Abstract. Background: Normalization of tumor blood vessels improves drug and oxygen delivery to cancer cells. The aim of this study was to develop a technique to normalize blood vessels in the hamster cheek pouch model of oral cancer. Materials and Methods: Tumor-bearing hamsters were treated with thalidomide and were compared with controls. Results: Twenty eight hours after treatment with thalidomide, the blood vessels of premalignant tissue observable in vivo became narrower and less tortuous than those of controls; Evans Blue Dye extravasation in tumor was significantly reduced (indicating a reduction in aberrant tumor vascular hyperpermeability that compromises blood flow), and tumor blood vessel morphology in histological sections, labeled for Factor VIII, revealed a significant reduction in compressive forces. These findings indicated blood vessel normalization with a window of 48 h. Conclusion: The technique developed herein has rendered the hamster oral cancer model amenable to research, with the potential benefit of vascular normalization in head and neck cancer therapy.

Tumor blood vessels resulting from angiogenesis and vasculogenesis are structurally and functionally abnormal. The blood vessels are leaky, tortuous and dilated. These abnormalities contribute to heterogeneous blood flow, interstitial hypertension, hypoxia and acidosis. Impaired blood supply and elevated interstitial fluid pressure hinder effective convective fluid transport and result in the poor distribution of blood-borne therapeutic agents (1, 2). Tumor blood vessel normalization by tailored administration of antiangiogenic agents that down-regulate vascular endothelial growth factor (VEGF) (overexpressed in the majority of solid tumors) would lead to less leaky, less dilated and less tortuous vessels, decreased interstitial fluid pressure, increased tumor oxygenation and improved penetration of drugs in tumors (3). Within this context, tumor blood vessel normalization would conceivably improve the outcome of chemotherapy and radiotherapy.

Despite the remarkable success of boron neutron capture therapy (BNCT) protocols employed in studies by our group, in the hamster cheek pouch model of oral cancer (65% to 95% tumor control with no normal tissue radiotoxicity) (4-8), there is still room for improvement. We are currently devoted to developing new BNCT protocols and assessing their therapeutic advantage. In particular, an unresolved challenge lies in achieving homogeneous targeting of all tumor cells (8). Within this context, an improvement in blood flow efficiency in tumor, would conceivably be contributory.

The hamster cheek pouch model of oral cancer is widely accepted (9). Carcinogenesis protocols mimic the spontaneous process of malignant transformation, inducing premalignant changes and carcinomas, that closely resemble human oral lesions (10). Conversely, experimental tumor models employed routinely for BNCT studies are based on the growth of implanted cells (11). The hamster cheek pouch model is unique in that in addition to neoplastic tissue, it also allows for the study of tissue with potentially malignant disorders (PMD) surrounding the tumors, an issue of clinical relevance due to the phenomenon of field cancerization (12, 13). Second primary tumor locoregional
recurrences that arise in field-cancerized tissue are a frequent cause of therapeutic failure (14). Furthermore, tissue with PMD can behave as a dose-limiting tissue in tumor control studies. In addition, the model allows for the study of clinically relevant healthy tissues. In this way, the hamster cheek pouch oral cancer model allows for the study of therapeutic modalities for head and neck cancer, their potential inhibitory effect on tumor development from tissue with PMD, and their potential toxic effects on tissue with PMD and healthy tissue. The clinical rationale for exploration of the potential therapeutic advantage of different techniques in this model lies in the need for alternative therapeutic strategies to improve the relatively poor 5-year survival rate of 38.3% – 63% for malignancies of the oral cavity, and to avoid the large tissue defect caused by radical surgery (6, 15).

BNCT is a binary treatment modality that involves the selective accumulation of 10-boron carriers in tumors, followed by irradiation with a thermal or epithermal neutron beam. The high linear energy transfer (LET) alpha particles and recoiling \(^7\)Li nuclei, emitted after the capture of a thermal neutron by a \(^{10}\)B nucleus (16), have a high relative biological effectiveness (17). In this way, BNCT targets neoplastic tissue selectively, sparing normal tissue. Boron content and distribution in tumor are pivotal to the efficacy of BNCT (18). Tumor cell areas that are poorly loaded with boron will be refractory to BNCT. It follows that reversible tumor blood vessel normalization prior to boron compound administration would conceivably improve boron targeting and BNCT efficacy.

