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CHAPTER 10

Changes in immunological markers and influx of macrophages following trans-scleral thermotherapy of uveal melanoma


ABSTRACT

Purpose: In trans-scleral thermotherapy (TSTT), heat is applied through the sclera in order to target an intraocular uveal melanoma. Previously, it had been shown that in uveal melanoma, hyperthermia and transpupillary thermotherapy influenced expression of immunologically relevant proteins, such as S100, HLA and heat-shock proteins (HSPs). We investigated whether TSTT induced similar changes.

Methods: Experimental TSTT was applied on eleven uveal melanomas prior to enucleation. Each tumour sample was processed for histopathological examination; immunohistochemical analysis was performed to determine expression of S100, HLA, HSPs and macrophage markers.

Results: In TSTT-treated areas, expression of S100 and different HSPs was lost, while an upregulated expression of HSP GP96 was observed at the border of these areas. Expression levels of HLA-A and HLA-B varied between tumours and were not influenced by TSTT. The borders of the TSTT-treated areas showed high numbers of infiltrating macrophages, which were predominantly of the M2 phenotype.

Conclusion: TSTT has an effect on immunological parameters with local loss of expression of HSPs and S100. The influx of M2 macrophages around the TSTT-treated areas indicates the presence of an innate immune reaction against the induced necrosis, suggesting that TSTT-treated tumour cells are removed by a macrophage-mediated tissue repair mechanism.
INTRODUCTION

Uveal melanoma is the most common primary intra-ocular malignancy in adults with an incidence that ranges from six to eight persons per million per year in Caucasians. Uveal melanoma arises most frequently in the choroid (80%) and less frequently in the ciliary body (12%) and iris (8%). Besides other therapies for choroidal melanoma such as enucleation and brachytherapy, heat treatment options are hyperthermia, transpupillary thermotherapy (TTT) and (experimental) trans-scleral thermotherapy (TSTT). Hyperthermia with a thermal range of 42–44°C was originally developed as adjuvant treatment to radiotherapy, allowing reduction of the radiation dose in an attempt to reduce radiation-induced complications. The combination of TTT at 45–60°C and brachytherapy can be used as a treatment of uveal melanoma. Peripheral tumours, however, cannot be treated by TTT because of the angle of the laser beam. Therefore, experiments were started to study the feasibility of applying thermotherapy transclerally for peripheral tumours. The aim of TSTT is to cause direct killing of choroidal melanoma cells without causing damage to the sclera. Studies in vitro and in vivo showed that at a cytotoxic temperature of about 60°C, the intrascleral tumour cells and the intraocular melanoma are destroyed without damaging scleral collagen, the main constituent of the sclera.

Heat may not only directly kill tumour cells, but may affect molecules that play an important role in immunological tumour cell recognition. Immunologically interesting molecules include HLA class I and II antigens, heat shock proteins (HSP) and tumour antigens. T cells will only recognize tumour cells which express the proper HLA Class I or II antigens as well as tumour antigens, such as S100, an immunogenic melanocyte antigen. One might thus hope that TSTT may have an additional effect, by stimulation of a local immune response.

Hypothetically, damaging tumour cells by heat may kill cells and induce immunization. This has been observed in experimental treatment of xenografted cutaneous melanoma in mice. Radiofrequency ablation induced upregulation of HSPs and helped boost immune responses against tumour cells lysate-pulsed dendritic cells. Prior to considering functional immunological studies, we first decided to ask the question which markers, relevant to induce immune responses, change because of local TSTT. Trans-scleral thermotherapy already had been used experimentally on uveal melanoma-containing eyes that were planned to undergo enucleation. We set out to investigate whether TSTT treatment of uveal melanoma led to changes in the expression of immunological parameters or the presence of tumour-infiltrating macrophages.
Chapter 10

MATERIALS AND METHODS

Trans-scleral thermotherapy

Experimental TSTT was applied by one single surgeon (author JK) and performed as previously described. Three different types of TSTT probes were used. The effect of different TSTT probes was investigated on uveal melanoma eyes planned to undergo enucleation. After each treatment, improvements were made to the probes. The overall description of the probes is as follows: episcleral conductive heating was achieved by perfusion of water from a closed pump-heater at 60°C (±0.3°C) with the tip of the probe. The tip of the applicator had a diameter of 5–7 mm. Laser-induced heating was obtained with an 810 mm infrared diode laser, at a laser power of 750–1250 MW. The diameter of the laser spot was 3 mm, the exposure time 1 min.

