

REVIEW

The role and potential use of oral transforming growth factor- β in the prevention of infant allergy

A. Nakao^{1,2}

¹Department of Immunology, Faculty of Medicine, University of Yamanashi, Yamanashi, Japan and ²Atopy Research Center, Juntendo University School of Medicine, Tokyo, Japan

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Summary

The increasing prevalence of allergic diseases in infants and children as well as adults has become an important issue in public health in industrial countries. However, few preventive measures are available to reduce the risk of allergic diseases in infants; e.g. the avoidance of smoking and alcohol consumption during pregnancy and lactation. Therefore, there is an enthusiasm to identify certain factors in foods, nutrients, and environment responsible for the primary prevention of allergic diseases during infancy. In the last decade, TGF- β in maternal milk has been implicated in the prevention of allergic diseases in infants and young children. This review summarizes the relevant epidemiological reports and highlights the recent animal studies to support the preventive role of orally administered TGF- β , such as TGF- β in human milk, in the development of allergic diseases in infants. We also provide suggestions for the potential use of dietary (oral) TGF- β for the primary prevention of allergic diseases. Further studies to address the scientific validity and mechanistic insight to this Mother Nature-inspired concept are clearly required and will be important to develop new approaches to prevent allergic diseases.

Correspondence:

Dr Atsuhito Nakao, Department of Immunology, Faculty of Medicine, University of Yamanashi, 1110 Shimokato, Chuo, Yamanashi 409-3898, Japan.
E-mail: anakao@yamanashi.ac.jp

Introduction

After birth, the gastrointestinal tract of the neonate is exposed to numerous environmental antigens such as bacteria and food. Maternal milk, the first food for neonates, is thought to play an irreplaceable role not only in infant nutrition but also in the regulation of infant immunity to such luminal antigens [1–5]. For example, mammalian milk contains a number of immunological substances (e.g. IgA) that contribute to infants' resistance to infectious diseases.

Mammalian milk, including the milk of humans and cows, contains abundant TGF- β , a potent regulator of the immune system [5, 6]. Although the precise roles of mammalian milk TGF- β remain uncertain, several epidemiological studies have suggested that TGF- β in human milk may be a protective factor against allergic diseases during infancy [7–9]. However, the molecular and cellular basis underlying these observations are still not fully understood.

In this article, we summarize previous epidemiological data and highlight recent evidence from animal studies demonstrating that mammalian milk (or orally adminis-

tered) TGF- β retains its activity in the gastrointestinal tract and its anti-allergic property is attributed to the presence of regulatory T cells, which are induced only when an oral antigen is co-ingested with TGF- β [10–13]. In light of these findings, we also herein discuss the potential use of orally administered TGF- β (simultaneously with an allergen) for the primary prevention of allergic diseases during infancy.

What is transforming growth factor- β ?

TGF- β was originally identified in 1981 as a growth factor that promoted the anchorage-independent growth of fibroblasts [14]. However, it is now known that TGF- β is a multifunctional cytokine that regulates cell growth, differentiation, and survival in virtually every cell type in our body [14]. There are three isoforms of TGF- β : TGF- β 1, TGF- β 2, and TGF- β 3 in mammals. Human TGF- β 2 displays a 71% and a 76% homology in amino acid sequences with human TGF- β 1 and TGF- β 3, respectively. The three isoforms have identical activity in *in vitro* growth-inhibition assays, but each isoform is expressed

in both a tissue-specific and a developmentally regulated fashion, thus suggesting their distinct roles *in vivo*. The different TGF- β isoforms all bind to the same receptor complex, termed type I and type II, and then transduce intracellular signals via the Smad family of proteins [14].

Transforming growth factor- β is evolutionally conserved across species

TGF- β -related proteins with their cognate receptors and signalling effectors are found in all vertebrates and are also operational in invertebrates [14]. As such, TGF- β is well-conserved in mammals.

