Medical Hypotheses 85 (2015) 141–144

Contents lists available at ScienceDirect

Medical Hypotheses

journal homepage: www.elsevier.com/locate/mehy

Transdermal delivery of bovine milk vesicles in patients with multiple sclerosis: A novel strategy to induce MOG-specific tolerance

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ARTICLE INFO

Article history:
Received 26 November 2014
Accepted 19 April 2015

ABSTRACT

Recently, butyrophilin (BTN) – a protein which shares cross-reactive epitopes with myelin oligodendrocyte glycoprotein (MOG) – has been found in milk. A high amount of milk BTN has been reported in the outer membrane of vesicular structures known as exosome and milk fat globule membrane (MFGM). These vesicles can act as Trojan horses, passing their BTN content through epidermis or other biologic barriers of the body. By altering the dose schedule and route of administration, the BTN-bearing vesicles (exosomes and MFGMs) may acquire enough potential to be used in MOG-specific immunotherapy program. Regarding above evidence and considering immunological characteristics of skin-associated lymphoid tissue (SALT), transdermal delivery of bovine milk vesicles, whether through topical administration of bovine milk or by using epicutaneous administration techniques, could be considered as an intriguing approach to induce MOG-specific tolerance in patients with multiple sclerosis.

Introduction

Multiple sclerosis (MS) is characterized as a demyelinating autoimmune disease of central nervous system (CNS) mediated by autoimmune responses of immune cells (mainly TCD4 cells) toward myelin components of neurons. Destruction of myelin sheaths of neurons following myelin-specific immune responses can lead to the delay or even complete failure of signal conduction and thus to neuro-physical disabilities [1,2].

Even though several myelin proteins including myelin basic protein (MBP), proteolipid protein (PLP) and myelin oligodendrocyte glycoprotein (MOG), have been identified as encephalitogenic, but expression of MOG on the external layer of myelin sheath makes it more accessible to both antibody and auto-reactive cell attacks [3,4]. Accordingly, induction of immunological tolerance to MOG may prevent subsequent autoimmune responses towards MBP and PLP in inner layers of myelin sheath.

Researches indicate that BTN – shows more than 50% amino acid homology with the corresponding domain of MOG. Immunological cross-reactivity of bovine milk BTN with human MOG may highlight the potential of BTN as a novel therapeutic protein for the immunotherapy of MS [5].

Antigen-specific immunotherapy opposes autoimmunity by restoring immune tolerance to target self-antigens without compromising immune function [6]. In the case of MS, MOG specific tolerance can be achieved by altering the dose schedule or route of administration [7–9]. In this regard, expression of milk BTN in the outer membrane of vesicular carriers such as exosomes and MFGMs [10] makes it possible to deliver an excess amount of BTN through the skin barrier following topical and/or epicutaneous administration of bovine milk. In this approach, interactions of BTN presenting cells with naive T cells in skin lymph nodes have proposed to contribute to a series of overlapping tolerogenic responses to MOG.

Hypothesis

The steps listed below describe the pathways by which topical and/or epicutaneous administration of bovine milk to MS patients may lead to MOG-specific immune tolerance:

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http://dx.doi.org/10.1016/j.mehy.2015.04.019
0306-9877/

1. The BTN content of bovine milk has mainly expressed in the outer membrane of the vesicular structures known as exosomes and MFGM.
2. Topical administration of bovine milk allows BTN-bearing vesicles to penetrate through the skin barrier. Transdermal delivery of bovine milk vesicles may be improved by using penetration enhancers or through epicutaneous administration techniques. Alternatively, bovine milk vesicles (whether MFGM or exosome) may be used.
3. Epidermal and/or dermal DCs capture vesicles and process their BTN content to desired peptides. A series of BTN-derived peptides produced by DCs cross-reacts with MOG epitopes.
4. After migration of DCs to nearest lymph node (LN), DC-mediated antigen presentation to naive T cells may induce generation of BTN-specific regulatory T cells and/or anergy in T cells. Due to cross-reaction between BTN and MOG, BTN-specific regulatory T cells terminate MOG-specific immune responses toward myelin.
5. Alternatively, the increased presentation of BTN-derived peptides to naive T cells may induce proliferative burst of BTN-reactive TCD4 cells in LN. The successive re-activation of the proliferating T cells can lead to activation induced cell death (AICD; a form of regulated cell death which is induced during lymphocyte activation) and then release of intracellular TGF-β from apoptotic T cells. The immunosuppressive milieu induced by TGF-β may support generation of BTN/MOG-specific regulatory T (Treg) cells. AICD may also be induced in MOG-specific T cells following recruitment to the SALT and successive reactivation by BTN-derived peptides.

