Tissue-specific ceruloplasmin gene expression in the mammary gland

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Using a ceruloplasmin cDNA clone in RNA blot analysis, a single 3.7 kb ceruloplasmin-specific transcript was detected in rat mammary gland tissue from pregnant and lactating animals. Ceruloplasmin gene expression in the mammary gland was tissue-specific, with no evidence of expression in brain, heart or other extrahepatic tissues. Ceruloplasmin mRNA was also detected in mammary gland tissue from male, virgin female and non-pregnant/multiparous animals, and the abundance of ceruloplasmin-specific transcripts in virgin female rats was independent of their stage of oestrus. In virgin female mammary gland the content of ceruloplasmin mRNA was 20 % of that in hepatic tissue from these animals and approx. 2–3-fold greater than that found in mammary gland tissue of pregnant or lactating animals. Development studies revealed ceruloplasmin gene expression in male and female mammary gland by only 2 weeks of age, prior to the onset of puberty. Biosynthetic studies indicated that the ceruloplasmin mRNA in mammary gland tissue was translated into a 132 kDa protein qualitatively similar to that synthesized in liver. By *in situ* hybridization, ceruloplasmin gene expression was localized to the epithelium lining the mammary gland alveolar ducts, without evidence of expression in the surrounding mesenchyme. Ceruloplasmin gene expression was also detected in a human breast adenocarcinoma cell line and in biopsy tissue from women with invasive ductal carcinoma. Taken together, these data indicate that the mammary gland is a prominent site of extrahepatic ceruloplasmin gene expression and add to the evidence that ceruloplasmin biosynthesis is associated with growth and differentiation in non-hepatic tissues.

INTRODUCTION

Development and differentiation of the mammary gland is determined by a complex interplay of hormones, growth factors, micronutrients, cell–cell interactions and the extracellular matrix [1]. This tissue provides a unique model for the study of the developmental regulation of gene expression. The mammary gland is one of only a few tissues which demonstrate significant developmental potential postnatally, undergoing multiple episodes of epithelial proliferation during pregnancy and lactation followed by involution at the time of weaning. Previous work on gene expression in the mammary gland has focused on those genes encoding milk proteins in the lactogenic gland, including β -casein and whey acidic protein, and has defined some of the mechanisms determining cell-specific expression [2,3]. Recently the hepatic plasma protein transferrin has been shown to be expressed in the rodent mammary gland independent of maternal ion status, suggesting a unique functional role for this protein in mammary gland development [4].

Ceruloplasmin is a blue-copper oxidase which is synthesized in the liver as a single polypeptide chain and is secreted into the serum as an α_2 -glycoprotein containing greater than 95 % of the copper circulating in the plasma [5,6]. Although the functions of ceruloplasmin have not been fully characterized, current data suggest a role for this protein in copper and iron metabolism [7], tissue angiogenesis [8], antioxidant defence [9] and the coagulation cascade [10]. Consistent with its role in host defence, ceruloplasmin is an acute-phase reactant, with the serum concentration increasing during inflammation, tissue injury and certain malignant disorders [11]. In addition, marked changes in serum copper and ceruloplasmin concentrations have been noted during pregnancy and in the *post partum* period, although the mechanisms of these changes have not been well characterized [12].

Although the liver is considered the primary source of ceruloplasmin, extrahepatic expression has been observed. Thus the function and regulation of ceruloplasmin must be considered in the context of extrahepatic tissue expression. Previous work has demonstrated expression of ceruloplasmin in human synovial tissue [13], rat Sertoli cells [14], rat testis and choroid plexus [15], and pregnant rat uterus [16]. Most recently, abundant extrahepatic ceruloplasmin gene expression has been demonstrated in rodent and human lung both during development [17] and during inflammation and hyperoxia-induced tissue injury [18]. Because of the profound changes in copper and ceruloplasmin metabolism during pregnancy and lactation, and the recent studies demonstrating extrahepatic ceruloplasmin gene expression, we undertook the current study to examine the mammary gland as a potential site of ceruloplasmin production.

