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Anti-Inflammatory Effect of the Peptide LKEKK

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Abstract

The review summarizes and systematizes data on the anti-inflammatory effect of the synthetic peptide LKEKK *in vitro* and *in vivo*. Based on the analysis, it was concluded that it has a significant therapeutic potential as an anti-inflammatory drug in Crohn's disease, various forms of colitis and contact dermatitis.

Keywords: Proteins; Peptides; Receptors; Cytokines; Inflammation; Drugs

Abbreviations: IFNs-α: Interferons-α; TM-α_1: Thymosin-α_1; CT-B: Cholera Toxin B Subunit; sGC: Soluble Guanylate Cyclase; GTP: Guanosin-5'-Triphosphate; cGMP: Cyclic Guanosin-3',5'-Monophosphate; NOS: NO-Synthase; iNOS: Inducible; eNOS: Endothelial; nNOS: Neuronal; NEC: Necrotizing Enterocolitis; rCT-B: Recombinant CT-B; TNBS: Tri Nitro Benzene Sulfonic acid; TPA: 12-O-Tetradecanoyl-Phorbol-13-Acetate.

Introduction

Several decades ago, in the course of structural and functional studies of interferons-α (IFNs-α), the octapeptide LKEKKYSP, fragment 131–138 of human interferon-α_2 (IFN-α_2) capable of binding with high affinity to mouse thymocytes [1] and human fibroblasts [2], was obtained. Binding of the labeled octapeptide was competitively inhibited by unlabeled IFN-α_2, and thymosin-α_1 (TM-α_1) and the cholera toxin B subunit (CT-B). Comparison of the amino acid sequences of the octapeptide and TM-α_1 showed that they contain the same five member fragment LKEKK corresponding to sequences 16-20 of TM-α_1 and 131-135 of IFN-α_2 (Figure). It was suggested that this fragment may be involved in the binding of TM-α_1 and IFN-α_2 to various cell types, and the corresponding synthetic peptide may have not only receptor, but also biological activity.

66 93
CT-B -ERMKNTLRIAYLTEAKVEKLCVWNNKTP-
1 16 20 28
TM-α_1 SDAAVDTSSEITTKD*LKEKK*EVVEEAEN
116 131 135 143
IFN-α_2 -VKRYYGRILHRITLY*LKEKK*YSPCAWEV-

The amino acid sequences of CT-B, human TM-α_1, and human IFN- α_2. Numbers of amino acid residues are shown in figures. The identical residues are underlined. The sequence of peptide LKEKK is shown in italics.

Recently we synthesized the peptide LKEKK, labeled it with tritium, and found that [^{3}H] LKEKK is able to bind with high affinity to donor blood T-lymphocytes [3] and membranes isolated from rat small intestine epithelial cells [4]. Binding of the labeled peptide was competitively inhibited by TM-α_1, IFN-α_2 and cholera toxin B subunit (CT-B). Treatment of cells and membranes with proteases did not affect binding, which indicated the non-protein nature of the receptor, or at least that part of it that is directly involved in

the binding. The results obtained indicated that lymphocytes and intestinal epithelial cells have a non-protein receptor common to TM-α_1, IFN-α_2, and CT-B. It has been suggested that this receptor may be the cholera toxin receptor ganglioside GM1.

Effects of CT-B and the Peptide LKEKK on Intestinal Epithelial Cells

Our studies have shown that ^{125}I-labeled CT-B binds with high affinity to rat IEC-6 [5] and human Caco-2 [6] intestinal epithelial cells (K_d 3.6 and 3.7 нМ, respectively). In both cases the binding of labeled protein was completely inhibited by TM- α_1, IFN-α_2 and the peptide LKEKK but was not inhibited by unlabeled peptide KKEKL with inverted sequence (Ki > 10 мкМ). Similar results were obtained in the study of the reception of ^{125}I-labeled CT-B with membranes isolated from epithelial cells of the rat small intestine: binding was characterized by high affinity (K_d 3.7 nM) and was inhibited by unlabeled IFN-α_2, TM-α_1 and the peptide LKEKK (K_i, 2.0 1.5 and 1.0 nM, respectively), the unlabeled peptide KKEKL was inactive (K_i > 1 μM). In addition, CT-B and the LKEKK peptide did not affect the activity of adenylate cyclase and particulate guanylate cyclase pGC [7]. At the same time, in the concentration range of 10–1000 nM, CT-B and the peptide LKEKK dose-dependently increased the activity of soluble guanylate cyclase (sGC) in rIEC-6 and Caco-2 cells and the production of the activator of this enzyme, NO, while peptide the KKEKL tested in parallel was inactive [6].

