

Original Articles

Novel Therapeutic Approach toward Inflammatory Diseases: Targeting Transglutaminase 2

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Transglutaminase 2 (TGase 2) is an enzyme that is widely used in many biological systems for generic tissue stabilization purposes or immediate defenses for wounds. Many reports have showed that TGase 2 is aberrantly activated in tissues and cells and contributes to a variety of diseases, including neurodegenerative diseases and autoimmune diseases. In most cases, the TGase 2 appears to be a factor in the formation of inappropriate proteinaceous aggregates that may be cytotoxic. However, in other cases such as celiac disease, arthritis, lupus, amyotrophic lateral sclerosis, TGase 2 is involved in the generation of autoantibodies. This suggests the possibility that the inappropriate expression and/or presentation of TGase 2 to T cells might contribute to these diseases in genetically predisposed individuals. Others and we have found that TGase 2 expression is also increased in the inflammation process. We also demonstrated reverse of inflammation by TGase inhibition. Furthermore we discovered the genuine role of TGase 2 in immune cell activation. Increase of TGase activity induces or exacerbates inflammation via NF- κ B activation without I- κ B α kinase signalings. This review will examine a possibility of TGase inhibitors as therapeutic agents in a variety of inflammatory diseases.

Key Words: Transglutaminase, transglutaminase inhibitor, inflammation, autoimmune disease

Introduction

Transglutaminase 2 (TGases, EC 2.3.2.13) is a particularly interesting enzyme to consider in the context of pathology because its many functions

generally involve both protection and prevention of bodily injury, or tissue remodeling and repair¹. Its most common function is to catalyze the formation of isopeptide linkages between the carboxamide group of protein-bound glutamine residues and the ϵ -amino group of protein-bound lysine residues (Fig. 1)². Aberrantly activated TGase 2 contributes to a variety of diseases, including neurodegenerative diseases, atherosclerosis, inflammatory diseases, autoimmune diseases, and fibrosis³. Although TGase 2 appears to be responsible for formation of insoluble deposits in neurodegenerative diseases, it is not clear to answer

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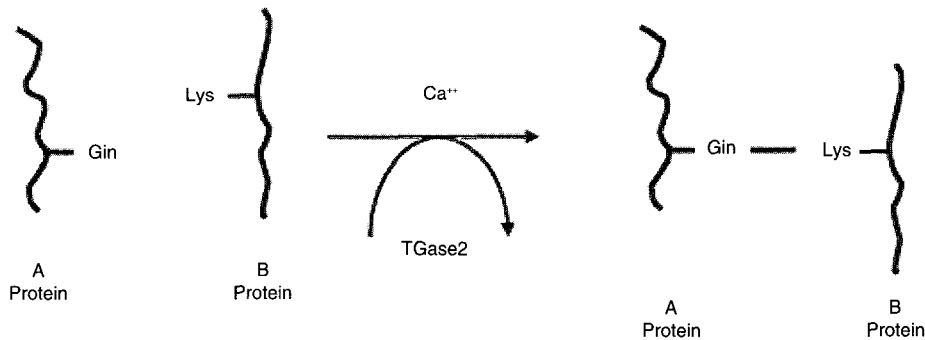


Fig. 1. TGase reaction².

Protein A is a glutaminy (Gln) residue in a protein/peptide substrate (acyl donor, amine acceptor). Protein B is a lysyl (Lys) residue in a protein/peptide substrate (acyl acceptor, amine donor). TGase catalysis with calcium results in a very strong covalent cross linking of the proteins/peptides by the isopeptide bond, N^ε-(γ-glutamyl)-L-lysine (GGEL).

whether cross linked inclusion itself is pathogenic. In other diseases, protein deposit is not a common factor for TGase 2 induction. Increased TGase activity is easily found both in the diseased tissues with inflammation and in the cells with inflammatory stress. We found that increase of TGase activity induces or exacerbates inflammation via NF- κ B activation without I- κ B α kinase signalings⁴. The mechanism of activation of NF- κ B by TGase 2 is unique and novel compared to the standard pathway. Increase of TGase activity reduces free I- κ B α in the cytosol by I- κ B α polymerization, which leads translocation of free NF- κ B into nucleus (Fig.1). Interestingly, TGase 2 also can be induced directly by NF- κ B activation because the TGase 2 promoter has a NF- κ B binding motif⁵. Therefore, if TGase activity is aberrantly activated, NF- κ B keeps activating inflammation. Reversely, if we can regulate TGase activity, we can stop the vicious inflammatory loop. Recently, we demonstrated a possibility that TGase inhibitors reverse the inflammatory process in the conjunctivitis⁶, uveitis⁷ and

LPS induce lung fibrosis⁸ models.

