The Potential of Tocotrienols in Cancer Immunotherapy and Wound Healing

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INTRODUCTION

Tocotrienol is a natural form of vitamin E found abundantly in palm oil (Nesaretnam *et al*., 1998). Palm tocotrienol extracted from palm oil contains approximately 70% of the tocotrienol-rich fraction (TRF) and 30% of tocopherol (Gapor, 1995; Choo *et al.*, 1997; Nesaretnam *et al.*, 2000). Tocotrienols can also be found in cereal grains such as wheat, barley and rice bran. This analogue of vitamin E possesses many health-enhancing effects and has been the focus of increasing research interest as a unique nutritional compound. Scientific evidence has shown that in addition to being powerful biological antioxidants tocotrienols may reduce cholesterol levels in people with hypercholesterolemia, and may slow down the progression of atherosclerosis (Gapor, 1995; Choo *et al.*, 1997; Nesaretnam *et al.*, 2000). Tocotrienols have been proven to have anti-cancer effects, and are useful as an adjuvant for enhancing an anti-tumour immune response in cancer immunotherapy (Abdul Hafid *et al.*, 2010; 2013). Other than that, tocotrienols have also been reported to have potential as wound-healing agents (Musalmah *et al.*, 2002; 2005). Both of these properties are discussed in this article.

CANCER IMMUNOTHERAPY

Immunotherapy is a promising treatment approach for cancer, including for advanced and recurrent forms of the disease (Zhou and Zhong, 2004). Cancer immunotherapy is a natural form of therapy by which the host immune system is activated to fight the cancer. The activated lymphocytes can then attack the tumour cells. This concept was first described by Rosenberg and Lotze (1986) and this approach is now widely used in various countries. There are three main groups of immunotherapy used to treat cancer, namely, cell-based therapies, antibody therapies and cytokine therapies. Cell-based therapies such as cancer vaccines involve the removal of immune cells from the patient either from the blood or the tumour, and the activation of the specific immune cells for the tumour. These cells are grown and returned to the patient to provoke an immune response against cancer. These cell types include natural killer cells, lymphokine-activated killer cells, cytotoxic T cells and dendritic cells.

Antibody therapies include the proteins that are produced by the immune system which bind to target antigens on the surface of a cell, and use the immune system to fight pathogens. The antibody therapies approved for treatment include Alemtuzumab, Brentuximab, Vedotin, Cetuximab, Trastuzumab and Rituzimab. Antibody-based immunotherapy, such as monoclonal antibody against Her2/neu, has been successfully used

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in the treatment of many breast cancer patients (Zhou and Zhong, 2004; Burris *et al.*, 2011). Interferon-gamma (IFN-γ) and Interleukin-2 (IL-2) are examples used for the cytokine therapies. These cytokines regulate and coordinate the behaviour of the immune system, and have the ability to enhance anti-tumour activity, thus can be used as cancer treatment (Abdul Hafid *et al.*, 2010).

Cancer Vaccines: Stimulating the Immune System to Attack Cancerous Cells

Cancer vaccine-based immunotherapy is a promising method to treat cancer patients. Cancer vaccines can be used to induce specific anti-tumour immunity in these patients (Zhou and Zhong, 2004). The aim is to assist in the treatment of cancer, and/or to prevent the cancer from recurring after the treatment (Zhou and Zhong, 2004). There are a few types of cancer vaccines being studied, such as tumour cell vaccines, antigen vaccines, dendritic cell (DC) vaccines, vector-based vaccines and DNA vaccines (Mayordomo *et al*., 1995; Zhou and Zhong, 2004). Non-specific immunotherapies do not target a certain cell or antigen. They stimulate the immune system in a very general way which still results in more activity against cancer cells (Mayordomo *et al*., 1995; Zhou and Zhong, 2004). The non-specific immunotherapies can be individually applied as a cancer treatment, or used as adjuvants to boost the immune system (Mayordomo *et al.*, 1995). They can be cytokines such as IL-12 or IFN-gamma, or drugs such as thalidomide, lenalidomide, bacillecalmette-guerin and also imiquimod (Mayordomo *et al.*, 1995). Other than that, natural compounds such as curcumin (Panahi *et al*., 2014), tocopherols (Ramanathapuram *et al*., 2004; 2005), tocotrienols (Abd Hafid *et al.,* 2010; 2013) and other antioxidants have been used as adjuvants in cancer immunotherapy.