In view of the fact that the antiangiogenic monoclonal antibodies employed to induce blood vessel normalization in humans, rats and mice [e.g. (2)] cannot be used in hamsters, due to lack of cross-antigenicity, the aim of the present study was to develop a technique to normalize aberrant blood vessels in the hamster cheek pouch model of oral cancer and thus establish an experimental model in which to assess the potentiating effect of tumor blood vessel normalization on head and neck cancer therapy in general, and BNCT in particular.

Materials and Methods

Tumor induction. Tumors were induced in the right cheek pouch of 52 noninbred 6-week-old Syrian hamsters by topical application of 0.5% of the complete carcinogen dimethyl-1,2-benzanthracene (DMBA) in mineral oil twice a week for 12 weeks, in keeping with a standard hamster cheek pouch carcinogenesis protocol (19), modified as previously described (8). Once the exophytic tumors, i.e. squamous cell carcinomas, developed and reached a diameter of approximately 3-5 mm, the animals were used for experiments. Animal experiments were carried out in accordance with the guidelines of the National Institute of Health (NIH) in the USA, regarding the care and use of animals for experimental procedures (20) and in accordance with local laws and regulations.

Treatment with thalidomide. Thalidomide is an antiangiogenic drug and has been reported to induce tumor blood vessel normalization in a mouse model. That study determined the importance of optimal scheduling to exploit the window for normalization of the tumor vasculature (21). Given the need for normalization protocols tailored and characterized for each experimental model, we performed several pilot studies employing different dose levels of thalidomide and time-points to identify the most promising protocol to be assessed in terms of potential normalizing capacity in the hamster cheek pouch oral cancer model (n=8 cancerized hamsters bearing a total of 48 tumors). Macroscopic and light microscopy examination of the everted pouch was performed in order to select the adequate dose of thalidomide and the window of normalization.

Based on preliminary data, we selected a protocol that consisted of the administration of two doses of 200 mg thalidomide/kg body weight in dimethyl sulfoxide (DMSO) (112 mg thalidomide/ml DMSO) intraperitoneally (i.p.) into tumor-bearing hamsters on two consecutive days. Thalidomide was a generous gift from Triquim S.A. (Argentina). Tumor-bearing hamsters not treated with thalidomide were used as controls. In addition, parallel DMSO-only controls were run to evaluate the potential effect of DMSO on tumor blood vessels in the cancerized hamster cheek pouch. Two doses of DMSO (1.8 ml/kg each dose) were administered i.p. on two consecutive days to match the thalidomide protocol (n=4 cancerized hamsters bearing a total of 20 tumors).

Macroscopic and light microscopic examination of the everted pouch. Prior to, and 1 to 15 days after the first injection of thalidomide, the pouch was everted for macroscopic and low-magnification (×2.5) light microscopic in vivo examination of the blood vessels under light i.p. ketamine (70 mg/kg body weight)-xylazine (10.5 mg/kg body weight) anesthesia. The transparency of the tissue with PMD made it possible to observe the blood vessels in vivo in this tissue. However, it was not possible to observe the blood vessels within the solid, opaque, exophytic tumors. The contralateral, normal (non-cancerized) pouch of each animal was also everted, in order to examine for potential changes in blood vessels of normal pouch tissue, also observable in vivo due to tissue transparency. Animals treated with thalidomide were assessed to qualitatively establish the time-point at which blood vessel morphology in tissue with PMD was most similar to normal tissue. As described in the Results section, 48 h after the first injection of thalidomide, the window of maximum normalization in terms of narrowing and straightening of blood vessels was considered. Thus, all subsequent experiments designed to assess the normalizing effect of the thalidomide protocol were performed 48 h after the first injection of the drug.

Toxicity studies. Tumor-bearing (n=8 cancerized hamsters bearing a total of 34 tumors) and non-cancerized hamsters (n=5) were treated under the thalidomide protocol, as described above. Body weight and clinical signs were monitored daily for a period of one week in both groups and thereafter once a week for one month in the non-cancerized group. The clinical response to anesthesia in thalidomide-treated animals at the dose level routinely employed to evert the cheek pouch, was also assessed in tumor-bearing (n=9 cancerized hamsters bearing a total of 76 tumors) and non-cancerized hamsters (n=5). These observations were compared to findings from our previous studies on tumor-bearing and non-cancerized hamsters not treated with thalidomide.