For example, the amino acid sequence of TGF- β 1 between mice and humans demonstrates >98% homology and human TGF- β can therefore act on mouse cells and vice versa. In addition, cow TGF- β was able to inhibit the growth of the human osteogenic cell line MG-63 [15] and commercial cow milk-borne TGF- β induced the activation of TGF- β /Smad signalling in human lung epithelial cell line A549 cells [16], thus suggesting that cow-derived TGF- β can potentially act on human cells.

Transforming growth factor- β and immunity

A pivotal role of TGF- β in the homeostatic regulation of the mammalian immune system was highlighted in the early 1990s when researchers demonstrated that TGF- β 1-null mice exhibited extensive autoimmune inflammation in various organs and died at approximated 3 weeks of age [17]. Recent comprehensive studies in the past several years using mice with T cell-specific deletions of TGF- β or TGF- β signalling have advanced our understanding of the main target of and the mechanisms of action by TGF- β in the immune system [18]. The prominent function of TGF- β is the maintenance of T cell tolerance to self or innocuous environmental antigens via its direct effects on the differentiation and homeostasis of regulatory and effector T cells [18]. An important novel finding in this context is that TGF- β has been shown to be required for the differentiation of naïve T cells into CD4⁺CD25⁺Foxp3⁺ regulatory T cells.

Mammalian milk is rich in transforming growth factor- β

In the mid 1990s, TGF- β was found to be abundantly present in mammalian milk, including the milk of cows and humans, with a predominance of the TGF- β 2 isoform over TGF- β 1 [19–21]. Raw cow milk and cheese whey contain total TGF- β (both TGF- β 1 and TGF- β 2) at concentrations of 4300 $\pm$ 800 and 3700 $\pm$ 700 pg/mL, respectively [19]. It is thought that TGF- β can act at concentrations of $\sim$ 1 pg/mL in the microenvironment of our body [14]. Some of the beneficial effects of cow milk or cow milk-derived whey proteins on human health,

particularly on inflammatory activity, have been associated with this immunoregulatory molecule [6].

Human milk also contains substantial amounts of TGF- β [20, 21]. A study using 257 samples collected longitudinally from 49 healthy mothers during the first 12 weeks of lactation showed that all colostrum/milk samples regularly contained TGF- β 1 (range, 43–7108 pg/mL) and TGF- β 2 (range, 208–57 935 pg/mL) with little change during the study period [21]. Another previous study suggested that colostrum samples showed higher concentrations of TGF- β than mature milk samples [20], but a recent study using a protein array did not confirm these results [22]. In any event, considerable variability in the concentration of human milk TGF- β has been consistently reported [20–23], although the reasons why such variability exists remain unclear. However, the variable breast milk TGF- β concentrations in each mother may result in varying degrees of risk for developing allergic disease in infants.

Mammalian milk transforming growth factor- β may be activated by gastric acid *in vivo*

TGF- β in human, cow, and rodent milk exists primarily as a latent form [19–21, 24] and the mechanism of its conversion to the active form *in vivo* has been unclear.

TGF- β is secreted extracellularly as a latent complex in which TGF- β homodimers are non-covalently associated with homodimers of a pro-peptide called the latency-associated peptide (LAP) [25, 26]. The release of TGF β from its LAP is required for the binding of TGF- β to cellular receptors and for the subsequent activation of the intracellular Smad signalling pathway. Latent TGF- β is known to be efficiently activated by extremely high or low pH *in vitro* due to a conformational change, which results in the liberation of TGF- β from the constraints of LAP [25, 26].

Consistent with this notion, a recent study has suggested the latent form of orally administered TGF- β (such as TGF- β in human milk) to be activated when it was exposed to the extremely low pH of gastric acid in the stomachs of mice [27]. The study showed that mice treated orally with human milk containing latent TGF- β for 2 weeks had an increased expression of TGF- β target genes in the small intestine and this was inhibited by the anti-acid treatment. Therefore, it is speculated that the passage through the acidic pH of the infant stomach might free the active moieties of TGF- β in human milk, thereby exposing the neonatal intestine to high concentrations of activated TGF- β .

