Thymosin α1

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Thymosin α1 (Tα1) is an immunostimulatory peptide that is commonly used as an immune enhancer in viral infectious diseases such as hepatitis B, hepatitis C, and acquired immune deficiency syndrome (AIDS). Tα1 can influence the functions of immune cells, such as T cells, B cells, macrophages, and natural killer cells, by interacting with various Toll-like receptors (TLRs). Generally, Tα1 can bind to TLR3/4/9 and activate downstream IRF3 and NF-κB signal pathways, thus promoting the proliferation and activation of target immune cells.Moreover, TLR2 and TLR7 are also associated with Tα1. TLR2/NF-κB, TLR2/p38MAPK, or TLR7/MyD88 signaling pathways are activated by Tα1 to promote the production of various cytokines, thereby enhancing the innate and adaptive immune responses. At present, there are many reports on the clinical application and pharmacological research of Tα1, but there is no systematic review to analyze its exact clinical efficacy in these viral infectious diseases via its modulation of immune function.

thymosin α1 virus infection immune regulation protein structure

1. Properties of Tα1

Tα1 (generic drug name: thymalfasin; trade name: Zadaxin) is a bioactive peptide with 28 amino acid residues, which is obtained by cutting the front part of prothymosin α (ProT α, composed of 109 amino acid residues) by asparagine endopeptidase [1][2][3]. The sequences of the Tα1 peptide are as follows: Ac-Ser-Asp-Ala-Ala-Val-Asp-Thr-Ser-Ser-Glu-Ile-Thr-Thr-Lys-Asp-Leu-Lys-Glu-Lys-Lys-Glu-Val-Val-Glu-Glu-Ala-Glu-Asn-OH. Additionally, Tα1 has some interesting characteristics: (1) it has a relative molecular weight of 3108 Da [4]; (2) it is highly acidic with an isoelectric point of 4.2; (3) the N-terminal of Tα1 is acetylated, and there are no disulphide bonds or glycosylation structures [5][6][7]; (4) the entire polypeptide molecule has six amino acid repeats (Ala-Ala, Ser-Ser, Thr-Thr, Lys-Lys, Val-Val, Glu-Glu); (5) it may be involved in the regulation of the cell cycle [8]; and (6) it can indirectly affect transcription and/or DNA replication [9].

2. The Structure of Tα1

Under natural conditions, Tα1 is a short, highly charged, and inherently disordered protein. At neutral pH and 37 °C, Tα1 typically exhibits intrinsic disorder, meaning that it does not have a stable, defined structure [6]. In the monolayer vesicle of dimiristoylphosphatidylcholine and dimiristoylphosphatidic acid (10:1), it showed a partially structured conformation [10]. Under low pH conditions, Tα1 has the ability to build ordered protein complexes through interaction with other naturally existing proteins. Moreover, in organic solvents such as trifluoroethanol, hexafluoropropanol, or sodium dodecyl sulfate (SDS), Tα1 is commonly observed to adopt a stable conformation. A

structured conformation of the peptide was observed through restrained molecular dynamic simulations with an explicit solvent comprising 40% TFE/60% TIP3P water (*v*/*v*), with two stable regions identified: an alpha-helix region spanning residues 14 to 26, and two double β-turns in the N-terminal twelve residues of Tα1, which form a distorted helical structure [5]. Additionally, two β-rotational conformations were detected at the N-terminal of Tα1, namely (I, I + 1)-double rotations of the residues ASP2-ASP6 and (I, I + 2)-double rotations of the residues Thr7-Thr12. In contrast to TFE mixed solvents, Tα1 in SDS displays a spiral folded conformation [11]. This conformation is characterized by a structural fracture between residues 1–9 and 14–25, with the acetylated N-terminal residues 1–5 of Tα1 often inserted into the hydrophobic region of the micelle. The investigation also revealed that the folded conformation of Tα1 in SDS closely resembled that in phospholipid vesicles, taking on a 3_{10} helical structure [11]. Nonetheless, a few differences were observed in the tertiary structure of Tα1 between these two environments.