Vascular permeability assay. Permeability studies with Evans Blue Dye (EBD) were performed according to a modification of the
technique by Chen et al. (22), in tumor-bearing hamsters treated with thalidomide (n=9 cancerized hamsters bearing a total of 13 tumors amenable to this analysis), in tumor-bearing hamsters not treated with thalidomide (n=10 cancerized hamsters bearing a total of 13 tumors amenable to this analysis) and in tumor-bearing hamsters treated with DMSO alone (n=4 cancerized hamsters bearing a total of 8 tumors amenable to this analysis). We sought to explore for potential differences in vascular permeability induced by the thalidomide protocol, as measured by the EBD extravasation. Briefly, 3 ml of EBD (Fluka, St. Gallen, Switzerland) per kg body weight (2% mass/volume) were injected in the surgically exposed jugular vein of hamsters under i.p. ketamine (140 mg/kg body weight)-xylazine (21 mg/kg body weight) anesthesia. EBD was allowed to circulate for 30 minutes. Transcardial perfusion (through the right auricle) was then performed with 0.9% NaCl solution at 120 mmHg for 8 min to remove the dye-containing blood. Samples of pouch tumor, tissue with PMD surrounding the tumor, contralateral normal pouch, liver and kidney were excised, weighed and frozen in liquid nitrogen until use. Tissue samples were homogenized in 2 ml trichloroacetic acid (50% mass/volume) and centrifuged at 10,000 rpm for 20 min. The EBD content in the supernatant was quantified by spectrophotometry at 620 nm. If necessary, the supernatant was diluted two- or three-fold in trichloroacetic acid (50% mass/volume) to ensure that measurements fell within the calibration curve. This technique allowed for the measurement of extravasated dye in tissues as an indicator of vascular permeability.

**Blood vessel morphology.** Immunohistochemical staining of Factor VIII in 12 μ-thick paraffin sections was used as a marker of vascular walls. As determined by Aromando et al. (23), of all markers studied, Factor VIII was the best in terms of vascular sections visualization in the hamster cheek pouch cancer model. Endogenous peroxidase activity was blocked with 100% H2O2 in methanol. Sections were transferred to phosphate buffered saline (PBS) of pH 7.4. After antigen unmasking by protease digestion with proteinase K (10% in PBS) and permeabilization of tissue with PBS-albumin, the sections were incubated with the secondary and tertiary antibodies using the biotin-streptavidine–peroxidase kit (Super sensitive multilink detection kit, Biogenex, San Ramon, CA, USA). The slides were then incubated with 3,3-diaminobenzidine (DAKO, Logan, UT, USA), counterstained with hematoxylin and mounted. Digital images of the vascular sections-immunostained with Factor VIII were obtained. Vessels were hand-traced employing the ImageJ image analysis software (ImageJ 1.42q Wayne Rasband NIH, USA. http://rsb.info.nih.gov/ij Java 1.6.0_10 (32-bit)/National Institutes of Health), as described by Padera et al. (25) and Hagedoorn et al. (26). The aspect ratio, i.e. the ratio of the maximum to the minimum diameter, of stained vessels was calculated as an indicator of vascular compression in keeping with Hagedoorn et al. (26). An aspect ratio of 1 represents a perfect circle. The larger the aspect ratio, the greater the amount of vessel compression. The aspect ratio was measured in sections of pouch tumors from thalidomide-treated animals (two cancerized hamsters bearing a total of four tumors, n=115 vascular sections). The values were compared with control data (corresponding to cancerized or non-cancerized animals not treated with thalidomide) obtained within the context of a parallel study (23), performed with the exact same methodology as herein, for tumor (n=149 vascular sections) and normal (non-cancerized) cheek pouch tissue (n=98 vascular sections). During evaluation, particular attention was attributed to the potential presence of glomeruloid microvascular proliferations (GMPs), that have been described in a variety of human tumors (27) and in hamster cheek pouch tumors (23).

**Statistical analysis.** The statistical significance of the differences between thalidomide-treated and control animals was assessed by the Student’s t-test (vascular permeability assay) or ANOVA (blood vessel morphology). Statistical significance was set at p=0.05.

**Results**

**Toxicity studies.** Overall, treatment with thalidomide exerted a mostly reversible, sedative effect on the animals, that was enhanced by anesthesia. Cancerized animals were more sensitive to this effect than non-cancerized animals. One of the eight tumor-bearing animals (13%) treated with thalidomide died within the experimental period, whereas none (0/5) of the non-cancerized animals treated with thalidomide died within the experimental period. Two of the nine tumor-bearing animals (22%) treated with thalidomide and anesthetized died within the experimental period, whereas none (0/5) of the non-cancerized animals treated with thalidomide and anesthetized died within the experimental period. Previous studies by our group (4-8) revealed an overall percentage of deaths (unrelated to specific treatment protocols under study in each case) within the experimental period, of approximately 5% in cancerized animals and approximately 7% in cancerized animals submitted to anesthesia. In the case of non-cancerized animals, only occasional deaths (approximately 1 to 2%) were observed, regardless of the administration of anesthesia. Although no deaths were observed in the present study in non-cancerized animals treated with thalidomide, the small sample size would be insufficient to detect the occasional deaths observed in previous studies.

