REVIEW

Prothymosin alpha: a ubiquitous polypeptide with potential use in cancer diagnosis and therapy

Kyriaki Ioannou · Pinelopi Samara · Evangelia Livaniou · Evelyna Derhovanessian · Ourania E. Tsitsilonis

Abstract The thymus is a central lymphoid organ with crucial role in generating T cells and maintaining homeostasis of the immune system. More than 30 peptides, initially referred to as "thymic hormones," are produced by this gland. Although the majority of them have not been proven to be thymus-specific, thymic peptides comprise an effective group of regulators, mediating important immune functions. Thymosin fraction five (TFV) was the first thymic extract shown to stimulate lymphocyte proliferation and differentiation. Subsequent fractionation of TFV led to the isolation and characterization of a series of immunoactive peptides/polypeptides, members of the thymosin family. Extensive research on prothymosin α (proTα) and thymosin α1 (Tα1) showed that they are of clinical significance and potential medical use. They may serve as molecular markers for cancer prognosis and/or as therapeutic agents for treating immunodeficiencies, autoimmune diseases and malignancies. Although the molecular mechanisms underlying their effect are yet not fully elucidated, proTα and Tα1 could be considered as candidates for cancer immunotherapy. In this review, we will focus in principle on the eventual clinical utility of proTα, both as a tumor biomarker and in triggering anticancer immune responses. Considering the experience acquired via the use of Tα1 to treat cancer patients, we will also discuss potential approaches for the future introduction of proTα into the clinical setting.

Abbreviations

AAK cells	Anti-CD3-activated killer cells
BRM	Biologic response modifier
CDDP	Cisplatin
CML	Cell-mediated lympholysis
CTL	Cytotoxic T lymphocyte(s)
CY	Cyclophosphamide
DC	Dendritic cell(s)
DL	Dalton's lymphoma
DTIC	Dacarbazine
5-FU	5-Fluorouracil
HMGB1	High mobility group box 1
HSP	Heat sock protein
IFN	Interferon
IL	Interleukin
LAK cells	Lymphokine-activated killer cells
3LL carcinoma	Lewis lung carcinoma
MHC	Major histocompatibility complex
NK cells	Natural killer cells
NSCL cancer	Non-small cell lung cancer
pI	Isoelectric point
PBL	Peripheral blood lymphocyte(s)
PBMC	Peripheral blood mononuclear cell(s)

K. Ioannou · P. Samara · O. E. Tsitsilonis (✉)
Department of Animal and Human Physiology,
Faculty of Biology, University of Athens, Panepistimiopolis,
15784 Athens, Greece
e-mail: rtsitsil@biol.uoa.gr

E. Livaniou
Immunopeptide Chemistry Lab, Institute of Radioisotopes
and Radiodiagnostic Products, National Centre for Scientific
Research "Demokritos", Patriarchou Gregoriou and Neapoleos,
Aghia Paraskevi, 15310 Athens, Greece

E. Derhovanessian
Tübingen Ageing and Tumour Immunology Group,
Center for Medical Research, University of Tübingen
Medical School, Waldhörnlestr. 22, 72072 Tübingen, Germany

PGE2	Prostaglandin E2
PMN	Polymorphonuclear(s)
ProTα	Prothymosin alpha
s.c.	Subcutaneous
TACE	Transarterial chemoembolization
TAM	Tumor-associated macrophages
Tα1	Thymosin alpha 1
Tβ4	Thymosin beta 4
TFV	Thymosin fraction V
TLR	Toll-like receptor(s)
TNF	Tumor necrosis factor
VP-16	Etoposide

Introduction

The thymus gland as a central lymphoid organ plays a crucial role in developing, maturing and differentiating many of the cellular components of the immune system and is considered essential for normal immune function. Until the early 60s, the gland was thought to be vestigial. At this time, its importance was documented by reports showing that the immune system of neonatally thymectomized rodents was generally crippled and an incapability to resist infections and histoincompatible transplants was noted [1–3]. The gland's involution with age results in a decrease of the levels of thymus-derived hormones [4] and a deficiency in immune reactivity, accompanied by an increase in cancer incidence and age-related diseases [5]. Thus, research efforts focused on the detection and identification of thymic humoral messages, namely complex thymic fractions and a series of structurally defined peptides [5].

