Detection of Melanocytes From Uveal Melanoma in Peripheral Blood Using the Polymerase Chain Reaction

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Purpose. - Uveal melanoma is the most common intraocular malignancy in adults and can cause loss of vision in the affected eye and death from metastasis, usually to the liver. The techniques currently used to detect cellular dissemination from the tumor are inadequate, and lack the sensitivity required for the detection of low levels of melanocytes in the peripheral blood of patients. The detection of circulating melanocytes is important as an early indication of the possibility of metastasis.

Methods. - The viability of reverse transcription/polymerase chain reaction amplification of the tyrosinase gene to detect circulating melanocytes was examined as a first sign of dissemination from uveal melanoma.

Results. - It was shown that it is possible to detect as few as ten circulating melanocytes in 5 ml of blood. Blood-borne dissemination was also detected in three of six patients with uveal melanoma examined. Two of these patients had clinically confirmed widespread metastases. A positive result was also recorded in one patient in whom there was no other evidence for tumor dissemination. Overt metastatic disease developed in this patient 9 months after blood collection.

Conclusions. - The success of this technique has important implications for the detection of circulating tumor cells from uveal melanoma, as an early indication of dissemination. This may be important when considering the administration of adjuvant therapy.

Uveal melanoma is the most common intraocular malignancy in adults. It causes loss of vision in the affected eye and death from metastasis, mostly to the liver. Although only about 1% of patients show evidence of metastases at presentation, approximately 40% of patients will subsequently develop metastases and die, up to 16 years after apparently successful enucleation of the affected eye. The mechanism of dissemination and metastasis for uveal melanoma is different from that for cutaneous melanoma, because metastasis from cutaneous melanoma initially spreads via the lymphatic system. There is no lymphatic drainage in the uveal tract and metastatic spread from uveal melanoma is hematogenous and generally to the liver. One of the major problems encountered in the treatment of uveal melanoma is the difficulty of detecting metastatic spread. A number of reports have been published of patients with normal liver enzymes and liver scans, but who have widespread metastases affecting the liver. Enucleation of the affected eye, which remains the only course of action possible in certain cases, has not been shown to improve survival. It has been suggested that the procedure itself may precipitate metastatic spread. The detection of tumor dissemination at an early stage would be of potential clinical benefit. Current methods are not adequately sensitive to detect the very small numbers of melanocytes...
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cytes present in the blood during the early stages of tumor dissemination.

The polymerase chain reaction (PCR) has been successfully employed to detect small numbers of circulating melanocytes from the peripheral blood of patients with cutaneous melanoma by the amplification of tyrosinase (a melanocyte-specific gene) transcripts. The technique relied on the fact that melanocytes are not normally present in the blood, and therefore the amplification of a melanocyte-specific gene in the blood is a suitable indicator of the presence of circulating melanocytes. Tyrosinase is an important enzyme in melanin biosynthesis, and is specific to melanocytes, which makes it an obvious candidate for such an approach. Melanocytes from uveal melanoma, however, differ in a number of ways from their cutaneous counterparts. Therefore, it was important for us to determine the validity of this approach using tyrosinase transcripts as a marker for the presence of melanocytes from uveal melanoma in the blood.

MATERIALS AND METHODS

Patients

Patients were recruited from Moorfields Eye Hospital and the Hospital for Sick Children in London. The research followed the tenets of the Declaration of Helsinki, and informed consent was obtained after the nature of the study was explained. The study received formal approval from the Moorfields ethical committee. The diagnosis of posterior uveal melanoma was made by experienced clinicians on the basis of binocular indirect fundoscopy and ultrasonography. All tumors were melanotic and their clinical details are summarized in Table 1. Peripheral venous blood was collected at routine clinic visits with the exception of patient 1. Patient 1 had undergone enucleation and the blood was collected before surgery for removal of local recurrence in the orbit. Patient 2 was known to have advanced metastatic disease at the time of blood collection on the basis of abnormal biochemistry and abdominal ultrasonography and was selected as a positive control. Of the other four cases, only in patient 3 did overt metastatic disease develop, 9 months after blood collection.