Patients and specimens

Between 2001 and 2003, eleven uveal melanoma-containing eyes received TSTT prior to enucleation, with a median interval of 14 days (range 6–15 days), and an outlier at 42 days, because of logistical problems. Six eyes had been treated with a conductive probe, three with a laser-probe and two with a combination probe (Table 1). Formalin-fixed, paraffin-embedded specimens from these eyes were available at the Leiden University Medical Center (LUMC). Each tumour sample was processed for histopathological diagnosis and immunohistochemistry. The research protocol had received permission from the Ethical Committee of the Leiden University Medical Center and followed the tenets

### TABLE 1. Characteristics of patients and tumours treated by trans-scleral thermotherapy.

<table>
<thead>
<tr>
<th>ID</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Localization</th>
<th>Cell type</th>
<th>VN Diameter (mm)</th>
<th>Probe</th>
<th>Days to enucleation</th>
<th>Spot (mm)</th>
<th>Follow-up (months)</th>
<th>Cause of death</th>
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<tr>
<td>UM1</td>
<td>F</td>
<td>53</td>
<td>Chor</td>
<td>E/S</td>
<td>14.6x7.4</td>
<td>C</td>
<td>6</td>
<td>5.3x1.2</td>
<td>57</td>
<td>Metastasis</td>
</tr>
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<td>UM2</td>
<td>F</td>
<td>56</td>
<td>Cil/Chor</td>
<td>E/S</td>
<td>17x17</td>
<td>C</td>
<td>15</td>
<td>5.0x0.2</td>
<td>101</td>
<td>-</td>
</tr>
<tr>
<td>UM3</td>
<td>F</td>
<td>58</td>
<td>Chor</td>
<td>S</td>
<td>11x11</td>
<td>C</td>
<td>8</td>
<td>4.7x0.3</td>
<td>98</td>
<td>-</td>
</tr>
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<td>UM4</td>
<td>F</td>
<td>81</td>
<td>Chor</td>
<td>S</td>
<td>15x13</td>
<td>C</td>
<td>8</td>
<td>5.0x0.7</td>
<td>10</td>
<td>Other</td>
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<td>UM5</td>
<td>F</td>
<td>35</td>
<td>Cil/Chor</td>
<td>S</td>
<td>17.9x17.4</td>
<td>C</td>
<td>15</td>
<td>5.9x0.5</td>
<td>26</td>
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</tr>
<tr>
<td>UM6</td>
<td>F</td>
<td>76</td>
<td>Chor</td>
<td>S</td>
<td>15</td>
<td>C</td>
<td>12</td>
<td>No</td>
<td>53</td>
<td>Metastasis</td>
</tr>
<tr>
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<td>F</td>
<td>57</td>
<td>Cil/Chor</td>
<td>S</td>
<td>14</td>
<td>L</td>
<td>12</td>
<td>4.5x0.9</td>
<td>36</td>
<td>Metastasis</td>
</tr>
<tr>
<td>UM8</td>
<td>M</td>
<td>64</td>
<td>Cil/Chor</td>
<td>S</td>
<td>14.7x12.4</td>
<td>L</td>
<td>7</td>
<td>3.2x1.0</td>
<td>40</td>
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<tr>
<td>UM9</td>
<td>M</td>
<td>44</td>
<td>Cil/ Iris</td>
<td>E/S</td>
<td>15</td>
<td>L</td>
<td>14</td>
<td>2.6x0.5</td>
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<td>-</td>
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<tr>
<td>UM10</td>
<td>F</td>
<td>69</td>
<td>Cil/Chor/Iris</td>
<td>E/S</td>
<td>12x5.1</td>
<td>Comb</td>
<td>13</td>
<td>7.2x3.0</td>
<td>77</td>
<td>-</td>
</tr>
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<td>UM11</td>
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<td>72</td>
<td>Chor</td>
<td>E/S</td>
<td>20</td>
<td>Comb</td>
<td>42</td>
<td>6.2x1.8</td>
<td>14</td>
<td>Metastasis</td>
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</tbody>
</table>

