothesized that α-TTP may not only play an important role in α-tocopherol transport in the human liver and brain, but also in the human placenta. As a control for the applicability of the antibodies created, sections of human liver, where α-TTP is known to be abundantly localized, were immunohistochemically stained to verify the presence of α-TTP in the human liver.

MATERIALS AND METHODS

Tissue preparation

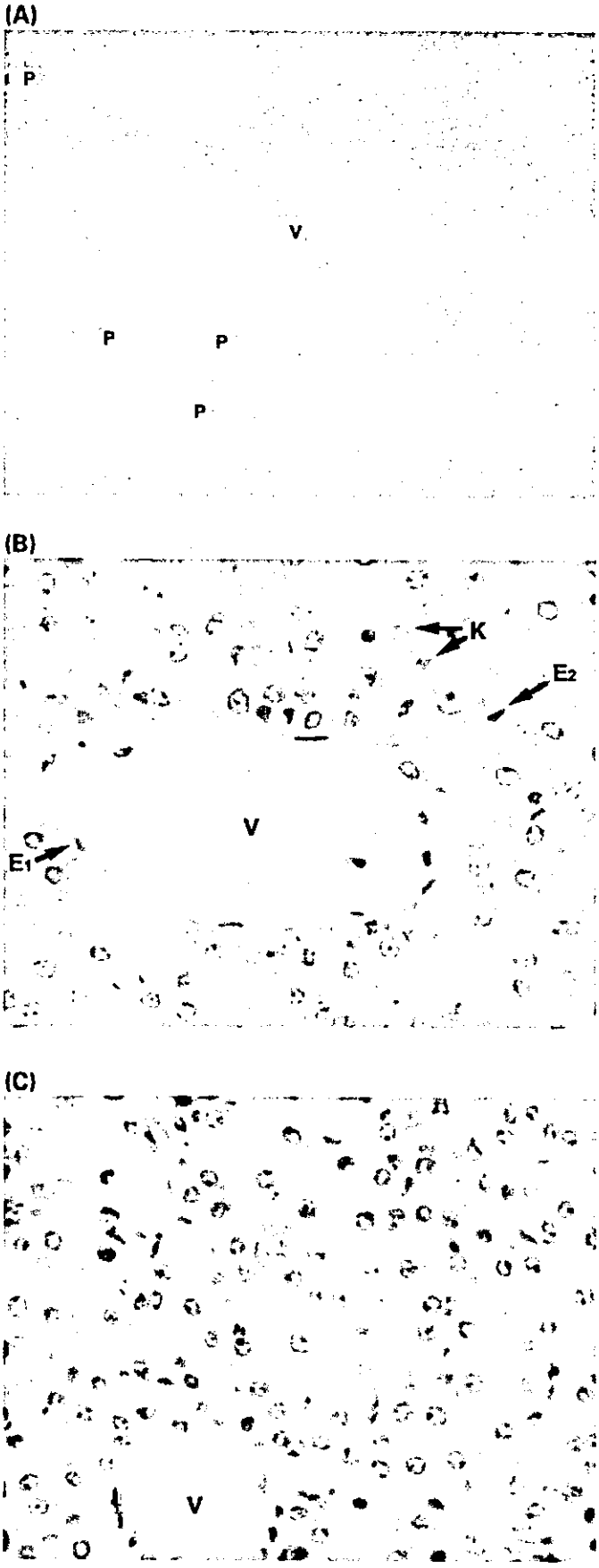
Term placental tissue was collected immediately after delivery from six normal pregnancies at Tokyo Women's Medical University. From each placenta, at least three pieces of placental tissue were collected: one from the maternal side and one from the foetal side near the umbilical insertion, another from the placental margin. Paraffin-embedded liver sections were a kind gift from Dr Y. Tanaka (Children's Medical Center, Kanagawa Prefecture, Japan). Paraffin block preparation of placental tissue was performed according to standard procedures after fixation in 4 per cent paraformaldehyde and subsequent dehydration and paraffin embedding. Sections (5 μm) were cut on a Microm HM 400R microtome and adhered to polylysine-coated microscope slides (Matsunami, Tokyo, Japan).

Preparation of human α-TTP-specific rat monoclonal antibodies

The coding region of human α-TTP cDNA was inserted into the *SalI*/*Eco*RI sites of the pET21a vector (pET system, Novagen, Madison, USA). After the plasmid was introduced into *E. coli* strain BL21 (DE3) (Novagen), the protein was expressed as a *His*-tagged protein by induction with 1 mM isopropyl-β-D-thiogalactopyranoside. The protein was purified using nickel column chromatography (Novagen) according to the manufacturer's protocol. Four rats (WKY strains, female, 8 weeks; SLC, Hamamatsu, Japan) were immunized by injecting the protein into the hind foot pads using Freund complex adjuvant. At 3-week intervals after the initial injection, the rats were injected twice with the purified protein mixed with Freund complex adjuvant. One week after the last booster injection, the two enlarged medial iliac lymph nodes from each rat were used for cell fusion with mouse myeloma cells, line PAI. Several monoclonal antibody-producing hybridoma cell lines were established and selected after checking the produced antibodies by ELISA, Western blotting and immunohistochemical screening of human liver sections. Only the clones highly positive in all three screening methods were selected, with special regard to immunohistochemical usefulness. In this study, the monoclonal antibody from clone 13-E9 (rat IgG2a) was used for immunohistochemistry.

Immunohistochemistry of human liver and placenta

Immunohistochemistry was performed based on avidin–biotin amplification and oxidation with 3,3′-diaminobenzidine tetrahydrochloride (DAB) (Sumner, 1988). Tissue sections adhered to polylysine-coated slides (Matsunami) were deparaffinized in xylene and rehydrated in a graded series of ethanol. The endogenous, tissue-specific peroxidase was blocked with 3 per cent H_2O_2 in methanol for 20 min followed by washing in 0.05 M Tris–HCl/0.15 M NaCl, pH 7.6 (TBS). Antigen retrieval was performed using a microwave oven: samples were microwaved at 750 W and boiled five times for 5 min in 0.01 M citrate buffer, pH 6.0, followed by brief washing in tap water and TBS prior to blocking with 10 per cent rabbit serum (Vector Laboratories, California, USA) in TBS for 30 min at room temperature. Excess fluid was allowed to drain from the sections, which were then covered with 50 μl of a 1 : 20 dilution of the human α-TTP-specific rat monoclonal antibodies. Negative control sections were left at the blocking stage and not covered with the primary antibodies. Incubation of all slides was carried out overnight at room temperature in humidified air-tight chambers. After three 5-min washes in



TBS, the sections were covered with a 1 : 1000 dilution of rabbit biotinylated anti-rat IgG (Vector Laboratories) for 60 min at room temperature. After three 5-min washes in TBS, the slides were covered with avidin–biotin-complex elite (Vectastain ABC Kit, Vector Laboratories) for 30 min and washed again three times for 5 min in TBS. After oxidation with DAB for 5 min and brief washing in tap water, slight counterstaining was performed with Mayers haematoxylin. The slides were then cleared and mounted with 60 per cent HSR solution (Kokusai Shiyaku, Kobe, Japan).

RESULTS

Immunostaining for α-TTP in human liver

The hepatic immunohistologic distribution of α-TTP is shown in Figure 1A (magnification 4 ×). Centrally, a terminal hepatic venule (V) can be seen, as well as several portal tracts (P), consisting of a hepatic portal vein branch, bile ductule and hepatic artery branch surrounded by fibrocollagenous tissue. It is evident that between the stained hepatocytes, the cells of the sinusoidal channels are not stained, as well as the cells of the portal tract including the surrounding fibrocollagenous tissue. No hepatocyte heterogeneity with regard to α-TTP distribution could be noted due to the fact that periportal and centrilobular hepatocytes were similarly stained. In Figure 1B, a terminal hepatic venule (V) is depicted with its surrounding hepatocytes (magnification 40 ×). It can be noted that the endothelial cells lining the hepatic venule (E_1) and the sinusoids (E_2) were not stained, as well as the scattered phagocytic Kupffer's cells (K), suggesting specific localization of α-TTP to the cuboidal hepatocytes in the liver. The fine granular staining of α-TTP in hepatocytes suggests an organelle-associated distribution of this cytosolic protein. A control histological section incubated with solely 10 per cent rabbit serum followed by anti-rat biotinylated antiserum showed no staining whatsoever (Figure 1C, magnification 40 ×).

