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

Benot 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^HH, COMT^HL and COMT^LL) 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.

Keywords: lymphangioleiomyomatosis; oestrogen metabolism; catechol-O-methyltransferase.
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. 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 catecholoestrogens 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). 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-
potential COMT inhibitor. Determination of the presence and absence of plasma.

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 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. The compounds were detected by fluorescence (Ex = 310 nm, Em = 420 nm). Retention times of dihydroxybenzoic acid and its methylated derivatives were obtained by injectng 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.

### 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 (years)</td>
<td>42.6 (9.2)</td>
<td>38.9 (9.8)</td>
</tr>
<tr>
<td>Mean (SD) BMI (kg/m²)</td>
<td>23.7 (5.5)</td>
<td>22.3 (2.7)</td>
</tr>
<tr>
<td>Lung biopsy*</td>
<td>9/13</td>
<td>2/17</td>
</tr>
<tr>
<td>Ovarectomy*</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>
</tr>
</tbody>
</table>

*Complete data were not available for two of the 15 patients.
**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 2 Distribution of COMT genotype

<table>
<thead>
<tr>
<th>COMT&lt;sup&gt;HH&lt;/sup&gt;</th>
<th>COMT&lt;sup&gt;LL&lt;/sup&gt;</th>
<th>COMT&lt;sup&gt;HL&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LAM</strong></td>
<td>40%</td>
<td>7%</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td>38%</td>
<td>6%</td>
</tr>
<tr>
<td><strong>COMT&lt;sup&gt;HH&lt;/sup&gt;</strong></td>
<td>6%</td>
<td>53%</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td>6%</td>
<td>56%</td>
</tr>
</tbody>
</table>

### 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<sup>HH</sup> 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<sup>HH</sup> or COMT<sup>LL</sup>, 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<sup>HH</sup>, COMT<sup>LL</sup>, and COMT<sup>HL</sup> 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<sup>HH</sup> cases than Thompson et al<sup>21</sup> who found 19% of COMT<sup>HH</sup>, perhaps because of the small numbers of subjects in our groups. Nevertheless, the low incidence of COMT<sup>HH</sup> 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 Ca<sup>2+</sup> 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 and the COMT activity was determined with an enzymatic assay using the 3,4-dihydroxybenzoic acid as a catechol substrate (table 3). The plasma used was not treated by Chelex-100 or any other procedure. Since the Ca<sup>2+</sup> 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
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 hydroxyoestrogen 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 catecholoestrogens in LAM disease, it remains to be established whether the blood and lung levels of 2- and 4-hydroxyoestriol and 2- and 4-hydroxyoestrone are increased. Higher activity of the 2-hydroxylase and 4-hydroxylase pathways leading to these oes-trogen metabolites have already been detected by catecholoestrogen in LAM disease, it remains to be established whether the blood and lung levels of 2- and 4-hydroxyoestriol 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, both between LAM and altered 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 hydroxyoestrogen derived ROS, molecules known to induce smooth muscle cell proliferation.

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