Inflammation

Recent studies by others and myself indicate that inhibition of TGases will be a profitable new approach to the treatment of at least some types of inflammation. Inflammation processes are complex biochemical phenomena that are manifested physiologically in tissues by edema, pain and leukocyte infiltration. Currently the most effective drugs for inflammation are glucocorticoids. Glucocorticoid induces many proteins such as lipocortins as inhibitory proteins of phospholipase A₂ (PLA₂, EC 3.1.1.4). PLA₂ plays a key role in the pathogenesis of allergic conjunctivitis. Miele et al identified a region of sequence similarity between uteroglobin and lipocortin⁹. Furthermore, they designed several synthetic peptides (nona peptides) corresponding to the region of highest similarity between uteroglobin and lipocortin-1. Miele et al named these peptides anti-flammins (AFs), corresponding to

uteroglobin residues 39-47 and lipocortin-1 residues 246-254. Both peptides were shown to be PLA₂ inhibitors in vitro and were effective in a classic model of acute inflammation in carrageenan-induced rat footpad edema.

Interestingly, treatment of purified PLA₂ with TGase 2 strikingly increased PLA₂ activity in vitro. TGase 2-catalyzed conformation of PLA₂ can be brought about by formation of an intramolecular ε-(γ-glutamyl)-lysine cross-link¹⁰ or by incorporation of polyamines¹¹. These observations suggest that TGase 2-mediated

modification may activate PLA₂ in vivo, following an influx of calcium. Increased TGase 2 expression has been reported for many inflammatory diseases, such as celiac's disease¹², Crohn's disease¹³, and sporadic inclusion body myositis¹⁴. Pathological roles of TGase 2 in those diseases might be associated with activation of PLA₂. Therefore we hypothesized that blocking both TGase 2 and PLA₂ activities may ameliorate PLA₂-mediated inflammation. To test this hypothesis, a series of new recombinant peptides using sequences from AFs and pro-elafin (cementoin) were constructed as

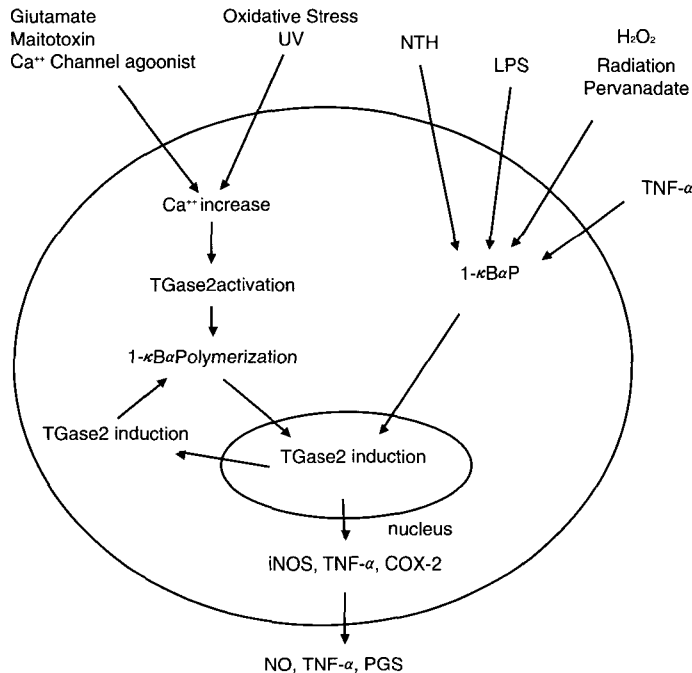


Fig. 2. Role of TGase 2 in NF-κB activation⁴.