Goldstein and colleagues were the first to report the purification of a thymic extract that stimulated lymphocytopoiesis and enhanced in vivo immune cell proliferation in mouse lymph nodes [6]. A year later, this lymphocytopoietic factor was purified, chemically characterized and given the name "thymosin" [7]. Subsequently, Goldstein's research team succeeded in further purifying a highly active preparation from bovine thymus, termed thymosin fraction V (TFV). In order to produce sufficient quantities of this fraction and thus explore TFV's properties, a new method, including 5 sequential steps of heat treatment, acetone and ammonium sulfate precipitation, ultrafiltration and gel filtration, was developed [8].

When tested in vivo, TFV and its component parts were able to enhance immune functions in newborn mice and to augment the survival of mice that had undergone thymectomy [9]. A similar activity was demonstrated in vitro in lymphocytes of immune-suppressed humans, where TFV restored, to almost normal levels, their deficient immune responses [10]. In addition, Dauphinee and colleagues reported that TFV administration in animals with autoim-

mune diseases resembling human lupus erythematosus led to the retreat of some of the symptoms [11]. Finally, TFV was shown to exert in vitro antiproliferative effects in several neoplastic cell lines, including murine neuroendocrine pituitary adenoma and glioma [12], human acute T lymphoblastic [13] and promyelocytic leukemias [14]. In animals, treatment with TFV conferred resistance to Dunning [15] and murine lymphoblastic leukemias [16].

All these in vivo and in vitro immunomodulatory properties of TFV became the pretext for further investigating and analyzing its components. Efforts toward this direction showed that TFV consisted of 10–15 basic components and 20 or more secondary ones, with molecular weights varying between 1,000 and 15,000 Da. A system of nomenclature was developed to distinguish and characterize the individual peptides in TFV. These were consequently divided into three groups (identified by the Greek letters alpha [α], beta [β] and gamma [γ]), according to their isoelectric point (pI). Group α comprised peptides with pI < 5, group β those with pI between 5 and 7 and group γ peptides with pI > 7. Peptides from each group were, and still are, indicated with a subscript, 1, 2, 3, etc., that denotes the order of their isolation and identification from TFV. Depending on whether they are biologically active or not, they are further characterized as thymosins or simple peptides/polypeptides, respectively [17].

The majority of the polypeptides, isolated so far from TFV, belong to α- and β-thymosins, whereas none of them belong to γ-thymosins. The family of α-thymosins is mainly represented by thymosin α1 (Tα1), a 28-amino acid peptide that was the first member of this family isolated from TFV and prothymosin α (proTα), a 109–112 amino acid long polypeptide (depending on the mammalian species), proven to be Tα1's precursor molecule, and the natural polypeptide present within the cells [18]. Thymosin β4 (Tβ4) is the major representative of the β-thymosin family [19]. Tβ4 along with proTα retains the major activity of TFV and this is the main reason why, more than 25 years after their isolation, they are still in the scientific spotlight (Table 1).

The dual—intracellular and extracellular—role of proTα

Although initially referred to as a "thymic hormone," proTα demonstrates some unique properties: it is not thymus-specific, as it is encountered in many lymphoid, but also non-lymphoid organs [20]; it is highly conserved during evolution, as it presents extended homology in its primary structure among mammalian species [19]; and it is a universal polypeptide surmounting approximately 10,000 copies per cell [21]. The ubiquitous and wide distribution of proTα in tissues and cells implied its participation in important cellular molecular circuits. Accumulating evidence available to date suggests a dual role for the polypeptide: in the

Table 1 Major representatives of structurally characterized α- and β-thymosins

Thymosin	Year of isolation/identification	Number of amino acids	Localization	Ref.
α-Thymosins				
Thymosin α1	1966	28	Ubiquitous, cytoplasm, serum, urine	[7]
Prothymosin α	1984	109–112	Ubiquitous, nucleus, cytoplasm, serum, urine	[18]
Parathymosin α	1985	101	Ubiquitous, nucleus, cytoplasm, serum	[19]
β-Thymosins				
Thymosin β4	1979	44	Ubiquitous, nucleus(?), cytoplasm, serum	[19]
Thymosin β9	1982	42	Ubiquitous in calf, cytoplasm	[19]
Thymosin β10	1983	42–44	Ubiquitous in man, mouse and rat, cytoplasm	[19]

cell, proTα controls the cell cycle, whereas extracellularly, the polypeptide exerts an immunomodulatory effect.

