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

Benjamin Paquette, Pierre-Karl Fortier, Julie Héroux, Paul A Thibodeau, Richard Wagner, Jiankang Liu, André Cantin

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<sup>HI</sup> and COMT<sup>LO</sup>) 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 catecholestoegens in LAM.

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Keywords: lymphangioleiomyomatosis; oestrogen metabolism; catechol-O-methyltransferase
Prevention of ROS generation by catechol-estrogens is closely related to the activity of the catechol-O-methyltransferase (COMT). This enzyme catalyses the methylation of hydroxylated sites on the aromatic ring of catechol compounds which prevents their conversion to semiquinones and quinones, and therefore blocks the generation of ROS. While kidney and liver cells are major sources of COMT, red blood cells and mononuclear cells are also rich in COMT and can convert significant amounts of catechol-estrogens to their inoffensive monomethoxy derivatives. Red blood cell COMT has been shown to play an important part in the detoxification of oestrogen catechols, as low red blood cell levels of COMT are thought to contribute to oestrogen carcinogenicity in hamster kidneys. Interestingly, kidney carcinogenesis is observed with increased frequency in patients with tuberous sclerosis, a disease in which the incidence of LAM is also increased.

Another possible correlation of COMT with LAM is abnormal HMB-45 antigen positive smooth muscle cell proliferation along the lymphatics, the key histopathological feature observed in the lungs of patients with LAM. The HMB-45 reactive antigen has been well characterised and studies have shown that it has full homology with the protein encoded by cDNA of Pmel 17, a protein thought to play a critical role in the conversion of indole-5,6-quinone and indole-5,6-quinone-2-carboxylic acid to eumelanin (fig 2). Eumelanin, a black-brown pigment, is one of two major classes of cutaneous melanin, the other being the yellow-red pheomelanin. Since the precursors of eumelanin are quinones with the potential to generate superoxide and hydroxyl radicals and since HMB-45 reactive antigen is homologous to the Pmel gene product, this raises the possibility that quinone derived oxidants may be directly involved in the pathophysiology of LAM.

We hypothesised that LAM may be associated with a deficiency in COMT activity. If correct, this hypothesis would help explain why LAM is found exclusively in women, and why the lung tissue stains positively for HMB-45. Patients with LAM may have defective COMT activity in peripheral blood cells such as mononuclear phagocytes. Blood mononuclear phagocytes migrate to the lung and mature to become resident alveolar and interstitial macrophages. A defect in COMT activity would result in the accumulation of melanin and/or catechol-estrogens with the potential of generating superoxide and hydroxyl radicals. These oxidants could then induce tissue destruction, tumours, and smooth cell proliferation.

To verify this hypothesis, blood samples were collected from women with LAM and compared with matched controls. COMT genotype was analysed to determine whether women with LAM disease would be prone to carrying the low activity alleles of COMT (COMT<sup>-/-</sup>). The level of COMT activity in blood cells was also measured, and the presence of a potential COMT inhibitor was verified. The plasma concentration of malondialdehyde (MDA) was also measured since defective COMT activity or altered catechol compound metabolism would generate more oxidative damage.

**Methods**

**STUDY POPULATION**

Fifteen women of mean (SD) age 42.6 (9.2) years with clinical characteristics of LAM were recruited through Canada and the USA with the assistance of the LAM Foundation. Seventeen healthy age matched women of mean (SD) age 38.9 (9.8) years in Canada and the USA were recruited as a control group. Experi-
COMT activity in blood cells was performed in 200 µl whole blood collected in 2 ml heparinised tubes. A volume of 800 µ1 ice cold distilled water was added to lyse the blood cells and 100 µ1 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. 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). 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 7000g 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 x 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. 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).