MATERIALS AND METHODS

Materials

Chemicals and reagents used in this study included: phenol from Anachemia Chemicals (Montreal, Canada), chloroform and isoamyl alcohol from Fisher Scientific (Fairlawn, NJ, U.S.A.), guanidinium isothiocyanate from Fluka (Ronkonkoma, NY, U.S.A.), Seakem GTG and ME agarose from FMC Bioproducts (Rockland, ME, U.S.A.), Dulbecco's modified minimal essential medium (DMEM) with and without methionine from GIBCO (Grand Island, NY, U.S.A.), bovine fetal calf serum from Flow (McLean, VA, U.S.A.), nylon membranes (Hybond-N) from Amersham Corp. (Arlington Heights, IL, U.S.A.), and nitrocellulose from Schleicher and Schuell (Keene, NH, U.S.A.). Additional chemicals were purchased from Sigma (St. Louis, MO, U.S.A.). RNA- and DNA-modifying enzymes were purchased from Promega Biotech (Madison, WI, U.S.A.) and used according to the manufacturer's specifications. 2,5-

Abbreviations used: DMEM, Dulbecco's modified minimal essential medium; RT, room temperature; SSC, 0.15 M-NaCl/0.015 M-sodium citrate.

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Fig. 1. RNA blot analysis of tissue-specific ceruloplasmin mRNA

Total cellular RNA (10 µg) was isolated from non-pregnant adult liver (lane 1), term uterus (lane 2), mammary gland at day 18 of pregnancy and at term (lanes 3 and 4 respectively), and non-pregnant adult brain and heart (lanes 5 and 6 respectively) of Sprague-Dawley rats. The RNA was electrophoresed, transferred to nylon and then hybridized with a cRNA antisense probe to rat ceruloplasmin as described in the text. The blot was exposed to X-OMAT film at -70°C for 20 h and subsequently re-hybridized with a cDNA probe to β -actin.

Diphenyloxazole (EnHance) was purchased from Du Pont-NEN Research (Wilmington, DE, U.S.A.), formalin-fixed *Staphylococcus* Protein A (IgG-sorb) from Enzyme Center (Malden, MA, U.S.A.); and DPX mountant was purchased from BDH Ltd. (Poole, Dorset, U.K.). ^{14}C -methylated protein standards were purchased from Amersham. Electrophoresis reagents including SDS, TEMED and ammonium persulphate were purchased from Bio-Rad (Richmond, CA, U.S.A.). $[^{32}\text{P}]\text{dCTP}$, $[^{35}\text{S}]\text{methionine}$, and $[^{35}\text{S}]\text{UTP}$ were purchased from ICN Radiochemicals (Costa Mesa, CA, U.S.A.).

Male and female Sprague-Dawley rats purchased from Harlan Sprague-Dawley (Indianapolis, IN, U.S.A.) were housed in the Animal Care Facility of the Children's Hospital at Washington University and were maintained on standard laboratory chow. Pregnancy was determined by the presence of a vaginal plug; this was considered day 0. The stage of oestrus was determined by vaginal swab; slides were prepared immediately before killing the animals [19]. All animal experiments were conducted to conform with the 'Guiding Principles for Research Involving Animals and Human Beings' of the American Physiological Society, as previously described [18]. Normal and pathological specimens of human breast tissue were obtained under approved protocol immediately post-surgery from patients undergoing radical mastectomy at the Jewish Hospital at Washington University Medical Centre, St. Louis, MO, U.S.A.

RNA isolation and analysis

Animals were killed and the organs were removed, rinsed in phosphate-buffered saline (0.01 M- $\text{Na}_2\text{HPO}_4/0.15$ M- NaCl , pH 7.2) and frozen in liquid nitrogen as previously described [20]. Tissue was homogenized in guanidinium isothiocyanate and RNA was isolated by caesium chloride gradient centrifugation [21]. Poly(A)⁺ RNA was selected by oligo(dT)-cellulose chromatography [22]. RNA was electrophoresed in 1% agarose/2.2 M-formaldehyde denaturing gels, transferred to nylon membranes by capillary blotting, immobilized by u.v.-cross-linking and subsequently hybridized with ^{32}P -labelled cRNA antisense probes as described [17]. ^{32}P -labelled cDNA probes were prepared with random priming and hybridized overnight at 42°C [23]. RNA samples were analysed for degradation after