F, female; M, male; Chor, choroid; Cil, ciliary body; E, epithelioid cell type; S, spindle cell type; VN, vascular networks; C, conductive-heating probe; L, laser-induced heating probe; Comb, combined conductive and laser-induced heating probe.
of the Declaration of Helsinki. All patients had given permission for experimental TSTT after explanation of the experimental nature of the treatment. Histological sections were prepared from tissues fixed in 4% buffered neutral formalin for 48 hr and embedded in paraffin. Hematoxylin- and eosin-stained 4-μm sections were prepared and reviewed.

**Immunohistochemistry**

Staining for S100, CD68, HCA2, HC10 and the HSPs was performed by the alkaline phosphatase-anti-alkaline phosphatase (APAAP) method, as described previously. In short, paraffin-embedded sections (4 μm), mounted on slides coated with aminopropyltriethoxysilane (APES; Sigma-Aldrich, St. Louis, MO, USA), were deparaffinized in xylene (four times, 5 min each) and ethanol 99% (three times, 5 min each). After the slides were washed, antigen retrieval was performed by boiling in citrate buffer (Dako, Glostrup, Denmark) for 20 min. After the slides were washed again in phosphate-buffered saline (PBS), they were incubated with the first antibody for 1 hr. The slides were labelled with fast red (Scytek, Logan, UT, USA) in naphthol-phosphate buffer (Scytek) and levamisole 50 mm. Slides were counterstained with Mayer’s hematoxylin and finally embedded in Kaiser’s glycerin.

A subtype of macrophage, the M2 subtype, was identified using double immunofluorescence histochemistry (IF), to enable evaluation of double positive cells in pigmented tumours, with monoclonal antibodies (mAbs) directed against CD68 and CD163, as described previously. When analysing the slides with the confocal laser microscope, we used the HE-stained slides/pictures for mapping the localization.

Optimal antibody concentrations of the mAbs (Table 2) were determined from titration studies of the primary reagents, diluted in phosphate-buffered saline/bovine serum

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Dilution</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD68*</td>
<td>PG-M1</td>
<td>1:100</td>
<td>Dako, Glostrup, Denmark</td>
</tr>
<tr>
<td>S100</td>
<td>AC88</td>
<td>1:400</td>
<td>Dako</td>
</tr>
<tr>
<td>HCA2</td>
<td></td>
<td>1:100</td>
<td>Dutch Cancer Institute</td>
</tr>
<tr>
<td>HC10</td>
<td></td>
<td>1:100</td>
<td>Dutch Cancer Institute</td>
</tr>
<tr>
<td>HSP90</td>
<td>C92F3A-5</td>
<td>1:400</td>
<td>Stressgen, Victoria, British Columbia, Canada</td>
</tr>
<tr>
<td>HSP70</td>
<td>G3.1</td>
<td>1:800</td>
<td>Stressgen</td>
</tr>
<tr>
<td>GRP94</td>
<td>9G10</td>
<td>1:800</td>
<td>Stressgen</td>
</tr>
<tr>
<td>HSP27</td>
<td></td>
<td>1:400</td>
<td>Stressgen</td>
</tr>
<tr>
<td>CD68†</td>
<td>514H12</td>
<td>1:50</td>
<td>Abcam, Cambridge, UK</td>
</tr>
<tr>
<td>CD163</td>
<td>10D6</td>
<td>1:100</td>
<td>Novocastra, Newcastle upon Tyne, UK.</td>
</tr>
<tr>
<td>Alexa fluor</td>
<td>IgG2a (488)</td>
<td>1:200</td>
<td>Invitrogen, molecular probes, Eugene, Oregon, USA</td>
</tr>
<tr>
<td></td>
<td>IgG1 (546)</td>
<td>1:200</td>
<td></td>
</tr>
</tbody>
</table>