IKK dependent NF-κB activation induces TGase 2 expression as well as iNOS and TNF-α. The induced TGase 2 may exacerbate the inflammation by continuous activation of NF-κB unless NF-κB induces regulatory molecules for TGase activity such as TGF-β. In addition, NF-κB is activated by increase of TGase 2 activity following increase of calcium uptake by multiple stresses. The regulation of TGase 2 activity is a new therapeutic approach in inflammatory diseases. NTHi: nontypeable Haemophilus influenza, NO: nitric oxide, PGS: prostaglandins, 1-κBα-P: phosphorylated 1-κBα by activated 1-κBα kinase (IKK).

competitive inhibitors of PLA₂ and TGase 2 respectively. We showed that the recombinant peptides abolish the TGase 2-catalyzed activation of PLA₂ *in vitro* and have a pronounced anti-inflammatory effect on allergic conjunctivitis *in vivo*⁶. In this study, we have shown that rationally designed peptide inhibitors for TGase 2 are potent anti-inflammatory agents in allergic conjunctivitis⁶. This concept can be applied to PLA₂-mediated (arachidonic acid-mediated) inflammatory diseases. Interestingly, Taggart et al demonstrated that over-expression of elafin containing pro-elafin domain (TGase inhibitory domain) prevents LPS-induced NF- κ B activation without proteasome inhibition¹⁵. This suggests that TGase 2 may be involved in the regulation of inflammatory signaling beyond activation of PLA₂. Interestingly, we found that the only pro-elafin sequence, E2, (a part of elafin, TGase inhibitor) itself also has dramatic anti-inflammatory effects⁶. This strongly suggests that TGase activity may play a key role in macrophage activation resulting from inflammatory stress. Recently, we found that TGase 2 activates the transcriptional activator NF- κ B and thereby enhances LPS induced expression of inducible nitric oxide synthase⁹. TGase 2 activates NF- κ B via a novel pathway (Fig. 2). Rather than stimulating I- κ B α phosphorylation and degradation, TGase2 induces the polymerization of I- κ B α . This polymerization results in dissociation of NF- κ B and its translocation to the nucleus where it is capable of upregulating a host of inflammatory genes, including iNOS and TNF- α .

Autoimmune Diseases

A) Celiac Disease (Inflammatory bowel disease)

TGase inhibitors may have a role in the treatment of celiac disease (CD). This disease is characterized by damage to the upper small intestine, causing effacement of the villi and producing a characteristically flat mucosa with markedly hypertrophic crypts (inflamma-

tory bowel disease). Consequently, patients with CD usually have difficulty with absorbing nutrition. This disease is found in genetically predisposed individuals (mostly HLA-DQ2 and -DQ8 positive) who mount heightened T cell-mediated and humoral immune responses to gluten. The immunological reaction to gluten in the upper small bowel, where ingested dietary components are concentrated, leads to inflammation and the characteristic features of disease¹⁶.

Progress in understanding the mechanism of the disease has been enhanced by the discovery that TGase 2 is an autoimmune antigen of CD¹⁷. Immunoprecipitation of proteins from fibrosarcoma cells with IgA from CD patients led to the identification of TGase 2 as the dominant endomysial autoantigen. Based on a series of *in vitro* experiments, TGase 2 is thought to be responsible for generating neoepitopes of gliadin through deamidation of glutamine residues. In this hypothesis, auto-antibody generation against TGase 2 cannot be explained at all. TGase 2, however, also cross-links itself onto gliadin *in vitro*. More likely the cross-linked TGase may act as a hapten for the formation of antibodies against gliadin *in vivo*. This is a plausible hypothesis because serum anti-TGase antibody titer falls dramatically when wheat products are removed from the diet. Thus, the presentation of fragments of gliadin cross-linked to TGase 2 results in antibodies against gliadin, TGase 2 and the cross-linked proteins. The greater preponderance of anti-TGase antibodies presumably reflects the greater antigenicity of this antigen.

TGase 2 expression in jejunal biopsies of CD patients is elevated by 3.2 fold¹⁸ and it is theoretically possible that this contributes to CD by increasing the frequency of gliadin-TGase 2 cross-linking and/or deamidation. Increase of TGase 2 expression by proinflammatory cytokines is a possible novel mechanism. A plausible hypothesis for the increase in TGase activity in CD

duodenal mucosa is induction of the enzyme by cytokines present in this inflamed tissue. The inflammatory infiltrates of the jejunal tissues of CD patients are rich in T cells. T cells are not the only source of cytokines as duodenal biopsies from CD patients show increases in $\text{INF-}\gamma$ of greater than a 1,000-fold relative to samples from normal individuals. Elevations in IL-2 , IL-4 , IL-6 and $\text{TNF-}\alpha$ have also been reported in the CD patients¹⁹. We have shown that

$\text{INF-}\gamma$ can induce expression of TGase 2 in the rat IEC-6 small intestinal cells, and interestingly, $\text{TGF-}\beta$ suppresses TGase 2 expression in that system²⁰. Therefore, it was suggested that the increased expression of TGase 2 leading to pathogenesis of CD in genetically predisposed individuals (HLA-DQ2+) could be due to increased $\text{INF-}\gamma$ signaling, loss of $\text{TGF-}\beta$ signaling, or both, in the small intestine.