Immunohistochemical localization of α-TTP in the villous trophoblast of term human placenta

Figure 2A shows staining with α-TTP monoclonal antibodies in the syncytiotrophoblast (S) of term human placenta (magnification 10 ×). Syncytiotrophoblast positive staining shows up as a dark lining against a less intense counterstain. The syncytiotrophoblastic lining of the mature intermediate villi

Figure 1. (A) Human liver paraffin section (5 μm), magnification 4 ×. Immunohistochemical binding of α-TTP-specific monoclonal antibody to the cuboidal hepatocytes is evident. (V: terminal hepatic venule. P: portal tract containing hepatic portal vein branch, bile ductule and hepatic artery branch.) (B) Magnification 40 × of A. (V: terminal hepatic venule. E1: endothelial cells lining hepatic venule. E2: endothelial cells lining sinusoid. K: Kupffer's phagocytic cell.) (C) Negative control to B. Magnification 40 ×. No staining of the hepatocytes is visible. (V: terminal hepatic venule.)

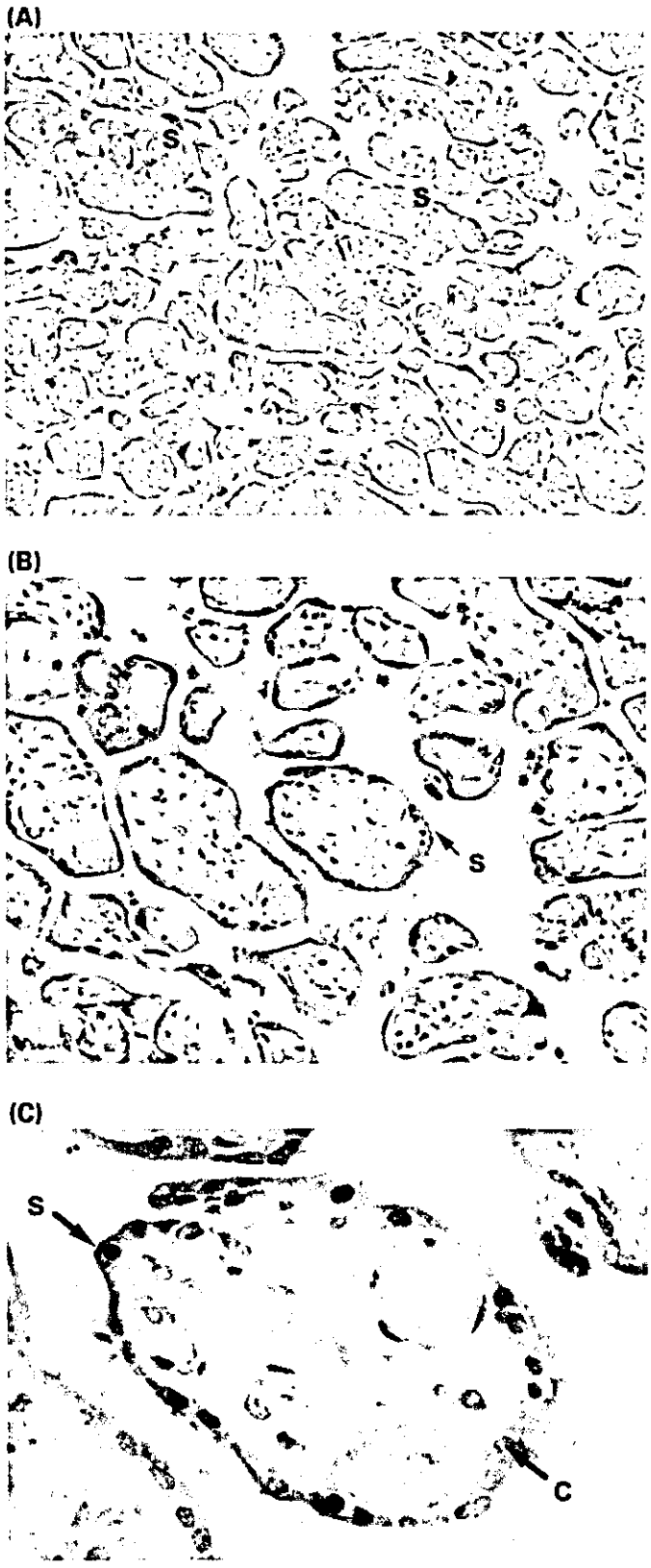


Figure 2. (A) Paraffin section (5 μm) through term, human placental tissue (magnification 10 ×). Immunohistochemical binding of α-TTP-specific monoclonal antibody to syncytiotrophoblast (S) can be seen. (B) Magnification 20 × of A. (C) Magnification 40 × of A shows staining of both syncytiotrophoblast (S) and cytotrophoblast (C) cells.

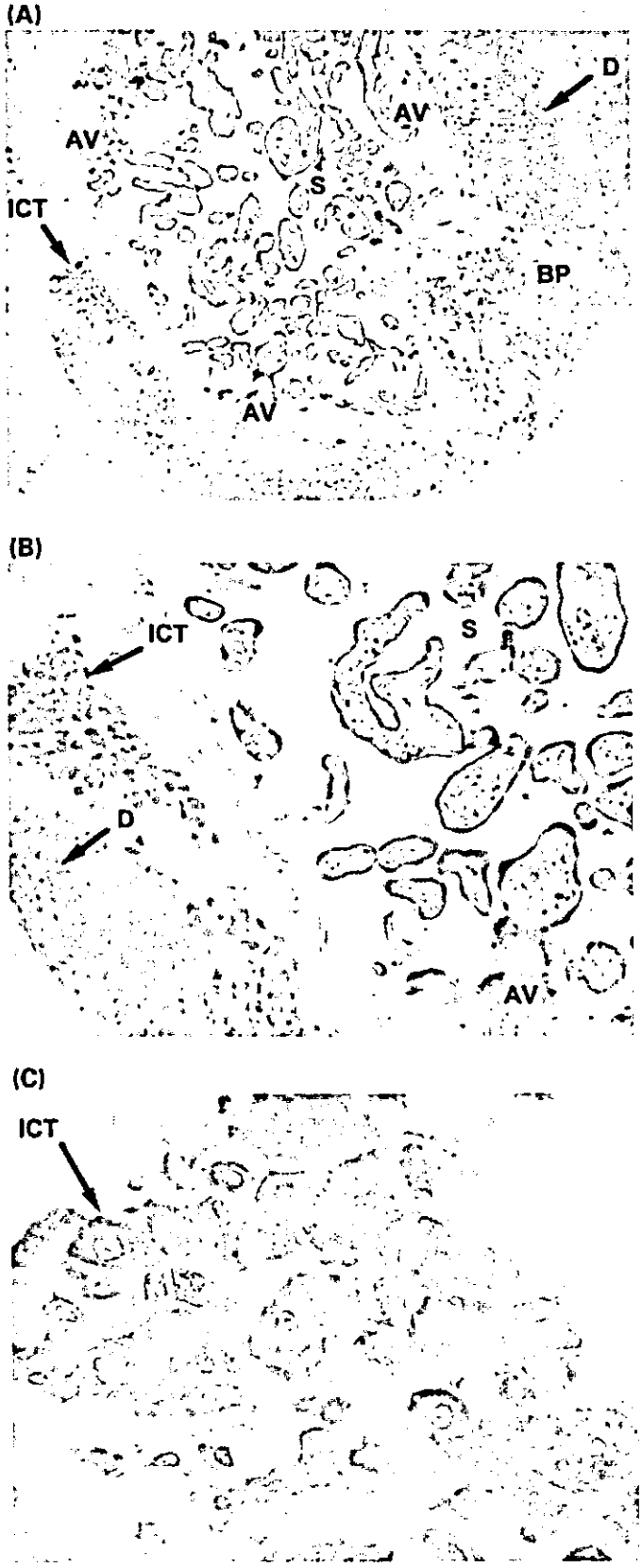
was homogenously stained throughout the sample section. The villous stroma, consisting of connective tissue fibres and fixed connective tissue cells, showed no staining, similar to the endothelial cells of the numerous foetal capillaries, small terminal arterioles and collecting venules. In 20 × magnification (Figure 2B), staining of the syncytiotrophoblast is evident, while some foetal erythrocytes seem stained as well. Figure 2C (magnification 40 ×) shows that the brush border of the syncytiotrophoblast cells was also homogeneously stained, as well as the cytoplasm of these cells. No evident granular distribution of α-TTP could be verified as in hepatocytes. Several cytotrophoblast cells (C) located at the base of the syncytiotrophoblast were stained as well. Negative controls showed no α-TTP staining (data not shown).