Vaccines are medicines that boost the immune system's natural ability to protect the body against foreign invaders, mainly infectious agents that may cause disease. Cancer treatment vaccines are designed to work by activating the important types of white blood cells, namely, the B cells and killer T cells, and directing them to recognise and act against specific types of cancer. One or more molecules known as antigens may be injected into the body in order to expose the immune system so that immune cells can recognise pathogens. Lymphocytes or leukocytes (white blood cells) play supporting roles to ensure that the B and killer T cells do their jobs effectively. These cells including the helper T cells and dendritic cells which help activate the killer T cells and enable them to recognise specific threats. A strong immune system is able to identify antigens and attack or eliminate them. The immune system is then left with a 'memory' that helps it respond to those antigens in the future.

Tocotrienols and Cancer Immunotherapy

Tocotrienols are members of the vitamin E family and can be found as essential fat-soluble vitamins that can induce favourable effects on the human immune system. Increasingly, research studies on tocotrienols have opened up new perspectives in cancer immunotherapy. Studies have shown that tocotrienols possess anti-cancer effects and have the ability to reduce tumour growth in prostate cancer (Aggarwal *et al*., 2008; Yap *et al*., 2008), breast cancer (Nesaretnam *et al.*, 1998; 2004; 2008), pancreatic cancer (Malafa and Klapman, 2008; Husain *et al.*, 2013) and colon cancer (Eitsuka *et al.*, 2006). Tocotrienols are also useful as an adjuvant for developing cancer vaccines or in cancer immunotherapy due to their ability to stimulate the immune system.

Using dendritic cell vaccine is one of the potent forms of cancer immunotherapy due to the ability of dendritic cells (DC) to process and present antigens to the T-cells and stimulate specific immune response (Chiarella *et al*., 2007; Her, 2008). DC are antigen-presenting cells that have a number of receptors that will enhance the uptake of antigens, and that are capable of activating the T cells and stimulating the growth and differentiation of the B cells by influencing their behaviour and thereby inducing immune activation (Chiarella *et al*., 2007; Her, 2008). To improve the efficacy or therapeutic use of DC vaccination strategies, potent adjuvants can be used to boost the immune system. Adjuvants are substances added to vaccines to increase the body's immune response, thus efficiently promoting a protective immune response to control a disease. A number of poorly immunogenic vaccines use some added substances in the preparations to increase the immune-enhancing potential of these vaccines (Chiarella *et al*., 2007; Her, 2008).

Adjuvants used in many cancer vaccines originate from many different sources. Some cytokines

have been used as adjuvants in tumour vaccines (Chiarella *et al*., 2007; Her, 2008). Other examples of adjuvants include complete tumour lysate, tumour antigens and tumour growth factor receptors combined with powerful natural compounds such as curcumin (Wilken *et al*., 2011), (Ravindran *et al*., 2009), squalene (Fox *et al*., 2009), tocopherol succinate (Ramanathapuram *et al*., 2004) and tocotrienols (Abdul Hafid *et al*., 2010; 2013). All of these natural compounds have been found to possess anti-cancer activities (Fox *et al*., 2009; Ravindran *et al*., 2009; Abdul Hafid *et al*., 2010; Wilken *et al*., 2011; Abdul Hafid *et al*., 2013). In our current study, we used tocotrienol as an adjuvant in developing cancer vaccines.