agarose gel electrophoresis by visualization of intact ribosomal bands with ethidium bromide. In some cases, blots were also hybridized with a cDNA clone for β -actin to ensure that equivalent amounts of RNA were present in each lane. RNA slot-blot analysis was performed using the Minifold II Slot-Blot System (Schleicher and Schuell). Total cellular RNA was prepared as above and diluted to 1 µg/µl. RNA (1, 2 or 4 µg) was dissolved in 100 µl of sterile water, mixed with 6.15 M-formaldehyde/10× SSC (1× SSC = 0.15 M- $\text{NaCl}/0.015$ M-sodium citrate) and applied to nitrocellulose filters under vacuum. RNA slot-blots were dried, baked in a vacuum oven at 80°C for 2 h and hybridized as described [24]. After washing, all blots were exposed to Kodak X-OMAT film at -70°C with a Du Pont intensifying screen. Autoradiographs within a linear range were quantified via laser densitometry. cDNA clones used in this study included rat ceruloplasmin [17], human ceruloplasmin [20], rat β -actin [25], rat β -casein [3], rat whey acidic protein [2] and mouse albumin [26]. A cDNA probe for rat transferrin was obtained by polymerase chain reaction cloning after first strand synthesis of liver RNA. Oligonucleotides used in cloning were derived from the published sequence for a partial rat transferrin cDNA [27].

In situ hybridization

Frozen sections (5 µm) of rat lactating mammary gland were mounted on poly(L-lysine)-coated slides at 56°C for 10 min and stored at room temperature (RT) under vacuum with desiccant overnight. Tissue sections were subsequently pre-hybridized in proteinase K and acetylated in acetic anhydride to expose mRNA and decrease non-specific binding of the probe as described [28]. Tissue sections were then hybridized with ^{35}S -labelled cRNA probes by coating the coverslip with hybridization solution and sealing with DPX mountant. Slides were incubated overnight at 56°C , allowed to cool to RT, and then washed four times in 4× SSC (5 min each). Slides were digested in RNAase A (10 mg/ml) at 37°C , rinsed with decreasing concentrations of SSC, dehydrated in ethanol, drained and vacuum-dried at RT for 30 min and subsequently dehydrated in ethanol, delipidized in xylene, rinsed and air-dried. Sections were then coated with Kodak NTB-2 liquid autoradiography emulsion and subsequently stored at 4°C in a sealed light-proof container with desiccant. Following exposure, slides were developed and counterstained with haematoxylin and eosin.

Biosynthetic labelling, immunoprecipitation and SDS/PAGE

Freshly removed liver and mammary gland were rinsed in phosphate-buffered saline, minced in methionine-free DMEM/10% fetal calf serum and incubated at 37°C in 5% CO_2 for 1 h. A 1 g portion of tissue was subsequently pulse-labelled with $[^{35}\text{S}]\text{methionine}$ for 2 h. After labelling, culture medium (extracellular) was removed and the tissue was lysed by freeze/thawing followed by solubilization (intracellular lysate) [29]. Total protein synthesis was determined by trichloroacetate precipitation of an aliquot of tissue lysate and medium [30]. Both lysate and medium were clarified by centrifugation at 4°C for 10 min at 10000 g and incubated overnight at 4°C in 1% Triton/1% SDS/0.5% deoxycholic acid with excess antibody. Immune complexes were precipitated with formalin-fixed *Staphylococcus* Protein A, released by boiling in sample buffer, and applied to SDS/7.5% PAGE under reducing conditions [31]. ^{14}C -methylated molecular size markers were included on all gels. Following electrophoresis, gels were soaked with 2,5-diphenyloxazole (EnHance), rinsed in cold water and vacuum-dried with heat for fluorography on Kodak XAR-5 film. Antibodies used in this study included rabbit anti-(rat ceruloplasmin) [32], rabbit anti-(human ceruloplasmin), anti-(rat transferrin) and rabbit anti-(rat albumin) [13].

Fig. 2. RNA blot analysis of ceruloplasmin gene expression in the mammary gland

(a) Total RNA (10 µg) was prepared from mammary gland tissues and hybridized with a cRNA antisense probe to rat ceruloplasmin (lanes 1–6) or transferrin (lanes 7–12); blots were exposed for 17 h at -70°C and for 0.5 h at RT respectively. RNA was analysed from two different adult males (lanes 1, 2, 7 and 8) and from four females at different stages of development: virgin (lanes 3 and 9), non-pregnant/multiparous, 3 months *post partum* (lanes 4 and 10), 18-day pregnant (lanes 5 and 11) and lactating, 3 days *post partum* (lanes 6 and 12). (b) Total RNA (10 µg) was prepared from mammary gland tissue and hybridized with β -casein cDNA and exposed for 16 h at -70°C . RNA was obtained from mammary glands from virgin females (lane 1), non-pregnant multiparous females (lane 7), males (lanes 8 and 9) and females during early pregnancy (day 7; lane 2), late pregnancy (day 18; lane 3), term (lane 4), early lactation (3 days *post partum*; lane 5) and late lactation (14 days *post partum*; lane 6). (c) RNA blot analysis as in (b), hybridized with a cDNA probe to whey acidic protein and exposed for 30 min at room temperature.