Treatment with thalidomide induced a transient loss in initial body weight of approximately 3 to 9% over the first three days after treatment in both tumor-bearing and non-cancerized animals. Cancerized animals treated with DMSO only exhibited a similar weight loss of 3 to 5%. The use of anesthesia somewhat enhanced this effect to approximately 5 to 11% of body weight loss. These weight losses closely resembled the approximate weight loss of 3 to 10% observed in the previous studies described above, for tumor-bearing and non-cancerized animals not treated with thalidomide but submitted to anesthesia.

**Macroscopic and light microscopic examination of the everted pouch.** Both naked eye and light microscopic examination of the everted cancerized pouch revealed progressive narrowing and straightening of the blood vessels.
and a qualitative reduction in blood vessel density in tissues with PMD in thalidomide-treated animals, resembling more closely the normal vasculature. The effect was maximal 48 h after the first dose of thalidomide and reverted progressively, regaining the pre-treatment phenotype six days after the first dose of thalidomide. No changes were observed in the contralateral normal (non-cancerized) pouch of animals treated with thalidomide. DMSO alone did not induce any visible changes in the aberrant blood vessels of the cancerized hamster cheek pouch.

**Vascular permeability assay.** Figure 1 shows the values (mean±S.D.) corresponding to extravasated EBD in tumor, in tissue with premalignant disorders (PMD), normal pouch tissue, liver and kidney in control and thalidomide-treated animals as indicated. Values are expressed as the mean±S.D. Statistically significant differences between thalidomide-treated and control animals were only observed for tumor tissue.

---

**Figure 1.** Extravasated Evans Blue Dye (μg EBD/100 mg tissue) in tumor, in tissue with premalignant disorders (PMD), normal pouch tissue, liver and kidney in control and thalidomide-treated animals as indicated. Values are expressed as the mean±S.D. Statistically significant differences between thalidomide-treated and control animals were only observed for tumor tissue.

---

**Discussion**

Tumor blood vessels that result from angiogenesis and vasculogenesis are functionally and structurally altered. They are tortuous and dilated, their walls exhibit fenestrae, vesicles and transcellular holes, widened interendothelial junctions and lack of or discontinuous basement membrane (29). These vascular alterations lead to interstitial hypertension and impaired heterogeneous blood supply, severely interfering with the delivery of therapeutic drugs and oxygen to tumors. It is widely accepted that antiangiogenic drugs for cancer therapy destroy tumor vasculature and deprive cancer cells of oxygen and nutrients. However, when administered as single agents, antiangiogenic drugs produce modest therapeutic benefits (30). It has been reported (2) that tailored application of antiangiogenic agents can normalize the abnormal tumor vasculature to yield more efficient delivery of drugs and oxygen to tumor cells, conceivably improving the outcome of chemotherapy and radiotherapy. Our studies on BNCT in the hamster cheek pouch oral cancer model have shown the pivotal role in

---

**Table I. Aspect ratio of blood vessel sections.**

<table>
<thead>
<tr>
<th>Blood vessels</th>
<th>Thalidomide</th>
<th>n</th>
<th>Mean±S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor +</td>
<td>115</td>
<td>1.60±0.58</td>
<td></td>
</tr>
<tr>
<td>Tumor –</td>
<td>149</td>
<td>2.48±1.35a</td>
<td></td>
</tr>
<tr>
<td>Normal pouch –</td>
<td>98</td>
<td>1.56±0.47a</td>
<td></td>
</tr>
</tbody>
</table>

aAromando et al. (23).
therapeutic success of homogeneous, efficient boron targeting of tumor cells (6, 31). Other authors have reported concurring findings in other experimental models (32). Within this context, and given that the hamster vasculature is not amenable to treatment with the monoclonal antibodies employed to induce blood vessel normalization in humans, rats and mice (2, 33), we sought to establish, for the first time, a blood vessel normalization technique in the hamster cheek pouch oral cancer model for future BNCT studies in particular, and cancer therapy studies in general.