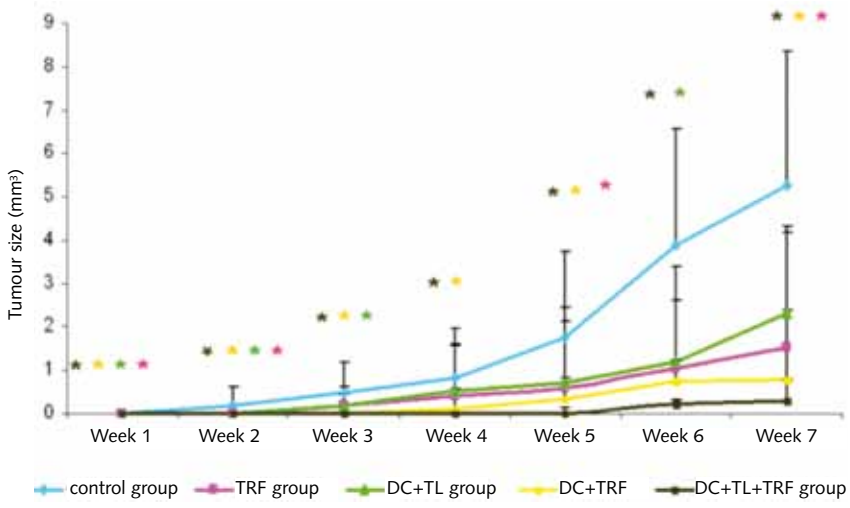
Our findings show that the tocotrienol-rich fraction (TRF), in combination with DC pulsed with tumour lysate and injected subcutaneously, significantly inhibited the growth of a 4T1 mammary tumour as compared with the control group and with mice injected with DC alone (*Figure 1*) (Abdul Hafid *et al*., 2010). Analysis of mice splenocytes revealed significantly higher levels of IFN-γ and IL-12 in the T- cells of those animals treated with TRF and DC compared with the other treatments (*Figure 2*) (Abdul Hafid *et al*., 2010). TRF supplementation combined with DC pulsed with tumour lysate, enhances the specific anti-tumour immune response, and has the potential to be used as an adjuvant in cancer immunotherapy. The approach may prove to be beneficial for future clinical testing.

WOUND HEALING

Wound healing, as a normal biological process in the human body (Guo and Dipietro, 2010), is a complex and dynamic process with the wound environment changing with the shifting health status of the individual. The physiology of a normal wound-healing trajectory, through the phases of hemostasis, inflammation, granulation and maturation, provides a framework for an understanding of the basic principles of wound healing. (Keast and Orsted, 1998; Larson *et al*., 2010; Howard *et al*., 2013).

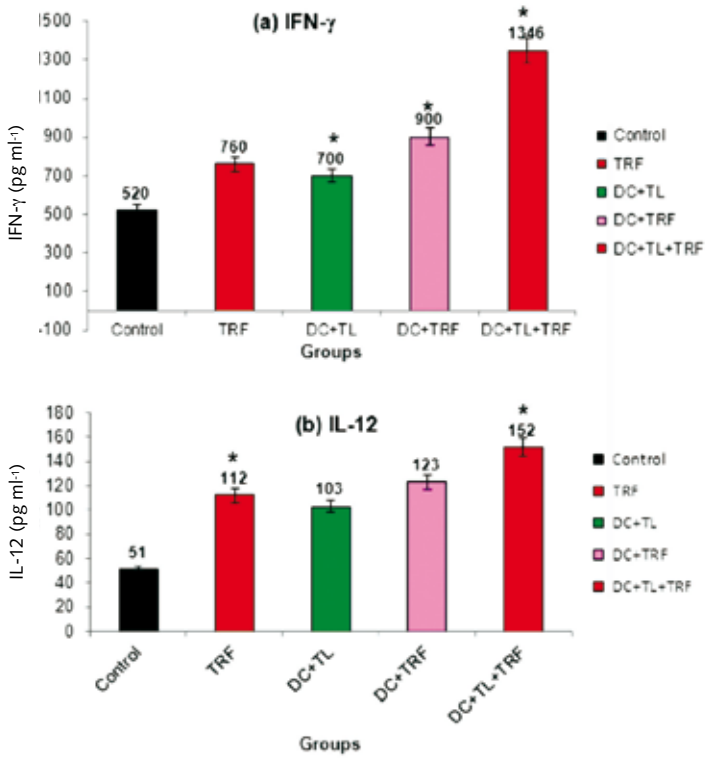
Causes of Wounds

In wound care, the basic underlying causes and factors affecting the healing process must be identified and controlled before wound repair can begin. The following are some of the common underlying causes or factors which may interfere with wound repair: trauma (initial or repetitive), scalds and burns (both physical and chemical), animal bites or insect stings, pressure, vascular compromise, arterial, venous or mixed injuries, immunodeficiency, malignancy, connective tissue disorders, metabolic diseases (including diabetes), nutritional deficiencies, psychosocial disorders as well as adverse effects of medications. In many cases, the underlying causes and factors interfering with wound healing may be multifactorial (Witte and Barbul, 1997; Keast and Orsted, 1998; Guo and Dipietro, 2010).