Evaluation of hypothesis

The present hypothesis can be examined in experimental autoimmune encephalomyelitis (EAE) as an animal model of MS. Indeed, apart from many clinical and pathophysiological features shared between EAE and MS, the cross-reactivity of human MOG with rat one may increase validity of experiment. EAE will be induced in female 8–10-week-old Dark Agouti (DA) rats by co-immunization with recombinant MOG^{Igd} (amino acids 1–120) emulsified in complete Freund's adjuvant and pertussis toxin [11]. After induction of disease and appearance of clinical symptoms, rats with the same physical condition and same clinical signs will be randomly divided into a control and three treatment groups. Subsequently, the whole body of rats will be shaved. After skin scraping using a safe detergent (to remove stratum corneum), rats in both control and treatment groups will receive topical administration of phosphate buffered saline (PBS), bovine milk, exosome, and MFGM, respectively. The administration will be repeated every day. During the procedure, restrictions should be made to avoid rats' oral access to PBS and/or bovine milk. Alternatively, rats in control and treatment groups may receive epicutaneous administration of PBS, bovine milk, exosome, and MFGM, respectively. In this case isolation and purification of exosomes and/or MFGM will be performed according to the approved protocol [10].

All rats will be monitored daily for the severity of EAE symptoms. At the end of experiment, rats will be euthanized and their serum samples as well as brain and spinal cord tissues will be harvested. The levels of inflammatory cytokines (IL-17 and IFN-γ) should be determined using commercial ELISA kits. In histopathological sections prepared from CNS samples, hematoxylin and eosin (H&E) and luxol fast blue (LFB) staining methods will be performed to explore mononuclear cell infiltration and demyelination.

It is expected that clinical scores, CNS pathology, and serum levels of inflammatory cytokines would significantly decrease in rats treated with bovine milk/vesicles compared with PBS-treated group.

Discussion

The development of safe and effective antigen-specific therapy is an urgent need to treat patients with autoimmune diseases. The functional consequence of such therapies should be a restored immune tolerance to target antigen without compromising host immunity against detrimental insults. Strategies to induce antigen specific tolerance are mainly based on the alteration in antigen structure, dose schedule, or route of administration [7,8]. In the case of multiple sclerosis, the candidate antigen(s) should have the same epitope(s) with myelin sheath antigens.

The molecular mimicry between BTN and MOG, along with the localization of BTN – reactive antibodies in cerebrospinal fluid (CSF) – suggests that exposure to BTN may influence generation of the MOG-specific autoimmune responses [5,11]. These may also highlight the potential of BTN as a novel therapeutic protein for the treatment of MS. Evidence derived from experimental studies indicates that BTN, not only inhibits the MOG-induced EAE, but also stimulates encephalitogenic T cell response to MOG and CNS pathology [11,12]. This result indicates that milk BTN can be considered as a good candidate antigen for MOG-specific immunotherapy of MS.

Considering structural and functional similarities between MOG and BTN, an effective strategy to induce MOG-specific tolerance could be conducted through altering the dose schedule and administration route of BTN.

Among various routes of administration, transdermal delivery of BTN-bearing vesicles overcomes the disadvantages associated with the other routes including hepatic clearance, gastrointestinal breakdown, and uncontrolled delivery [13,14]. Moreover, presence of a high number of potent antigen-presenting cells (APCs), makes epidermis an ideal application route for antigen immunotherapy. This is because these APCs enhance efficacy and thus shorten treatment duration; in addition, the antigen administration site is ideally non-vascularized, so that inadvertent systemic distribution of the allergen and consequent systemic allergic side effects are minimized [15].

More recently, a number of clinical trials have introduced epicutaneous immunotherapy as an effective approach to induce antigen-specific gastrointestinal Treg and to protect against food-induced allergies [15]. Accordingly, using bovine milk and/or its derived vesicles, epicutaneous immunotherapy may partly terminate the concern about possible adverse effects of milk consumption in patients with MS.