Only patients 1 and 5 had undergone enucleation: in patient 1 more than 4 years, and in patient 5 more than 1 year before collecting the blood sample.

Reverse Transcription/PCR Amplification

For the amplification of tyrosinase transcripts in peripheral blood, we used primers HTYR1, HTYR2, HTYR3, and HTYR4.

The sequences of the primers are as follows:

**HTYR1 = TTGGCAGATTGTCTGTAGCC**

(outer, sense)

**HTYR2 = AGGCATTGTGCATGCTGCTT**

(outer, anti-sense)

**HTYR3 = GTCTTTATGCAATGGAACGC**

(nested, sense)

**HTYR4 = GCTATCCCAGTAAGTGGACT**

(nested, anti-sense)

The outer primers HTYR1 and HTYR2 produce a PCR fragment of 284 bp and the inner primers

<table>
<thead>
<tr>
<th>No.</th>
<th>Age</th>
<th>Sex</th>
<th>Diagnosis</th>
<th>Size of Base (mm)</th>
<th>Height (mm)</th>
<th>Treatment</th>
<th>Histology</th>
<th>Outcome</th>
<th>Detection of mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>F</td>
<td>Choroidal MM</td>
<td>40 × 40</td>
<td>45</td>
<td>Enucleation</td>
<td>Mixed spindle and epitheloid cells</td>
<td>Widespread metastases</td>
<td>Detected</td>
</tr>
<tr>
<td>2</td>
<td>54</td>
<td>F</td>
<td>Choroidal MM</td>
<td>14 × 14</td>
<td>11</td>
<td>Proton beam irradiation</td>
<td>Not available</td>
<td>Widespread metastases</td>
<td>Detected</td>
</tr>
<tr>
<td>3</td>
<td>44</td>
<td>F</td>
<td>Ciliary body MM</td>
<td>15 × 10</td>
<td>14</td>
<td>Proton beam irradiation</td>
<td>Not available</td>
<td>Developed widespread metastases 9 mo later</td>
<td>Detected</td>
</tr>
<tr>
<td>4</td>
<td>72</td>
<td>F</td>
<td>Ciliary body MM</td>
<td>Ring growth</td>
<td>4</td>
<td>Declined treatment</td>
<td>Not available</td>
<td>Well</td>
<td>Not detected</td>
</tr>
<tr>
<td>5</td>
<td>60</td>
<td>F</td>
<td>Choroidal MM</td>
<td>7 × 7</td>
<td>11</td>
<td>Enucleation</td>
<td>Spindle B</td>
<td>Well</td>
<td>Not detected</td>
</tr>
<tr>
<td>6</td>
<td>62</td>
<td>F</td>
<td>Choroidal MM</td>
<td>16 × 9</td>
<td>5</td>
<td>Proton beam irradiation</td>
<td>Not available</td>
<td>Well</td>
<td>Not detected</td>
</tr>
</tbody>
</table>
HTYR3 and HTYR4 produce a fragment of 207 bp. These primers are represented on different exons of the gene, enabling a distinction to be made between the amplification of tyrosinase messenger RNA and any possible amplification of tyrosinase from the DNA.9