As a result, when Tα1 is folded on a membrane with negative charge exposed on the surface, it may connect with receptors on or near the membrane and insert the N-terminal of Tα1 into the hydrophobic area of the membrane, resulting in a bio-signaling cascade response [11]. For instance, the activation of the phosphorylation pathway of I(κ)B kinase (IKK) via the tumor necrosis factor (TNF) receptor-related factor 6 (TRAF6) can be induced by Tα1 [12]. Nevertheless, due to the limitations of full peptide encapsulation, the potential interaction between Tα1 and the cell membrane remains unknown.

3. The Protein Binding Properties and Biosafety of Tα1

Despite the pleiotropic effects of Tα1 on immune regulation, the lack of particular receptors remains one of the fundamental factors leading to the inefficacy of therapy with Tα1. However, recent research has demonstrated that the C-terminal portion (residues 11–20) of Tα1, which is defined by the amino acid sequence “LKEKK”, is capable of binding to human serum albumin (HSA) [13]. HSA is a serum protein that can serve as a carrier for a variety of medicines and polypeptides. The C-terminal sequence of Tα1 can be delivered to the vicinity of a target membrane exposing phosphatidylserine (PS) through the assistance of HSA, under conditions where the membrane region is negatively charged. The N-terminal region of Tα1 can then enter into the hydrophobic region of the cell membrane, producing a cascade reaction of biological signals [7][13]. As a result, plasma proteins may act as carriers of Tα1 targeting areas. However, the combination of the two is not close; it just creates conditions for the binding and diffusion of Tα1.

Furthermore, electrostatic interactions may enhance the binding of Tα1 to hyaluronic acid (HA) and interfere with the binding of HA to CD44 and the motor receptor RHAMM, inhibiting viral infection progression [14]. HA is a glycosaminoglycan found on the cell surface and in extracellular media that interacts with RHAMM and CD44 via a shared BX7B motif, where “B” is an Arg or Lys residue and “X” is any amino acid with no basic properties. Nevertheless, no definitive BX7B motif is found in the amino acid sequence of Tα1. When the sequence of Tα1 was compared to the HA receptor sequence, it was discovered that the C-terminal portion of Tα1, specifically at the lysine residue position in the “LKEKK” sequence, shows sequence resemblance to the HA receptor. Specifically, the sequence of CD44 at positions 41–45, 153–162, and 711–719, as well as the sequence of RHAMM at positions 743–750 and 721–731, all exhibit similarities with the sequence of Tα1. The positively charged lysine residue side

chains on Tα1 may create ion bridges with negatively charged HA, thereby potentially interfering with the binding of HA to certain receptors such as CD44 or RHAMM and their complicated interactions.

Tα1 has garnered substantial clinical therapeutic attention due to its various biological effects. There are currently three primary approaches for the production and purification of Tα1: biological extraction, chemical solid-phase synthesis, and gene engineering expression [15]. Solid-phase synthesis is the only technique approved for the clinical production of Tα1. Tα1 is commonly provided twice a week through subcutaneous injection, with a conventional dosage range of 0.8–6.4 mg and a multi-dose range of 1.6–16 mg [16]. A pharmacokinetic study showed that after subcutaneous injection, Tα1 is well-absorbed in the body, and its peak blood drug concentration (Cmax, the highest blood drug concentration after administration) is reached at 1–2 h, with a plasma half-life ($t_{1/2}$, an estimate of the time it takes for the concentration or amount in the body of that drug to be reduced by exactly one-half (50%)) of less than 3 h [17]. Tα1 usually has good security. The most common adverse reactions include local irritation, redness, or discomfort at the injection site. However, Tα1 is often banned in immunocompromised individuals due to its immunomodulatory action (such as organ transplant patients).

4. The Immunomodulatory Mechanism of Tα1

Tα1 is a well-known polypeptide with immunoregulatory effects as well as biochemical features [18]. Tα1 has shown encouraging outcomes in viral infectious disorders such as hepatitis B, either alone or in combination with other medications. The direct effect of Tα1 on lymphoid cells might explain some of the reported effects. Tα1 exerts an immune modulatory activity on T cell and NK cells, and impacts the functions of mature lymphocytes, such as stimulating cytokine production and cytotoxic T-lymphocyte-mediated cytotoxic responses [19]. Presently, the non-specific immune regulation mechanisms of Tα1 are now classified as direct and indirect immunological processes [20].

