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Systemic effects of oral tolerance reduce the cutaneous scarring

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ABSTRACT

Background: Immunological tolerance refer to the inhibition of specific immune responsiveness and the ingestion of proteins previous to immunization is a reliable method to induce (oral) tolerance. Parenteral exposure to tolerated antigens, in adjuvant, trigger indirect and systemic effects that inhibits concomitant immune responses to other unrelated antigens and also decrease unrelated inflammatory responses. Interesting, intraperitoneal (i.p.) exposure to orally-tolerated proteins soon before an incisional linear skin wound improves the healing by primary intention in mice. An important clinical and surgical objective is to identify strategies to improve wound healing and reduce scarring.
Objective: To evaluate whether i.p. injection of an orally-tolerated protein improves wound healing by secondary intention and reduce scarring of full-thickness excisional skin injury.
Methods: C57Bl/6 mice were turned tolerant to ovalbumin (OVA) by drinking a solution containing OVA; seven days later, they received an i.p. injection of OVA plus $Al(OH)_3$ adjuvant immediately before two full-thickness excisional skin wounds, under anesthesia. The wound healing process was evaluated macro and microscopically after H&E, toluidine blue and Gomori's Trichrome staining. The presence of granulocytes, macrophages, miofibroblasts, fibronectin, collagen I and collagen III was investigated by immunofluorescence and the levels of cytokines by flow cytometry or ELISA. Mice not tolerant to OVA were included as controls.
Results: The i.p. injection of OVA + $Al(OH)_3$ in mice orally tolerant to OVA reduced the subsequent inflammatory response in the wound bed and the cutaneous scarring. There was a change in the pattern of collagen deposition making it more similar to the pattern observed in intact skin. In tolerant mice, mast cells and granulocytes (Ly-6C/G+), were reduced, while lymphocytes (CD3+) were increased in the wound bed. Time course analysis of Th1/Th2/Th17 cytokines and growth factors showed slightly differences between tolerant and control groups.
Conclusion: Parenteral injection of an orally-tolerated protein has systemic consequences that impair the inflammatory response triggered by skin injury and reduce the cutaneous scarring.

1. Introduction

Oral tolerance is a T-cell mediated phenomenon defined by inhibition or down-regulation of immune responsiveness to a protein previously contacted by the oral route (Richman et al., 1978; Noval Rivas et al., 2015). Orally tolerant animals form less specific antibodies and display weak delayed-type hypersensitivity (DTH) reactions in the presence of the cognate antigen (Hanson et al., 1977; Frossard et al., 2015). In addition to the local specific effects, oral tolerance has also systemic effects that affects the migration of leukocytes and bone-marrow eosinopoiesis (Rodrigues et al., 2006). It has been known for a long time that oral pretreatment with one protein does not affect immune responsiveness to other unrelated proteins (Thomas and Parrott, 1974). Surprisingly, however, par-

Abbreviations: $Al(OH)_3$, aluminium hydroxide; DTH, delayed-type hypersensitivity; ELISA, enzime-linked immunosorbent assay; GM-CSF, granulocyte macrophage-colony stimulating factor; HBSS, Hank's balanced salt solution; IFN, interferon; IL, interleukin; i.p., intraperitoneal; OPD, orthophenylene-diamine; OVA, ovalbumin; PBS, phosphate-buffered saline; Relm-α, resistin-like molecule, alpha; Th, T helper; TNF, tumor necrosis factor.

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enteral immunization with a tolerated protein, in adjuvant, triggers systemic and indirect effects that result in significant reduction in the responsiveness to a second unrelated antigen injected concomitanly or a few days later (Carvalho et al., 1994; Carvalho et al., 1997). The effects of the injection of tolerated proteins into tolerant mice also significantly block inflammation triggered by carrageenan injection into footpads (Ramos et al., 2009), DTH to unrelated antigens (Ramos et al., 2009), granuloma formation around *Shistosoma mansoni* eggs (Azevedo et al., 2012) and experimental autoimmune disease induced to unrelated antigens (Miller et al., 1991)

Inflammation normally occurs after tissue injury but its role in the wound healing process is not fully understood. The inflammatory response is considered important to protect against wound infection but the absence of inflammation has been correlated with regeneration and better, scarless healing (Eming et al., 2007; Martin and Leibovich, 2005; Martin and Nunan, 2015).