Immunohistochemical localization of α-TTP in the extravillous trophoblast and decidua of term human placenta

Figure 3A (magnification 4 ×) shows some syncytiotrophoblastic villi (S) and anchoring villi (AV) attached to the basal plate (BP). Marked staining of the extravillous, invasive cytotrophoblast cells (ICT) in cell columns and cell islands as well as a less marked, but evident staining of decidual basal cells (D) was seen. In Figure 3B (magnification 10 ×), the characteristic polygonal cells of the ICT were stained, as compared to the more slender cells of the decidual stroma that showed slighter staining. ICT exhibits granular staining similar to hepatocytes (Figure 3C, magnification 40 ×).

DISCUSSION

The present study for the first time provides data on the expression and cellular localization of the 30 kDa α-TTP, the major cytosolic carrier protein for α-tocopherol, in term human placenta, namely in syncytiotrophoblast, different cytotrophoblast subtypes and in basal decidual cells. This was done using human liver sections as positive control for the applicability of the anti-human α-TTP antibodies obtained. Vitamin E, of which the biologically most active form is α-tocopherol, was initially identified as a factor required for animals to have offspring and to prevent miscarriage (Evans and Bishop, 1922). Furthermore, vitamin E deficiency in prematurely born infants has been intensely discussed as a factor possibly leading to neonatal oxygen radical diseases such as bronchopulmonary dysplasia or retinopathy of prematurity (Ehrenkranz, Bonta, Ablow et al., 1978; Phelps, Rosenbaum, Isenberg et al., 1987). It therefore seems most important that infants at delivery are equipped with sufficient stores of vitamin E, namely its biologically most active form α-tocopherol, to prevent oxygen radical injury of diverse sensitive tissues. So far, little is known about the transport and maximum delivery time point of vitamin E via the placenta to the foetus during pregnancy. As a lipid-soluble vitamin, simple



diffusion across the foetal–maternal interface has been suggested, similar to the rapid process of fatty acid transfer (Kamp, Zakim, Zhang et al., 1995). On the other hand, several intracellular and membrane-bound α-tocopherol binding proteins have been discussed as playing a role in α-tocopherol transport (Dutta-Roy, 1999), of which a 15 kDa cytosolic protein has been described to be expressed in human placenta (Gordon, Campbell and Dutta-Roy, 1996). This 15 kDa tocopherol-binding protein has been reported to be present in all major tissues, while the 30 kDa α-TTP has only recently been identified to be present in tissues other than animal liver, namely in rat brain (Hosomi, Goto, Kondo et al., 1998) and pregnant mouse uterus (Jishage, Arita, Igarashi et al., 2001; Kaempf-Rotzoll, Igarashi, Aoki et al., 2002), the latter reports making it most likely that α-TTP is involved to a major degree in pregnancy maintenance during the entire span of pregnancy. It should be noted that in mouse placenta, α-TTP could neither be identified by Northern blotting (Jishage, Arita, Igarashi et al., 2001) nor immunohistochemistry (Kaempf-Rotzoll, Igarashi, Aoki et al., 2002), documenting once again the dilemma that animal data cannot simply be transferred to the human situation (Rinkenberger and Werb, 2000). Although the labyrinth of the murine placenta has been compared to the syncytiotrophoblast on human chorionic villi (Hemberger and Cross, 2001), α-TTP is not localized in the murine labyrinth, in contrast to human syncytiotrophoblast. This implies that transport mechanisms across the human syncytiotrophoblast may require different active transport systems than the labyrinthine trophoblast in the mouse.

α-TTP in the hepatocyte fulfils the role of transporting α-tocopherol after uptake from the chylomicron fraction and facilitating its secretion into plasma (Kayden and Traber, 1993; Traber, 1994; Arita, Nomura, Arai et al., 1997). It is plausible that the human trophoblastic cells can be compared to the hepatocyte regarding their expression of α-TTP, facilitating the secretion of maternal α-tocopherol to the foetus via the placenta and consecutively supplying the foetus with this antioxidative substance.

The expression of α-TTP in term human placenta may be linked to the fact that the foetus most likely must accumulate large amounts of α-tocopherol in the last trimester of pregnancy and is therefore dependent on a continuous supply by the mother, which may be supported by an active transport via α-TTP to accumulate α-tocopherol, since dietary supplies can be insufficient post partum (Specker, De Marini and Tsang, 1992). Foetal tissue concentrations of α-TTP are extremely low in foetal rat liver, where this protein is practically not

Figure 3. (A) Paraffin section (5 μm) through term, human placental tissue(magnification 4×). Immunohistochemical binding of α-TTP-specific monoclonal antibody to syncytiotrophoblast (S) and syncytiotrophoblast of anchoring villi (AV) as well as invasive trophoblast cells (ICT). Decidual basal cells (D) also show staining, but to a lesser degree than S and ICT. (B) Magnification 10× of A. Stronger staining of characteristic polygonal cells of the invasive cytotrophoblast (ICT) as opposed to the less marked staining of the decidual basal cells (D) can be seen. (C) Magnification 40× of A. Granular staining of the invasive cytotrophoblast (ICT) is evident.

detectable (Kim, Arai, Arita et al., 1996). It is therefore likely that in the hepato-placental unit, where the foetal liver and maternal placenta form an interactive system (Battaglia, 1997), placental α-TTP is responsible for tocopherol transport and foetal tocopherol nutritional status in the human. This is of great clinical importance with respect to the nutritional requirements in premature infants and their supplementation with vitamin E (Kaempf, Miki, Ogihara et al., 1994; Kaempf and Linderkamp, 1998). Premature infants without major clinical symptoms are born with adequate vitamin E levels with respect to their gestational ages, but deplete very quickly, if a sufficient supply with vitamin E is not feasible. In utero, the supply of α-tocopherol to the foetus is at least in part dependent on α-TTP in the trophoblastic cells of the human placenta, resulting in adequate foetal vitamin E levels. By premature birth, this accumulation in near-term pregnancy is cut off and deficiency states can occur in sick, premature infants. Further investigations will therefore have to elucidate to what degree α-TTP is expressed in preterm placentae and if α-TTP expression levels are positively linked to α-tocopherol levels in preterm infants.

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Vitamin E and transfer proteins

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Purpose of review

Recently, the intracellular transport as well as cellular uptake and excretion of α-tocopherol, the major representative of vitamin E, have been elucidated.

Recent findings

Alpha-tocopherol transfer protein has been identified as the major intracellular transport protein for vitamin E, mediating α-tocopherol secretion into the plasma via a non-Golgi-dependent pathway, while other binding proteins seem to play a less important role. New information has accumulated concerning the role of this protein in the transport and supply of vitamin E to tissues such as the central nervous system and the feto–maternal unit. The scavenger receptor class B type I receptor, a membrane-bound protein, is capable of transferring vitamin E into the cell, while the ATP-binding cassette transporter A1 can excrete vitamin E out of the cell. Advances in the area of vitamin E metabolism have shown that α-CEHC (2,5,7,8-tetramethyl-2-(2'-carboxyethyl)-6-hydroxychroman) and γ-CEHC (2,7,8-trimethyl-2-(2'-carboxyethyl)-6-hydroxychroman) are formed by a cytochrome p450-mediated process, important for α and γ-tocopherol excretion.

Summary

Insights into the regulation of vitamin E transport and metabolism on the cellular level have made enormous advances, showing the complex interplay of influx, trafficking, efflux and metabolism of this crucial antioxidant.

Keywords

α-tocopherol transfer protein, tocopherol binding proteins, α-tocopherol, Sec 14 family, SPF, SR-B1, ABCA1, vitamin E metabolism

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Abbreviations

α-CEHC	2,5,7,8-tetramethyl-2-(2'-carboxyethyl)-6-hydroxychroman
γ-CEHC	2,7,8-trimethyl-2-(2'-carboxyethyl)-6-hydroxychroman
ABC	ATP-binding cassette transporter
AVED	ataxia associated with vitamin E deficiency
Sec14p	*Saccharomyces cerevisiae* phosphatidylinositol transfer protein
SPF	supernatant protein factor
SR-B1	scavenger receptor class B type I
TTP	tocopherol transfer protein

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Introduction

Vitamin E is the most important lipid-soluble antioxidant in humans. It is therefore necessary that the transport and metabolism of this substance are tightly regulated, involving various pathways that are only partly elucidated to date. Due to its lipophilicity, vitamin E is transported in the circulation in association with lipoproteins. It is likely that tissues contain various proteins related to vitamin E that are required for intracellular trafficking, secretion and metabolism.