Human HepG2, Hep3B, Hs242T and MCF-7 cell lines were obtained as stocks from the American Tissue Culture Collection and were maintained in culture according to specifications.

Fig. 4. Immunoprecipitation with a specific anti-ceruloplasmin antibody of biosynthetically labelled tissues

Tissue from the mammary gland and liver (1 g) of a lactating (day 4 *post partum*) female and an adult male rat was minced and biosynthetically labelled with [^{35}S]methionine as described in the text. Intracellular lysates from HepG2 (lane 1), mammary gland and liver from a lactating female (lanes 2 and 3 respectively) and male mammary gland (lane 4) were immunoprecipitated with rabbit anti-rat ceruloplasmin followed by SDS/PAGE and fluorography. Molecular mass markers are indicated on the left. The dried gel was exposed to XAR-5 film at -70°C for 30 h.

Biosynthetic labelling of cell lines and immunoprecipitation from cellular lysates was performed as previously described [33].

RESULTS

The ceruloplasmin gene is expressed in the rat mammary gland

Ceruloplasmin gene expression in the mammary gland was initially examined by RNA blot analysis of total RNA from mammary gland tissue of rats at day 18 of gestation and at term. As can be seen in Fig. 1, a single 3.7 kb ceruloplasmin transcript was detected in the mammary gland from both of these animals which is qualitatively similar to that present in liver and in term uterus. No ceruloplasmin-specific transcript was detected in total RNA from brain and heart tissue, despite prolonged exposure of the gels. The ceruloplasmin mRNA content in mammary gland

Fig. 3. RNA slot-blot analysis of ceruloplasmin and transferrin mRNA in prepubertal pups and their lactating dam

(a) Total RNA (lane 1, 1 µg; lane 2, 2 µg; lane 3, 3 µg) from mammary gland (M) and livers (L) of two male and two female 2-week-old pups and their lactating dam was applied to nitrocellulose membranes and subsequently hybridized with a cRNA antisense probe to ceruloplasmin. (b) RNA slot-blot as in (a) hybridized with a cRNA antisense probe to transferrin. Blots were exposed to X-OMAT film at -70°C for 3 h.

Fig. 5. *In situ* hybridization of mammary gland tissue with ceruloplasmin

Lactating rats (1 day *post partum*) were killed and 5 μ m frozen sections of mammary gland were prepared and mounted on slides as described in the text. Gene expression was examined by light (*a–c*) and corresponding dark-field (*d–f*) microscopy. (*a*), (*d*) Lactating mammary gland hybridized with an 35 S-labelled cRNA antisense probe to rat ceruloplasmin. Large arrowheads outline alveolar ducts; the surrounding stroma is indicated by small arrowheads. (*b*), (*e*) Slides of frozen sections from lactating mammary gland were hybridized with an 35 S-labelled cRNA antisense probe to rat ceruloplasmin. Again, large arrowheads outline alveolar ducts; small arrowheads identify a mast cell. (*c*), (*f*) Lactating mammary gland tissue hybridized with an 35 S-labelled sense strand probe for ceruloplasmin. Slides were coated with Kodak NTB-2 liquid autoradiography emulsion and stored in a light-proof container with desiccant at 4 °C for 1–3 weeks (exposure time determined empirically). Magnifications: $\times 100$ for (*a*), (*c*), (*d*) and (*f*); $\times 400$ for (*b*) and (*e*).

at term was 18–20 % of that observed in liver (results not shown). Subsequent hybridization of these blots with a cDNA to rat β -actin confirmed that these differences were tissue-specific and not the result of variation in the amount of RNA per lane.

