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Electroporation in veterinary oncology

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ABSTRACT

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Introduction

The therapeutic armamentarium available for the control of solid neoplasms relies on the well-established applications of surgery, radiotherapy and chemotherapy to control both local and systemic disease. Our understanding of the role of the immune response against cancer is expanding and opening new horizons for intervention into the malignant process (Galon et al., 2006; O'Brien et al., 2014).

Local application of electroporation to tumours enhances the absorption of specific chemotherapeutic drugs, the most commonly used of which are cisplatin and bleomycin. This 'reversible' electroporation procedure, where the cell membrane recovers, induces a strong tumour immune response (Gothelf et al., 2003; Fridman et al., 2013; Gerlini et al., 2013). The chemotherapeutic agent is delivered at a single low dose on the day of treatment, greatly reducing the side effects associated with standard intravenous chemotherapy, where dose frequency and dose intensity are maximised for cell death. In contrast, the surrounding healthy tissue structures are preserved after electroporation. Electroporation can be conducted in seconds to minutes and, together with a preparation and observation period of several hours, the overall process can be completed as an outpatient procedure in most cases (Marty et al., 2006; Mir et al., 2006; Whelan et al., 2006).

In 'irreversible' electroporation, no chemotherapy is applied, but more energy is delivered, rendering the tumour cell membrane unable to recover, inducing localised cell death (Bower et al., 2011; Charpentier, 2012; Cannon et al., 2013). Electroporation also can be used to introduce genetic material into cells; in this process, the innate charge on the DNA molecule brings it into contact with the cell membrane and the electrophoretic pulse induces entry into the cell (Mir et al., 1999; Gothelf and Gehl, 2012). These applications of electroporation have been studied over the last 20 years in human and, more recently, veterinary clinical trials. Promising data have been published on this technology, which is the subject of this review.

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Electroporation to enable targeted drug absorption

Mechanism of electroporation

Cancer treatments in veterinary medicine continue to evolve beyond the established standard thera-

pies of surgery, chemotherapy and radiation therapy. New technologies in cancer therapy include a targeted mechanism to open the cell membrane based on electroporation, driving therapeutic agents, such as che-

motherapy (electro-chemotherapy), for local control of cancer, or delivery of gene-based products (electro-

gene therapy), directly into the cancer cell to achieve systemic control. This review examines

electrochemotherapy and electro-gene therapy in veterinary medicine and considers future directions

The cell membrane acts as a physical barrier to prevent the influx of hydrophilic drugs, macromolecules and peptides. The application of an electric field has been used to overcome this barrier by allowing the cells to become 'permeable' to these molecules, which normally would not be able to cross the plasma membrane. This phenomenon is referred to as electroporation (Fig. 1).

One of the most commonly accepted theories to explain this phenomenon is that electroporation results in the formation of hydrophilic pores due to the rearrangement of lipid molecules, which have hydrophilic tails embedded within the cell membrane (Mir et al., 1991, 2003; Mir and Orlowski, 1999; Mir, 2001). These pores are responsible for the transport of molecules across the cell membrane.

Electroporation is achieved once the applied voltage is greater than the threshold voltage for the cell, which ranges from 0.2 V to 1 V. Square wave pulses offer the advantage of independently controlling the pulse amplitude and pulse length; the optimised pulse



Review





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Fig. 1. Electroporation sequence. In the resting state (A) the therapeutic agent (drug or DNA) is present around the interstitial spaces between the cells, but has poor access across the cell membrane. The application of a square wave electrical pulse (B) delivered directly to the tissue causes the cell membrane to become porous allowing for the influx by passive diffusion of larger macromolecules (C and D). DNA enters via an endocytotic type mechanism where the charged DNA comes into contact with the cell wall after the electric pulse.

parameters for electroporation consist of eight square waves of 100 µs duration at a frequency of 1 Hz (Pucihar et al., 2002; Mir et al., 2006).

