Assessment of melanoma extent and melanoma metastases invasion using electron paramagnetic resonance and bioluminescence imaging

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The clinical outcome of melanoma depends on the local and distant spread of the disease at the time of diagnosis, as the estimated 5-year survival rate is about 100% for superficial melanoma diagnosed early, but less than 10% for melanoma that has disseminated to major organs such as lungs. There is a crucial need for new effective methods for the detection and the characterization of melanomas. In the pre-clinical setting, this will help to understand the factors that contribute to the malignancy while the transfer into the clinic will contribute to an early effective treatment of patients. Melanoma lesions can be detected by electron paramagnetic resonance (EPR) using paramagnetic properties of melanin pigments. As part of the development of EPR imaging to characterize melanomas, we evaluated in the present study the usefulness of EPR to report on the extension of lung metastases by comparing the method with bioluminescence imaging using B16 melanoma cells expressing luciferase. B16 melanoma cells were injected subcutaneously or intravenously in C57/BL6 mice. The primary tumors or the lung colonization by melanoma cells was measured after several delay periods to obtain several degrees of invasiveness. The animals were measured in-vivo with bioluminescence after i.v. injection of luciferin. The primary tumors or lungs were then excised. After freeze-drying, the content of melanin in lungs was measured and imaged by EPR at 9 GHz. We observed a direct relationship between the EPR intensity and the bioluminescence intensity. Another tumor model (KHT sarcoma), non-pigmented but expressing luciferase, was used to confirm that the EPR signal was directly linked to the melanin pigment present in the tumors. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: EPR; bioluminescence; melanoma; melanin; luciferase; metastases

1. Introduction

The clinical outcome of malignant melanoma strongly depends on the local and distant spread of the disease at the time of diagnosis, as the estimated 5-year survival rate is about 100% for superficial melanoma diagnosed early, but less than 10% for melanoma that has disseminated to major organs (lungs, brain). There is a crucial need for new effective methods for the detection and the characterization of melanomas.

Melanoma lesions can be detected by electron paramagnetic resonance (EPR) using paramagnetic properties of melanin pigments. Melanins are polymeric pigments that contain a mixture of eumelanin and pheomelanin, which can be distinguished by EPR spectrometry because their EPR spectra are different (1). In melanoma, eumelanin seems to be the major component of the pigment mixture (2), even if some reports point out that melanoma can also be composed by pheomelanin (3). The melanin pigments can be detected by EPR spectroscopy (4–6) using the specific signal of stable free radicals contained in melanin. First EPR studies concerning melanin in melanoma were made on extracted melanin and on ex-vivo entire melanomas (7) and successfully revealed the potential of EPR to detect such lesions. Later, Katsuda et al. demonstrated that it was possible to image an ex-vivo melanoma using a X-band (9 GHz) EPR spectrometer (8). In 2005, Timmins submitted a patent for the concept of detecting melanoma by electron paramagnetic resonance (9). More recently, our group was able to get the first image of endogenous pigments from an in-situ B16 mouse melanoma using a L-band (1 GHz) EPR spectrometer (10).
In the pre-clinical setting, EPR imaging could help to understand the factors that contribute to the malignancy, while the transfer into the clinic will allow a non-invasive and early diagnosis, and contribute to effective treatment of patients. Indeed, EPR imaging (EPRI) could provide a non-invasive image of the lesion thickness (Breslow index) and invasion depth (Clark’s level) before biopsy, which could speed up the diagnosis process. Moreover, EPRI could possibly demonstrate the presence of metastasis in sentinel lymph nodes.

Melanin has an intrinsic signal with a rather large linewidth (LW = 5.5 G), which could be a limiting factor affecting both sensitivity and resolution in EPR imaging studies. Nevertheless it was demonstrated in our previous proof-of-concept study that melanin could be detected directly in-vivo and that its EPR signal is convenient for EPR spectrometry and imaging studies in melanoma (10). Exogenous probes such as trityls or nitroxides giving a strong EPR signal are not adapted to this situation so that direct detection of melanin remains the best solution to investigate melanoma.