In order to characterize more fully the extent of ceruloplasmin gene expression in the mammary gland, we examined the ceruloplasmin mRNA content in the mammary glands from pregnant and lactating rats as well as male, virgin female and non-pregnant/multiparous rats (Fig. 2*a*). A single 3.7 kb ceruloplasmin transcript was readily identified in mammary gland tissue from each of these sources. The abundance of ceruloplasmin mRNA was roughly the same in male as in pregnant and lactating rats, whereas in the non-pregnant (virgin or multiparous) rats, ceruloplasmin gene expression was approx. 2–3-fold greater. Similarly, transferrin gene expression was also readily detectable in mammary glands from male and non-pregnant female animals as well as during pregnancy and lactation. The expression of ceruloplasmin and transferrin in the mammary glands from these animals was distinctly different

from that of β -casein (Fig. 2*b*) and whey acidic protein (Fig. 2*c*). The expression of β -casein-specific and whey acidic protein-specific mRNA was entirely confined to mammary gland tissue from female animals during pregnancy and lactation. In addition, no difference in the abundance of ceruloplasmin-specific mRNA in mammary gland tissue was observed in virgin female rats throughout the oestrus cycle (results not shown).

The ceruloplasmin gene is expressed in the mammary glands of prepubertal rats

Having demonstrated abundant ceruloplasmin gene expression in mammary gland tissue from adult male and female animals, we next examined ceruloplasmin-specific mRNA in the livers and mammary glands of prepubertal pups. Initial studies revealed no qualitative changes in ceruloplasmin gene expression in mammary gland tissue during development, and thus ceruloplasmin-specific mRNA was examined in 2-week-old rat pups and their lactating dam by slot-blot analysis. As can be seen in Fig. 3(*a*), the ceruloplasmin gene was expressed in both mammary gland

Fig. 6. *In situ* hybridization of mammary gland tissue with transferrin

Slides of lactating mammary gland tissue sections were prepared as described in the text and hybridized with an ^{35}S -labelled cRNA antisense (a, b, d and e) or sense (c and f) probe to rat transferrin. Gene expression was examined by light (a–c) or dark-field (d–f) microscopy. (a), (d) Large arrowheads outline alveolar ducts; small arrowheads identify an artery. (b), (e) Large arrowheads indicate ducts. (c), (f) Lactating mammary gland tissue. Slides were exposed 4–7 days as described in the text. Magnifications: $\times 100$ for (a), (c), (d) and (f); $\times 400$ for (b) and (e).

and liver as early as 2 weeks, and the abundance of ceruloplasmin mRNA in each of these tissues was comparable with that seen in the lactating dam. Although ceruloplasmin gene expression was readily detected in male and female pups at 2 weeks of age, the abundance of ceruloplasmin mRNA in male glands was consistently less than that in the glands from female littermates at this developmental stage (results not shown). Interestingly, transferrin-specific mRNA was also detected in the mammary gland by 2 weeks of age (Fig. 3b), in contrast with β -casein and whey acidic protein, which were not present in this tissue until pregnancy (result not shown).

Ceruloplasmin gene expression in the mammary gland is associated with production of ceruloplasmin protein

To determine if the expression of ceruloplasmin mRNA in mammary gland tissue resulted in the production of ceruloplasmin protein, we isolated mammary gland and liver tissue from adult male and day 4 *post partum* female rats and immunoprecipitated ceruloplasmin from the medium (extracellular) and tissue lysate (intracellular) after biosynthetic labelling with [^{35}S]methionine (Fig. 4). Mammary glands from both male and female rats synthesized and secreted a 132 kDa protein,

consistent with the single chain size of ceruloplasmin. Determination of total protein biosynthesis from each tissue followed by quantification of specific ceruloplasmin synthesized indicated that equivalent amounts of ceruloplasmin were synthesized by male and female mammary gland tissue (results not shown). The relative amounts of ceruloplasmin synthesized by liver could not be quantified in these experiments because of inefficient labelling of this tissue. In all cases, immunoprecipitation of a 132 kDa protein was inhibited by pre-addition of excess unlabelled ceruloplasmin, but not albumin (results not shown).