We now know that proTα is crucial for the survival and proliferation of normal cells [21]. Several reports have demonstrated that proTα mRNA increases in lymphocytes [21, 22] and in NIH3T3 cells stimulated to divide [23], and proTα antisense oligomers inhibit myeloma cell-division [24]. ProTα prevents apoptosis through inhibiting apoptosome formation [25], regulates DNA remodeling during proliferation, controls gene expression by directly binding to free-core histone H1 [26] and increases the accessibility of micrococcal nuclease to chromatin [27]. An association between the oncogene *c-myc* and *proTα* has also been reported; Eilers and coworkers showed that activation of *c-myc* in quiescent rat and mouse fibroblasts led to rapid increase in transcription of *proTα* and elicited reentry into and progression through the cell cycle [28]. Most recently, proTα-mediated nuclear import of Keap1, an inhibitor of the stress-sensor Nrf2, was proposed as a novel mechanism promoting cell survival [29].

Extracellularly, it has been suggested that proTα acts as a biologic response modifier (BRM) stimulating immune responses. The initial observations came from in vivo studies in animals, where proTα was able to protect immunosuppressed mice against infections caused by *Candida albicans* and other opportunistic infections [30]. When added in lymphocyte cultures, proTα enhanced antigen or mitogen-induced T cell proliferation, increased the production of interleukin (IL)-2 and the expression (both in number and in density) of its receptor on T cells [31, 32] and upregulated major histocompatibility complex (MHC) class II antigen expression on human monocytes [33]. ProTα was also shown to stimulate the cytotoxicity of natural killer (NK) cells [34] and the induction of lymphokine-activated killer (LAK) cells activity [35]. To clarify the molecular mechanisms underlying these immunomodulatory functions, Cordero and colleagues used radioiodinated, biologically active proTα to study the binding sites of the molecule on human lymphoblasts [36]. Two binding sites, of low and high affinity, were identified, both of which were specific for proTα, but not Tα1. In a subsequent study, Piñeiro and colleagues revealed three binding "partners" for proTα in the membrane of lymphoblasts, which formed a cap-like structure on one of the cell's poles and associated with lipid rafts [37, 38]. Most recently, proTα was suggested to ligate toll-like receptor (TLR)-4, signal through the TRIF-dependent pathway and induce interferon (IFN)-β production [39]. The concept currently under investigation is that proTα functions as an alarmin, which when experimentally proven would explain the proTα-induced activation of innate and adaptive immunity [40] and provide a link between the two discrete functions of the molecule.

Nevertheless, in view of the two aforementioned activities of proTα, over the years, several research groups have explored the possibility that the polypeptide may serve as a molecular marker for cancer prognosis and/or as a therapeutic agent for treating immunodeficiencies, autoimmune diseases and malignancies [41]. Herein, we will review data acquired with respect to proTα's (in comparison with Tα1) potential use as a cancer biomarker and as an option in the treatment of cancer.

ProTα as a cancer (bio)marker. Use in cancer diagnosis/prognosis

ProTα is probably the most well-studied thymosin with respect to its relevance to malignant cell transformation, and several studies have been carried out to investigate the levels of proTα in a variety of cancer cells.

In view that cancer cells are highly proliferative, it was not surprising to detect high proTα content, both at mRNA and protein level, in malignant tissues. With respect to the former, in rats, high proTα mRNA expression was detected in mitogen-stimulated thymocytes and splenic T cells [42], in proliferating pituitary tumors [43] and during the initial stage (first 6 weeks) of hepatic carcinogenesis, where proTα mRNA remained almost tenfold higher as the neoplasm progressed. In situ hybridization revealed that overexpressed proTα mRNA was restricted to neoplastic nodules in the hepatic tissue and to tumor cells invading blood vessels [44].

Table 2 Prothymosin α as a biomarker for cancer prognosis in humans

Type of cancer	Material detected	Correlation with	Ref.
Bladder	Protein in tissue and urine	Cancer detection; cancer grade; follow-up	[58, 67]
Breast	Protein in tissue and serum	Cancer grade; tumor recurrence; lymph node metastasis; distant metastases; risk of death; overall survival	[52–55, 65]
Colon	mRNA in tissue	*c-myc* expression	[45, 52]
Gastric	Protein in tissue	–	[63]
Head and neck	Protein in tissue	Tumor recurrence	[62]
Liver	mRNA in tissue; protein in tissue	*c-myc* expression; poor prognosis	[46, 60]
Lung	mRNA in tissue	Poor prognosis	[48]
Neuroblastoma	mRNA in tissue	*N-myc* expression	[47]
Pituitary	Protein in tissue	Tumor recurrence	[61]
Prostate	Protein in tissue	Cancer cell differentiation; tumor progression	[56, 57]
Rectal	mRNA in tissue	Effectiveness of radiotherapy	[51]
Rhabdomyosarcoma	mRNA in tissue	–	[49]
Thyroid	mRNA in tissue; protein in tissue and serum	Cancer cell differentiation	[50, 66]
Upper urinary tract	Protein in tissue	Tumor recurrence	[59]