The purpose of the present study was to assess the suitability of ex-vivo X-band EPR (9 GHz) to characterize the extent of melanoma lesions by comparison with bioluminescence imaging (BLI). BLI is a well-documented method, commonly used to assess the number of viable cells in in-vivo experimental tumors and follow their evolution (11). Mice with non-pigmented tumors (KHT sarcoma) expressing luciferase were also used as a control.

The second part of the study was focused on the determination of the threshold level of melanin that can be detected by L-band EPR spectrometry (1 GHz), which can be used in-vivo, and X-band EPR spectrometry (9 GHz), whose utilization is limited to ex-vivo samples. This experiment was performed in order to characterize fully the abilities of this new technique applied to melanin.

### 2. Results and discussion

#### 2.1. Lung metastases

BLI and EPR intensities measured in melanoma lung metastases are reported in Table 1. For BLI, the mean intensity ranged from $2.52 \pm 0.51 \times 10^3$ (mean ± standard error of the mean) photons for control mice ($n = 2$) to $6.75 \pm 1.99 \times 10^6$ photons for the mice bearing 18-day tumors ($n = 5$). For EPR experiment, the basal signal was $0.79 \pm 0.02$ ($n = 2$) and rose to $7.72 \pm 0.70$ intensity units ($n = 5$) at day 18. The EPR signal measured in control lungs was probably due to the presence of organic free radicals in the tissue (12). The correlation between BLI and EPR is shown in Fig. 1. BLI and EPR results were well correlated, with a Pearson correlation coefficient of 0.7292.

EPR imaging was then applied to lung samples for each tumor growth state. EPR signal intensity on 2D images (Fig. 2c) and 3D images (Fig. 2e) correlates with the known density of metastases in each sample (Fig. 2a and b). EPR imaging provides thus a good confirmation of the results obtained with EPR spectroscopy.

#### 2.2. Cutaneous melanomas

As shown in Fig. 3, a different correlation was observed between intensities measured by EPR and BLI for the cutaneous melanomas. Here, the correlation coefficient of the trendline is 0.4214. This relative lack of correlation undeniably means that at least one of the two techniques provides values that do not reflect exactly reality.

In order to determine if BLI or EPR was providing questionable results, it was decided to correlate, for each technique, the intensity values with the weight of the associated samples. Indeed, as cutaneous melanomas are only constituted by melanoma cells, the weight of the tumor is directly linked to the number of cells present in the tumor, which is directly linked to the quantity of melanin detectable by EPR and to the luciferase activity detectable by BLI. A strong relation between the weight and the two different kinds of intensities should be observed. Results are summarized in Fig. 4. Figure 4(a) shows the correlation between the weight of the samples and EPR intensity and Fig. 4(b) shows the correlation for BLI intensity and the weight of the samples. The $R^2$ coefficient of the trendline is 0.9844 for EPR and 0.4993 for BLI. These results tend to prove that EPR is an efficient tool for quantification of melanin inside melanomas. When it is possible to work ex-vivo, EPR is then more adapted than BLI for measurements of melanoma burden.

Some factors might explain the lower correlation obtained by BLI on cutaneous melanomas. Amongst them, two biological factors seem the most likely to be involved: the low oxygenation of the cutaneous tumor (as oxygen is acting in the transformation of luciferin in oxyluciferin) and the inhomogeneous biodistribution of luciferin. These two factors are linked to the poor perfusion of skin tumors.

#### 2.3. Non-pigmented KHT tumors

In order to verify if the increase of EPR intensities was not an artifact linked to the increase in the weight of the samples, additional controls were performed with nonpigmented tumors (KHT sarcoma). Evolution of EPR and BLI intensities regarding the weight of the samples is summarized in Fig. 5. Figure 5(a, b) shows the evolution of EPR intensity, respectively for lung metastases (KHT and B16) and subcutaneous tumors (KHT and B16), regarding the weight of the samples. On both figures, we can see that EPR values for nonpigmented KHT tumors do not present any notable evolution regarding the weight of the samples, while an increase in EPR values regarding the weight is clearly visible for pigmented B16 tumors. These results demonstrate that the evolution of EPR intensities is definitely linked to the melanin content of the samples and is not a consequence of the increase in tumor weight.

