Oestrogen metabolism in lymphangioleiomyomatosis: catechol-*O*-methyltransferase pathway is not involved

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Abstract

Background—Lymphangioleiomyomatosis (LAM) is an uncommon lung disease for which no effective method of treatment has been found. The predilection of LAM for premenopausal women has led to the assumption that hormonal factors play an important role in the pathogenesis of this disease. The aim of this study was to determine if women with LAM manifest alterations in the catechol-*O*-methyltransferase (COMT) pathway which is essential for preventing the generation of oestrogen derived reactive oxygen species (ROS).

Methods—Blood samples were collected from 15 women with LAM and compared with appropriate controls. The distribution of high and low activity alleles of COMT was determined with a PCR based RFLP assay. The enzymatic activity of COMT was measured in each sample and the potential presence of a circulating inhibitor of COMT was determined. Since an alteration in the COMT pathway could increase the oxidative stress, the plasma concentration of malondialdehyde (MDA), a secondary product generated from lipid peroxidation, has been used as an internal marker.

Results—The distribution of high and low activity alleles of COMT (named $COMT^{HH}$, $COMT^{LL}$, and $COMT^{HL}$) was similar in the two groups with proportions of 40%, 7%, and 53%, respectively, in the women with LAM and 38%, 6%, and 56% in the control subjects. The mean (SD) COMT activity was 24.2 (12.3) pmol/min/mg protein in women with LAM and 24.1 (6.3) pmol/min/mg protein in the control group. Incubation of plasma from women in the two groups with a preparation of commercial COMT showed that no detectable COMT inhibitor was present. The plasma concentration of MDA in the women with LAM was also not significantly different from control subjects.

Conclusions—This study shows that there are no significant alterations in the COMT pathway of women with LAM. It is therefore unlikely that alterations in oestrogen mediated cell signalling pathways are mediated by oxidants derived from an excess of catecholestrogens in LAM.

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Keywords: lymphangioleiomyomatosis; oestrogen metabolism; catechol-*O*-methyltransferase

Lymphangioleiomyomatosis (LAM) is an uncommon lung disease with an incidence of less than 1/100 000 per year which affects almost exclusively women in their childbearing years.¹ Although much information has been accrued about the pathological and clinical manifestations of LAM, the molecular and cellular mechanisms responsible for the disease have remained a mystery and an effective treatment has yet to be defined. The predilection of LAM for premenopausal women has led to the assumption that hormonal factors play an important role in the pathogenesis of the disease.² At least three different lung transplantation groups have reported that LAM can recur in the transplanted lung.³⁻⁵ These reports point to a circulating factor, possibly related to oestrogen metabolism, that could play an important part.

Oestradiol is converted to oestrone by the 17 β -hydroxysteroid dehydrogenase (17 β -HSD) oxidase and vice versa by the 17 β -HSD reductase. Both oestradiol and oestrone can efficiently be hydroxylated by the 2- and 4-hydroxylation pathways. These 2- or 4-hydroxyoestrogens, also called catecholestrogens, can be oxidised to form semiquinones and quinones. During this latter conversion oxygen is reduced, producing the superoxide anion which can generate hydroxyl radical in the presence of iron or copper (fig 1).⁶ Hydroxyoestrogens derived from either oestradiol or oestrone show the same capacity to generate reactive oxygen species (ROS), as determined by induction of oxidative damage to DNA.⁷ At low concentration these ROS can behave as second messengers activating transcription factors such as NF- κ B⁸ and, interestingly, mediate mesenchymal cell proliferation.⁹ In light of these studies, ROS continuously produced at low levels by hydroxyoestrogens may contribute to the induction of smooth muscle cell proliferation associated with LAM. Thus, alterations in oestrogen metabolism in LAM may conceivably involve an increase in catecholestrogen production or a decrease in the expression of enzymes involved in their elimination.