Most of the information concerning vitamin E-related proteins have been studied in the liver because this organ is responsible for the processing of dietary vitamin E, especially α and γ-tocopherols. This review will describe the first tocopherol binding protein, some associated proteins, and other factors regulating vitamin E concentrations in plasma and tissues that have recently been identified.

Tocopherol binding proteins

So far, several intracellular transport proteins that can bind α-tocopherol with varying affinities have been described. Of these, by far the best studied and described is α-tocopherol transfer protein (TTP), on which this review will focus. Other proteins capable of binding α-tocopherol include the 45 kDa supernatant protein factor and a 14.2 kDa tocopherol-binding protein.

Alpha-tocopherol transfer protein

Hepatic intracellular, cytosolic transport of α-tocopherol is regulated by a 32 kDa protein named α-TTP, first described by Catignani *et al.* [1]. Alpha-TTP was purified from rat liver by Sato *et al.* [2]; complementary DNA cloning from rat liver and isolation of the human α-TTP followed [3,4]. The function of α-TTP in the body is shown in Figure 1.

Alpha-TTP belongs to a novel family of cytosolic lipid-binding and transfer proteins, known as the Sec 14 protein family, including *Saccharomyces cerevisiae* phosphatidylinositol transfer protein (Sec14p), cellular retinaldehyde binding protein (CRALBP) and supernatant protein factor (SPF). Alpha-TTP exhibits a marked ligand specificity and selectively recognizes α-tocopherol, the biologically most active form of vitamin E.

Hosomi *et al.* [5] assessed α-TTP ligand specificity and determined relative affinities of various tocopherol analogs. They showed that the three methyl groups on

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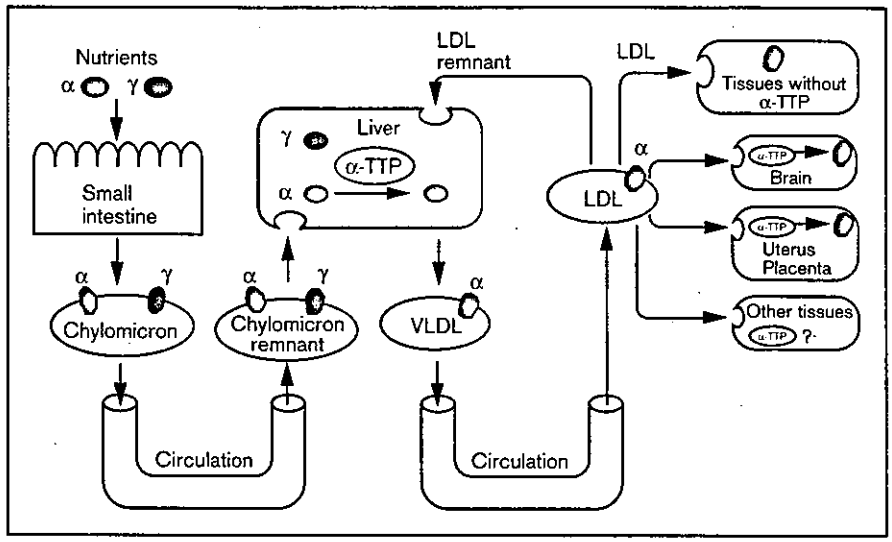
Figure 1. Function of α-tocopherol transfer protein

Alpha and γ-tocopherol are taken up into the small intestine and transported via the chylomicron fraction and enter the hepatocyte in chylomicron remnants. There, α-tocopherol transfer protein (α-TTP) transports preferentially α-tocopherol in the cytosol of the hepatocyte, from where it is mainly incorporated into the VLDL lipoprotein fraction. Alpha-tocopherol is then transported to various tissues, of which several exhibit α-TTP activity. These tissues (brain, uterus, placenta) are likely to be most dependent on efficient α-tocopherol transport.



the chromanol ring are important for recognition by α-TTP, and that among these, the methyl group at position five plays the most essential role for recognition. The hydroxyl group on the chromanol ring as well as the structure and orientation of the phytyl side chain are important determinants of tocopherol affinity to α-TTP.

While α-TTP was first described as being solely present in the liver, it is now accepted that α-TTP messenger RNA is also present in rat brain, spleen, lung and kidney [6] as well as in human brain [7]. Furthermore, α-TTP is present in pregnant mouse uterus and human placenta [8–10].

A specific human gene defect associated with α-TTP deficiency was first described 10 years ago and termed ataxia associated with vitamin E deficiency (AVED). Subsequently, many point mutations in the *α-TTP* gene have been described, leading to similar clinical symptoms [11]. It is interesting to note that no hot spots in the *α-TTP* gene exist where these mutations are primarily located, but that the mutations leading to AVED are distributed homogeneously in this gene. This shows how unstable a protein *α-TTP* is, being easily influenced by a single point mutation in its function. AVED patients are identified by their extremely low plasma vitamin E concentrations. Most develop symptoms at between 4 and 18 years of age. Vitamin E deficiency symptoms include cerebellar ataxia, dysarthria, absence of deep tendon reflexes, sensory loss and pyramidal signs with a positive Babinski sign.

Alpha-TTP physiological function in the hepatocyte was described in detail by Arita *et al.* [12]. In α-TTP-expressing McARH7777 cells, α-TTP stimulated α-tocopherol secretion, although this transport protein is overtly a cytosolic protein. It was also an unexpected finding that this α-TTP-mediated α-tocopherol secretion was not associated with VLDL secretion, which had until then been regarded as the major pathway for α-tocopherol secretion into plasma. Instead, a new pathway, not mediated by the Golgi apparatus, was suggested to be involved in α-tocopherol secretion.

Alpha-tocopherol transfer protein and the central nervous system

Alpha-TTP likely plays a major role in regulating cerebral vitamin E status. Recently, the importance of α-TTP in the central nervous system was stressed by the observation that α-TTP knockout mice are especially susceptible to vitamin E depletion and only extremely low α-tocopherol levels can be detected in knockout mouse brains [13,14]. Yokota *et al.* [13] used an α-TTP knockout mouse model which showed ataxia and retinal degeneration after 1 year of age, symptoms similar to those seen in the human AVED disease. Vitamin E deficiency symptoms were much more severe in α-TTP knockout mice on a normal diet than in wild-type mice on an α-tocopherol-deficient diet, highlighting the relevance of α-TTP in cerebral tocopherol maintenance. Parameters of lipid peroxidation (biochemical measurement of malondialdehyde determined by the thiobarbituric reactive substances, TBARS, method and Western blotting with anti-hydroxynonenal antibody) were significantly increased in α-TTP knockout mouse brains and histological abnormalities became evident when the mice were 20 months of age. Not only could this histological process be successfully reversed by α-

tocopherol supplementation, but also the neurological symptoms in these mice were reversed, making these α-TTP knockout mice excellent animal models for a delayed onset, slowly progressive neuronal degeneration disease caused by oxidative stress. Hosomi *et al.* [6] found α-TTP in normal rat cerebella by an in-situ hybridization method, while Copp *et al.* [7] did not find α-TTP in normal human brain, but only in brains of patients with diseases possibly linked to oxidative stress, such as Alzheimer's disease or Down's syndrome. It is unclear whether α-TTP expression is induced in cerebellar Purkinje cells under oxidative stress [7] or if α-TTP is present in normal cerebellar Bergmann glia cells [6], and thereby regulates α-tocopherol delivery to Purkinje cells.