Pulses are usually delivered using plate or needle electrodes. Plate electrodes are non-invasive and their use is usually limited to the treatment of cutaneous lesions. Needle electrodes have the advantage of penetrating the lesion, thereby decrseasing the impedance caused by the skin. The electric field facilitates the delivery of drugs into cells without the intention to kill the cells directly (Soden et al., 2004; Miklavcic et al., 2012).

Cell recovery

Electroporation is a transient process that allows the cell membrane to become 'electroporous' and then return to a normal state. Membrane resealing in vitro usually occurs in minutes and depends on the electrical parameters used; 63% of membrane resealing in mouse muscle in vivo occurred within 9 min of application of the electrophoretic pulse (Mir and Orlowski, 1999; Mir, 2001).

Electrochemotherapy

Electrochemotherapy (ECT) is the local potentiation, by means of permeabilising electric pulses, of the anti-tumour activity of a non-permeant (or a low permeant) anticancer drug possessing a high intrinsic cytotoxicity. It is essentially a therapeutic approach that increases the internalisation of non-permeant or poorly permeant molecules into the cytosols of the target cells. When the principle of electroporation is combined with certain chemotherapeutic drugs, the cytotoxicity of these drugs is increased by several-fold, leading to improved and dramatic responses in the treated tumours.

'Vascular lock' phenomenon

Electroporation induces vascular changes in the region being treated, with a transient decrease in blood flow. The most accepted theory explaining these vascular changes is reflex vasoconstriction of afferent arterioles mediated by the sympathetic nervous system. This hypoperfusion state can be transient in normal tissues, but can last from 12 h when using electroporation only to 5 days when ECT is used (Gothelf et al., 2003; Gehl and Geertsen, 2006).

The vascular changes can be advantageous; if the drug is already present at the time of electroporation, the induced vasoconstriction 'traps' the drug in the treated area, creating a so-called 'vascular lock' and maintaining a high local concentration, since there is a delay in the dispersal of the drug by the local vasculature. The 'vascular lock' also has another practical advantage in that it decreases and interrupts local bleeding at the treatment site.

Drugs and mechanism of action

Drugs suitable for electrochemotherapy

Ideal molecules for ECT use are those which are lipophilic, nonpermeant or poorly permeant and have a high intrinsic toxicity (Mir and Orlowski, 2000). To date, the two most effective candidates are bleomycin and cisplatin. Bleomycin is a non-permeant molecule, which cannot diffuse across the cell membrane because of its size and physical and chemical properties. Under normal circumstances, internalisation of bleomycin requires binding to a transmembrane protein; this 'mechanism of entry' is limited by the number of proteins involved and by the speed at which these proteins can withdraw from the cell membrane. Following electroporation, there is almost free diffusion of bleomycin into the cells for as long as the cells remain permeable (up to 60 min). The cytotoxic effect of bleomycin can be increased by up to 700-fold when used in ECT (Mir and Orlowski, 1999; Mir, 2001).

Cisplatin and other platinum based agents are important chemotherapeutic agents (Dasari and Tchounwou, 2014). These platinum complexes react in vivo, binding to and causing cross-linking of DNA, which leads to apoptosis. Cisplatin complexes appear to be poorly permeant, but the mechanisms of internalisation are not yet fully understood. In vitro, electroporation can increase the toxicity of cisplatin up to eight-fold. This increase in cytotoxicity results directly from increased cisplatin uptake. While this increase in toxicity of cisplatin seems modest compared to the 700-fold increase seen with bleomycin, it is important to note that cisplatin delivered as a single agent is active against several types of tumours, while bleomycin, without electroporation, is an inefficient drug (Mir et al., 2003; Moller et al., 2009).

Human clinical applications

Substantial amounts of clinical data have published on the application of electroporation in human beings. The European Standard Operating Procedures for Electro-Chemotherapy (ESOPE), a prospective, non-randomised, multi-institutional study, set the benchmark for protocols governing clinical electroporation; it demonstrated a successful treatment response regardless of (1) tumour histology; (2) drug utilised (cisplatin or bleomycin); (3) route of administration (intratumoral versus intravenous administration); or (4) type of electrode employed (needle or plate) (Belehradek et al., 1993; Marty et al., 2006; Miklavcic et al., 2006; Mir et al., 2006; Sersa, 2006; Snoj et al., 2006).