However, since BTN as a protein has a large size and is hydrophilic in nature, it cannot permeate passively across the skin due to the stratum corneum which allows the transport of only small molecules [13].

Recently, vesicular carrier systems such as liposomes, niosomes, and ethosomes have been developed to provide alternative tools for protein delivery across the biological barriers [16].

Interestingly, exosomes and MFGM as biological versions of liposomes have been found in milk [10]. Identification of proteomes of bovine milk exosome and MFGM has provided strong evidence for the presence of high amounts of BTN in these vesicles [10]. Since the ability of exosomes and MFGMs to penetrate through the biologic barriers has been proved [17,18], these

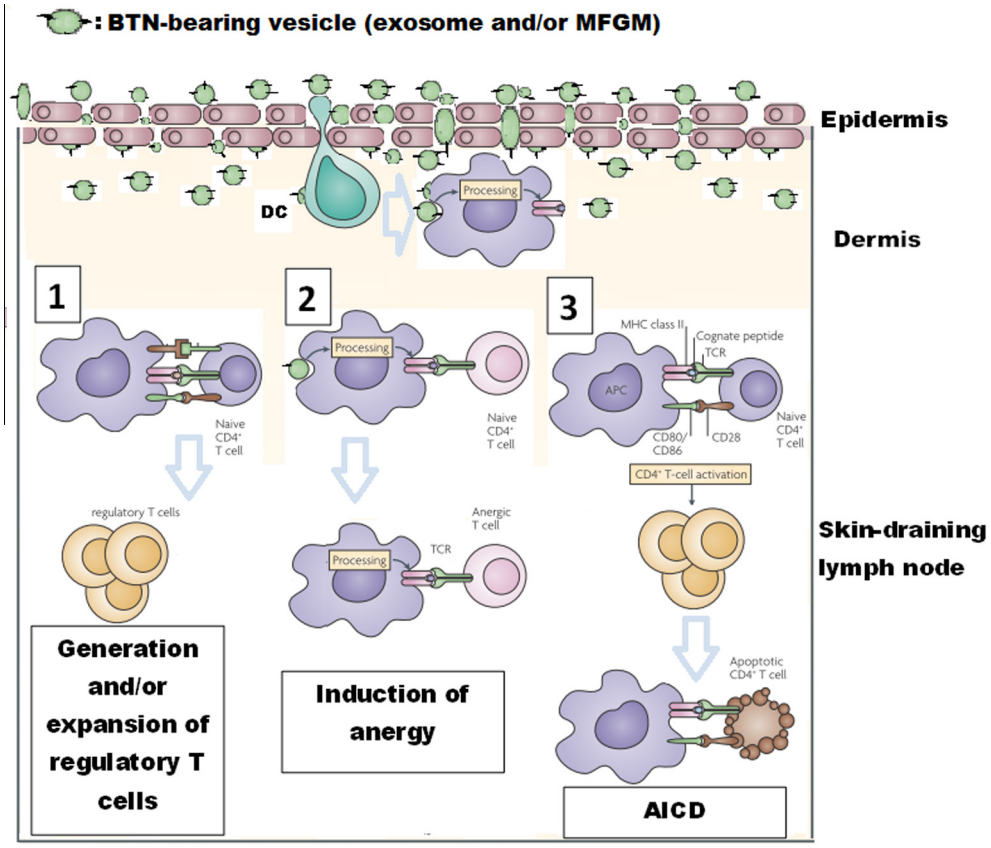

Fig. 1. Schematic representation of pathways by which transdermal delivery of bovine milk vesicles may lead to MOG-specific tolerance.

biological carriers may partly overcome the difficulties associated with BTN delivery across the skin barrier.

Even though BTN content of MFGM is significantly higher than exosome [10], considering that particle size of vesicles inversely influences their penetration into the skin [19], exosomes (30–100 nm in diameter) seem to have more penetration ability than MFGMs which are quite variable in size (ranging from 100 nm to several micrometers in diameter). In this regard, penetration of intact vesicles (with sizes up to 300–500 nm) to inner layers of the skin has been documented in previous studies [19–21]. However, transdermal delivery of bovine milk vesicles can be improved by using penetration enhancers or through epicutaneous administration techniques. Considering the barrier breaching property of bovine milk vesicles and immunological characteristics of skin-associated lymphoid tissue (SALT), transdermal delivery of BTN-bearing vesicles is thought to be an appropriate approach to induce MOG-specific tolerance in patients with MS. In this approach, the repeated administration of bovine milk or vesicles in a large body surface area may provide enough transdermal bioavailability of BTN to exert its tolerogenic effects.