Alpha-tocopherol transfer protein and the feto–maternal unit

Vitamin E was first described in 1922 as a factor relevant for normal fetal development in rats and the fetal resorption assay has long been a standard to test for vitamin E deficiency [15]. Vitamin E concentrations in neonates and premature infants are far lower than in adults [16,17]. The fact that the stereoisomer of α-tocopherol, which is preferentially transported by α-TTP, namely RRR-α-tocopherol, is also preferentially transferred to cord blood [18] points to the possibility that α-TTP is important for α-tocopherol transport in the feto–maternal unit as well. Jishage *et al.* [8] showed in α-TTP knockout mice that pregnancy develops normally until day 9.5 *post coitum*, the day when the labyrinthine trophoblast of the murine placenta begins to take over a relevant role in nutritional supply to the fetus. In female α-TTP knockout mice, however, a phenomenon also described in other transgenic or knockout mouse models affecting fetal development [19] occurs, namely thinning of the labyrinthine layer with marked reduction of embryonic vessels in this region. Furthermore, prior to fetal death and resorption between days 11.5 and 14.5 *post coitum*, the fetuses showed marked impairment of brain development with neural tube defects. Fetal death even resulted when fertilized eggs of wild-type mice were transferred into α-TTP knockout mice, pointing to the importance of adequate α-TTP function and consecutive adequate supply of vitamin E in the mother animal. When excess α-tocopherol or a synthetic antioxidant (BO-653) was administered to the α-TTP knockout females, pregnancies developed normally until term, suggesting that the role of α-tocopherol in the feto–maternal unit is an antioxidative one. From determining which part of the feto–maternal unit expresses α-TTP, it could be shown that the pregnant mouse uterus expresses the protein at the messenger RNA level [8] as well as immunohistochemically at the protein level [9], with a peak in α-TTP expression on day 4.5 *post coitum*, coinciding with the day of implantation in the mouse. On day 4.5 *post coitum*, α-TTP is localized in the secretory luminal and glandular epithelial cells of the uterus as well as in the inner decidual reaction zone, which later develops into a part of the murine placenta. The secretory columnar epithelial cells are relevant for the initial histiotrophic nutrition of the developing fetus prior to the beginning of hemotrophic nutrition through the placenta. Endometrium-derived secretions are important for the growth and development of the embryo and it can therefore be postulated that α-TTP and its transport of α-tocopherol are required in histiotrophic nutrition. This suggests that α-TTP and its ligand α-tocopherol not only play an outstanding role in the maintenance of pregnancy until term, but also in the first days after egg fertilization. So far, little is known concerning this topic, and no α-TTP has been detected by Northern blots in the murine placenta [8,20].

Active regulation of α-tocopherol in the human placenta seems very likely due to the fact that the vitamin accumulates in the amniotic fluid of term pregnancies (Burton GJ, personal communication). By monoclonal antibody studies, α-TTP was localized in the syncytiotrophoblast and invasive trophoblast of the term human placenta, suggesting that α-tocopherol accumulation in amniotic fluid involves the presence of α-TTP [10]. This once again documents that the labyrinthine layer of the murine placenta is similar, but by no means identical, to the chorionic villi of the human. Differences in active transport across the feto–maternal barrier are present. The expression of α-TTP and its transport of α-tocopherol in the feto–maternal unit make it likely that these factors are relevant for protection of the fetus against oxidative stress in the mouse and human.

Supernatant protein factor/tocopherol-associated protein

As another member of the newly described lipid-binding and transfer protein family, SPF, a 46 kDa protein also referred to as tocopherol-associated protein, has been described as binding tocopherol. The complementary DNA cloning and protein expression of SPF from rat and human has been reported [21], human tissue SPF levels being highest in adult liver. SPF enhances microsomal squalene monooxygenase activity and promotes intermembrane transfer of squalene *in vitro*. While in TTP knockout mice α-tocopherol levels are low, SPF levels are not influenced in these mice, suggesting that there is no direct correlation between α-tocopherol and SPF levels. SPF is, therefore, not likely to be a determinant of plasma vitamin E levels (Arai H, unpublished data).

It was shown that both the liver and small intestine express SPF messenger RNA, both sites being important for cholesterol biosynthesis. It was therefore postulated that SPF plays an important role in cholesterol

biosynthesis [21]. Recently, the crystal structure of human SPF was reported [22]. This structure analysis revealed that, unlike the crystal structure of Sec14p, a lipid-exchange loop exists in a closed conformation, which makes this region likely to be involved in a mechanism for lipid exchange. Short-term modulation of cholesterol biosynthesis by the phosphorylation of SPF has been reported [23], but its role in vitamin E transport other than its ability to transiently bind tocopherol was not described.

Other tocopherol-binding proteins

Reports on other tocopherol-binding proteins have not been made in the last 7 years. Dutta-Roy *et al.* [24] found a 14.2 kDa cytosolic protein in various tissues of different species as well as in term human placenta [25]. So far, only partial sequencing of this protein has been reported and no function has been described other than tocopherol binding, making it unclear if a separate protein or a degradation product of α-TTP was assayed here. Further characterization is necessary to clarify this point.

Membrane-bound tocopherol transport proteins

While the intracellular transfer and packaging of α-tocopherol into nascent VLDL is under the tight control of α-TTP, its uptake into and excretion from the cell are still an enigma. Several recent studies have addressed this point, showing that the scavenger receptor class B type I (SR-B1) is a major anchor in α-tocopherol uptake through a selective lipid uptake mechanism and that the ATP-binding cassette transporter (ABC)A1 mediates cellular secretion of this essential micronutrient.

Scavenger receptor class B type I

SR-B1 mediates cellular selective cholesteryl ester uptake from lipoproteins, namely HDL. While mechanisms such as lipoprotein lipase-mediated uptake or the activity of phospholipid transfer protein for the selective uptake of α-tocopherol have been discussed in the past, Mardones *et al.* [26•] suggested that SR-B1 efficiently facilitates transfer of α-tocopherol from HDL to cultured cells. Furthermore, using an SR-B1 knockout mouse model, they showed that plasma α-tocopherol levels in these animals were significantly increased compared with wild-type animals. This phenomenon was accompanied by a major decrease in α-tocopherol concentrations in bile and several specific tissues such as ovaries, testis, lung and brain, with no effect on α-tocopherol levels in other tissues such as liver, kidney, spleen or fat. The authors concluded that depending on the tissue involved, SR-B1-dependent and SR-B1-independent pathways are effective in α-tocopherol cellular uptake. These experiments showed once again that α-tocopherol levels in plasma are by no means adequate parameters for the assessment of α-tocopherol nutritional status. In this knockout mouse model, high plasma α-tocopherol levels could be misinterpreted as suggesting a highly normal vitamin E status, although tissue levels of α-tocopherol are partly low. The tissues showing low α-tocopherol concentrations due to SR-B1 deficiency are, amazingly, identical to the ones affected by α-TTP deficiency: the central nervous system, lungs and gonads. Also, this knockout mouse model is prone to atherogenesis, like the hyperlipidemic α-TTP knockout mouse model reported by Terasawa *et al.* [27]. No clinical symptoms associated with SR-B1 deficiency leading to α-tocopherol deficiency in the central nervous system or lungs have so far been reported. Goti *et al.* [28] demonstrated that SR-B1 promotes the uptake of HDL-associated α-tocopherol in porcine brain capillary endothelial cells, and concluded that this receptor is essential in the α-tocopherol crossing of the blood–brain barrier. On the other hand, female infertility involving production of abnormal oocytes and zygotes has been seen in SR-B1 knockout mice [29,30]. The observation was made that α-tocopherol supplementation in SR-B1 knockout mouse females does not restore their fertility [27], which can be well explained by the function of SR-B1: without this receptor, α-tocopherol cannot be delivered in adequate amounts to the key reproductive tissues, emphasizing the major role of SR-B1 in cellular α-tocopherol uptake in these tissues. On the cellular level, a tight inverse control of vitamin E over the expression of the SR-B1 receptor was reported [31]. In this study, rats were fed a vitamin E-depleted diet, leading to an 11-fold increase in SR-B1 protein levels in liver tissue, while the administration of vitamin E-enriched feeding could reverse this phenomenon. Increasing evidence therefore points to the fact that SR-B1 is an essential component in facilitating α-tocopherol uptake into cells.

ATP-binding cassette transporter A1

ABCA1 is an ATP-binding cassette protein mainly known for its function in transporting cellular cholesterol and phospholipids to lipid-poor HDL apolipoproteins such as apolipoprotein A-I. Recently, Oram *et al.* [32] demonstrated the role of ABCA1 in cellular α-tocopherol secretion. After induction of ABCA1 expression in human fibroblasts and RAW264 macrophages, enhanced apolipoprotein A-I-mediated α-tocopherol efflux was observed. This phenomenon could not be induced when cells lacked the ABCA1 transporter, such as in fibroblasts from Tangier disease patients. To what extent the impaired transport of α-tocopherol is relevant for the clinical manifestations seen in ABCA1-deficient mice has not been addressed so far. Christiansen-Weber *et al.* [33] reported that in their ABCA1 knockout mice, severe placental malformation, aberrant lipid distribution, kidney glomerulonephritis and HDL-cholesterol

deficiency occurred. It is interesting to note that similarities in α-TTP-deficient and ABCA1-deficient mouse pregnancies exist. In ABCA1-deficient mice, the labyrinthine layer is affected by day 14 of gestation. It is also the labyrinthine layer that shows abnormalities in α-TTP-deficient pregnant mice, though the morphology differs from ABCA1-deficient mice. Both knockout mouse models may therefore show these placental abnormalities due to inadequate α-tocopherol supply in the feto–maternal unit.