The ceruloplasmin gene is expressed in epithelial cells of the alveolar ducts

Cell-specific expression of the ceruloplasmin gene in mammary gland tissue was determined by *in situ* hybridization. Fig. 5(a) demonstrates that ceruloplasmin-specific mRNA was confined entirely to the epithelium of mammary gland ducts (large arrowheads), with no evidence of expression in the surrounding mesenchyme (small arrowheads). Ceruloplasmin gene expression was homogeneous, being detected throughout the epithelium of small alveoli and larger ducts (Fig. 5b, large arrowheads) and

Fig. 7. Ceruloplasmin biosynthesis in human cancer lines

Lanes 1–5 show immunoprecipitation of intracellular lysate from Hep3B cells with rabbit anti-(human ceruloplasmin) in the absence (lane 1) or the presence (lane 2, 50 μ g; lane 3, 5 μ g; lane 4, 0.5 μ g; lane 5, 0.05 μ g) of decreasing amounts of excess ceruloplasmin prior to the addition of antibody. Lanes 6–11 show immunoprecipitation of intracellular lysates from Hep3B (hepatoma, lanes 6 and 7), Hs242T (lung adenocarcinoma, lanes 8 and 9) and MCF-7 (breast carcinoma, lanes 10 and 11) in the absence (lanes 6, 9 and 10) or the presence (lanes 7, 8 and 11) of excess human ceruloplasmin. Immunoprecipitates were analysed by SDS/PAGE followed by fluorography as described in the text. Molecular mass markers are indicated on the right. The dried gel was exposed to XAR-5 film at -70°C for 25 h.

Fig. 8. RNA blot analysis of ceruloplasmin-specific mRNA in human breast tissue

Normal and pathological specimens of breast tissue were obtained at the time of surgery for invasive ductal carcinoma and snap frozen in liquid nitrogen. Total and poly(A)⁺ RNA were prepared as described in the text and subsequently hybridized with a cRNA antisense probe to human ceruloplasmin. Lanes 1–3 contain 10 μ g of total RNA from normal breast tissue (lane 1), invasive ductal carcinoma (lane 2) and HepG2, a human hepatoma cell line (lane 3). Lanes 4–7 contain 1 μ g of poly(A)⁺ RNA from normal (lanes 4 and 5) and malignant (lanes 6 and 7) breast tissue.

again absent in stromal cells such as mast cells (Fig. 5b, small arrowheads). A similar cell-specific pattern of gene expression was observed for transferrin (Fig. 6), with expression confined to the epithelium (Fig. 6a, large arrowheads) and absent in surrounding mesenchyme and blood vessels (Fig. 6a, small arrowheads). Dark-field examination was used in these studies to illustrate silver grains in order to allow demonstration of homogeneous gene expression throughout the mammary gland. Higher magnifications clearly showed silver grains in individual

epithelial cells on bright field for both ceruloplasmin and transferrin (results not shown).

The ceruloplasmin gene is expressed in human cancer cell lines and tissue

Having demonstrated ceruloplasmin gene expression in rat mammary gland, we next analysed human cancer cell lines and normal and pathological human breast tissue for ceruloplasmin-specific mRNA content and protein biosynthesis. Cell lines were biosynthetically labelled and immunoprecipitated with a specific antibody to human ceruloplasmin. The specificity of the ceruloplasmin antibody was evaluated by blocking immunoprecipitable ³⁵S-labelled protein by the addition of increasing amounts of unlabelled ceruloplasmin (Fig. 7). As can be seen from the data, abundant ceruloplasmin biosynthesis was found in the human breast adenocarcinoma cell line (MCF-7) as well as in liver (Hep3B) and lung (Hs242T) cell lines. To determine if ceruloplasmin was expressed in primary tissues from the human mammary gland, we isolated RNA from normal and pathological tissue samples removed during radical mastectomy for invasive ductal carcinoma. Ceruloplasmin-specific transcripts were readily detected in total and poly(A)⁺ RNA samples from malignant tissue, but not in RNA from the normal breast tissue surrounding the carcinoma (Fig. 8). In addition to the 3.7 and 4.3 kb ceruloplasmin-specific transcripts normally present in human liver and HepG2 cells, an additional 3.9 kb transcript was observed in mammary tissue from patients with invasive ductal carcinoma (Fig. 8). Ceruloplasmin gene expression and biosynthesis were identical in Hep3B and HepG2 cells (results not shown).