The soluble guanylate cyclase (sGC) is shown to be a heterodimer that consists of α- and β-subunits, catalyzes; conversion of guanosin-5'-triphosphate (GTP) into the cyclic guanosin-3', 5'-monophosphate (cGMP), and is activated by a direct interaction of NO with a hem of the β-subunit [8]. Besides sGC, there are at least seven plasma membrane enzymes that synthesize the second-messenger cGMP. All membrane (particulate) GCs (pGC-A through pGC-G) share a basic topology, which consists of an extracellular ligand binding domain, a short transmembrane region, and an intracellular domain that contains the catalytic (GC) region [9].

NO is a diffuse messenger, which mediates a wide spectrum of physiological and pathological processes in the nervous, cardiovascular, and immune systems [10]. It has several protective functions: improves tissue perfusion, inhibits thrombocyte aggregation [11], decreases leucocyte adhesion to endothelial cells and proliferation of cells of the smooth muscles [12,13] and contributes to the preservation of tissues and organs [14]. Along with regulation of normal physiological functions, NO participates in the development of a number of pathological states, such as septic shock, stroke, and neurodegenerative diseases [15,16]. NO is synthesized from L-arginine by several isoforms of the NO-synthase (NOS): inducible (iNOS), endothelial (eNOS), and neuronal (nNOS) [10,17] and activates sGC by the binding with heme of its β-subunit [8]. cGMP accumulating in the cell transmits signals to downstream elements of the signaling cascade: cGMP-dependent protein kinases, cGMP-regulated cation channels, and cGMP-activated phosphodiesterases [8,18].

Notably, NO functions in intestinal cells as a major inhibitory neuromediator; endothelial NO is involved in local regulation of mucous circulation, at the same time, high NO concentrations produced in inflammation promote loss of mucus integrity [19,20]. It is believed that NO hyperproduction by mucus epithelial cells is one of the mechanisms of necrotizing enterocolitis (NEC) [21]. In contrast to constitutively expressed eNOS and nNOS [22], a high level of iNOS expression is induced in intestines during inflammation, leading to elevated level of NO formation [21]. In experimental models of NEC, iNOS inhibition has been shown to weaken inflammatory damage of intestines [10,23-26].

There is compelling evidence that the effects of low NO concentrations (~5 50 μM) are mediated by cGMP [27,28]. According to our results, an increase in the presence of 100 nM CT-B or LKEKK peptide in IEC-6 cells from 16 in control to 28 and 26 μM, and in Caco-2 cells from 18 to 33 and 30 μM, led to an almost twofold increase activity of intracellular sGC [6]. These data also suggest that low levels of NO activate sGC and its mediated signal transduction pathway from the receptor into the cell.

Thus, binding of CT-B and the peptide LKEKK to a common receptor on intestinal cells leads to an increase in NO production by cells and sGC activation.

Action of CT-B and LKEKK Peptide in Experimental Colitis

It has been established that oral administration of recombinant CT-B (rCT-B) to mice with tri nitro benzene sulfonic acid (TNBS)-induced colitis (a model of Crohn's disease) suppresses the development of the inflammatory process in the early and late stages of the disease. Dose-response studies showed that 68% of mice treated with rCT-B at 100 μg four times a day and 30% treated with rCT-B at a dose of 10 μg four times a day, there was a complete inhibition of the progression of colitis; however, in both cases, the remaining mice showed a slight decrease in the severity of inflammation. Administration of rCT-B was accompanied by a decrease in IFN-γ secretion due to a pronounced inhibition

of IL-12 secretion by Th1 cells. In addition, administration of rCT-B resulted in increased apoptosis of lamina propria cells of the thin layer of connective tissue that is part of the mucosa, an effect previously shown as a sign of IL-12 deprivation. It follows from these studies that rCT-B is a potent inhibitor of Th1-cell driven inflammation [29]. It should be noted here that, according to Kulig, et al. [30], binding of IL-12 to the receptor on keratinocytes initiates a protective transcription program that limits inflammation.

The anti-inflammatory activity of rCT-B has been confirmed in clinical trials, which showed that the protein is able to suppress inflammation in mild to moderate Crohn's disease: seven out of fifteen patients who received rCT-B 5 mg orally every other day for two weeks responded to treatment remission; there were no side effects. The authors of the study concluded that rCT-B has significant therapeutic potential as a safe anti-inflammatory agent, and randomized trials are needed to establish its clinical efficacy [31].