BLI results for the same KHT tumors are reported in Figs 5(c and d), respectively, for lung metastases and subcutaneous tumors. As expected, we observe an increase in BLI intensities regarding the weight of the tumors. However, we did not put KHT and BLI data

<table>
<thead>
<tr>
<th>Growth stage (days)</th>
<th>BLI intensity (number of emitted photons ± SEM)</th>
<th>EPR intensity (arbitrary unit) ± SEM</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>$2.52 \pm 0.51 \times 10^3$</td>
<td>$0.79 \pm 0.02$</td>
</tr>
<tr>
<td>D = 9</td>
<td>$0.33 \pm 0.13 \times 10^6$</td>
<td>$2.72 \pm 0.11$</td>
</tr>
<tr>
<td>D = 15</td>
<td>$4.54 \pm 0.69 \times 10^6$</td>
<td>$5.16 \pm 0.34$</td>
</tr>
<tr>
<td>D = 18</td>
<td>$6.75 \pm 1.99 \times 10^6$</td>
<td>$7.72 \pm 0.70$</td>
</tr>
</tbody>
</table>
Figure 1. Evolution of EPR intensity according to BLI intensity in lung melanoma metastases samples.

Figure 2. Three lung samples coming from the three different growth states (9, 15 and 18 days after injection of B16 melanoma cells) (a) placed in an EPR quartz tube and separated from each other by a thin slice of cotton (b). The most invaded sample is placed on the top and the less invaded one on the bottom of the tube. Two- (c) and three-dimensional (d and e) images were acquired with the EPR imager working in X-band mode. Parameters were: power, 3.2 mW; modulation amplitude, 2.5 G; gradient field, 45 G cm$^{-1}$; conversion time, 10.24 ms; time constant, 5.12 ms; pixel size, 0.6 mm.

Figure 3. Evolution of EPR intensity according to BLI intensity in cutaneous B16 melanomas.
together on the same graphs because the expression level of luciferase might be different from one kind of tumor to another, as they were not transfected in the same way. Moreover, the animal model was different and we know that the difference in skin pigmentation between C57Bl6 (black) mice and C3H (white) mice might interfere with BLI results.

2.4. Limit of detection

The first step of the study demonstrated that EPR spectrometry can be successfully applied to the detection and quantification of melanin. In the next step, we determined the threshold level of melanin that can be detected with this method.

The limit of detection was assessed for EPR X-band spectrometry and EPR L-band spectrometry, with two kinds of samples: synthetic melanin and B16 melanoma powder. The density of spins inside the synthetic melanin used was estimated to be $1.65 \times 10^{18}$ spins g$^{-1}$ by comparison with a TEMPO standard. Decreasing quantities of melanin or melanoma powder were measured until the signal-to-noise ratio was 2, which is generally admitted as the smallest significant EPR signal. The quantity that led to this signal-to-noise ratio was considered as the limit of detection.

The results are summarized in Table 2. The limits of detection range from 2 μg for X-band EPR applied to synthetic melanin to 60 mg for L-band EPR applied to melanoma powder. On one hand, EPR L-band spectrometry (1 GHz) is by definition less sensitive than X-band EPR spectrometry (9 GHz), this explains the difference between X-band and L-band limitations. On the other hand, melanoma powder contains not only melanin but also all the non-resonant cellular components, which explains the difference between synthetic melanin and melanoma powder limitations.

The tiny values that we were able to detect with EPR X-band spectrometry indicate that this method is a very sensitive tool to detect and quantify melanin. However, EPR X-band spectroscopy cannot be applied to in-vivo studies because of the poor penetration (1 mm) of 9 GHz microwaves inside water-containing samples like biological tissues. For this purpose, we need to use EPR L-band spectrometry despite the decrease in sensitivity.

The limit of detection for the application of EPR L-band spectrometry to freeze-dried melanoma powder was 60 mg. This weight corresponded to a wet melanoma of 300 mg, with a diameter of 5–6 mm. These results indicate that L-band in-vivo spectrometry is more than 1000 times less sensitive than X-band spectrometry for the detection of melanin. It also indicates that L-band in-vivo spectrometry would unfortunately not be able to detect early melanomas for the moment. Nevertheless, improvements in EPR signal and image treatment processing (14,15), and development of new of resonators dedicated to in-vivo experiments (16) are ongoing and should soon allow

Figure 4. Evolution of EPR intensity (a) and BLI intensity (b) according to the weight of the cutaneous B16 melanoma samples.
Figure 5. Control nonpigmented KHT tumors: (a) evolution of EPR intensity regarding the weight of the samples for KHT (□) and B16 (●) lung metastases. Intensity values are calculated as the mean of three independent measurements. (b) Evolution of EPR intensity regarding to the weight of the samples for KHT (□) and B16 (●) cutaneous tumors. (c) Evolution of BLI intensity regarding to the weight of the samples for KHT lung metastases. (d) Evolution of BLI intensity regarding to the weight of the samples for KHT cutaneous tumors.
achievement of the performance level required for routine in-vivo EPR studies.