Vitamin E metabolism

Once tocopherols are absorbed and delivered to the liver, their fates are largely unknown; however, metabolism or excretion are likely because the liver does not accumulate toxic levels of vitamin E. In the last 5 years, substantial advances in the understanding of vitamin E metabolism have been made. Vitamin E metabolites α-CEHC (2,5,7,8-tetramethyl-2-(2'-carboxyethyl)-6-hydroxychroman) and γ-CEHC (2,7,8-trimethyl-2-(2'-carboxyethyl)-6-hydroxychroman) are derived from α and γ-tocopherols, respectively. This process does not involve α-TTP because AVED patients are able to metabolize α-tocopherol to α-CEHC [34].

Alpha and γ-CEHCs are formed by a cytochrome p450-mediated process, followed by β-oxidation [35,36,37••]. Sesamin appears to inhibit tocopherol metabolism by this process [35,38]. Cytochrome p450s, including CYP4F2 [37••], CYP3A4 [35,36] and CYP3A5 [36,39] have been implicated in the metabolic pathway. Which cytochrome p450 plays the major role in this process remains to be elucidated. All forms of vitamin E have been shown to activate gene expression via the pregnane X receptor, a nuclear receptor regulating a variety of drug metabolizing enzymes [39].

Future areas of study include the determination of the quantitative conversion of α and γ-tocopherols to their respective metabolites and how the amount of metabolites converted is related to the amount of tocopherol ingested. Also, the major route of excretion remains to be elucidated, as well as the temporal sequence of appearance of circulating CEHCs relative to tocopherols.

Conclusion

Vitamin E is critical for pregnancy, normal nerve function and possibly prevention of chronic diseases, such as atherosclerosis. While α-TTP was identified as a key regulatory factor in vitamin E status 12 years ago, only recently proteins involved in the uptake and excretion of α-tocopherol have been described, such as the SR-B1 receptor and ABCA1 transport protein. Advances in the knowledge of vitamin E metabolism have also been made: α and γ-CEHCs have been identified as metabolic products of α and γ-tocopherols, and cytochrome p450s have been implicated in this metabolic pathway. Therefore, the understanding of intracellular and metabolic vitamin E regulation has increased tremendously in the last few years. However, the interaction of these regulatory proteins has yet to be clarified to complete the picture of vitamin E function and metabolism in humans.

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- • of special interest
- •• of outstanding interest

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pH-dependent translocation of α-tocopherol transfer protein (α-TTP) between hepatic cytosol and late endosomes

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Abstract

Background: **α-Tocopherol transfer protein (α-TTP), a member of the Sec14 protein family, plays an important role in transporting α-tocopherol, a major lipid-soluble anti-oxidant, in the cytosolic compartment of hepatocytes and is known as a product of the causative gene for familial isolated vitamin E deficiency. It has been shown that the secretion of hepatocyte α-tocopherol taken up with plasma lipoproteins is facilitated by α-TTP. To explore the mechanism of α-TTP mediated α-tocopherol secretion, we investigated drugs which may affect this secretion.**

Results: **We found that, in a hepatocyte cell culture system, intracellular α-tocopherol transport is impaired by chloroquine, an agent known for its function of elevating the pH in acidic compartments. Under chloroquine treatment, the diffuse cytosolic distribution of α-TTP changes to a punctate pattern. Double-staining experiments with endocytosis markers revealed that α-TTP accumulates transiently on the cytoplasmic surface of late endosomal membranes. This phenomenon is specific for hepatoma cell lines or primarily cultured hepatocytes. Other members of the Sec14 family, such as cellular retinaldehyde-binding protein (CRALBP) and supernatant protein factor (SPF), do not show this accumulation. Furthermore, we elucidate that the obligatory amino acid sequence for this function is located between amino acids 21 and 50, upstream of the N-terminal end of the lipid-binding domain.**

Conclusion: **We hypothesize that a liver-specific target molecule for α-TTP exists on the late endosomal membrane surface. This transient binding may explain the mechanism of how α-tocopherol is transferred from late endosomes to cytosolic α-TTP.**

Introduction

Recently, a new cytosolic lipid-binding/transfer protein family has been described. This protein family has been named the Sec14 family, after its primarily described member, the Sec14 protein. Sec14p is known to bind and transfer phosphatidylcholine and phosphatidylinositol and by this means it may determine the diacylglycerol level in Golgi membranes, which is likely to be responsible for adequate vesicular transport in the Golgi apparatus (Kearns *et al.* 1998a). Database searching reveals that approximately 20 members of this protein family exist in mammals (SMART program package, http://smart.embl-heidelberg.de/), among others, α-tocopherol transfer protein (α-TTP), cellular retinaldehyde-binding protein (CRALBP), supernatant protein factor (SPF), and megakaryocyte protein-tyrosin-phosphatase (MEG2). CRALBP is expressed exclusively in the retina and carries 11-*cis*-retinal and 11-*cis*-retinol as endogenous ligands (Intres *et al.* 1994; Crabb *et al.* 1998). The deficiency of CRALBP has been linked to the autosomal recessive disorder retinitis pigmentosa (Maw *et al.* 1997; Saari *et al.* 2001). SPF was primarily described in 1957 by Konrad Bloch's group to stimulate squalene mono-oxygenase in rat liver cytosol, and its cloning has recently been reported (Shibata *et al.* 2001). SPF, as a cytosolic squalene transfer protein, enhances cholesterol biosynthesis and stimulates the conversion of squalene to lanosterol.

α-TTP, another member of this specific cytosolic lipid-binding and transfer protein family, was first purified

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from rat liver in 1991 (Sato *et al.* 1991). α-TTP is mainly expressed in liver tissue and binds preferentially to α-tocopherol, the major lipid-soluble, biologically active anti-oxidant. α-TTP regulates plasma α-tocopherol levels by influencing hepatic α-tocopherol secretion (Arita *et al.* 1997). The physiological importance of α-TTP in humans was shown after the description of neurologically symptomatic patients with α-TTP gene mutations, known as familial isolated vitamin E deficiency (Gotoda *et al.* 1995; Ouahchi *et al.* 1995). Aside from extremely low plasma α-tocopherol levels, these patients develop severe neurological symptoms such as ataxia, tremor and muscular weakness, often not reaching their 30th year of life. Similar symptoms can be observed in α-TTP knockout mice which were developed in our laboratory (Yokota *et al.* 2001).

α-Tocopherol is transported in plasma liporoteins (Kayden & Traber 1993). α-Tocopherol associated with circulating plasma lipoproteins such as chylomicron remnants and low density lipoproteins (LDLs) are endocytosed to a great extent by the liver. Endocytosed plasma liporoteins are transported to late endosomes/lysosomes, where the lipoproteins are hydrolysed and α-tocopherols associated with them are released. These α-tocopherols are repackaged in the liver and secreted into the plasma in nascent very low density lipoproteins (VLDLs). These processes ensure the efficient recycling of plasma α-tocopherol (Traber & Arai 1999).

In our laboratory, we studied α-TTP function using a cultured liver cell line (Arita *et al.* 1997). We devised an assay system using α-[^{14}C]tocopheryl acetate and α-TTP-expressing McARH7777 cells. α-TTP does not recognize α-[^{14}C]tocopheryl acetate itself (Hosomi *et al.* 1997); it recognizes it only after the acetate group has been hydrolysed intracellularly. When α-[^{14}C]tocopheryl acetate was added to the cell cultures, it was taken up by the cells, hydrolysed to produce α-[^{14}C]tocopherol in the cells and free α-[^{14}C]tocopherol is resecreted into the culture medium by the action of α-TTP. It is therefore possible to quantify the activity of α-TTP in living cells by measuring the amount of α-[^{14}C]tocopherol appearing in the medium, even in the presence of excess amounts of added α-[^{14}C]tocopheryl acetate. With this system we proved that the secretion of α-tocopherol is greatly stimulated in cells expressing α-TTP.

This study focuses on the possible functions of α-TTP in hepatic intracellular α-tocopherol trafficking. We demonstrate that the cytosolic protein α-TTP has the unique tendency to compartmentalize to intracellular acidic compartments such as late endosomes/lysosomes, when cells are treated with drugs that elevate the pH in these compartments. Furthermore, we show that, for this translocation of α-TTP, the region located upstream of the lipid-binding domain plays a crucial role.