To find out if the peptide LKEKK, like CT-B, can have an anti-inflammatory effect in vivo, we studied the effect of both the protein and the peptide on the course of the inflammatory process in the intestines of mice with DSS-induced colitis. Animals were orally administered CT-B or the peptide LKEKK (5 20 mg/kg body weight for 14 days) and 5% dextran sulfate sodium (DSS) was added to the drinking water on day 7 to induce colitis. On day 14, tissues were collected for analysis. Experiments showed that by day 7, DSS-treated mice exhibited the characteristic signs of colitis: weight loss, diarrhea, and rectal bleeding. At the same time, in animals treated with CT-B or the peptide LKEKK (20 mg/kg) for 14 days, all clinical signs of the disease were much less pronounced, and weight loss and shortening of the colon were practically absent. In parallel, it was shown that a decrease in the severity of the disease is accompanied by a significant decrease in the production of pro-inflammatory cytokines TNF-α and IL-6 by intestinal cells [32].

Since, as mentioned above, CT-B and the peptide LKEKK increase the activity of sGC in target cells, we assumed that this enzyme could be involved in their effects. To test this assumption, we investigated the effect of CT-B and the peptide LKEKK on TNF-α-induced secretion of the pro-inflammatory cytokine IL-8 in Caco-2 cells with partial or complete absence of sGC activity. The enzyme was inhibited with a specific ODQ inhibitor (1H-[1,2,4]oxadiazolo[4,3-alpha]quinoxalin-1-one), which oxidizes the NO-binding heme prosthetic group [33]. As a result, it was found that a decrease in sGC activity is accompanied by a loss of the ability of the protein and peptide to inhibit the secretion of IL-8 [32]. Thus, the effect of CT-B and the peptide LKEKK on the secretion of the pro-inflammatory cytokine IL-8 in TNF-α-stimulated Caco-2 cells is mediated by sGC.

Action of CT-B and the Peptide LKEKK on Human Keratinocytes

Keratinocytes are the main cells of epidermis; they react to various environmental factors and are directly involved in the regulation of the inflammatory response of the skin [34]. It has been shown that five pro-inflammatory cytokines produced by keratinocytes are directly involved in the induction and development of the inflammatory process in the epidermis: IL-17A, IL-22, oncostatin M, TNF-α, and IL-1α [35]. The role of individual cytokines in the development of inflammation has been established: IL-22 and oncostatin M control epidermal hyperplasia and loss of differentiation, while IL-1α, IL-17A, and TNF-α provide activation of innate immunity [36].

The study of the reception of the peptide LKEKK by normal human keratinocytes showed that the tritium-labeled peptide was able to bind to these cells reversibly, with high affinity and specificity (K_d 2.6 nM). Unlabeled TM-α_1, IFN-α_2 and the peptide with the inverted KKEKL sequence were tested as potential competitors of the labeled peptide. Experiments showed that TM-α_1 and IFN-α_2 competitively inhibited the binding, while the peptide KKEKL was inactive. The inability of the peptide with the inverted sequence to displace [^{3}H]LKEKK from the complex with the receptor indicates a high binding specificity of the labeled peptide [37].

Next, we examined the anti-inflammatory activity of the peptide LKEKK using an in vitro model of IL-17A-induced inflammation of human keratinocytes: IL-17A (20 ng/mL) was added to cells pretreated with the peptide to induce inflammation. Experiments have shown that the LKEKK peptide in the concentration range of 50-1000 nM dose-dependently reduces the IL-17A-induced production of three pro-inflammatory cytokines (TNF-α, IL-6 and IL-1α) and simultaneously increases the production of the anti-inflammatory cytokine IL-10.

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of the action of the LKEKK peptide [37,38].

According to our data, the peptide LKEKK at concentrations of 50–1000 nM dose-dependently increases the activity of sGC in keratinocytes, but does not affect the activity of pGC; the peptide KKEKL tested in parallel was inactive. Thus, binding of the peptide LKEKK to human keratinocytes leads to an increase in sGC activity. We also investigated the effect of the peptide on the ability of keratinocytes to IL-17A-induced production of TNF-α and IL-1α with partial or complete loss of sGC activity. ODQ, a specific sGC inhibitor, was used to inhibit the enzyme activity [33]. Experiments showed that ODQ dose-dependently reduced sGC activity and the inhibitory effect of 500 nM peptide LKEKK on IL-17A-induced keratinocyte production of TNF-α and IL-1α. Thus, the decrease in enzyme activity was accompanied by the loss of the ability of the peptide LKEKK to inhibit the secretion of anti-inflammatory cytokines, and, therefore, the action of the peptide is mediated through sGC.