Accumulation of these hydroxyoestrogens is not important in healthy women. However, in diseases such as breast cancer, local alterations in oestrogen metabolism have been detected which favour the production of catecholestrogens,¹⁰ and these oestradiol metabolites are suggested to be related to breast cancer progression.¹¹

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Table 1 Characteristics of LAM and control populations

	LAM	Control
Mean (SD) age (years)	42.6 (9.2)	38.9 (9.8)
Mean (SD) BMI (kg/m ²)	23.7 (5.5)	22.3 (2.7)
Lung biopsy*	9/13	—
Ovariectomy*	4/13	0/17
Hysterectomy*	3/13	0/17
Contraceptive pill	0/15	2/17
Hormone replacement therapy	9/15	2/17
Diabetes	0/15	1/17
Liver disease	0/15	0/17
Kidney disease	2/15	0/17
Osteoporosis	7/15	0/17
Thyroid disease	1/15	1/17

*Complete data were not available for two of the 15 patients.

mental protocols were approved by the institutional review board for human studies. Informed consent was obtained from all participants. Questionnaires were completed to determine whether participants had other diagnosed diseases, physiological conditions, or medication that could modify oestrogen metabolism. All subjects were non-smokers. The characteristics of the studied population are summarised in table 1.

COMT GENOTYPE

The presence of the G-to-A transition on exon 4 was used to analyse the polymorphic COMT genotype. Genomic DNA from LAM women and controls was subjected to a PCR based RFLP assay.¹⁷ A 237 bp fragment of the COMT gene was first amplified by PCR using the forward primer TACTGTGGCTACTCAGCTGTGC and the reverse primer GTGAACGTGGTGTGAACACC. A 25 µl PCR reaction containing 50 ng genomic DNA, 250 µM each deoxynucleotide triphosphates, 300 nM each primer, 1 X reaction buffer (Perkin-Elmer, Branchburg, New Jersey, USA), and 0.6 unit Taq polymerase (Perkin-Elmer) was placed in a thermocycler (PTC-100, MJ Research Inc, Waltham, Massachusetts, USA). After denaturing for three minutes at 94°C the DNA was amplified for 35 cycles at 94°C for 30 seconds, at 60°C for 20 seconds, and at 72°C for 30 seconds, followed by a five minute extension at 72°C. An 8 µl sample of the PCR product was then digested for three hours at 37°C with 10 units of *Nla*III (New England Biolabs, Beverly, Massachusetts, USA). The products of the restriction digest were combined with 1 µl of loading buffer (10% Ficoll and 0.25% xylene cyanol) and run on a 10% non-denaturing polyacrylamide gel in 1 X Tris-borate-EDTA buffer at 50 V for three hours. Restriction fragments of 27, 42, and 54 bp were present in every digested sample. The high and low activity alleles were detected by the presence of 114 bp and 96 bp fragments.

BLOOD SAMPLE PREPARATION

Blood samples were sent by courier and received within 24 hours. Precautions were taken to maintain the temperature at about 4°C using ice packs. Control blood samples were subjected to similar handling. Each sample was divided in two. Plasma was isolated from heparinised blood and used to assay for a potential COMT inhibitor. Determination of

COMT activity in blood cells was performed in 200 µl whole blood collected in 2 ml heparinised tubes. A volume of 800 µl ice cold distilled water was added to lyse the blood cells and 100 µl of a suspension of sodium Chelex-100 was added to eliminate the calcium ion (Ca²⁺), a strong inhibitor of the COMT enzyme. The lysed samples and the resin were mixed with a tube rotator at 12 rpm for one hour at 4°C. Samples were then centrifuged at 7000g for 10 minutes and the supernatant was removed for the determination of COMT activity.

DETERMINATION OF COMT ACTIVITY

Determination of COMT activity was performed according to an established procedure.¹⁸ The 3,4-dihydroxybenzoic acid was used as a catechol substrate to measure the level of COMT dependent methylation activity. Briefly, 20 µl of the supernatant from each Chelex-100 treated sample of lysed whole blood was added to 180 µl Tris-Mg buffer (0.08 M Tris-HCl, pH 7.5, 1 mM MgCl₂) and 100 µl of reaction buffer (0.08 M Tris-HCl, pH 7.5, 1 mM MgCl₂, 2.8 µM S-adenosyl-L-(Me-¹⁴C)methionine, 20.2 µM non-radioactive S-adenosyl-L-methionine, 1 mM 3,4-dihydroxybenzoic acid, 4.2 mM dithiothreitol, and 0.64 units of adenosine deaminase.¹⁹ The reaction mixture was incubated for 90 minutes at 37°C in a shaker water bath and the reaction was stopped by the addition of 100 µl of 1.0 N HCl; 2 ml of toluene was then added to each tube. The tubes were vortexed for 10 seconds, centrifuged at 700g for 10 minutes, and the organic phase was added to counting vials containing 10 ml toluene fluor based liquid scintillation. To verify that the Me-¹⁴C compound extracted with toluene was the methylated dihydroxybenzoic acid, compounds were separated by HPLC on a Spherisorb ODS-2 column (5 µm, 25 cm × 0.46 cm) with a mobile phase containing 15% methanol and 85% 30 mM sodium citrate at pH 4.75 eluted at a flow rate of 1.0 ml/min.²⁰ The compounds were detected by fluorescence (Ex = 310 nm, Em = 420 nm). Retention times of dihydroxybenzoic acid and its methylated derivatives were obtained by injecting appropriate standards.