DISCUSSION

These data demonstrate that the mammary gland is a prominent site of extrahepatic ceruloplasmin gene expression. This expression is qualitatively similar to that previously demonstrated in liver, lung and uterus [17], and results in the production of ceruloplasmin protein. The abundant expression of ceruloplasmin mRNA in the mammary glands from male and non-pregnant female animals, as well as in mammary gland tissue during prepubertal development, suggests that this protein plays a role in mammary gland function independent of, or in addition to, pregnancy and lactation. Our biosynthetic data support this conclusion, demonstrating equivalent amounts of ceruloplasmin biosynthesis in male and lactating female mammary gland relative to total protein synthesis. Although previous studies have demonstrated transferrin gene expression in the pregnant and *post partum* mammary gland, the data in this paper clearly demonstrate expression of the transferrin gene in this tissue independent of lactogenesis [27]. Consistent with these data, previous studies have shown that the majority of transferrin present in milk during lactogenesis is derived via transfer from maternal plasma as opposed to local synthesis [34]. Overall, these data do not support a role for either ceruloplasmin or transferrin as a milk product. The expression of these metalloproteins in the mammary gland is not due to non-specific expression, since specific mRNAs for other plasma protein genes, including albumin and haemopexin, were not detected [35]. Furthermore, ceruloplasmin gene expression in the mammary gland is not the result of acute-phase induction within tissue macrophages as previously described in lung [18], because *in situ* hybridization revealed that ceruloplasmin mRNA was localized to the epithelial cells lining mammary gland alveolar ducts. Taken together, these data suggest a fundamental role for ceruloplasmin and transferrin in the biology of the mammary epithelial cell.

The mechanisms which determine expression of the cerulo-

plasmin gene in the mammary gland are unknown. Previous studies have shown that oestrogen and copper can increase ceruloplasmin biosynthesis in primary cultures of rat hepatocytes [32]. However, the data presented here suggest that mammary gland ceruloplasmin gene expression is independent of the female hormonal milieu, and are therefore not supportive of the concept that ceruloplasmin gene expression in the mammary gland accounts for the changes in ceruloplasmin and copper metabolism observed during pregnancy and lactation. Transferrin gene expression in the mammary gland has been shown to be independent of lactogenic hormones, being instead mediated, at least in part, by the nature of the extracellular matrix underlying the mammary epithelium [27]. Future studies will need to determine if mammary gland epithelial cell expression of ceruloplasmin is similarly affected. Recent experiments in transgenic mice have defined *cis*-acting elements in the 3'- and 5'-flanking regions of whey acidic protein and β -casein genes that are responsible for determining mammary-gland-specific gene expression. It is reasonable to anticipate that analogous sequences will be found in the flanking regions of the ceruloplasmin gene. Defining these elements will be essential in elucidating the molecular mechanisms of extrahepatic tissue-specific ceruloplasmin gene expression, and also may be useful in directing mammary-gland-specific expression of transgenes independent of the state of pregnancy or lactogenesis.

The studies in this paper do not specifically address the role of ceruloplasmin in the mammary gland. The similarities of expression of ceruloplasmin and transferrin in this tissue suggest a possible functional relationship between these proteins. Such a relationship is supported by data demonstrating a role for ceruloplasmin ferroxidase activity in mediating iron binding to transferrin and ferritin [7,36]. A potential role for ceruloplasmin in the biology of the mammary epithelial cell is also suggested by the increased expression of this protein in invasive ductal carcinoma (Fig. 8). Although the lack of expression in normal tissue does not parallel the findings in rodents, this may be due in part to a relative paucity of epithelial cells from the normal tissue surrounding the tumours. The increased expression of ceruloplasmin in anaplastic tissue supports previous data suggesting a role for this protein in angiogenesis during development and tumour formation [8,37]. While it is possible that the mammary-gland-specific ceruloplasmin transcript encodes a unique isoform of ceruloplasmin, such a concept is not supported by data indicating that the nucleotide sequences of ceruloplasmin cDNA in lung and liver are identical. Finally, it is important to note that, although each of these putative roles involves the presence of copper within the protein, the current studies have not determined that holoceruloplasmin is synthesized. This latter point is important because distinct functional differences may exist for apo- and holo-ceruloplasmin, and recent studies clearly demonstrate marked difference in the ratios of apo- to holo-ceruloplasmin synthesized by human liver and transformed cell lines [33]. Future studies will be needed to determine if the holoprotein is synthesized in mammary gland tissue, and to identify the ultimate destination of ceruloplasmin (tissue versus serum versus milk) synthesized locally within mammary gland.

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