Anti-Inflammatory Effect of the Peptide LKEKK on Models of Acute and Chronic Skin Inflammation

We evaluated the anti-inflammatory activity of the peptide in mouse models of acute chronic contact dermatitis induced by 12-O-tetradecanoylphorbol-13-acetate (TPA). Increased skin thickening is the first sign of irritation and local inflammation, which occurs due to increased vascular permeability, dermal edema, and proliferation of epidermal keratinocytes.

To create a model of acute skin inflammation, the inner and outer surfaces of the ear of mice were treated with LKEKK or KKEKL peptides (10 300 μg in 20 μL of medium containing 2% dimethyl sulfoxide, 20% propylene glycol and 70% acetone, three times with an interval of 15 minutes). Animals of the negative control group received the base medium according to the same scheme. Mice in the positive control group were injected with dexamethasone (0.05 mg/ear in acetone). After 15 minutes, 20 μl of TPA (2.0 μg/ear in acetone) was applied to the skin. Edema (an increase in ear thickness) was measured using a digital thickness gauge (Mitutoyo Corporation, Japan) before and 5 hours after TPA application.

Experiments have shown that local exposure to TPA leads to a significant (more than 300 μm) increase in ear thickness against the background of the application of the base medium (negative control). At the same time the peptide, LKEKK (50–300 μg/ear) or dexamethasone (0.05 mg/ear, positive control) reduced edema to 200–140 and 60 μm, respectively. The peptide KKEKL tested in parallel was inactive. Histological studies of hematoxylin and eosin-stained sections of ear tissue from TPA-treated mice showed a marked increase in ear thickness with clear signs of edema and the appearance of a significant number of inflammatory cells in the dermis. Thus, topical application of the peptide LKEKK (50–300 μg/ear) significantly reduced ear edema and associated pathological indicators [39–40]. It should be noted that the effectiveness of the peptide at concentrations of 150–300 μg/ear was comparable to that of dexamethasone [39,40].

It is known that local exposure to TPA leads to a marked increase in the levels of pro-inflammatory cytokines (TNF-α, IL-6 and IL-1β) in mouse ear biopsy samples [41,42]. Our results are fully consistent with these data: the levels of TNF-α, IL-6 and IL-1β increased dramatically in the area of TPA-induced inflammation. At the same time, treatment with the peptide (50 300 μg/ear) dose-dependently reduced the levels of these cytokines, and hence the development of the inflammatory process mediated by them. Therefore, the peptide LKEKK may be effective as an anti-inflammatory agent in acute contact dermatitis.

To conclusively confirm that the peptide LKEKK has anti-inflammatory activity in vivo, we examined its effect in the model of chronic skin inflammation caused by repeated application of TPA to the ears of mice developed by Stanley, et al. [43]. The inflammatory response in this model is characterized by ear weight gain, inflammatory cell infiltration, and epidermal hyperplasia. The experiments were carried out according to the following scheme: 20 μL of a TPA solution (2.0 μg/ear in acetone) or acetone (filler) was applied to the inner and outer surfaces of the ear 6 times every other day. On days 7–9, mice were treated on the inner and outer surfaces of the right ear with peptide (100–300 μg/ear) or dexamethasone (0.05 mg/ear in acetone, positive control) twice a day. Ear thickness was measured with a digital thickness gauge 5 hours after the last TPA treatment. Experimental results showed that TPA treatment led to a significant increase in the thickness and weight of the ears, and the peptide LKEKK (100–300 μg/ear) significantly reduced the severity of these changes, while the peptide activity was comparable to that of dexamethasone. Histological examination of stained ear tissue sections from several TPA-treated mice showed that repeated application of TPA resulted in a marked increase in ear thickness and epidermal hyperplasia. Peptide treatment (300 μg/ear) significantly reduced ear thickness and associated pathological features. These data confirm the ability of the peptide to suppress persistent inflammatory lesions with repeated topical application.

Thus, LKEKK, a short peptide with a simple structure, has a pronounced anti-inflammatory activity in vitro and in vivo and has a significant potential as an anti-inflammatory drug.

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