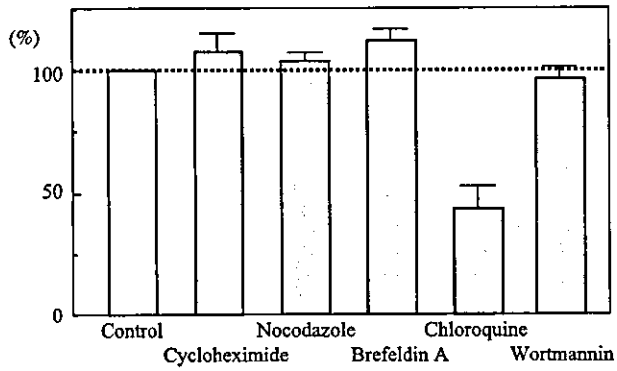

Figure 1 Influence of diverse pharmacological substances on α-tocopherol secretion via α-TTP. McA-TTP cell monolayers were incubated with α-[^{14}C]tocopheryl acetate integrated in liposomes for 4 h in the absence or presence of cycloheximide (35 μM), nocodazole (35 μM), brefeldin A (100 nM), chloroquine (100 μM) or wortmannin (1 μM). After 4 h incubation, the amounts of α-[^{14}C]tocopherol in cell and medium fractions were measured. The rate of α-tocopherol secretion was calculated as follows: α-tocopherol secretion (%) = α-[^{14}C]tocopherol in medium/(α-[^{14}C]tocopherol in cell + α-[^{14}C]tocopherol in medium) × 100 (%). The results are expressed as percentages of the secretion level obtained in the absence of pharmacological substances. The data are shown as the means ± SD of three independent experiments.

Results

Chloroquine inhibits α-TTP-mediated α-tocopherol secretion

To explore the mechanism of α-TTP-mediated α-tocopherol secretion, we investigated drugs which may affect this secretion. Twelve drugs were tested in the McA-TTP cell system, of which Fig. 1 shows the relative secretion rate of α-tocopherol from these cells treated with five of the drugs tested, compared with a control. Brefeldin A, a Golgi apparatus disrupting agent, as well as nocodazole, an α-tubulin polymerization inhibitor, showed no reduction in α-tocopherol secretion. Similarly, cycloheximide, a protein synthesis inhibitor, and wortmannin, a phosphatidylinositol 3-kinase inhibitor, did not lead to diminished α-tocopherol secretion. In contrast, 100 μM chloroquine partially but significantly inhibited the secretion of α-tocopherol. Chloroquine is a hydrophobic tertiary amine and is a well-known anti-malarial drug. It permeates biological membranes rapidly and accumulates within acidic compartments, such as late endosomes/lysosomes, neutralizing their lumenal

pH. Due to the fact that chloroquine inhibits α-tocopherol secretion, we investigated this pathway in detail to shed light on the mechanism of hepatic α-tocopherol secretion by α-TTP.

Redistribution of α-TTP after chloroquine treatment

To elucidate the effect of chloroquine on α-TTP in McA-TTP cells, an immunofluorescence assay was performed. When the cells were incubated without chloroquine, α-TTP was distributed homogenously throughout the cytosol (Fig. 2A, a and a′). The addition of 100 μM chloroquine surprisingly caused a remarkable conglomeration of α-TTP after 4 h, which now showed a punctate pattern (Fig. 2A.b.b′). After chloroquine was washed out, α-TTP showed a diffuse, homogenous distribution in the cell (Fig. 2A.c.c′), similar to the distribution prior to treatment. To verify if there was a change in α-TTP protein expression level during the three time points shown, Western blotting was performed, indicating no change (Fig. 2B). Therefore, this set of experiments implied that the accumulation of α-TTP by chloroquine is reversible.

As chloroquine has multiple effects on cellular physiology, such as acidic compartment neutralization (Wibo & Poole 1974) and sterol synthesis (Chen & Leonard 1984), we then investigated which mechanism induced by chloroquine caused this specific localization of α-TTP. Treatment of McA-TTP cells with 100 nM bafilomycin A1, a specific inhibitor of the vacuolar type H^+-ATPase, caused chloroquine-like localization of α-TTP (Fig. 2A.d.d′). As chloroquine is a specific lanosterol synthase inhibitor, we tested other agents showing this function, such as 2-isopropyl-4-dimethylamino-5-methylphenyl-piperidine carboxylate methyl chloride (Amo 1618) and *N,N*-dimethyldodecylamine *N*-oxide (LDAO), but no such effect was observed (unpublished data). These strongly support our hypothesis that neutralization of the lumenal pH of the acidic compartments was the cause of the localization change of α-TTP. The difference of chloroquine and bafilomycin on the localization pattern of α-TTP can be explained by the fact that chloroquine leads to swelling of the acidic compartments, while bafilomycin A1 does not, due to their different modes of action.

Accumulation of endogenous α-TTP after chloroquine treatment in rat primary culture hepatocytes

Following these observations, we investigated whether the localization of α-TTP in McA-TTP cells is also observed in hepatic parenchymal cells. In an *in vitro* primary culture of rat hepatocytes, the cellular localization of α-TTP after chloroquine treatment was determined. In Fig. 3 (left panel), rat hepatocytes are seen in primary culture, showing homogenous α-TTP distribution in the cytosol using anti-rat α-TTP mAb. In Fig. 3 (right panel), accumulation of endogenous α-TTP after chloroquine treatment in primarily cultured rat hepatocytes after 4 h is shown. This result indicated that α-TTP accumulation not only occurs in McA-TTP cells, but also in hepatic parenchymal cells.

α-TTP accumulates in late endosomes after chloroquine treatment

To determine the subcellular organelle in which α-TTP is localized, double staining studies with well-known organelle markers were performed. Figure 4(b, e and h) show a dual-label fluorescence experiment in which McA-TTP cells were incubated with chloroquine and fluorescein isothiocyanate-conjugated bovine serum albumin (FITC-BSA). FITC-BSA is a fluorescent tracer molecule for endocytosis, accumulating in acidic compartments (Kim *et al.* 1996b). Incubation of McA-TTP cells with FITC-BSA for 30 min most likely results in accumulation of this fluorescence probe in early endosomes (Fig. 4b), while incubation for 4 h showed slight (Fig. 4e), and incubation for 12 h, main probe localization in a compartment probably corresponding to late endosomes (Fig. 4h). Figure 4(a, d and g) show corresponding time points of α-TTP localization with chloroquine treatment. At 0.5 h α-TTP and FITC-BSA localization were not identical (Fig. 4c), while, with time, accumulation of both proteins in the same compartment gradually evolved (Fig. 4f and i). These results implied that the accumulation site of α-TTP after chloroquine treatment is predominantly late endosomes/lysosomes.

To confirm this finding, a dual-label fluorescence experiment with α-TTP (Fig. 5a) and lysobisphosphatidic acid (LBPA, Fig. 5b) was performed. LBPA is a unique acidic phospholipid which is specifically localized in late endosomes and accounts for approximately 15% of late endosomal total phospholipids (Kobayashi *et al.* 1998). α-TTP co-localized to a great extent with LBPA when McA-TTP cells were incubated with chloroquine (Fig. 5c). These observations stress the fact that α-TTP is localized in late endosomes after chloroquine treatment.