In humans, high proTα mRNA levels were correlated with those of *c-myc* in colon [45] and liver cancer [46] and *N-myc* in neuroblastoma [47] (Table 2). However, although lung cancer tissues showed similarly elevated proTα mRNA expression, this was not paralleled by c-myc mRNA increase [48], indicating that simultaneous transcription of the two genes does not occur in all cancer types. In the last report, the possibility of using proTα levels as a biomarker was proposed, as high proTα mRNA was associated with poorer prognosis of lung cancer patients. ProTα mRNA was also found significantly elevated in rhabdomyosarcoma [49] and in well-differentiated thyroid carcinomas compared to thyroid adenomas and goiters [50]. By developing a highly sensitive real-time polymerase-chain reaction assay able to determine proTα levels in very small samples, such as those acquired through fine-needle aspiration, the authors suggested the potential usage of proTα as a novel proliferation marker for cancer development. From another aspect, of particular interest is the study of Ojima and coworkers [51], which opens new prospects for the use of the polypeptide in the clinic: upregulation of proTα mRNA in rectal cancer, predicts resistance to and effectiveness of radiotherapy.

At the protein level, results from cancer tissues verified the fact that aggressive tumors contain more proTα. We showed that proTα levels, as quantified by highly sensitive and specific radioimmunoassays for the amino- and the carboxy-termini of the polypeptide, were highly elevated in breast and colon cancers compared to the nearby healthy tissues [52]. At the same time, Dominguez and coworkers

confirmed our observation, reporting high proTα content in primary ductal carcinomas of the breast [53], and went further correlating tumor proTα levels with disease outcome. Specifically, they estimated that patients with cancer tissue proTα content over 124 ng/mg had a higher probability of developing distant metastases.

The clinical significance of these findings was next investigated. The aforementioned research teams showed that expression of proTα in human breast cancer (a) depends on the proliferation status of the tumor [54], (b) is associated with already established prognostic factors describing the metastatic potential of the tumor [54], (c) predicts the rate of tumor recurrence [55] and (d) relates to the overall survival of the patient [54, 55]. Therefore, both studies, although performed independently, came to the same conclusion that high proTα levels in primary breast tumors can be considered as an early "sign" correlated with a worse outcome of the disease.

Later on, Suzuki and colleagues studied proTα expression with respect to the differentiation and progression of human prostate cancers. By immunohistochemistry and immunoblotting, they showed that proTα levels consecutively increased as prostate tissue progressed from normal epithelium, through prostatic intraepithelial neoplasia to carcinoma [56]. Particularly, in prostate cancer lesions, intense nuclear staining for proTα was also reported [57]. Subsequent complementary research investigated proTα expression in human bladder cancer using tissue microarrays [58]. A statistically significant enhancement of proTα expression, also from the clinical aspect, was

recorded in bladder tumors compared with their normal adjacent tissues, which was most prominent in tumors of a higher grade. In upper urinary tract cancer, tumor recurrence was associated with proTα overexpression, but only when the polypeptide was localized in the tumor-cell cytoplasm [59]. These findings support the potential usage of proTα as a diagnostic tool, maybe also as a therapeutic target, for cancers of the urinary tract. Finally, immunostaining of hepatocellular carcinomas [60], pituitary tumors [61] and head and neck carcinomas [62], but not of gastric cancers [63], associated high proTα content with aggressive tumors and poor prognosis.

As proTα is an intracellular protein, studies to reveal its relevance to cancer were performed until recently in malignant tissues and their extracts. Such analysis of proTα content has the advantage of directly "viewing" the mRNA or protein content of the tumor itself, but also has the main disadvantage that sufficient amounts of tissue are not always available. In principle, cancer biomarkers are determined in biologic fluids, usually blood plasma/serum or urine. Indeed, soon after its identification, proTα was detected in human blood [64], its presence attributed either to leakage from damaged leukocytes or "secretion" from proTα-rich cells via an as yet unknown mechanism. Nevertheless, to date, proTα detection in cancer patients' sera is reported in only two studies (a) in breast cancer patients [65] and (b) in a secretome analysis of thyroid carcinomas [66]. Unfortunately, no correlation of blood proTα levels with cancer development and/or prognosis was found.