Note: * DC+TL+TRF group; *: DC+TRF group; *: DC+TL group; *: TRF group.

Figure 1. Tumour size of tumours induced in in each mice group. The mice in the control group were not given any treatment whilst the mice in the TRF treatment were fed daily with 1 mg TRF once the tumour was palpable. The mice in the DC-treated groups received an injection of BM-derived DC once a week for three weeks before they were inoculated with the 4T1 murine mammary cancer cells. The animals that received DC primed with tumour lysate from the 4T1 cells were divided two groups: one receiving daily supplementation of 1 mg TRF (DC+TL+TRF), and the other only the vehicle (DC+TL). The mice in the DC+TRF group received unprimed DC and daily supplementation of TRF.



Note:* significantly different from control group ($p<0.05$).

Figure 2. Splenocytes ($1x10^5$ in each well) harvested from each group. The splenocytes were cultured in the presence of 1 mg ml^{-1} Con A for 72 hr in a 96-well plate. After 72 hr, the culture supernatant was collected and the production of (a) IFN-γ and (b) IL-12 by the mitogen-stimulated splenocytes was determined using ELISA.

How does Healing Work?

Research on acute wounds in an animal model shows that wounds heal in four phases (*Figure 3*):

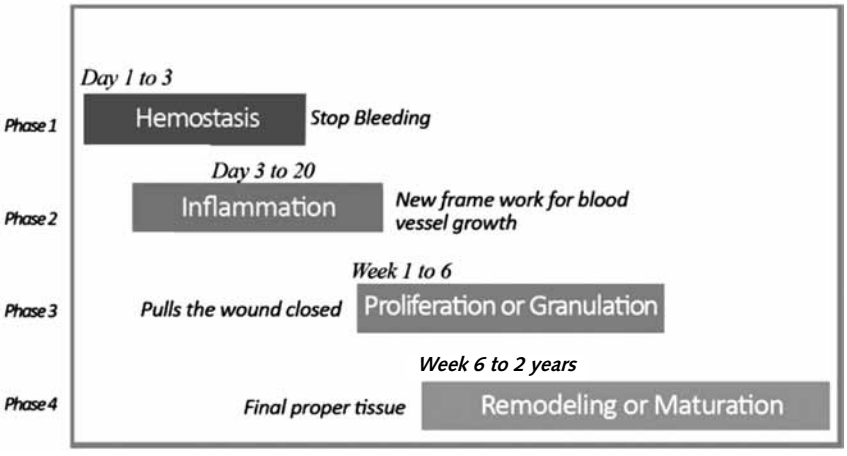
- Hemostasis;
- Inflammation;
- Proliferation or granulation; and
- Remodelling or maturation.

Hemostasis

Clotting is the term generally applied when discussing hemostasis, and it has to do with coagulation factors, platelets, and plasma (Zhong *et al*., 2010) in blood tissue that begin to act when a vascular injury occurs, working to stop the bleeding, thus preventing entry of micro-organisms, while still permitting blood to flow through the injured vessel. Platelets secrete vasoconstrictive substances, forming a stable clot to seal off the damaged vessel. Hemostasis occurs within minutes of the initial injury unless there are underlying clotting disorders (Levy *et al*., 2012; Versteeg *et al*., 2013).

Inflammation

The inflammatory phase occurs right after injury and initiates the wound-healing cascade. The main function of this phase is to remove debris and prepare the wound for the regeneration of new tissue. The inflammatory response causes the blood vessels to become leaky, releasing plasma and polymorphonuclear leukocytes (PMNs) into the surrounding tissue. The neutrophils phagocytize debris and microorganisms and provide the first line of defence against infection. Macrophages are able to phagocytize bacteria and provide a second line of defence. They also secrete a variety of chemotactic and growth factors such as the fibroblast growth factor (FGF), epidermal growth factor (EGF) and the transforming growth factor beta (TGF-β) as well as interleukin-1 (IL-1) which appears to direct the next stage (Larson



Source: http://skinqd.com/how-it-works.