Transforming growth factor- β in human milk may be a protective factor against allergic diseases during infancy

Although mammalian milk contains substantial amounts of TGF- β , the role of orally ingested TGF- β in infants

remains unclear. Several epidemiological studies in the past several years have suggested that TGF- β in human milk is associated with the prevention of allergic diseases during infancy and it may thus provide protection against allergic diseases in infants. Most recently, this topic has been reviewed by Oddy and Rosales[28] in a thorough and systemic manner. Therefore, this article briefly introduces an original report by Kalliomäki et al. [7].

Forty-seven infants with several atopic family members during their first year of life were followed and the concentrations of TGF- β in maternal colostrum, mature milk, and the infants' sera were determined [7]. The study found that the concentrations of TGF- β were higher in maternal colostrum, but not in mature milk and infants' serum, in infants with postweaning-onset atopic diseases (atopic dermatitis and cow's milk allergy) as compared with those with preweaning-onset disease. TGF- β in colostrum may, therefore, prevent the development of atopic diseases during exclusive breastfeeding.

Orally administered transforming growth factor- β retains its activity in the mouse intestine

Although several epidemiological findings strongly suggest the potent influences of human milk TGF- β on the infant allergic status, there is still a question with regard to whether orally administered TGF- β , such as TGF- β in human milk, can retain and mediate its activity in the gastrointestinal tract where inactivation of glycoproteins by gastric acid or protease digestion generally occurs.

A previous study demonstrated that ^{125}I -labelled active TGF- β administered orally by a gavage to the stomach of 5-day-old mouse pups was found in the neonatal lung, heart, and liver tissues within 15 min and in kidney tissues within 45 min following the oral challenge [29]. The orally administered TGF- β was, therefore, not completely degraded in the gastrointestinal tract of mouse pups and at least some of it was absorbed into the circulation.

A recent study using transgenic mice expressing a Smad-responsive luciferase reporter construct (SBE-luc mice) clearly showed that orally administered TGF- β can indeed retain and mediate its activity in the intestine [12]. The study showed a robust increase in luciferase activity in the intestine of SBE-luc mice following the oral administration of active TGF- β . In addition, the phosphorylation of Smad2 and TGF- β target gene mRNA expressions such as Smad7 were detected in the intestine of wild-type mice following the oral administration of active TGF- β . Therefore, orally administered TGF- β was able to induce the activation of TGF- β /Smad signalling in the mouse intestinal mucosa. Furthermore, a recent study demonstrated the activation of latent TGF- β by gastric acid *in vivo* as discussed above [27]. These findings suggest that the TGF- β activity can, therefore, be preserved in the hostile

gastrointestinal environment following an oral administration.

Mammalian milk transforming growth factor- β can inhibit allergic immune responses via the induction of regulatory T cells in mouse pups

Recently, Verhasselt et al. [13] provided a mechanistic insight into how breast milk TGF- β inhibits allergic T-helper type 2 (Th2)-type immune responses. They initially observed that an airborne antigen was efficiently transferred from the mother to the neonate through breast milk in mice, and this transmission resulted in the antigen-specific tolerance and the prevention of antigen-induced allergic airway inflammation (a mouse model of human asthma) in the offspring. The authors subsequently demonstrated that the neutralization of breast milk TGF- β activity by injecting an antibody to TGF- β into lactating mothers abrogated such breastfeeding-induced oral tolerance in the offspring, thus suggesting that the breastfeeding-induced oral tolerance relies on the presence of TGF- β during lactation. They further revealed that breastfeeding-induced oral tolerance was mediated by CD4⁺CD25⁺ regulatory T cells and was dependent on TGF- β signalling in T cells in the offspring.

These findings strongly suggest that breast milk-borne allergens ingested by nursing offspring are accompanied by TGF- β , which results in the induction of CD4⁺CD25⁺ regulatory T cells and provides the offspring with allergen-specific protection from asthma. Importantly, this mechanism appeared to function only when the antigen was administered together with endogenous (milk-borne) TGF- β [13]. This finding may be consistent with the requirement for activation of both the T cell receptor and TGF- β receptor signalling for *in vitro* induction of the differentiation of CD4⁺CD25⁺ regulatory T cells [30]. Mammalian milk TGF- β may therefore act to inhibit allergic immune responses to oral antigens during infancy via the induction of regulatory T cells.

