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

Benoit 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>HM</sup>, COMT<sup>LM</sup>, and COMT<sup>HL</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 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. 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 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<sup>ll</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-
were subjected to similar handling. Each sample using ice packs. Control blood samples taken to maintain the temperature at about °C. An 8 µl sample of the PCR product was denatured for three minutes at 94 °C, followed by a five minute extension at 72 °C for 30 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 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 supernatant was removed for the determination of COMT activity.

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. A 237 bp fragment of the COMT gene was first amplified by PCR using the forward primer TACTGTGGCCTACTCAGCT and the reverse primer TACTGTGGCTACTCAGCT. The gene was first amplified by PCR using the forward primer TACTGTGGCCTACTCAGCT and the reverse primer TACTGTGGCTACTCAGCT. The products of the PCR reaction containing 50 ng genomic DNA, 250 µM each deoxynucleotide triphosphates, 300 nM each primer, 1X reaction 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. COMT activity was expressed as pmol 4-hydroxy-3-methoxybenzoic acid formed per hour at 37°C. Samples were then centrifuged at 7000g for 10 minutes and the supernatant was removed for the determination of COMT activity.

The enzymatic assay was performed as mentioned previously. The presence of a circulating 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 was performed according to an established procedure. 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 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>Age (years)</td>
<td>42.6 (9.2)</td>
<td>38.9 (9.8)</td>
</tr>
<tr>
<td>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>9/13</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.

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. A 237 bp fragment of the COMT gene was first amplified by PCR using the forward primer TACTGTGGCCTACTCAGCT and the reverse primer TACTGTGGCTACTCAGCT. 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 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).

**DETERMINATION OF COMT ACTIVITY**

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 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>LAM 40% 7% 53%</td>
<td>Control 38% 6% 56%</td>
<td></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 using a Hewlett Packard 5890 Series II gas chromatograph interfaced to a 5989A mass spectrometer. The results were expressed as pmol MDA/ml plasma.

Table 3 Cumulative results

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

*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.

Discussion

LAM is an uncommon lung disease for which the etiology 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 hydroxyoestradiol 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 catechol-oestradiol in LAM disease, it remains to be established whether the blood and lung levels of 2- and 4-hydroxyoestradiol and 2- and 4-hydroxyoestron 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 (catechol-oestrogens) 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 catecholoestrogens reacts very rapidly with nitric oxide (rate constant 7 × 10^9 M/s) 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.

This research project has been supported by the LAM Foundation.

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

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

Thorax 2000 55: 574-578
doi: 10.1136/thorax.55.7.574

Updated information and services can be found at:
http://thorax.bmj.com/content/55/7/574

These include:

References
This article cites 24 articles, 7 of which you can access for free at:
http://thorax.bmj.com/content/55/7/574#BIBL

Email alerting service
Receive free email alerts when new articles cite this article. Sign up in the box at the top right corner of the online article.

Topic Collections
Articles on similar topics can be found in the following collections
Molecular genetics (211)

Notes

To request permissions go to:
http://group.bmj.com/group/rights-licensing/permissions

To order reprints go to:
http://journals.bmj.com/cgi/reprintform

To subscribe to BMJ go to:
http://group.bmj.com/subscribe/