4.1. The Effects of Tα1 on Immune Cell

Tα1 increases the expression of major histocompatibility complex (MHC) antigens and B-2 microglobulin on the cell surface, resulting in increased expression of virus-specific antigens and a reduction in viral replication [9][21][22][23]. A previous study has shown that Tα1 can increase the expression level of glutathione [24]. Simultaneously, one study discovered a negative association between glutathione concentration and influenza virus replication, indicating that glutathione may play an essential role in inhibiting influenza virus replication [25]. Tα1 may indirectly decrease virus multiplication and increase immune response by raising glutathione content via glutathione-dependent antiviral mechanism. However, further research is needed to confirm this possibility. Studies conducted in vitro have indicated that Tα1 can significantly impact T cell production and maturation. Additionally, Tα1 can stimulate the production of cytokines in T-helper 1 (Th1) cells, such as interferon-γ (IFN-γ) and interleukin-2 (IL-2), and activate NK cell-mediated cytotoxicity [26]. In cancer and cyclophosphamide immuno-suppressed mice, Chen et al. suggested that Tα1 administration can increase NK cell activity [27]. Furthermore, by stimulating NK cells, Tα1 can enhance the body's ability to eliminate virus-infected cells. Tα1 has also accelerated the recovery rate of NK activity in bone marrow-reconstituted murine chimeras [28].

Dendritic cells (DCs) are powerful antigen-presenting cells (APCs) that play an important role in the immune response [29]. Tα1 has activated subsets of myeloid DCs (mDCs) and plasmacytoid DCs (pDCs). Both immature and mature DC subsets are capable of phagocytosing conidia. Tα1 enhances the phagocytic activity of immature DCs, modifies the morphology of DCs, and increases the expression of HLA class Ⅱ antigens and costimulatory molecules in response to conidia. Regarding cytokine production, it has been demonstrated that Tα1 significantly promotes the release of IL-12 p70 by immature mDCs in response to conidia and zymosan, and increases the production of IL-10 by immature pDCs in response to conidia [30]. Interestingly, DCs are important not only in eliciting immunological responses but also in promoting immune tolerance [9]. Tα1 can activate TLR9, induce the expression of indoleamine 2,3-dioxygenase (IDO) in DCs, and then activate the tryptophan catabolism-induced immune suppression pathway in vivo [31][32]. As a result, the production of IL-10 in CD4CD25 regulatory T cells is stimulated [33].++

It is evident that Tα1 can affect the maturation, differentiation, and function of T cells. Recent research has also found that DC subsets have a significant polarizing impact on T-helper differentiation. Tumor necrosis factor-alpha (TNF-α) is one of the substances capable of stimulating DC maturation and IL-12 production in vitro. Huang et al. demonstrated that, during the maturation of bone marrow-derived DCs (BMDCs), Tα1 promoted the differentiation of CD4-expressing DCs and the expression of activation markers, but without affecting the production of IL-12, as well as the T cell-stimulatory capacity of DCs in the absence of TNF-α [29]. However, in the presence of TNF-α, Tα1 has been shown to not only raise the expression of CD4 on MHC class Ⅱ DCs and boost the up-regulation of mature markers caused by TNF-α, but also to decrease the up-regulation of IL-12 production. These effects were most noticeable at the therapeutic doses of Tα1.+

Furthermore, Tα1 can affect immune function by inducing and regulating the maturation of T and NK cells, activating lymphocytes, and regulating the secretion of inflammatory cytokines such as IL-2, IL-4, IFN-γ, TNF-α, et al. [31][34][35]. Upon binding to TLR receptors located on the surface of precursor T cells, Tα1 promotes their differentiation into cytotoxic T lymphocytes (CD8 T cells, CTL) [19][21][36]. These CTLs can recognize damaged or low-expression MHC-Ⅰ cells and trigger the release of IFN-γ, thereby controlling viral replication [37]. In conjunction with NK cells, CTLs form an integral defense line of antiviral immunity.+