Table 1  Characteristics of LAM and control populations

<table>
<thead>
<tr>
<th></th>
<th>LAM</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (SD) age</td>
<td>42.6 (9.2)</td>
<td>38.9 (9.8)</td>
</tr>
<tr>
<td>Mean (SD) BMI</td>
<td>23.7 (5.5)</td>
<td>22.3 (2.7)</td>
</tr>
<tr>
<td>Lung biopsy*</td>
<td>9/13</td>
<td>–</td>
</tr>
<tr>
<td>Ovariecotomy*</td>
<td>4/13</td>
<td>0/17</td>
</tr>
<tr>
<td>Hysterectomy*</td>
<td>3/13</td>
<td>0/17</td>
</tr>
<tr>
<td>Contraceptive pill</td>
<td>0/15</td>
<td>2/17</td>
</tr>
<tr>
<td>Hormone replacement therapy</td>
<td>9/15</td>
<td>2/17</td>
</tr>
<tr>
<td>Diabetes</td>
<td>0/15</td>
<td>1/17</td>
</tr>
<tr>
<td>Liver disease</td>
<td>0/15</td>
<td>0/17</td>
</tr>
<tr>
<td>Kidney disease</td>
<td>2/15</td>
<td>0/17</td>
</tr>
<tr>
<td>Osteoporosis</td>
<td>7/15</td>
<td>0/17</td>
</tr>
<tr>
<td>Thyroid disease</td>
<td>1/15</td>
<td>1/17</td>
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*Complete data were not available for two of the 15 patients.

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 without plasma = 4.35 pmol 4-hydroxy-3-methoxybenzoic acid formed per minute.

* A plasma volume of 10 µl or 50 µl was added to 12 units of a commercial preparation of COMT.

**A plasma volume of 10 µl or 50 µl was added to 12 units of a commercial preparation of COMT.

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

Table 3 Cumulative results

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Mean (SD)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>COMT activity*</td>
<td>24.2 (6.3)</td>
<td>0.996</td>
</tr>
<tr>
<td>COMT inhibitor in plasma**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 µl</td>
<td>0.48 (0.12)</td>
<td>0.44</td>
</tr>
<tr>
<td>50 µl</td>
<td>0.36 (0.10)</td>
<td>0.30</td>
</tr>
<tr>
<td>MDA (pmol/ml plasma)</td>
<td>178.5 (80.1)</td>
<td>0.40</td>
</tr>
</tbody>
</table>

Table 2 Distribution of COMT genotype

<table>
<thead>
<tr>
<th>COMT* and COMT** and COMT***</th>
<th>LAM</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>COMT*</td>
<td>40%</td>
<td>38%</td>
</tr>
<tr>
<td>COMT**</td>
<td>7%</td>
<td>6%</td>
</tr>
<tr>
<td>COMT***</td>
<td>53%</td>
<td>56%</td>
</tr>
</tbody>
</table>

MDA

Using gas chromatography-mass spectrometry (GC-MS), the MDA can be detected in femtomole quantities in biological samples. 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* or COMT**, 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* and COMT**, and COMT*** 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*** cases than Thompson et al. who found 19% of COMT***, perhaps because of the small number of subjects in our groups. Nevertheless, the low incidence of COMT*** 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 catecholoestrogens 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 154.5 (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...
potential relationship with hormonal factors has frequently been suggested since LAM affects almost exclusively premenopausal women. 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 hydroxyoestrone 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 catecholœstrogen 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. It has been suggested that these alterations play an important part in the development of breast cancer, and that a higher level of these hydroxyoestrogens (catecholoestrogens) induces the development of resistance against the anticancer agent methotrexate.

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. 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 catecholœstrogens reacts very rapidly with nitric oxide (rate constant 7 x 10^9 M^-1 s^-1) to form the highly reactive peroxynitrite anion, a product not detected by the MDA assay. Since the concentration of nitric oxide can be important in the lung, 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, an alternative hypothesis may be related to a lower activity of 17β-HSD oxidase or a higher activity of 17β-HSD reductase in women affected by LAM, both leading to overproduction of oestradiol.

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25. Thiodeau PA, Bissnouette N, Kocsis Bedard S, et al. Production or elimination of oestrogens. Since smooth muscle cell proliferation can be stimulated by oestradiol, an alternative hypothesis may be related to a lower activity of 17β-HSD oxidase or a higher activity of 17β-HSD reductase in women affected by LAM, both leading to overproduction of oestradiol.