COMT activity was expressed as pmol 4-hydroxy-3-methoxybenzoic acid formed per minute per mg protein quantified in the lysed whole blood with the Bio-Rad protein assay (Bio-Rad, Hercules, California, USA).

PRESENCE OF POTENTIAL COMT INHIBITOR

The presence of a circulating inhibitor of the COMT enzyme was verified as follows. Volumes of 10 µl or 50 µl plasma from LAM patients or controls were added to 12 units of a commercial preparation of COMT (Sigma, # C1897, porcine liver extract, 2500 U/mg). The enzymatic assay was performed as mentioned previously. The presence of a circulating inhibitor was determined by comparing the level of COMT activity measured in the presence and absence of plasma.

Figure 3 PCR-based RFLP analysis for the *COMT* genotype. Representative polyacrylamide gel of the *COMT* exon 4 amplified by PCR and digested by the *NlaIII* restriction enzyme. Lanes 1, 3, 4, and 6 contain DNA from heterozygote individuals (*COMT*^{HL}) and lanes 2, 5, and 7 contain DNA from homozygote individuals for the high activity allele (*COMT*^{HH}).

Table 2 Distribution of *COMT* genotype

	<i>COMT</i> ^{HH}	<i>COMT</i> ^{HL}	<i>COMT</i> ^{LL}
LAM	40%	7%	53%
Control	38%	6%	56%

MDA

Using gas chromatography-mass spectrometry (GC-MS), the MDA can be detected in femtomole quantities in biological samples.²¹ The analysis was performed with a Hewlett Packard 5890 Series II gas chromatograph interfaced to a 5989A mass spectrometer. The results were expressed as pmol MDA/ml plasma.

STATISTICAL ANALYSIS

The results are expressed as mean (SD). Statistical comparison between the two groups was analysed using the paired *t* test.

Results

The *COMT* activity is dependent on the presence of high and low activity alleles of this enzyme. A PCR based RFLP assay was used to determine whether women with LAM were preferentially carrying the low activity alleles (fig 3). Heterozygous *COMT* was detected by the presence of two bands of 114 bp and 96 bp on the polyacrylamide gel while a single band of 114 bp or 96 bp indicated the presence of homozygous *COMT*^{HH} or *COMT*^{LL}, respectively. Since LAM is a very rare disease, each group represents a small number of subjects. Nevertheless, the proportion of *COMT* alleles was similar for the two groups (table 2). The distribution of *COMT*^{HH}, *COMT*^{HL}, and

COMT^{LL} genotype was 40%, 7%, and 53%, respectively, in the LAM group, which is in close agreement with the control group which had a distribution of 38%, 6%, and 56%. Interestingly, we found fewer *COMT*^{LL} cases than Thompson *et al*²² who found 19% of *COMT*^{LL}, perhaps because of the small numbers of subjects in our groups. Nevertheless, the low incidence of *COMT*^{LL} in this LAM group (1/15) suggests that the presence of low activity alleles of *COMT* is not required for the expression of this disease.

If alterations in oestrogen dependent mesenchymal cell signalling in LAM result from defective *COMT* activity, then catechol-oestrogen metabolism may be associated with the generation of ROS. To verify this hypothesis the enzymatic activity of *COMT* in whole blood of LAM women was measured. The blood samples were treated with Chelex-100 to eliminate the Ca²⁺ ion, a potent inhibitor of *COMT*, and the *COMT* activity was determined with an enzymatic assay using the 3,4-dihydroxybenzoic acid as a catechol substrate (table 3). No significant difference in *COMT* activity was detected between the two groups (24.2 (12.3) pmol/min/mg protein in the LAM group and 24.1 (6.3) pmol/min/mg protein in the control group). The capacity of blood cells from women with LAM to prevent the generation of ROS by methylating the catechol-oestrogens was therefore not altered.