Tissue injury triggers a complex cascade of cellular and molecular interactions necessary to restablish tissue integrity of which inflammation is just one aspect (Gurtner et al., 2008). Just after cutaneous wounding the clotting cascade forms a fibrin clot that stops blood loss and initiate the rescue of tissue integrity (Martin, 1997). The interruption of skin integrity activates tissue resident cells to proliferate and secret inflammatory factors that atract leukocytes into the wound bed (Martin and Nunan, 2015). Concomitantly, fibroblasts are also atracted to the wound bed, proliferate and start to deposit extracellular matriz that is important for tissue reorganization. Fibroblasts may differentiate into myofibroblasts that are contractile cells characterized by expression of smooth muscle actin and contribute to wound contraction (Martin, 1997; Werner et al., 2007). In normal conditions during skin wound healing in adult mammals, in a few days keratinocytes restore the epidermis and the inflammatory phase resolves. However, the dermis and the skin appendages are not reconstituted to their original form. In the last phase of wound healing, the deposition and reorganization of the extracellular matriz continues for many days after wounding and the neodermis is caractherized by a remodeling of collagen which is realigned in a pattern that, however, is different from the intact skin and results in scar formation (Wynn, 2008).

The most commonly used models to study skin wound healing are the full-thickeness excisions left open (healing by secondary intention) and full-thickeness incision with the borders approximated (healing by first intention) performed on mouse dorsal trunk (Ansell et al., 2012). The phases of wound healing normally occurs in the same and predictable manner, either in healing occurring by primary or secondary intention, with differences in the extension (duration) of each phase, specially in the inflammatory phase and granulation tissue formation, that is extended during healing by secondary intention (Rivera and Spencer, 2007). During healing by primary intention, when the borders of the lesion are brought together, in normal conditions the resulting scar is reduced. On the other hand, after healing by secondary intention granulation tissue formation results in a broader scar.

We have previously shown, that i.p injection of a tolerated protein plus adjuvant minutes before a full-thickness linear incisional skin wound, reduces the inflammatory response and the cutaneous scarring during healing by primary intention (Costa et al., 2011).

In the present work we utilized a full-thickness excisional model of wound that involves the removal of a significant volume of the target tissue to evaluate the consequences of an i.p. injection of a tolerated antigen upon cutaneous wound healing. The lesions were left to heal by secondary intention allowing more ample material for the investigation of histological and cytokine parameters. Our findings indicate that systemic and indirect effects of immune oral tolerance is able to accelerate wound healing and reduce scar formation.

2. Materials and methods

2.1. Animals

8 week-old male C57BL/6 mice were bred and maintained in the animal breeding unit at the Institute of Biological Sciences, Universidade Federal de Minas Gerais (UFMG), Brazil and treated according to the guidelines of the Ethics Committee of Animal Experimentation of UFMG. The groups contained five to six mice per time point.

2.2. Feeding regimens for oral tolerance induction

Oral tolerance to ovalbumin (OVA) was induced by requiring mice to drink, *ad libitum*, a 1:5 solution of hen egg white in drinking water for three consecutive days. The egg white solution was prepared in our laboratory from commercially available eggs, and contained an average of 4 mg OVA/ml. Daily estimated average consumption was 20 mg OVA/mouse and this resulted in significant levels of tolerance measured either at the level of antibody production or cellular activation (Cunha et al., 2009; Faria et al., 1993). Bottles were changed every day to avoid contamination. Control groups received filtered tap water. Oral treatment was discontinued 7 days before parenteral immunization.

2.3. Parenteral immunizations

Purified OVA was obtained commercially (grade V, Sigma, St. Louis, MO). Mice which had been pre-treated orally with egg white (Tolerant) and control mice (Immune) received one intraperitoneal (i.p.) injection of 0.20 ml of a suspension containing 10 μg OVA plus 1.6 mg $Al(OH)_3$ immediately before the wounding procedure. The other control group (lesion) was not i.p. immunized.

2.4. Bleeding

Blood was collected in the absence of anti-coagulant from the tail veins 14 days after i.p. immunization, immediately frozen and later used in a serum antibody assay to test for tolerance induction.

2.5. Antibody assay

Anti-OVA antibody titres were determined by standard enzyme-linked immunosorbent assay (ELISA) using an automatic ELISA reader (BioRad, Hercules, CA). ELISA scores were computed by calculating the sums of the optical densities obtained from the six serum dilutions between 1:100 and 1:3200 of individual mice. The details of the assay method have been described previously (Rodrigues et al., 2006; Cunha et al., 2009). Each score shown represents the mean ± SEM, $n = 5$.

2.6. Wounding

Mice were anesthetized with ketamine (97 mg/kg) and xilazine (15 mg/kg) and their dorsal thoracic skin was shaved and cleaned with 70% ethanol before wound. Two circular through-and-through full-thickness excisional wounds (each with 6.5 mm diameter, 0.33 cm^2 area) were made by picking up a fold of skin and using a biopsy punch, resulting in generation of one wound on each side of the midline. Mice were caged individually and lesions were left unsutured to allow the evaluation of the process of healing by secondary intention.