However, an interesting report revealed that urine proTα has the potential of being a useful tumor marker for the detection and follow-up of bladder cancer [67]. Urine samples, obtained from healthy volunteers, patients with urinary infection or bladder transitional and non-transitional cell carcinomas, were assayed for their proTα levels by a specific enzyme-linked immunosorbent assay. Subsequent comparison of the results showed that (a) urine proTα was higher in newly diagnosed bladder cancer patients, (b) patients with non-transitional cell carcinoma tumors had lower proTα in their urine compared to patients with bladder tumors, and (c) during a 3-month follow-up, urine proTα content increased when residual or a recurrent tumor was present after treatment. Although the cross-reactive material present in urine was not identified as intact proTα, this study opens new perspectives for the introduction of proTα as a novel cancer marker in the clinic, particularly for urinary tract carcinomas (Table 2).

The use of Tα1 as a cancer biomarker

As already mentioned, Tα1 was the first α-thymosin isolated, and therefore, its relevance to human disease, including cancer, was investigated early on. In contrast to the study of proTα expression mainly in cancer tissues, Tα1 content was investigated, almost exclusively, in plasma of patients with cancer. In the initial report by Hersh and colleagues [68], plasma Tα1 levels were higher in patients with various solid tumors, hematologic malignancies and immunodeficiencies. In lung cancer patients, mean Tα1 plasma levels, as determined by a sensitive radioimmunoassay specific for Tα1, were elevated compared to normal individuals, suggesting that plasma Tα1 could be used as a biomarker for lung cancer prognosis [69]. Further investigations aimed at determining the role of Tα1 in lung cancer revealed that its levels correlated neither with the stage nor the pathological subtype of the cancer. Similarly, patients with gastric cancer had elevated plasma Tα1, which did not correlate with the clinical stage of the disease [70]. Nevertheless, plasma Tα1 was higher in patients with lymph node metastases that stained positive for Tα1. The latter finding led the authors to conclude that higher Tα1 expression may be related to the more aggressive nature of the tumor.

It is of interest to note that the relevance of Tα1 levels with cancer development and/or prognosis is not as established as that of proTα. In our opinion, this observation is reasonable and results from the fact that the natural polypeptide, present intracellularly and participating in cell proliferative events, either normal or abnormal, is proTα [52]. Consequently, the presence of Tα1 in malignant tissues or in cancer patients' plasma could be either the result of extensive proTα cleavage and release, possibly associated with uncontrolled cancer cell growth, or the outcome of a randomly produced by-product of abnormal processes often seen in malignant lesions.

A-thymosins in cancer therapy

During the last decade, scientific interest has turned toward therapies that may restore immunity under conditions of immune-suppression associated with cancer, based on the assumption that a strong immune response will result in successful tumor growth inhibition. Among other thymic peptides tested alone or in combination with other BRMs such as cytokines, proTα and Tα1 have been suggested as candidate molecules for cancer immunotherapy. Actually, synthetic Tα1 formulated for subcutaneous (s.c.) injection (thymalfasin), under the trade name Zadaxin (SciClone Pharmaceuticals) [71], is the only α-thymosin widely tested in the clinical setting in humans for over 25 years, for the treatment of a variety of diseases, from cancer to viral infections and AIDS. Because of its excellent safety record, Zadaxin is currently approved in over 30 countries worldwide, as an immunostimulant. The drug is proposed to act by increasing the expression of MHC class I molecules, the numbers of CD4+, CD8+ T and NK cells and the

production of IL-2 and IFN-γ, while reducing T cell apoptosis and decreasing IL-4 and IL-10 production.