CD4 T-helper2 (Th2) cells are important in immune response regulation because they activate T-dependent B cells and promote the generation of virus-specific antibodies. It should be noted that CD4 T cells are more susceptible to viral infections than other types of immune cells [38]. By increasing the number of CD4 T cells and effectively maintaining the CD4/CD8 T cell ratio, Tα1 is capable of exerting a significant positive impact on the immune system of the host organism [39].+++++

The monocytic/granulocytic system (including the differentiated macrophages) and the principal cellular effectors of the immune response, play a crucial role in identifying and eliminating foreign entities such as pathogenic microorganisms [40]. Research by Peng et al. demonstrated that Tα1, as a weak immune modulator, can directly activate bone marrow-derived macrophages (BMDMs) to produce IL-6, IL-10, and IL-12 [41]. Moreover, Tα1

promptly stimulates the assembly and disassembly of podosomal structures, thus affecting the motility, invasion, and chemotaxis of BMDMs [42].

4.2. The Effects of Tα1 on Inflammation Related Signaling Pathways

Tα1 is also the major activator of Toll-like receptors (TLRs) in myeloid and plasma cell-like DCs. DCs, a type of mononuclear phagocyte, are often regarded as the most efficient antigen-presenting cells and play a critical role in modulating both innate and adaptive immune responses [43]. TLRs, which belong to the class I transmembrane receptor family, are present on the cell membrane surface or expressed on organelle membranes. The typical signaling pathways for TLRs include myeloid differentiation factor 88 (MyD88), IL-1 receptor-related kinase activator (IRAK), and TRAF6 [44]. MyD88 serves as the key adaptor protein that triggers the activation of Nuclear Factor Kappa B (NF-κB) in the signaling cascade induced by Tα1. Tα1 can directly induce functional maturation of DCs via TLRs (such as TLR2, TLR3, TLR5, TLR9, et al.), activate signal transduction pathways, such as the MyD88-dependent pathway and the p42/44 mitogen-activated protein kinase (MAPK)/c-Jun NH2 terminal kinase (JNK) pathway, and enhance the secretion of cytokines, such as IL-6, IL-10, IL-12, IL-13, and IL-17, thereby conferring protection against viral infections [30][45][46]. Studies have indicated that Tα1 can induce the expression of IL-6 through the TRAF6/atypical protein kinase C (PKC)/IKK/NF-κB pathway [12].

Tα1 can stimulate the expression of IL-6, IL-10, and IL-12 by activating the IRAK4/1/TRAF6/PKCζ/IKK/NF-κB and TRAF6/MAPK/AP-1 signaling pathways [41]. Furthermore, the p38 MAPK/NF-κB and TLR9/MyD88/IRF7 pathways are also potential mechanisms by which Tα1 activates DCs, inducing IFN-α/IFN-γ-dependent pathways and antiviral responses in vivo [47][48][49]. Moreover, Sodhi et al. suggested the activation of the p42/44 MAPK/JNK pathways in response to in vitro treatment with Tα1 in murine BMDMs [45]. The maximal expression of phospho-p42/44 MAPK was observed after 5–15 min following stimulation with 100 ng/mL of Tα1. Moreover, Tα1 can activate a TRAF6-atypical PKC-IκB kinase signaling pathway that activates NF-κB, which in turn triggers cytokine gene expression in murine BMDMs [50].

In summary, the antiviral effect of Tα1 can be summarized into two aspects: on the one hand, Tα1 can directly inhibit virus replication and viral protein expression by increasing the expression of cell surface-related antigens [9][21][22][23]; on the other hand, after the virus enters the body, Tα1 can treat viral diseases by enhancing T cell function, activating dendritic cells and macrophages, increasing the phagocytic activity of dendritic cells and the cytotoxicity of NK cells, activating TLRs, and starting MAPK, Jak, NF-κB, and other signaling pathways [12][41][44][45][47][48][49].

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