The ESOPE and other subsequent studies (Whelan et al., 2006; Larkin et al., 2007; Matthiessen et al., 2012; Campana et al., 2013; Caraco et al., 2013; Solari et al., 2014) achieved an objective response rate of ~85% with a single treatment in tumours (melanoma, head and neck cancer, squamous cell carcinoma, breast cancer skin metastases, cutaneous Kaposi's sarcoma) of 3 cm in diameter or smaller. Larger tumours have less favourable outcomes, but the technology has compared favourably with other options for late stage disease management (Marty et al., 2006; Whelan et al., 2006). Clinically, the application of ECT has been focussed on the treatment of cutaneous or semi-cutaneous tumours with palliative intent (melanoma, head and neck cancer, squamous cell carcinoma, basal cell carcinoma, breast cancer skin metastases, cutaneous Kaposi's sarcoma). Tumours from a variety of histological origins and anatomical locations, including perineal sites, have responded successfully to ECT, including tumour masses previously resistant to chemotherapy or radiotherapy.

Minimally invasive procedures: Endoscopic electroporation

The technology available for delivery of ECT has to date been reliant on macroelectrodes, limiting its application to surface tumours. The ability to safely and efficiently deliver electroporation minimally invasively to intra-abdominal, intra-thoracic or genitourinary tumours presents an exciting opportunity for the treatment of surgical inoperable cases. ECT could also be applied to cancers that are recalcitrant to radiation therapy, thus allowing a more targeted tumoricidal therapy, with less collateral tissue injury.

The endoscopic vacuum electrode (EndoVe) device and ePORE generator (Mirai Medical) were developed for endoscopic delivery of electroporation and have the advantage of attaching to the end of a conventional endoscope, thereby allowing both direct tumour visualisation and targeting. The device has been used for endoluminal delivery of electroporation to gastrointestinal cancers (Figs. 2,3). The creation of a vacuum effect draws tissue into a chamber within the EndoVe, thus bringing the tumour into contact with plate electrodes contained with this chamber. The design and size of the chamber can be altered according to the tumour size and location to allow for optimum tumour accessibility and tumour/electrode contact (Miklavcic et al., 2012). The EndoVE is currently undergoing human clinical evaluation in a multicentre phase II study for patients with inoperable colorectal and oesophageal cancer.

Veterinary experience with electrochemotherapy

Experience to date in veterinary species has taken place primarily in Europe (Table 1). The paucity of generators available outside Europe has, until recently, limited case accrual in North America. Several studies support the veterinary use of ECT with tumour types traditionally known to have limited or no treatment options, or responses with standard therapy of less than 10% (Spugnini et al., 2007b, 2007c, 2008a, 2008b; Tozon et al., 2014) (Fig. 4). In our



Fig. 2. Delivery of electroporation with the EndoVE device and ePORE generator (Mirai Medical). A standard endoscope is used to bring the device into contact with the tumour. The use of vacuum brings tumour tissue into direct contact with the electrodes and facilitates chemotherapy drug perfusion around the cellular interstitial spaces. The electrical field extends to tissue within the device chamber and underneath.



Fig. 3. Canine colorectal adenocarcinoma pre- and post-treatment with EndoVE electroporation in combination with intravenous bleomycin. Complete resolution of disease was achieved over 1 month using two treatments 15 days apart. No recurrent disease was detected in the 3 year follow up.

experience, unresectable tumours or those in areas of anatomical limitations are amenable to this treatment.