ProTα as an immunotherapeutic tool

As already stated, proTα exhibits some intriguing extracellular properties and is now classified in the family of BRMs. In the early 90s, evidence with respect to the anticancer potential of proTα came to light, although the mode of action of the polypeptide still remained obscure (Table 3). ProTα's efficacy was studied in three successive reports in an aggressive in vivo animal tumor model. Specifically, therapeutic administration of proTα (ca. 3 μg/mouse over 3 weeks) in DBA/2 mice inoculated with syngeneic leukemic L1210 cells inhibited the development of ascites in 20% of the animals and prolonged the survival of 40–60% of them by several weeks compared to controls (10 vs 2 weeks, respectively) [72]. A potential mechanism of action of the polypeptide was linked to the activation of peritoneal exudate cells in proTα-treated mice, since they were shown to produce six to eight times higher levels of tumor necrosis factor (TNF)-α and thus exhibit cytotoxicity against various cell lines, both TNF-α-sensitive and TNF-α-resistant. A detailed analysis of the immunological alterations induced upon proTα treatment was subsequently performed in the same animal model [73]. As expected, splenocytes from proTα-treated mice exhibited significantly higher ex vivo cytotoxic activity against NK-sensitive and NK-resistant targets and most importantly against the syngeneic tumor cells. Indeed, proTα was shown to increase both the number and the activation level of NK and CD3+ T cells, as well as of the CD8+ T cell population. In vitro, proTα-induced stimulation of splenocytes additionally revealed that lymphocyte proliferation and cytotoxicity were sustained by the high concentrations not only of TNF-α but also of IL-2 produced in response to proTα, and this most likely also occurs in vivo [74]. Of utmost importance was the observation that when proTα was administered in vivo simultaneously with the L1210 tumor cells, the polypeptide provoked the generation of tumor-specific CD8+ cytotoxic T lymphocytes (CTL), which, subsequently, lysed the syngeneic targets in a MHC-restricted fashion [74]. This enhanced CTL activity was supported by concomitantly induced L1210-specific CD4+ T cells and by the production of IL-2 and TNF-α in the spleen, peritoneal cavity and probably other lymphoid organs. This last report clarified an important issue regarding the activity of proTα; mice receiving only proTα in principle developed non-MHC-restricted cytotoxic activity (mediated by NK and LAK cells), whereas mice concomitantly receiving proTα and L1210 tumor cells developed both MHC-restricted (CTL) and non-MHC-restricted cytotoxic activities and survived longer. The overall results suggest that in vivo,

proTα acts upstream lymphocyte activation and its mode of action is IL-2-dependant. Moreover, depending on the presence of tumor-specific antigenic peptides or not, proTα can "manoeuver" anticancer immune responses shifting them toward the appropriate, most effective route of stimulation.

Following the studies in mice, the effect of proTα was next tested in humans (Table 3). Since cancer patients are generally characterized by decreased cytotoxic activity and dysfunction in lymphokine production, proTα's ability to restore these deficiencies was investigated in vitro, using immune-impaired peripheral blood lymphocytes (PBL) from cancer-bearing individuals [75]. ProTα normalized, to a great extent, the significantly reduced cell-mediated lympholysis (CML) and NK cell activity, which were by definition much lower in patients with advanced malignancies of various types compared to normal individuals. This restoration of immune responses was attributed to the appropriate modification of the levels of prostaglandin E2 (PGE2) and IL-2, cytokines that were down- and up-regulated by proTα, respectively. Accordingly, both CML and NK cell activity improved almost to normal, indicating that, in vitro, the polypeptide is capable of partially or even fully restoring cancer-induced immune deficiencies [75].

With respect to a specific type of cancer, results from a study in cells from melanoma patients showed that monocyte disorders could be partially normalized by proTα [76]. In fact, both normal donor- and melanoma patient-derived monocytes preincubated with proTα alone or in the presence of IFN-γ to enhance antigen presentation inhibited SK-MEL-28 melanoma cell growth in vitro. Moreover, monocytes from patients at earlier stages (I/II) of the disease showed higher tumoristatic activity than those at stage III and tended to respond better to preincubation with proTα followed by IFN-γ activation. The restorative effect of proTα on the impaired activity of LAK cells derived from melanoma patients was additionally shown [77]. However, lymphocytes from individual patients demonstrated a broad range of LAK cell responsiveness to proTα, which was dependant, among other factors, on the stage of the disease; cells from patients with early stage I/II melanomas were those that responded better to proTα treatment in vitro.

The effect of proTα was also studied on PBL and monocytes derived from colorectal tumor patients [78–80]. Once more, proTα in synergy with IL-2 could efficiently stimulate patients' LAK cell activity only at early stages (Dukes A/B) of the disease [78, 80]. ProTα acted via increasing the adhesion of lymphocytes to tumor targets (namely of SW620 colon carcinoma), and this effect was mediated by increased secretion of the deficient IL-2-induced IFN-γ. Phenotypic analysis of the proTα (in synergy with IL-2)-activated lymphocytes revealed that the polypeptide enhanced the expression of CD56, CD16/56 on NK cells, of