* Used for immunohistochemical staining.
† Used for immunofluorescence staining.
albumin 1% (PBS/BSA). Sections of colon carcinoma (GP96), gastric carcinoma (HSP70), normal skin (HSP27 and HSP90), and tonsils (CD68 and CD163) were used as positive controls. In negative controls, the primary antibody was replaced by PBS/BSA 1%.

All scores were assessed visually by two masked operators to ensure accuracy of the semi-quantitative assessment, by the percentage of stained tumour cells, with – for no positive cells; ± for 1–5% positive cells; ++ for 5 to < 50%; +++ for more than 50% of cells positive; ++++ for more than 50% of cells positive, with very bright staining. In case of a difference, consensus was reached during a simultaneous session.

RESULTS

Experimental trans-scleral thermotherapy

Examining the hematoxylin–eosin slides, ten of the eleven enucleated eyes histologically showed a TSTT spot (areas with local visible changes owing to heat application) in the uveal melanoma (Fig. 1). The tumour from patient number six was excluded from the results, because of lack of any visible effect. The area of treatment could not be identified histopathologically: no necrosis, areas of cell degeneration, haemorrhages or oedema were detected at the scleral side of the tumour. Lack of the function of the probe was the most likely reason for absence of effect.

Histological evaluation

Trans-scleral thermotherapy-treated tumours showed necrotic areas along the scleral side of the tumours, which were demarcated from the viable part (tumour cell areas composed of either spindle or mixed cell type) by a wall of melanophages (Fig. 1). The sharpness of

**Figure 1.** Trans-scleral thermotherapy spot in uveal melanoma (UM10) in a hematoxylin–eosin stained slide. The sclera (S) can be observed as well as a sharply demarcated border (b) between the necrotic part of the tumour (N) and the viable part (V). Magnification 25×.
the demarcation varied between the three probes: the sharpest demarcations between layers were observed in the two lesions made by the combination probe. The largest TSTT spot had a diameter of 7.2 mm, which was because of treatment with the combination probe. The combination probe also caused the deepest heat effect, with a penetration from the sclera downwards into the tumour tissue of 3.0 mm.

**Immunohistochemistry**

CD68-positive macrophages were found inside eight of the spots, and especially at a high density at the borders of nine of the spots (Fig. 2). The remaining part of the tumour contained some CD68-positive macrophages, but with a scattered distribution pattern. Further examination of the macrophages by immuno-fluorescent labelling with anti-CD68 and CD163, which identifies a subtype of macrophages, i.e. the M2 type macrophage,
demonstrated the presence of many double-positive cells in the border area (Fig. 3). In the necrotic part, the staining was highly CD163 positive, possibly an artefact of the large number of dead cells as CD163 can also react with dead cells and debris.

HLA Class I expression, observed with the mAb’s HC10 and HCA2, showed a variable staining pattern in the spots themselves and the border areas, as well as in the remaining tumour, but expression was not changed by the TSTT. The use of the three probes did not lead to different HLA staining patterns.

Nontreated areas from nine of the ten tumours stained positively for S100. In contrast, seven TSTT spots were not stained by the S100-antibody.

Expression of HSPs was not observed in the TSTT-spots except for one case, which stained positively for HSP90; however, the rest of this tumour also stained positive for...
HSP90. The expression of HSP27, HSP70 and HSP90 outside the spots was variable. Seven TSTT borders had very little or no staining of HSP90, while seven TSTT borders stained positively for GP96, with the surrounding tissue showing less staining (Fig. 4). Diagrams of the results are shown in Fig. 2.