Feline studies

Canine studies

Mast cell tumours (MCTs) of the skin in dogs have been treated successfully with ECT (Spugnini et al., 2011). A long term study (>2 years) with this modality by two of the authors support its use for treatment of MCTs (Lowe et al., 2016). ECT has been beneficial against MCTs in one study when combined with surgery (Spugnini et al., 2011); even with recurrence and retreatment with ECT, survival ranged from 6 to >28 months. Using a combination of surgery and ECT, we have had success in the treatment of a canine perianal gland adenoma, achieving a complete response after treatment for >18 months (Fig. 5; Spugnini et al., 2008a), and a canine sarcoma (Spugnini et al., 2008b). Tumours with behaviour limited to local recurrence are well suited to ECT and it is hoped that the use of ECT in combination with immunotherapy may be a successful approach to treatment. Experience has also been gained with endoscopic application of electroporation for the treatment of canine colorectal tumours using the EndoVE device (Figs. 2,3; Forde et al., 2016).

ECT has elicited favourable responses against cutaneous squamous cell carcinoma in cats treated with bleomycin administrated intravenously at least 8 min prior to the needle delivery of electroporation pulses directly to the tumour tissue (Tozon et al., 2014). One author (JAI) has personal experience with partial responses from feline oral and sublingual squamous cell carcinomas, which are notoriously difficult to treat using conventional therapies.

Spugini et al. (2015) conducted a non-randomised prospective study to evaluate the efficacy of bleomycin with electroporation compared to bleomycin alone in the treatment of 21 feline cases of periocular carcinoma, comprising 17 squamous cell carcinomas (SCCs) and four anaplastic carcinomas, as well as to 26 cases with advanced SCC of the head. In the periocular cohort, 12 were treated with bleomycin and electroporation (ECT), with nine receiving bleomycin alone. In the advanced SCC group, 14 received ECT, while 12 had bleomycin only. Of the 26 ECT treated cases, there were 21 complete responses and two partial responses, with the treatment being well tolerated and having minimal reported toxicity. In contrast, the bleomycin only group (21 cases) had four complete responders and three partial responses.

Table 1

Overview of veterinary studies using electrochemotherapy.

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Species	Drug	Route ^a	Cases (n)	Type of tumour	Stage	Response	Reference
Feline	Bleomycin	IT	58	Soft tissue sarcoma	T2-4	Median time to recurrence 12–19 months	Spugnini et al., 2007a
Canine	Bleomycin	IT	28	Mast cell tumour	T1-3	82% complete response	Spugnini et al., 2006
Canine	Bleomycin	IT	10	Mucosal melanoma	T2-3	70% complete response	Spugnini et al., 2006
Canine	Bleomycin/Cisplatin	IT	12	Perianal adenoma	T1-2	65% complete response	Tozon et al., 2014
Equine	Cisplatin	IT	83	Sarcoid	T1-4	100% complete response	Tamzali et al, 2012
Feline	Bleomycin	IV	12	Soft tissue sarcoma	T1-2	Stable disease	Mir et al., 1997
Feline	Bleomycin	IT	17	Squamous cell carcinoma	T1-4	87% complete response	Tozon et al., 2014

Route of administration: IT, Intratumoral; IV, Intravenous.



Fig. 4. Application of electroporation via needle electrode directly to a cutaneous tumour mass. First image is the treatment of a peritonsillar squamous cell carcinoma with a finger electrode. This electrode provides excellent dexterity and is suited for access inside narrow cavities. The second image is of a feline sarcoma undergoing treatment with the larger needle electrodes.

DNA vaccines

Tumour immunotherapy using DNA vaccines is achieved by the intramuscular or intradermal injection of plasmid DNA encoding a protein antigen of interest. DNA vaccines present a number of advantages: (1) good manufacturing practice (GMP) grade DNA is stable and easy to produce; (2) absence of viral elements; and (3) DNA vaccines can be administered repeatedly because they do not engender specific anti-vector immunity and there are no pre-existing antibodies against DNA (Aurisicchio et al., 2007; Peruzzi et al., 2010b). However, there are limitations to efficient immunisation following injection of plasmid DNA: (1) there is a low efficiency of entry



Fig. 5. Treatment images of a perianal adenoma pre- and post-electrochemotherapy. The initial volume was 6.5 cm × 4.5 cm, with a reduction in size to 1.8 cm × 1.5 cm 8 weeks post-procedure. Biopsy of the remaining mass confirmed fibrotic tissue.