Accumulation in late endosomes is α-TTP specific

α-TTP belongs to a family of lipid-binding and transfer proteins, which includes supernatant protein factor

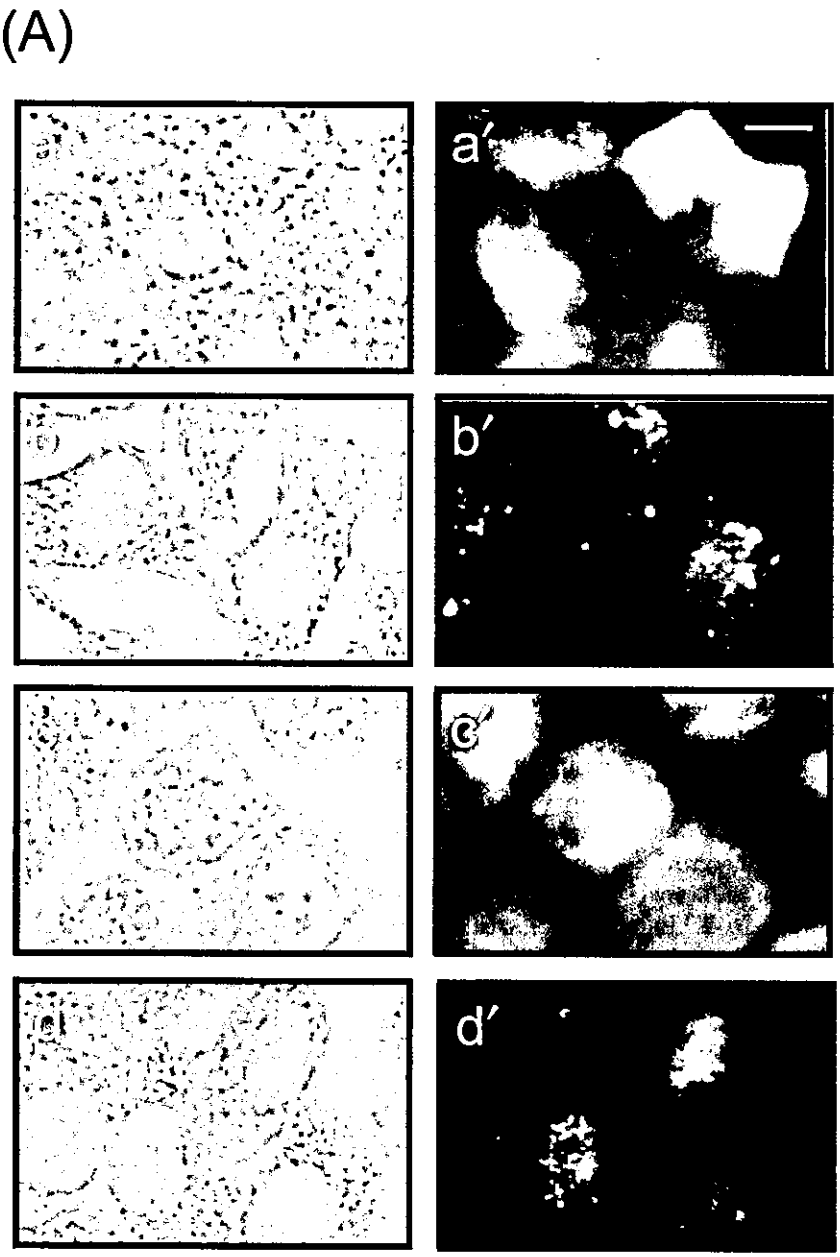

Figure 2 Effect of chloroquine and bafilomycin A1 on cellular localization and amount of α-TTP in McA-TTP cells. (A) McA-TTP cells were incubated for 4 h in the absence (a, a′) or presence of 100 μM chloroquine (b, b′) or 100 nM bafilomycin A1 (d, d′). McA-TTP cells which were incubated with 100 μM chloroquine were washed out and then incubated without drugs for 4 h at 37 °C (c, c′). These cells were fixed and immunostained (a′, b′, c′, d′) with anti-α-TTP mAb AT-R1 as described in Experimental procedures. Bar, 10 μm (B) The cells prior to, during and after chloroquine treatment were homogenized and subjected to Western blotting with mAb AT-R1 for the detection of α-TTP.

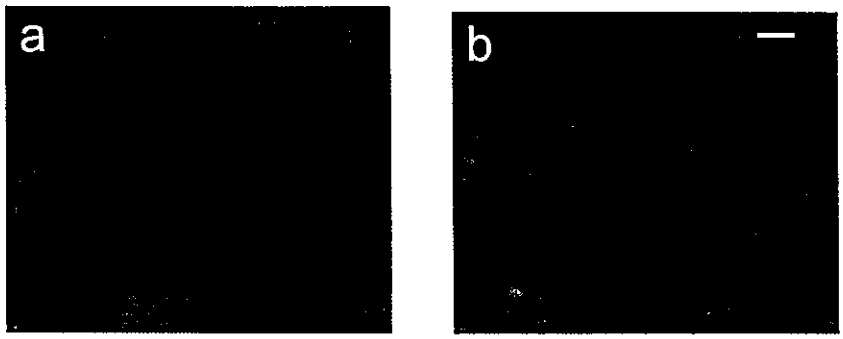

Figure 3 Effect of chloroquine treatment on cellular α-TTP localization in primary culture rat hepatocytes. Primary culture hepatocytes were obtained from rat livers and incubated as described in Experimental procedures. Rat hepatocytes were incubated for 4 h with 100 μM chloroquine followed by immunostaining with mAb AT-R1. Rat hepatocytes prior to (a) and after (b) chloroquine treatment. Bar, 10 μm.

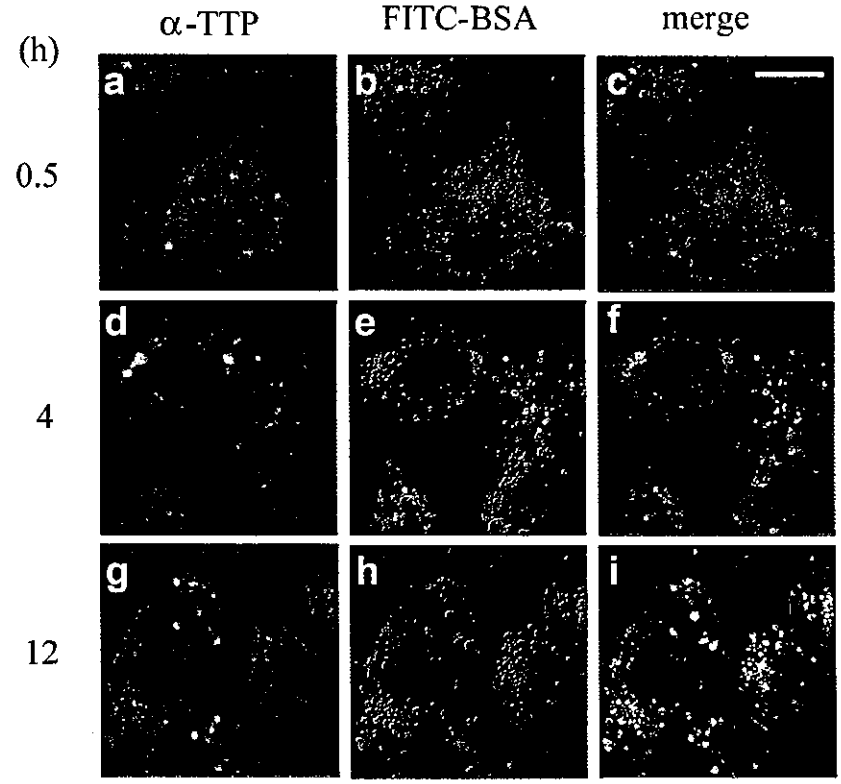

Figure 4 Accumulation of α-TTP in late endosomes after chloroquine treatment. A dual-label fluorescence experiment using McA-TTP cells was performed according to Experimental procedures. McA-TTP cells were incubated with 0.75 mg/mL FITC-BSA for 0.5, 4 and 12 h, respectively. Incubation with chloroquine for 4 h followed. The cells were then fixed and immunostained with primary mAb AT-R1 and secondary the Alexa 594 fluorescence-conjugated antibody. α-TTP (red) and FITC-BSA (green) showed dissimilar distribution (a, b) as shown in the merge (c) after 0.5 h. After 4 h, the distribution became similar (d, e and f), while after 12 h, they showed a practically identical distribution pattern (g, h and i). Bar, 10 μm.

(SPF), cellular retinal binding protein (CRALBP), yeast Sec14p and others. We investigated whether the proteins belonging to this family also accumulate in late endosomes after chloroquine treatment. For this experiment, we used rat hepatoma McARH7777 cells which do not express any of these proteins endogenously. Cultured McARH7777 cells were transiently transfected with an expression vector containing the cDNAs of N-terminal myc-tagged α-TTP, SPF and CRALBP, respectively. After 24 h, chloroquine was added to the culture, and the cells were incubated for 4 h. When the cells were stained with mAb against myc tag, myc-tagged α-TTP appeared as a punctate pattern similar to treated McA-TTP cells as described above (Fig. 6a). In contrast, both SPF and CRALBP remained homogenously in the cytosol, and did not accumulate (Figs 6b and c). These results revealed that pH-dependent translocation from cytosol to late endosomes is unique to α-TTP, as opposed to other members of this protein family.

α-TTP localization to acidic compartments may be liver specific

α-TTP is expressed mainly in the liver as previously mentioned. We therefore examined whether pH-dependent