Another possibility is that the source of TGase 2 may

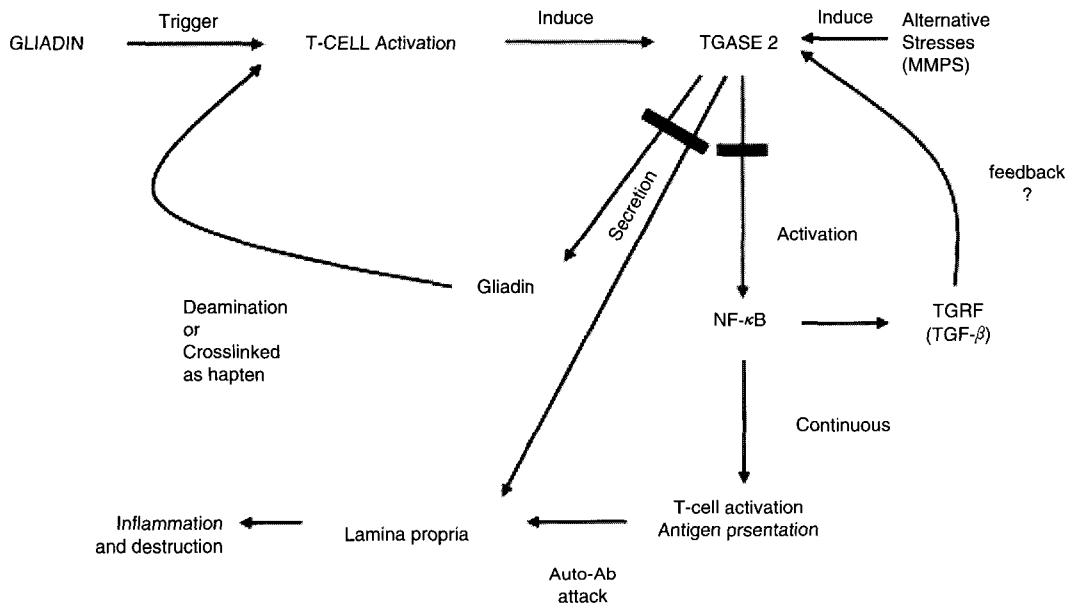


Fig. 3. Proposed model for beneficiary effect of TGase inhibition in CD

Gliadin is the major dietary component of wheat and rye. CD patients with HLA-DQ2⁺ type start inflammatory reaction when they uptake wheat or rye. TGase 2 in the activated T cells is secreted out to the matrix of lamina propria so that gliadin can be modified by TGase 2 to increase antigenicity. Increased TGase activity in the jejunum of CD patients appears to be responsible for the inflammatory chain reaction. TGase inhibition may prevent and/or reverse the consequence of T-cell activation (allergic reaction). MMPs: matrix metalloproteinases, TGRF: TGase regulatory factor, $\text{TGF-}\beta$: transforming growth factor- β , auto-Ab: auto-antibody of TGase 2. Black bar represents TGase activity inhibition.

be macrophages and T cells. TGase 2 is known as a marker of activated macrophages²¹⁻²³. Activated T cells increase TGase 2 expression. They are also able to release over-produced TGase 2 into the extracellular matrix. Immunohistostaining of TGase 2 in CD biopsy supports this idea with strong staining on the subepithelial membrane where macrophages and T cells are rich²⁴. In this case, gliadin may act as a responsible hapten for triggering a milieu of inflammation against autoimmune antigen, TGase 2. This theory is supported by which the severe inflammation of CD is not triggered until gliadin is delivered although autoimmune antigen (TGase 2) is abundant in the lamina propria and subepithelial membrane.

Taken together, the above observations suggest that TGase 2 is involved in the pathogenesis of CD. More work is necessary to ascertain whether its role is direct or indirect. In either case, TGase 2 inhibitors could be useful in the treatment of this fairly common chronic disease via blocking vicious consequences after induction of TGase activity (Fig. 3).