and nuclear localisation of plasmid DNA into living cells: and (2) naked DNA injection does not result in a level of local inflammation that is sufficient for recruitment and activation of professional antigen presenting cells and therefore this does not create the necessary conditions for efficient priming of immune responses. Although positive results can be obtained in laboratory animals, in which proportionally high doses of DNA can be injected at high pressures into small muscles, the limitations of this technology were particularly evident when naked DNA vaccines were scaled up to non-human primates and human beings, in which clinical trials failed to show strong vaccine immunogenicity (Kennedy et al., 2008; Trimble et al., 2009). The reason why DNA vaccines were unable to induce potent and effective immune responses when scaled up has not yet been fully clarified. (Wolff and Budker, 2005; Wooddell et al., 2011). However, it is reasonable to speculate that the combination of inefficient cellular delivery of DNA plasmids, low levels of antigen production and lack of stimulation of the innate immune system are together accountable for the low potency of naked DNA vaccines. Further optimisation is required, particularly for cancer vaccines, in view of local and systemic impairment of immunity in animals with cancer.

DNA electro-gene transfer: Mechanisms of action

In addition to the increased permeability of target cells, EGT may enhance immune responses through increased protein expression, secretion of inflammatory chemokines and cytokines, and recruitment of antigen presenting cells at the site of electroporation. Antigen expression in muscle is usually enhanced 100-1000-fold upon gene electroporation in comparison with naked DNA vaccines, mainly because of increased cellular uptake (Mathiesen, 1999; Mir et al., 1999; Rizzuto et al., 2000). Furthermore, in vivo electroporation causes transient and reversible cell damage, resulting in local inflammation and release of cytokines, which further facilitate the induction of immune responses (Babiuk et al., 2004; Liu et al., 2008). As a result, antigen-specific humoral and cellular immune responses are increased by electroporation mediated delivery of plasmid DNA in comparison with levels achieved by intramuscular injection of DNA alone (Aurisicchio and Ciliberto, 2012).

In vivo electro-gene transfer of plasmid DNA (DNA-EGT) has been shown to be a safe methodology, resulting in greater DNA cell uptake, enhanced protein expression and concomitant increases in longer term immune responses against the target antigen compared to naked DNA injection in a variety of species, including large animals such as dogs, pigs cattle and non-human primates (Cappelletti et al., 2003; Capone et al., 2006; Luckay et al., 2007; Reed and Li, 2009; Fowler et al., 2012). The other organ used for electro gene transfer is the skin; due to the presence of antigen presenting cells, the skin is an immunocompetent site and an excellent target for vaccinations. Another advantage of the skin is its easy accessibility and the possibility to develop devices where the electrodes are minimally invasive, do not penetrate skin and can operate at low voltage (Hirao et al., 2008; Ansaldi et al., 2011).

Current veterinary cancer vaccines (Oncept)

An important breakthrough in the field of tumour vaccination and in the treatment of canine melanoma was achieved with a DNA vaccine encoding the human tyrosinase (TYR) gene (Oncept, Merial). Currently, this is the only veterinary therapeutic tumour vaccine licensed by the United States Department of Agriculture (USDA) for the treatment of oral melanoma. The licensing followed a successful study that demonstrated prolonged survival compared to historical control dogs (Bergman et al., 2006). Vaccination with a plasmid encoding murine TYR generated similar results within off-label use for canine digital melanoma (Manley et al., 2011). The plasmid encoding the xenogenic TYR is administered by a transdermal device and the protocol consists of four biweekly injections, followed by booster doses every 6 months (Grosenbaugh et al., 2011). An antibody response against human TYR was present in 3/9 tested dogs, two of which were also positive for antibodies against canine TYR (Liao et al., 2006). A correlation between antibody response and clinical response was observed. Recently, the efficacy of Oncept has been questioned and therefore further prospective studies are necessary (Ottnod et al., 2013).