The mechanism of tolerance induction following transdermal delivery of BTN may partly be due to the interaction of BTN with tolerogenic cells resident at the site of administration. Recent studies have shown that peripheral conversion of naive TCD4 cells to regulatory T cells can be conducted via a specialized dendritic cell (DC) population known as "migratory DC" which traffics tissue antigens from peripheral tissues to the draining lymph nodes (LN). This subpopulation is in contrast to lymphoid resident DCs which arise from blood-born precursors and present antigens from the lymph and blood stream to T cells (DC CD8+ DEC 205+ and DC CD8− DCIR2+). Skin-resident DCs including both epidermal Langerhans cells and Dermal DCs (with two main subsets: CD103+ and CD11b+ DCs) have been identified to be migratory and are involved in the induction of tolerogenic responses [22–24]. Accordingly, several studies have focused on the skin-resident migratory DCs to induce immunological tolerance to target antigens [25,26].

In accordance with the above findings, prophylactic targeting of Langerin + DCs to MOG peptide has been shown to cause decreased severity of EAE (experimental autoimmune encephalomyelitis) as an animal model for MS disease [26].

Additionally, regardless of DC phenotype, skin DCs remain immature in the steady-state (absence of danger signal). Therefore, immature DC-mediated antigen presentation to naive T cells may contribute to the generation of regulatory or anergic T cells [9,27].

Collectively, the mechanisms proposed to be involved in the induction of antigen-specific tolerance following transdermal delivery of BTN can be illustrated at three scenarios (Fig. 1).

In the first scenario, after breaching the skin barrier, BTN-bearing vesicles will be taken up and processed to achieve desired peptide by skin migratory DCs. After migration of DCs to LNs, antigen presentation to naive TCD4 cells, whether through migratory DC or by immature DC, will lead to the generation of antigen-specific regulatory T cells. Regulatory T cells which are identified with various phenotypes (such as CD4+ CD25+^{high}, CD4+ IL-10+ Foxp3+, CD4+ CD25+ Foxp3+, CD4+ TGF-β+, CD8+ CD25+, and CD8+ IL-10+) exert their suppression of activated immune cells either through secretion of anti-inflammatory cytokines (IL-10 or TGF-B), ger enzymes, or inhibitory surface molecules. Moreover, cross-presentation of MHC-I-restricted myelin epitopes by skin migratory DCs may lead to deletional tolerance in cytotoxic TCD8 cells which have been found to contribute to CNS plaques [22–26].

In the second scenario, excessive presentation of BTN peptides via dermal and epidermal DCs in the absence of pro-inflammatory signals (IL-12, IL-6, and TNF-α) or co-stimulatory molecules (CD80 and CD86) may lead to T cell anergy (state of unresponsiveness to antigen) after TCR (T cell receptor) stimulation [9]. Simultaneously, extensive incorporation of membrane-bound BTN into neighbor cells' membrane (both non-immune and immune cells) is the other possible mechanism by which BTN-bearing vesicles may contribute to T cell anergy [28–34].

In the third scenario, the increased presentation of BTN may induce IL-2 secretion and an initial proliferative burst of BTN-reactive TCD4 cells in LNs. However, the successive re-activation of the proliferating TCD4 cells will be inhibited due to the activation-induced cell death (AICD; a form of regulated cell death which is induced during lymphocyte activation) [9]. AICD may also be induced in MOG-specific T cells following recruitment to the SALT and successive re-activation by BTN-derived peptides. Consequently, TGF-β released from apoptotic T cells may contribute to immunosuppressive milieu which support the generation of regulatory T cells (Treg) [35].

Conclusion

According to above evidence, transdermal delivery of bovine milk vesicles, whether through topical administration of bovine milk or by using epicutaneous administration techniques, could be considered as an intriguing approach to induce MOG-specific tolerance in patients with multiple sclerosis. This approach prevents problems and costs associated with antigen purification required in conventional antigen-specific immunotherapies.

Conflict of interest

The authors claim no conflict of interest.

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