B) Sporadic Inclusion Body Myositis and Inflammatory Myopathies

Sporadic inclusion body myositis (SIBM) is the most common progressive muscle disorder that affects older individuals. This disease is characterized by a progressively worsening weakness in the proximal and distal limbs that does not respond to steroid therapy. There is as yet no useful therapy for this disease. Askanas and coworkers hypothesized that the overexpression of the β -amyloid precursor protein and its abnormal deposition may precipitate the muscle-fiber destruction characteristic of inclusion body myositis²⁵. This hypothesis further suggested a role for TGases in inclusion body myositis based on the putative involvement of these enzymes in the formation of the β -amyloid aggregates in Alzheimer's disease. Total

TGase enzyme activity is elevated by 16 fold in SIBM tissue¹⁴. Interestingly, pharmacological agents designed to attenuate the progression of symptoms of AD also have an inhibitory effect on TGase induced β -A cross-linking in vitro, for instance, indomethacin, phenelzine, tacrine, and deferoxamine²⁶. This suggests that appropriate TGase inhibitors may be already available for treating this relatively common disease.

To test whether increase of TGase 2 expression in SIBM is common factor in muscle inflammation, idiopathic inflammatory myopathies (IMs), including dermatomyositis (DM), polymyositis (PM), and sporadic inclusion body myositis (s-IBM) were analyzed. Using immunocytochemistry and quantitative RT-PCR, the level of TGase 2 expression was found to be significantly increased in DM and PM²⁷. DM and PM do not present any deposition of inclusion bodies containing highly cross-linked amyloid and other proteins. Therefore a plausible role of TGase 2 in the cascade of debilitating muscle diseases may be a contribution to the inflammatory process.

C) Other Autoimmune Diseases

Autoantibodies against TGase 2 are found in the blood of patients with dermatitis herpetiformis²⁸. Dermatitis herpetiformis is characterized by blistering of the extensor surfaces and sensitivity to gluten. Thus, the presence of anti-TGase 2 antibodies in CD and dermatitis herpetiformis may reflect a common etiology. In addition, the antibodies in dermatitis herpetiformis may arise in a similar manner to that described above for CD. Consistent with this view is the observation that the anti-TGase 2 antibodies found in dermatitis herpetiformis are mainly of the IgG class. The IgA immunoglobulins are predominantly associated with external secretions including those of the digestive tract. Antibodies against TGase 2 also occur in the blood of patients with type 1 diabetes²⁹.

Table 1. Autoantibodies against TGase Substrates in Autoimmune Disease

TGase substrate /autoantigen	Autoimmune disease	References for	
		Autoimmunity	TGase substrate
Actin	Hepatitis Necrobiosis lipoidica Lupus (human)	Orth <i>et al.</i> ³² Haralambous <i>et al.</i> ³⁴ Adyel <i>et al.</i> ³⁵	Nemes <i>et al.</i> ³³
Keratins	Hepatitis Buxman <i>et al.</i> ³⁶ Necrobiosis lipoidica		
Histone H2B	Lupus (human and murine)		Ballestar <i>et al.</i> ³⁷
Lipocortin I	Lupus (murine)	Ikai <i>et al.</i> ³⁸	Ando <i>et al.</i> ³⁹
Myosin	Lupus (human) Myasthenia gravis	Cardona <i>et al.</i> ⁴¹	Eligula <i>et al.</i> ⁴⁰
Troponin	Necrobiosis lipoidica		Bergamini <i>et al.</i> ⁴²
Tubulin	Hemolytic anemia Lupus (human)	Hentati <i>et al.</i> ⁴³	Piredda <i>et al.</i> ⁴⁴
Acetylcholine receptor	Myasthenia		Hucho <i>et al.</i> ⁴⁵
Gliadin	Celiac disease	Dieterich <i>et al.</i> ¹¹	Schuppan <i>et al.</i> ⁴⁶
Collagen	Autoimmune disease with subepidermal blisters Bullous pemphigoid Goodpasture disease Rheumatoid arthritis	Ghohestani <i>et al.</i> ⁴⁷ Chimanovitch <i>et al.</i> ⁴⁹ Borza <i>et al.</i> ⁵⁰ Diab <i>et al.</i> ⁵¹	Bowness <i>et al.</i> ⁴⁸
Myelin basic protein	Multiple sclerosis	Wajgt <i>et al.</i> ⁵²	Selkoe <i>et al.</i> ⁵³

Type 1 diabetes is a common disease, although not as common as late onset type 2 diabetes. Given the above observations, I would like to suggest that TGase 2 generates autoantibodies in a variety of autoimmune disorders by cross-linking potential autoantigens and acting as a hapten. This hypothesis is supported by numerous reports of the participation of TGase substrates in autoimmune diseases (Table 1). They occur in the blood in systemic lupus erythematosus (SLE)³⁰ and in the synovial fluid in rheumatoid arthritis³¹. TGase 2 involvement in the pathogenesis of common autoimmune diseases requires further investigation. However accumulating data suggest that specific TGase inhibitors might be very useful in treating chronic autoimmune diseases, some of which

are very common chronic diseases.