Another option could be the use of S-band (3 GHz) EPR spectrometry as a compromise between the sensitivity of X-band spectrometry and the penetration depth of L-band spectrometry. Indeed, the penetration depth of S-band spectrometry is 4 mm, which would enable the study and characterization of superficial skin melanomas (13).

### 3. Conclusion

In this study, we demonstrated that electron paramagnetic resonance could be a valuable tool in the detection and characterization of melanoma. Not only is it as efficient as BLI for the lung metastases study, but it provides more accurate results for the characterization of primary melanomas. Moreover, EPR suits the detection of every pigmented tumor while BLI is limited to the study of luciferase-encoding melanocytes. However, some questions and problems remain. Firstly, for now, no data demonstrates whether it is possible to distinguish melanoma from non-malignant nevi using EPR. Some studies are now performed in our laboratory to assess the capability of EPR imaging to sort different types of melanoma lesions and non-malignant nevi. Secondly, as demonstrated, in-vivo analyses seem limited by the actual low sensitivity of L-band spectrometry. The minimal quantity of freeze-dried melanoma powder that can be detected by EPR L-band spectrometry is around 62 mg, corresponding to a cutaneous melanoma with a weight of 300 mg. That means that the tumor has to be well developed to be detected with L-band EPR. This is consistent with our previous attempts to image melanoma models in mice (9). This lack of sensitivity restrains the in-vivo utilization of EPR to big tumors and slows down the development of EPR as a clinical diagnostic tool for human melanoma. Moreover, in-vivo measurements at L-band EPR are expected to be even less sensitive due to additional microwave losses from water present in the organism. However, in the future, technical improvements and other strategies like the use of S-band EPR will be helpful and could bring EPR to the forefront of melanoma study.

<table>
<thead>
<tr>
<th>EPR method</th>
<th>Kind of sample</th>
<th>Limit of detection</th>
</tr>
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<tbody>
<tr>
<td>X-band EPR spectrometry (9 GHz)</td>
<td>Synthetic melanin</td>
<td>2.0 µg</td>
</tr>
<tr>
<td></td>
<td>Cutaneous melanoma powder</td>
<td>37.5 µg</td>
</tr>
<tr>
<td>L-band EPR spectrometry (1 GHz)</td>
<td>Synthetic melanin</td>
<td>3 mg</td>
</tr>
<tr>
<td></td>
<td>Cutaneous melanoma powder</td>
<td>62 mg</td>
</tr>
</tbody>
</table>

### 4. Experimental

#### 4.1. Cell cultures and mouse models

Luciferase-encoding B16F10 melanoma cells (B16F10Luc) were routinely cultured in serum-containing DMEM. As a model of primary tumors, 750 000 melanoma cells were injected subcutaneously (s.c.) to 8-week-old male C57Bl6 mice (Elevage Janvier, France, n = 8). As model of lung metastasis, 750 000 melanoma cells were injected intravenously (i.v.) using a tail vein of the mice (n = 17). Two mice that were not injected were used as negative controls. Additional controls were performed by injecting KHT sarcoma cells expressing luciferase (KHT-luc) into 8-week-old C3H mice. Twelve mice were injected s.c. at the top of the back with 10⁶ cells and 9 mice were injected i.v. with 10⁶ cells using a tail vein of the mice. Mouse care and experimental procedures were approved by the local ethics committee of Université Catholique de Louvain according to national animal care regulations.