Another possibility is that the activity of *COMT* may be reduced by the presence of a circulating inhibitor in the plasma. This hypothesis was verified by incubating 10 µl or 50 µl plasma from the two groups of women with a preparation of commercial *COMT* (table 3). The plasma used was not treated by Chelex-100 or any other procedure. Since the Ca²⁺ was still present, the activity obtained with the commercial *COMT* was reduced by 6–33 times according to the volume and sample of plasma tested. There was no statistical difference between the final *COMT* activity obtained by the addition of plasma from LAM or control women, which indicates that women with LAM do not carry a specific inhibitor for *COMT* enzyme in their plasma.

According to the *COMT* pathway hypothesis, altered catechol compound metabolism would result in overproduction of ROS which could increase the level of oxidative damage found in blood. The plasma concentration of MDA, a secondary product generated from lipid peroxidation, has been used as an internal marker of oxidative stress (table 3). Using a GC-MS analysis, the average MDA level in the women with LAM was 178.5 (80.1) pmol/ml plasma, which was not significantly different from the control group in whom a mean level of 155.4 (75.0) pmol/ml plasma was detected. Therefore, according to this assay, LAM is not associated with an increased level of oxidative stress, as detectable in blood.

Discussion

LAM is an uncommon lung disease for which the aetiology remains a mystery and an effective treatment has yet to be defined. The

Table 3 Cumulative results

Analysis	Mean (SD)		<i>p</i> value
	LAM	Control	
<i>COMT</i> activity*	24.2 (6.3)	24.2 (12.4)	0.996
<i>COMT</i> inhibitor in plasma**			
10 µl	0.48 (0.12)	0.43 (0.20)	0.44
50 µl	0.36 (0.10)	0.31 (0.13)	0.30
MDA (pmol/ml plasma)	178.5 (80.1)	155.4 (75.0)	0.40

**COMT* activity = pmol 4-hydroxy-3-methoxybenzoic acid formed per minute per mg protein.

**A plasma volume of 10 µl or 50 µl was added to 12 units of a commercial preparation of *COMT*. *COMT* activity without plasma = 4.35 pmol 4-hydroxy-3-methoxybenzoic acid formed per minute.

potential relationship with hormonal factors has frequently been suggested since LAM affects almost exclusively premenopausal women.² It has been hypothesised that alterations in oestrogen metabolism may be involved in the pathophysiology of LAM. This study is the first to verify this hypothesis, and specifically addresses whether the catechol-O-methyltransferase pathway is defective in LAM. This pathway is particularly important since a failure in COMT activity would result in a continuously higher production of hydroxy-oestrogen derived ROS, molecules known to induce smooth muscle cell proliferation.

Our results have shown that the genotype of COMT, as well as the enzymatic activity of COMT, did not differ significantly between the women with LAM and the control subjects, and that no specific inhibitor of COMT was detectable in blood. To eliminate definitively the possible involvement of ROS overproduction by catecholestrogen in LAM disease, it remains to be established whether the blood and lung levels of 2- and 4-hydroxyoestradiol and 2- and 4-hydroxyoestrone are increased. Higher activity of the 2-hydroxylase and 4-hydroxylase pathways leading to these oestrogen metabolites have already been detected in breast carcinoma.²³ It has been suggested that these alterations play an important part in the development of breast cancer,²⁴ and that a higher level of these hydroxyoestrogens (catecholestrogens) induces the development of resistance against the anticancer agent methotrexate.²⁵

Our results also indicated that the level of MDA was not modified, which suggests that there was no increase in systemic oxidative stress. However, we cannot exclude the possibility that ROS are present at a higher level in lung tissue with the potential to act as second messengers.⁸ It would therefore be of interest to determine whether some signalling pathways sensitive to ROS are activated in women with LAM. On the other hand, the superoxide anion generated by catecholestrogens reacts very rapidly with nitric oxide (rate constant 7×10^9 M/s) to form the highly reactive peroxynitrite anion,²⁶ a product not detected by the MDA assay. Since the concentration of nitric oxide can be important in the lung,²⁷ alteration in the production of either nitric oxide or superoxide anion could have deleterious effects and might deserve to be investigated.

Thus, this study showed that there were no significant differences in the COMT pathway in women with LAM. The relationship between LAM and altered oestrogen metabolism should now therefore be focused on other oestrogen metabolic pathways responsible for the production or elimination of oestrogens. Since smooth muscle cell proliferation can be stimulated by oestradiol,²⁸ an alternative hypothesis may be related to a lower activity of 17 β -HSD oxidase or a higher activity of 17 β -HSD reductase in women affected by LAM, both leading to overproduction of oestradiol.

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