Fibrosis Including Liver Fibrosis (Cirrhosis)

TGase 2 is involved in the pathogenesis of fibrosis since TGase 2 plays a key role in the process of wound healing and scar formation⁵⁴. Fibrosis, like inflammation, is a physiological process that becomes pathological when it goes too far. TGase inhibitors have been proposed as agents that may prevent hypertrophic scar tissue in human skin - for instance, non-toxic amine compounds that are aminoacetonitrile, cadaverine, putrescine, and spermidine⁵⁵. TGase activity is increased in pathological (paraquat-induced) pulmonary fibrosis⁵⁶. In 1997, Johnson *et al* showed that characteristic TGase cross-linking product is highly increased in the renal

fibrosis model induced by rat subtotal nephrectomy. This model disorder may be associated with loss of renal tubule integrity⁵⁷.

TGases are involved in the stabilization of the extracellular matrix by cross-linking matrix proteins. TGase 2 is associated with the extracellular surfaces, although the mechanism by which this protein traverses plasmalemma membranes is not known⁵⁸. A number of the major matrix proteins have been identified as TGase substrates including fibronectin⁵⁹, fibrinogen⁶⁰, osteonectin⁶¹, osteopontin⁶², collagens⁶³⁻⁶⁶, vitronectin⁶⁷, collagen-tailed acetylcholinesterase⁶⁸, elafin⁶⁹ and plasminogen activator inhibitor 2⁷⁰. Another TGase substrate of interest, which is inserted into the extracellular matrix, is latent transforming growth factor β (LTGF- β)^{71,72}. LTGF- β cannot be activated without binding to matrix by TGase 2. Active TGF- β is released from the insoluble LTGF- β . TGase 2 is important in the development of the extracellular matrix since TGase 2 regulates the availability of this cytokine in the matrix. Perturbation of the formation of the extracellular matrix has been implicated in a number of pathological conditions including cancer metastasis⁵, as well as several already mentioned conditions including pathological fibrosis, atherosclerosis, and CD.

Liver fibrosis represents the response of liver to damage by toxic, infectious or metabolic agents. The process leading to liver fibrosis resembles the process of wound healing, including the three phases following tissue injury: inflammation, synthesis of collagenous and noncollagenous extracellular matrix components, and tissue remodelling (scar formation). In 1997, Mirza et al showed that TGase activity is increased at the early stage of hepatic fibrogenesis induced by CCl₄ in the rat. That finding suggests a possible role for this enzyme in stabilizing the fibrotic bands during liver fibrogenesis⁷³. Rapamycin (immunosuppressive agent) inhibited extracellular matrix deposition in the rat model of liver

fibrogenesis as determined by mRNA levels of procollagen and TGF- α 1, and inhibited TGase 2 activity as well⁵⁴. Recently, Grenard *et al*⁷⁴ found that TGase-mediated cross-linking occurs in liver extracellular matrix during the early, inflammatory, stage of liver fibrosis, whereas cross-linking by pyridinoline occurs mostly later in the fibrotic process. TNF- α expression is upregulated in early hepatic inflammation, and Kuncio *et al* showed that liver cells treated with TNF- α increase TGase 2 expression⁷⁵. TGase 2 appears to be involved in the early pathogenesis of fibrogenesis, indicating that it is an excellent target for the design of novel inhibitors to treat this very common condition.

Conclusion

We suggest that TGase 2 is strongly associated with the pathogenesis of many inflammatory diseases. Others have also come to this conclusion as evidenced by the large number of publications devoted to TGase inhibitors as potential therapeutic agents. While generic TGase inhibitors do exist (e.g., thiol-reactive reagents), they are non-specific and even highly toxic. Therefore, it seems reasonable that some effort should be devoted to the development of safer and more specific inhibitors in near future. Furthermore, screening TGase inhibitors from traditional medicine is imminent because many safe TGase inhibiting substances may exist in nature. I believe that if there is a dreadful spear against us, always an almighty shield is in nature since we have all evolved in a closed environment for very long time. In other words, nature and our defense system have known each other for over 500 million years.

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