Thalidomide is described as an immunomodulatory, anti-inflammatory and antiangiogenic drug. Its action as an inhibitor of angiogenesis is associated with its capacity to inhibit basic fibroblast growth factor (bFGF)- and vascular endothelium growth factor (VEGF)-induced angiogenesis (34-36). It has been shown that the short-term administration of antiangiogenic agents could “prune” inefficient vascular sprouts (37), yielding more efficient flow paths. After the tragic withdrawal of thalidomide from the market, due to its teratogenic effects (38, 39), it was approved in 1997 by the FDA to combat a variety of dermatological conditions (40, 41). It is still a promising candidate drug for cancer prevention and treatment (36). Yang et al. (36) showed the inhibitory effects of thalidomide against malignant transformation in the hamster cheek pouch oral cancer model. Within this context, and based on pilot studies, we selected a thalidomide administration protocol that would potentially induce blood vessel normalization in the hamster cheek pouch oral cancer model. Based on qualitative assessment of the blood vessel morphology in tissues with PMD in situ, we were able to establish a tentative window of normalization at 48 h, to employ in subsequent experiments. Macroscopically observable narrowing and straightening of blood vessels in tissue with PMD in the everted pouch were maximal 48 h after the first injection of thalidomide. This finding was interpreted as potential blood vessel normalization in the cancerized pouch to be confirmed by two additional end-points.

A significant reduction in blood vessel permeability to EBD in pouch tumors of thalidomide-treated animals indicated a reduction in the characteristically aberrant tumor blood vessel hyperpermeability that compromises blood flow and consequently impairs drug delivery. Thus, the reduction in extravasation of EBD was interpreted as a sign of blood vessel normalization (28). The normal blood vessels of normal pouch tissue, liver and kidney would not undergo changes as a result of thalidomide treatment. Only abnormal blood vessels would be sensitive to normalization. In this sense, the blood vessels of tissue with PMD are aberrant versus normal pouch tissue, but are less functionally and structurally altered than those of tumor tissue (42). Within this context, the small degree of normalization that blood vessels in tissue with PMD are liable to undergo would not be sufficient to be unequivocally demonstrated. The qualitative light microscopic observations indicating the presence of less tortuous, narrower blood vessels in the tissue with PMD of thalidomide-treated hamsters, were used as a guideline to establish the window of normalization in tumors but are not intended to be quantitative evidence of blood vessel normalization in tissues with PMD.

The aspect ratio values showed that treatment with thalidomide significantly reduced the blood vessel compression in tumors to values that resemble those of normal vasculature. Compressive forces can cause vessel lumen collapse and restrict blood flow. Blood vessel normalization would reduce vascular hyperpermeability and interstitial fluid pressure (26), reducing compressive forces and contributing to adequate delivery of therapeutic agents in tumors (43). Given that GMPs have been described in a variety of human tumors (27) and in hamster cheek pouch tumors (23), the absence of GMPs in
thalidomide-treated animals was interpreted as an additional sign of blood vessel normalization and could be attributed to the inhibitory effect of thalidomide on VEGF (34).

The side-effects of thalidomide therapy should not be disregarded. In particular, cancertized animals submitted to anesthesia were particularly sensitive to toxic effects, with a 22% incidence of deaths. Anesthesia would exacerbate the effect of thalidomide, known to be a potent non-barbiturate sedative-hypnotic drug (35, 44, 45). Similar toxic effects have been previously described (36). In particular, Yang et al. (36) reported that only cancertized animals exhibited these thalidomide-induced side-effects and suggested that the pharmacokinetics of drugs in animals exposed to a carcinogen were different to those of normal animals. This aspect must be borne in mind when experimental studies are planned.

Herein we have developed, for the first time, a model of tumor blood vessel normalization in the hamster cheek pouch model of oral cancer, amenable to examination regarding the effect of normalization on head and neck cancer therapy in general, and BNCT in particular. Tailored normalization of aberrant vasculature will conceivably improve the delivery of oxygen and drugs to the tumor site, contributing to therapeutic success.

Acknowledgements

Funding was received as a grant of Agencia Nacional de Promoción Científica y Tecnológica, Argentina (Principal Investigator A.E. Schwint, PICT2006-00700). This study was partially supported by the Department of Energy through Idaho National Laboratory (USA).

The Authors wish to express their gratitude to Dr. Sandra Renou for software assistance and gratefully acknowledge the generous gift of thalidomide by Triquim S.A. and Laboratorio Lazar (Argentina).

References


27 Nagy JA, Chang SH, Dvorak AM and Dvorak HF: Why are tumour blood vessels abnormal and why is it important to know? Br J Cancer 100: 865-869, 2009.


Received March 6, 2012

Revised April 3, 2012

Accepted April 5, 2012