Five-milliliter samples of peripheral blood from normal subjects and from six patients with uveal melanoma underwent centrifugation at 1000 g for 5 minutes, the plasma was discarded, and the samples were stored at −70°C. Total RNA was extracted by the guanidinium thiocyanate method.10 Reverse transcription was carried out on 5 g of RNA. RNA was heated to 90°C for 5 minutes, cooled on ice, and diluted to a volume of 20 μl, containing a final concentration of 1 X PCR buffer (10 mmol/l tris-Cl pH 8.3, 50 mmol/l KCl, 0.01% gelatin), 8 mmol/l MgCl2, 1 mmol/l of each dNTP (dATP, dCTP, dGTP, dTTP), 1 U/μl human placental ribonuclease inhibitor (BRL), 25 pmol HTYR2, and 100 units Moloney murine leukemia virus reverse transcriptase (BRL). After incubation at 37°C for 1 hour, the reaction was stopped by heating the samples to 95°C for 5 minutes. Ten microliters of each sample were then diluted to volumes of 50 μl, each containing a final concentration of 1 X PCR buffer, 200 μmol/l of each dNTP, 1.5 mmol/l MgCl2, 30 pmol HTYR1 and HTYR2, 0.1% Triton X-100, and 2 units of Taq DNA polymerase (Stratagene). Thirty cycles of PCR were carried out (at 95°C for 65 seconds, 55°C for 65 seconds, and 72°C for 50 seconds). For reamplification, 5 μl of a 1 in 100 dilution of the PCR product was amplified in a 25-μl reaction volume containing 30 pmol of each primer (TYR3 and TYR4) for a further 30 cycles. The integrity of the blood RNA was checked by reverse transcription/PCR with primers for β-globin. To minimize contamination, all preparation steps were performed in a laminar flow cabinet.

The VUP cell line, a human metastatic choroidal melanoma cell line, was used to analyze the sensitivity of the technique and as a positive control. Five milliliters of normal blood was spiked with different numbers of VUP cells—5, 10, 100, and 1000 cells, taken from confluent cultures. Total RNA was then extracted from the different dilutions and reverse transcription/PCR amplification was carried out on each as detailed above. The procedure was repeated to confirm the results.

RESULTS
Using reverse transcription/PCR amplification, tyrosinase messenger RNA was detected in the blood of two patients with overt metastatic disease (cases 1 and 2), and in one out of four patients (case 3) without any other evidence of metastases (Figure 2). This patient went on to develop overt metastatic disease, nine months after blood collection. Control blood samples showed no amplification of the tyrosinase gene.

We were able to detect the presence of as few as ten cells of the VUP cell line in 5 ml of normal blood—i.e. 2 cells ml⁻¹ (Figure 1). This was the limit of the sensitivity of the technique, and we could not detect cells at a lower concentration than this. Amplification of cDNA from human cDNA clones Pmel34 (tyrosinase) and JW102 (-globin) confirmed that the PCR products were of the expected sizes.

DISCUSSION
We were able to successfully employ the reverse transcription/PCR amplification of tyrosinase messenger RNA,9 to detect circulating melanocytes in the peripheral blood of two patients with choroidal melanoma who had overt metastatic disease. A positive result was also recorded in one patient in whom there was no other evidence of tumor dissemination. Overt metastatic disease developed in this patient 9 months after blood collection.

We were able to detect the presence of tyrosinase transcripts from 10 cells of the VUP cell line in 5 ml of normal blood. This is potentially more sensitive than
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FIGURE 2. Detection of tyrosinase messenger RNA in blood from patients with uveal melanoma. Lanes 1, 2, and 5: uveal melanoma patients (cases 4, 5, and 6) with no overt metastatic disease, showing no tyrosinase expression. Lane 3: choroidal melanoma patient (case 2) with overt metastatic disease, showing tyrosinase expression. Lane 4: ciliary body melanoma patient (case 3) with no overt metastatic disease, but showing tyrosinase expression. This patient went on to develop overt metastatic disease, 9 months after blood collection. Lane 6: choroidal melanoma patient (case 1) with overt metastatic disease, showing tyrosinase expression.

made at a late stage, and a third view is that enucleation itself may trigger metastatic spread. The reverse transcription/PCR amplification of tyrosinase messenger RNA in the blood could be used to study dissemination from uveal melanoma in detail and to help investigate the many questions relating to the disease.

Key Words

reverse transcriptase, PCR, tyrosinase, metastasis, uveal melanoma

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References