Figure 3. Phases of wound healing.

et al., 2010; Peplow *et al*., 2010; Maxson *et al*., 2012; Versteeg *et al*., 2013).

Proliferative (Proliferation, Granulation and Contraction)

The proliferative phase, also called the granulation tissue formation phase, occurs between three and 12 days (Huang *et al*., 2014). Granulation tissue formation involves the proliferation of fibroblasts, the deposition of collagens and other extracellular matrices (ECM), and the development of new blood vessels. This phase is characterised by the deposition of connective tissue, collagen cross-linking and epithelial cell migration across the wound surface. It is characterised clinically by the presence of pebbled red tissue in the wound base, and involves the replacement of dermal tissues and sometimes subdermal tissues in deeper wounds as well as the contraction of the wound. In addition, fibroblasts will secrete a collagen framework on which further dermal regeneration occurs. Specialised fibroblasts are responsible for wound contraction. In the final stage of epithelialisation, contracture occurs as the keratinocytes differentiate to form a protective outer layer or stratum corneum (Larson *et al*., 2010; Peplow *et al*., 2010; Maxson *et al*., 2012).

Remodelling or Maturation

Once the basic structure is completed in wound repair, the healing process involves remodelling the dermal tissues to produce greater tensile strength. The principal cells involved in this process are the fibroblasts. Remodelling can take up to two years after wounding and explains why apparently healed wounds can break down so dramatically and quickly if attention is not paid to the initial causative factors (Larson *et al*., 2010; Maxson *et al*., 2012; Howard *et al*., 2013; Huang *et al*., 2014).

Palm Tocotrienol and Wound Healing

A wound is another condition that results in a decrease in antioxidants (Musalmah *et al*., 2005). There is great consumer interest these days in 'natural products', which include compounds derived from fruits, plants and herbs. Indeed, these contain a rich diversity of antioxidants, are widely available and used for a multitude of cutaneous ailments (Fitzmaurice *et al*., 2011; Kilík *et al*., 2014). A number of journals have already given a considerable level of attention to the review of these compounds as potential wound-healing agents. Furthermore, preclinical studies have also shown their potential usefulness in wound healing due to their versatility as antioxidants (Fitzmaurice *et al*., 2011). Thus, supplementation of wounds with antioxidants should help to prevent oxidative damage of cells and enhance healing.

Palm oil is a rich source of α-, γ- and δ-tocotrienols (Musalmah *et al*., 2002; 2005). The tocotrienol-rich fraction (TRF) derived from palm oil is a potent natural antioxidant preparation that consists of a mixture of α-, β-, γ- and δ-tocotrienols and α-tocopherol. Several studies have shown that tocotrienols are beneficial in the treatment of wounds resulting from radiation exposure, including excessive exposure to sunlight. Musalmah *et al*., (2005) showed that diabetic rats receiving oral palm TRF exhibited accelerated healing of 6-mm punch biopsy wounds compared with animals treated with α-tocopherol. Tocotrienols have beneficial effects on scar formation of acute surgical wounds, suggesting that these compounds alleviate contracture following breast implantation, and diminish adhesion formation in animals with experimental peritoneal lesions. The oral administration of palm TRF reduced the deposition of collagen and fibronectin in chronic pancreatitis.

The beneficial effects of vitamins on wound healing have mainly been studied using animal models (Ringsdorf and Cheraskin, 1982; Witte and Barbul, 1997; Musalmah *et al*., 2005; Maxson *et al*., 2012). Animal models provide vital insights into the mechanisms and pathophysiology of cutaneous wound repair. Many different aspects of the healing process can be characterised and quantified in a reproducible, controlled environment. To mimic clinical problems, rates of repair can be compromised by surgical impairment of blood supply or by metabolic manipulations. Although animal wound repair is an imperfect reflection of human wound healing and its clinical challenges, these models continue to be crucial tools for the development of new strategies and approaches to rational wound

therapy (http://www.medscape.com/viewarticle/407568).

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