**Follow-up**

Patient data and survival were updated till September 2010. The mean follow-up at the time of analysis was 54 months (range 10–101 months). During this period, seven patients died, six because of metastatic disease and one patient because of another, but unknown cause. There were no patients with metastasis still alive at the end of follow-up and no patients were lost to follow-up. According to Kaplan–Meier analysis and log rank testing, there was no association between survival and the type of probe used (p = 0.94).

**DISCUSSION**

This study presents the results of uveal melanoma antigen expression analysis after experimental laser treatment, i.e. TSTT, in eyes enucleated shortly after treatment. We hypothesized that TSTT could sensitize the patient immunologically against the intraocular tumours, by making the melanoma cells more susceptible for the immune system.

Because studies with hyperthermia and TTT demonstrated an effect on immunological parameters, we wondered whether trans-scleral application of heat on uveal melanomas would also result in upregulation of immunological relevant molecules and an influx of antigen-presenting cells. Extracellular S100 proteins have been shown to exert regulatory effects on inflammatory cells. In addition, HSPs are synthesized in response to stress such as a hyperthermic treatment. Heat-shock proteins are released following necrotic
cell death, and released HSPs stimulate macrophages and dendritic cells to secrete cytokines, and activate antigen-presenting cells. An increase of HSPs on the cell surface has also been associated with an increase of the level of MHC class I antigens and higher immunogenicity. As we know there is natural variation of expression between tumours, we used the viable part of the same tumour as an internal control. Our present observations reveal that TSTT provides a similar histological image as the TTT treatment had shown, but with sometimes different staining results: TSTT induced a greater loss of S100 than TTT. A possible explanation could be that a high concentration of heat coagulated the S100 proteins, which prevented binding of antibody. Unfortunately, the S100 antibody does not differentiate between the two subunits alpha and beta, and thereby limits the ability for further analysis.

In this study, all TSTT spots were necrotic and did not stain for heat HSP. Heat-shock proteins are believed to be regulators of normal cell physiology and of the cellular stress response. They are induced in order to prevent immediate apoptosis of the cell when it undergoes various types of environmental stresses like heat, cold and oxygen deprivation. HSP chaperone denatured proteins in the stressed cells, which gives the proteins time to re-establish their normal structure and escape the risk of cell death. Heat-shock protein GP96 has been identified as a tumour-specific antigen, expressed on the surface area of various tumour cells. The borders of the TSTT spots stained positively for GP96: the induction of intracellular HSP may lead to HSP migration to the tumour cell surface. The induction of GP96 expression could imply that TSTT treatment may sensitize tumour cells for immune attack.

All the borders of the TSTT spots, regardless of the different type of probe, showed an increase in the presence of CD68-positive macrophages. Numbers of macrophages vary between tumours, but the local high density of CD68-positive macrophages as seen at the borders of the TSTT spots can clearly be differentiated from the known variability. Further analysis revealed that the macrophages in the border of the TSTT-treated areas zone were both CD68 and CD163 positive and thus typical M2 macrophages, which are associated with tissue repair and angiogenesis. The increased presence of specifically the M2-type macrophage and not of M1-type macrophages around the necrotic area would be detrimental for the induction of a specific immune response. However, as M2-type macrophages have a specific phagocytosis effect, and that role may be the most important function to help clear treated tumour cells and prevent the induction of intraocular inflammation.

In our study, there was no difference in immunological parameters observed between the three thermal probes. However, the combination probe has a deeper effect and seems to have more effect on the expression of HSPs. As mentioned earlier, the presence of a massive macrophage influx may provide an explanation that in spite of the upregulation of HSP antigens, the heat-treated lesions are being carefully removed by the innate im-
mune system, without an intense ocular inflammation. Based on current results, we cannot give advice of clinical use of TSTT. When applying treatment to intraocular tumours, one should realize that local changes occur that are not seen by the eye of the beholder.

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