Exogenously supplied transforming growth factor- β can also inhibit allergic immune responses in mouse pups and adult mice

Consistent with the findings by Verhasselt et al. [13], Penttilä et al. showed previously that supplementation of a cow's milk-based formula with TGF- β at a level comparable to that in breast milk inhibited elevations of total serum IgE and cow's milk-specific IgG1 antibodies when compared with the unsupplemented formula in allergy-prone rat pups [11]. Therefore, oral intake of TGF- β via either breast milk or formula likely acts to induce immunological tolerance to food antigens in the neonatal period. Because some commercially available formulas do not contain TGF- β , possibly due to the manufacturing

process (e.g. heating) [31], an effort not to destroy TGF- β in the formula during the manufacturing process might therefore be important for allergy prevention.

Similarly, the oral administration of recombinant TGF- β at a high-dose (5 μ g/mouse) together with an antigen in adult mice has been shown to inhibit oral sensitization to the antigen in a food-allergy model and also to enhance the induction of oral tolerance to a food antigen in wild-type mice [10, 12]. These actions of orally administered TGF- β in adult mice were observed only when an oral allergen was co-ingested with TGF- β , which was consistent with the studies in mouse or rat pups as described earlier [11, 13].

Low expression of transforming growth factor- β in the infant intestine until weaning

In adult mice, a higher dose of orally administered TGF- β (5 μ g/mouse) was necessary to achieve anti-allergic effects [10, 12] when compared with the physiologically (maternal milk)-related level of TGF- β (~100 ng/mL/mouse during lactation) that imparted anti-allergic effects in mouse or rat pups [11, 13]. These findings suggest that infant mice are more susceptible to the treatment of orally administered TGF- β .

Although the expression levels of TGF- β in the infant digestive tract have not been fully investigated, a report showed that the TGF- β 1-positive cell number in the small intestine was low in 3- and 10-day-old rats and had increased markedly by 19 days of life (weaning time of rat pups) [32]. In humans, the TGF- β 1 levels in the stool filtrates of 21 children born to healthy mothers were reported to range from 578 to 604 pg/mL from birth (4 days) to 6 months and then dramatically increased to 2829 pg/mL at 12 months [33]. Therefore, endogenous TGF- β production in the infant intestine may initially be low and thereafter increases during the weaning period, thus resulting in the adult levels. The increased susceptibility to orally administered TGF- β in mouse or rat pups as compared with adult mice may be due to an insufficient TGF- β production in the intestines of the pups. Until the full production of endogenous TGF- β in the gastrointestinal tract is achieved, then mammalian milk TGF- β might act as an important exogenous source of TGF- β in infants.

Dietary transforming growth factor- β could be a potential new approach for allergy prevention

The epidemiological and animal studies described above suggest that the oral administration of TGF- β together with an allergen may be a useful and simple strategy to prevent the development of allergic diseases, particularly during infancy.

Previously, the efficacy of a polymeric diet containing TGF- β (approximately at a level comparable to that in

human or cow milk) was tested in children and adolescents with Crohn's disease [34]. The TGF- β -enriched polymeric diet was tolerant, safe, and effective in inducing remission and mucosal healing. Therefore, similar dietary approaches (e.g. a TGF- β -enriched diet or formula supplemented with TGF- β) might be utilized in infants for allergy prevention. Recombinant TGF- β protein, as described in the animal studies [10, 12], could be used for this purpose although legal, regulatory, and manufacturing concerns still need to be overcome.

In any event, exogenous TGF- β could theoretically be orally administered with an allergen to efficiently develop regulatory T cells and prevent allergen-specific immune responses. In addition, based on the findings published previously described above [10, 12, 34], both TGF- β 1 and TGF- β 2 isoforms can be given equal consideration for such dietary usage.