#### 4.2. Bioluminescence measurements

Mice were depilated 1 day before the experiment. They were anesthetized using 0.1 ml of ketamine/xylazine and then received an intraperitoneal injection of luciferin (150 mg kg⁻¹). Mice with lung colonization by melanoma cells were imaged 9 (n = 6), 15 (n = 6) and 18 days (n = 5) after the i.v. administration of tumor cells. In vivo bioluminescence was measured 15 min after luciferin injection, a delay commonly used in the literature (11,17), when the light emission seems the most stable. Mice were measured on an IVIS50 imaging system (Xenogen) using a 16-bit digitizer, with an exposure time of 60 s. The animals bearing s.c. tumors were measured after 8 days (n = 8). BLI intensity was measured as the total amount of the photons emitted by the tumor. For animals bearing lung metastases, it was calculated as a mean of two intensities: one with the animal placed in the ventral position, one with the animal placed in the dorsal position. For mice bearing s.c. tumors, a single image was sufficient. After each bioluminescence measurement, the mice were sacrificed. Primary tumors or lungs were excised, cooled at −80°C, and then freeze-dried for further EPR experiments. It was decided to use freeze-dried samples instead of frozen samples as it was more convenient for EPR imaging.

Control mice injected with KHT-luc cells underwent the same experiment. For KHT lung metastases, the first group of mice (n = 5) was measured and sacrificed 7 days after injection and the second group (n = 4) was measured and sacrificed 10 days after injection. For KHT cutaneous tumors, the first group of mice (n = 6) was measured and sacrificed 7 days after injection and the second group (n = 6) was measured and sacrificed 10 days after injection.

#### 4.3. EPR measurements

All spectra and images were recorded at room temperature on a Bruker E540 Elexys system (Bruker Biospin GmbH, Germany). For X-band studies, it was equipped with a Super High Sensitivity Probe (10 mm diameter, 30 mm long) operating at ~9.5 GHz and 100 kHz modulation frequency. Parameters were chosen to provide the best signal-to-noise ratio with no distortion of the signal shape. For X-band spectrometry, the whole lungs and cutaneous tumors were measured one by one using the following
parameters: microwave power, 3.2 mW; modulation amplitude, 0.25 mT; conversion time, 10.24 ms; time constant, 5.12 ms; sweep width, 100 G; number of points, 1024; number of scans, 3. For EPR spectrometry measurements, spectra intensities were normalized by comparison with the EPR intensity of a DPPH standard. The variation of DPPH spectrum intensity was less than 5%. EPR intensity values for lung metastases (KHT and B16) regarding the weight were calculated as the mean of three independent measurements. For the X-band EPR imaging studies, a lung sample from each of the three growth stages was selected. The three samples were introduced together into the same quartz tube and isolated from each other with a thin slice of cotton. The parameters for imaging were the same as for EPR spectrometry, with a gradient field of 0.45 mT m\(^{-1}\) and field of view = 20 mm. The pixel size was 0.6 mm.

For L-band spectrometry studies, spectra were recorded on a Bruker E540 Elexys system (Bruker Biospin GmBH, Germany) equipped with an E540R23 L-band EPR head-coil resonator (internal diameter 2.3 cm) operating in L-band mode at \(1.1 \text{ GHz}\). The following parameters were used: microwave power, 45 mW; modulation amplitude, 3G; conversion time, 10.24 ms; time constant, 5.12 ms; sweep width, 100 G; number of points, 1024; number of scans, 100.

The limits of detection were assessed by measuring synthetic melanin (Sigma, cell culture tested synthetic melanin) and cutaneous melanoma powder with EPR X-band spectrometry and EPR L-band spectrometry. The synthetic melanin was characterized. Its density of spins was determined by a comparison of its spectrum with 4-hydroxy-TEMPO (3.49 \(10^2\) spins g\(^{-1}\)). Melanoma powder was obtained by crushing freeze-dried cutaneous melanoma. Decreasing quantities were obtained by dry dilution of the both samples with D-sucrose and then measured with the two methods until signal-to-noise ratio was 2. A vial containing pure D-sucrose was used as a control.

Acknowledgements

This work was supported by grants from the Belgian National Fund for Scientific Research, the Televie, the Fonds Joseph Maisin, the Saint-Luc Foundation, the Actions de Recherches Concertées-Communauté Française de Belgique-ARC 09/14-020, the Fondation contre le Cancer and the Pôle d’attraction Interuniversitaire PAI VI (P6/38).

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