Table 3 Prothymosin α as a therapeutic agent for cancer treatment

Cancer/species	Synergy with	Effect	Ref.
In vitro			
Breast/human	Anti-CD3	Improvement of MHC- or non-MHC-restricted cytotoxicity	[81, 86]
	±fMLP	Increase of chemotaxis, oxidative responses and cytotoxicity of PMN	
Colorectal/human	IL-2	Enhancement of LAK activity; increased expression of cytotoxic surface molecules on NK, NKT-like cells and lymphocytes	[78–80, 86]
	±IFN-γ	Decrease of monocyte-derived TGF-β and PGE2 production; increase of monocyte-derived IL-1β and TNF-α production; monocyte-induced colon cancer cell cytotoxicity	
	±fMLP	Increase of chemotaxis, oxidative responses and cytotoxicity of PMN	
Lung/human	Anti-CD3	Improvement of MHC- or non-MHC-restricted cytotoxicity	[81]
Melanoma/human	±IFN-γ	Enhancement of PBMC-induced melanoma cell cytotoxicity	[76, 77, 86]
	IL-2	Restoration of LAK cell activity	
	±fMLP	Increase of chemotaxis, oxidative responses and cytotoxicity of PMN	
Ovarian/human	Anti-CD3	Improvement of MHC- or non-MHC-restricted cytotoxicity	[81]
Various tumors/human	–	Restoration of CML and NK cell activity; increase of PBMC-derived IL-2 production; decrease of PBMC-derived PGE2	[75, 84]
	IL-2	Increase of CD4+ T cell proliferation; generation of tumor-specific CTL	
In vivo			
Leukemia/mouse	–	Prolongation of survival	[72, 74]
		Enhancement of NK cell- and induction of LAK cell- activity in splenic lymphocytes	[73]
		Increase of TNF-α production; induction of tumoricidal activity by peritoneal exudate cells; selective expansion of leukemia-specific T cells	[74]

CD3/16/56 on NK-like T cells and of CD25, CD18 and CD11a on lymphocytes. Colon cancer patient-derived monocytes in vitro stimulated with proTα and/or IFN-γ exhibited elevated average antitumor activity against SW620 colon carcinoma cells, paralleled by production and secretion of reduced concentrations of transforming growth factor-β and PGE2 and increased levels of the proinflammatory cytokines IL-1β and TNF-α, as determined in the culture supernatants [79, 80]. The aforementioned results suggested that in vitro proTα can partially, however significantly, restore lymphocyte deficiencies present in cancer patients, by selectively regulating some functions of monocytes. The latter, in response to proTα, secrete cytokines and create a suitable cytokine milieu facilitating lymphocyte activation. It should be noted that proTα's activity highly depends on the stage of the disease, at least in melanoma and colon cancer patients, suggesting that the polypeptide can reverse mild but not severe tumor-induced immune-suppression.

A combination of two or more immunomodulatory molecules acting synergistically has often been shown to induce improved anticancer effects. Anti-CD3 monoclonal antibody has been reported to augment the cytotoxic functions of lymphocytes and has been successfully used for the large-scale expansion of non-MHC-restricted cytotoxic subsets for adoptive cellular immunotherapy, referred to as anti-CD3-activated killer (AAK) cells. Baxevanis and coworkers combined anti-CD3 with proTα and demonstrated that anti-CD3 plus proTα can improve AAK cytotoxicity of peripheral blood mononuclear cells (PBMC) derived from cancer patients with various malignancies, such as breast, ovarian and lung [81]. ProTα increased PBMC MHC- and non-MHC-restricted killing of tumor targets, but most importantly of autologous tumor cells by anti-CD3-activated CD8+ and CD56+ lymphocytes, implying that this thymic polypeptide might be active in vivo also.

Along the same line, combining proTα with low-dose IL-2 enhanced the induction of LAK cell activity [82], potentiating in particular the cytotoxicity of the CD16+ CD2- subset [83], whereas synergy between IL-2 and proTα increased the generation of tumor-specific cytotoxic T lymphocytes against autologous human carcinomas [84]. In the study of Voutsas and colleagues [84], PBMC obtained from cancer patients were cocultured in vitro with autologous tumor cells in mixed lymphocyte-tumor cultures in the presence of proTα and/or IL-2. The combination proTα/IL-2 increased the proliferation of CD4+ T cells and the generation of MHC class I-restricted autologous tumor-specific CTL. An important issue clarified by this study was that in order for proTα to exert its beneficial effect on CTL, the concomitant presence of autologous

CD4+ T cells and monocytes in the cultures was required. Indeed, the absence of either subpopulation resulted in strong impairment of both proliferative and cytotoxic responses, which could not be reversed even if proTα was added later on, suggesting that proTα-stimulated responses demand initial collaborative interactions between CD4+, CD8+ T cells and antigen-presenting cells.