Probiotics may increase maternal milk transforming growth factor- β levels

It is possible that, in addition to a direct oral administration of TGF- β , indirect approaches to increase breast milk concentration of TGF- β may become useful strategies to prevent allergic diseases during infancy.

The probiotic *Lactobacillus* GG was reported to increase the amounts of TGF- β 2 in breast milk in a randomized, placebo-controlled study [35]; however, the use of a different probiotic strain, *L. reuteri*, had the opposite effect [36]. Therefore, specific strains of probiotics could be utilized in mothers during pregnancy and lactation to increase TGF- β concentrations in maternal milk, thereby preventing allergic diseases in infants. Because breast milk concentrations of TGF- β were reportedly lower in allergic mothers than in healthy mothers [9, 37], such approaches might be reasonable to prevent allergic diseases in infants with a high risk of allergy.

Issues to be solved

Many basic, epidemiological, and clinical issues must be solved in order to realize the practical clinical use of dietary (oral) TGF- β for allergy prevention.

Basic

The first target for mammalian milk TGF- β in the gastrointestinal tract (e.g. intestinal epithelial cells, dendritic cells, or some other cell type) remains unclear. In addition, the mechanism underlying how CD4⁺CD25⁺ regulatory T cell development is facilitated locally or systemically by mammalian milk TGF- β is still not understood.

Recent new studies have revealed that a unique subset of gut-associated dendritic cells drive the differentiation of regulatory T cells in TGF- β - and retinoic acid-

dependent manners and this process plays an important role in the induction of immunological tolerance to inert oral antigens (oral tolerance) [38, 39]. It is therefore possible that orally administered TGF- β such as maternal milk TGF- β may promote the differentiation of regulatory T cells in the intestine by acting directly or indirectly (possibly by inducing endogenous TGF- β from the intestinal epithelial cells) on T cells. To identify the cellular target(s) of mammalian milk or dietary TGF- β in the intestinal microenvironment would therefore be of paramount importance in this context.

It will also be an important issue to investigate the synergistic effects of TGF- β with other bioactive milk components (e.g. prostaglandins) on T cell development in the intestine. Because mammalian milk contains retinoic acid [40], it might also contribute to the development of regulatory T cells in concert with mammalian milk TGF- β .

Epidemiology

The epidemiological findings linking human milk TGF- β and infant allergy development are controversial [41]. Therefore, larger, more detailed, and well-controlled studies should be performed in the future. In addition, investigations for environmental and intrinsic factors influencing maternal milk concentrations of TGF- β will be important.

Clinical

Dose and duration studies regarding the oral administration of TGF- β are required. We do not yet know precisely how much and how long TGF- β should be orally administered for the development of regulatory T cells or for the prevention of allergic diseases during infancy. In particular, the unfavourable effects of orally administered TGF- β when used at high doses should be investigated. These issues are very important for performing translational research.

Conclusion

After birth, the immature neonatal immune system displays Th2-skewed immune responses [42]. As these Th2 responses are involved in the onset of allergic diseases, the regulation of such skewed Th2 responses is therefore normally favourable during infancy. TGF- β in mammalian milk may thus act as a natural 'brake' for unfavourable allergic Th2-type immune responses to food antigens, thereby preventing the development of allergic disease during infancy. This mechanism might work efficiently in infants due to the insufficient production of TGF- β in the gastrointestinal tract until the weaning period and it may

also represent a beneficial post-partum interaction between the mother and child.

Importantly, this concept suggests that the oral administration of TGF- β simultaneously with an allergen might potentially be a useful approach for the primary prevention of allergic diseases in infants and, hopefully, also in adolescents or even adults. Because of its simple strategy, this Mother Nature-inspired approach can be easily tested clinically. The challenge remains as to elucidate whether or not such an approach can really work in humans as well as in animals in near future.

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Conflict of interest: A. N. has filed a patent entitled 'Methods of treating allergies using TGF- β 1 and allergens'.

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