Targeting dTERT: Demonstration of clinical efficacy in dogs by DNA electro-gene therapy

We have recently focussed our studies on the sequential administration of plasmid DNA and an adenoviral vector in different combinations, and have shown synergistic immune activation and a higher degree of protection from tumour development. In preclinical murine and primate models, we have shown that this heterologous prime-boost regimen induces 10 to 100-fold higher frequencies of T cells than naked DNA or recombinant viral vectors alone (Aurisicchio and Ciliberto, 2012). A further advantage of heterologous prime/boost protocols comprising the sequential use of adenoviral vector and plasmid DNA is that one can exploit the strong immunogenicity of adenovirus as the best priming agent to break tolerance, while DNA can be used for repeated boosting because of the lack of anamnestic responses against the vector (Nasir et al., 2001; Argyle and Nasir, 2003).

Telomerase reverse transcriptase (TERT) is an ideal target for cancer immunotherapy and its activity has been reported in the majority (>90%) of canine tumours (Argyle and Nasir, 2003; Nasir, 2008). We have shown that a genetic vaccine targeting dog telomerase (dTERT) and based on adenoviral/DNA-EGT heterologous prime/ boost (two adenoviral vector injections and repeated DNA-EGT boosts) can induce strong immune response and increase overall survival of dogs with B cell malignant lymphoma when combined with a cyclophosphamide, vincristine and prednisone (COP) chemotherapy regimen (Peruzzi et al., 2010a, 2010b). dTERT-specific cell mediated immune (CMI) responses were detected by ELISPOT assay in almost all treated animals.

Other DNA electro-gene therapy based cancer vaccines

A new DNA vaccine expressing the TAA chondroitin sulphate proteoglycan 4 (CSPG4) has been proposed for the treatment of dogs with oral malignant melanoma (Riccardo et al., 2014). CSPG4 is an early cell surface progression marker involved in tumour cell proliferation, migration and invasion (Price et al., 2011). It is expressed in ~80% of human melanomas (Campoli et al., 2010) and ~60% of canine melanomas (Mayayo et al., 2011). The vaccine is a DNA plasmid encoding the human CSPG4 sequence, administered monthly through EGT. When tested in dogs with surgically resected stages II–III CSPG4-positive oral melanomas, it extended the overall and disease free survival times of vaccinated dogs compared to control dogs. All vaccinated dogs developed antibodies against both human and canine CSPG4, showing that xenogeneic vaccination was able to overcome host unresponsiveness to the self-antigen (Riccardo et al., 2014).

Conclusions

As with any new technology, it is important to understand both its capability and limitations. Delivering a voltage for a millionth of a second directly into tissue induces previously impervious cancer cells to accept whichever 'Trojan horse' has been selected as the therapeutic agent of choice. Local application of electroporation, in combination with certain chemotherapeutic agents, is an effective tool for the control of some types of primary and metastatic disease. The treatment can be provided with curative intent or as an adjuvant treatment to surgery. Although no comparative prospective studies have been documented, the low toxicity and minimal damage to surrounding healthy tissues due to the non-thermal nature of the treatment is a significant advantage, along with the efficient delivery and reasonable cost of treatment.

The delivery of electroporation to veterinary patients requires sedation and/or general anaesthesia, which removes the simplicity and speed of treatment. The electrodes available are largely limited to cutaneous tumours, with improved devices for intraluminal and laparoscopic approaches under development. The next generation of electroporation generators promise to overcome some of these issues through the employment of more complex pulse waveforms.

The expansion of immunotherapy as the fourth arm of veterinary therapeutics is an exciting approach. The most appropriate endpoints in veterinary oncology are overall survival (OS), but also a better quality of life. As observed in human clinical trials, the 'build up' of an immune response leading to disease stabilisation and improved survival requires evaluation of cell-mediated and antibody responses, since target therapies are not expected to shrink tumours, but inhibit metastasis and have an impact on the quality of life and survival.

Conflict of interest statement

L. Aurisicchio is a director at Takisbiotech, which has a commercial interest in the dTERT vaccine. None of the other authors has any other financial or personal relationships that could inappropriately influence or bias the content of the paper.

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