Our research team assembled this accrued data on the mode of action of proTα in a cellular model. We used proteomics to detect intracellular protein changes induced in mononuclear cells, derived either from normal donors or cancer patients, over the course of a 3-day stimulation with proTα [85]. Based on the identification of immunologically significant molecules overexpressed downstream proTα activation, we proposed that initially, proTα triggers monocyte stimulation via TLR-ligation and signaling and enhances antigen presentation through MHC class II molecule upregulation. This event consequently promotes monocyte-T cell immune synapse. Subsequently, activated monocytes produce proinflammatory cytokines (IL-1), and highly activated, via T cell receptor triggering, T cells produce IL-2 and proliferate. Finally, proTα-stimulated PBMC express high levels of surface integrins (CD2) and of intracellular cytotoxic molecules (perforin), both of which contribute to the enhancement of their cytotoxic activity. Our results are in agreement with most recent data, suggesting that proTa is an endogenous TLR-4 ligand, which can induce the production of type I IFNs from macrophages [39].

ProTα exerts multiple immunological activities in vitro, and with this in mind, proTα's ability to enhance polymorphonuclear leukocyte (PMN) properties was also investigated [86]. ProTα-stimulated PMN isolated from patients with colorectal, breast and melanoma tumors showed increased chemotaxis and enhanced oxidative responses. ProTα-stimulated PMN became also more cytotoxic versus HCT-116 colon tumor target cells. The outcome of the latter study, in conjunction with the already analyzed effects of proTα, implies that the polypeptide acts pleiotropically and is able to improve distinct functions of immune cells under particular conditions of immunosuppression.

Although extensive research on the mechanisms of action underlying proTα's anti-tumor immunological effect has been performed [36], the polypeptide has not yet been introduced in immunotherapeutic protocols for treating cancer patients. There are several reasons for this ambiguity, one of the main being the unclear extracellular molecular mechanism of action of proTα. Another is proTα's length and acidic nature, as it cannot be easily synthesized and/or purified [87]. Most recent reports demonstrate that various areas of the molecule possess diverse activities, for example, amino acids 50–89 of proTα inhibit HIV-1 activity [39], proTαΔ1–29 and proTαΔ102–112 exert anti-necrosis effect [88] (Table 4). We recently reported that the decapeptide spanning residues 100–109 of proTα's carboxy-terminus (proTα(100–109); TKKQK TDEDD) is the actual immunoactive area of the polypeptide and a most potent lymphocyte stimulator [89]. ProTα(100–109) induces PBMC proliferation and cytotoxicity, promotes the maturation of dendritic cells (DC), adopts a β-sheet conformation, and its effects are sequence-specific and comparable to that of intact proTα. In an earlier study, we had shown that a slightly smaller segment (103–109) was also effective in restoring the immune function of PBMC obtained from cancer patients in vitro [90]. As for today, the peptide TKKQKTDEDD is known to be generated in vivo upon caspase-cleavage of proTα during apoptosis [91, 92], and additional mechanisms of its generation and immunoreactivity are currently being investigated in our laboratory. Therefore, besides the scientific interest in elucidating the immunological role of proTα and revealing a link between its two distinct functions, intracellular/proliferative and extracellular/immunomodulatory (Fig. 1), the identification of a smaller proTα-related peptide with immunomodulatory, possibly also with anticancer activity, may provide a novel compound that could help improve immune deficiencies associated with cancer.

Table 4 Prothymosin α peptides with recorded activity

Fragment (amino acid residues)	Nomenclature	Activity	Ref.
ProTα(1–28)	Tα1	Immunomodulatory; DC activation; anticancer; antiviral; antifungal; vaccine enhancement	[17]
ProTα(1–24)	Des (25–28) Tα1	n.t.	[19]
ProTα(1–35)	Tα11	Antifungal	[19]
ProTα(50–89)		Anti-HIV-1	[39]
ProTα(100–109)		Immunomodulatory; DC maturation; anticancer	[89]
ProTα(30–109)	ProTαΔ1–29	Anti-necrotic	[88]
ProTα(1–101)	ProTαΔ102–112	Anti-necrotic	[88]

n.t. not tested
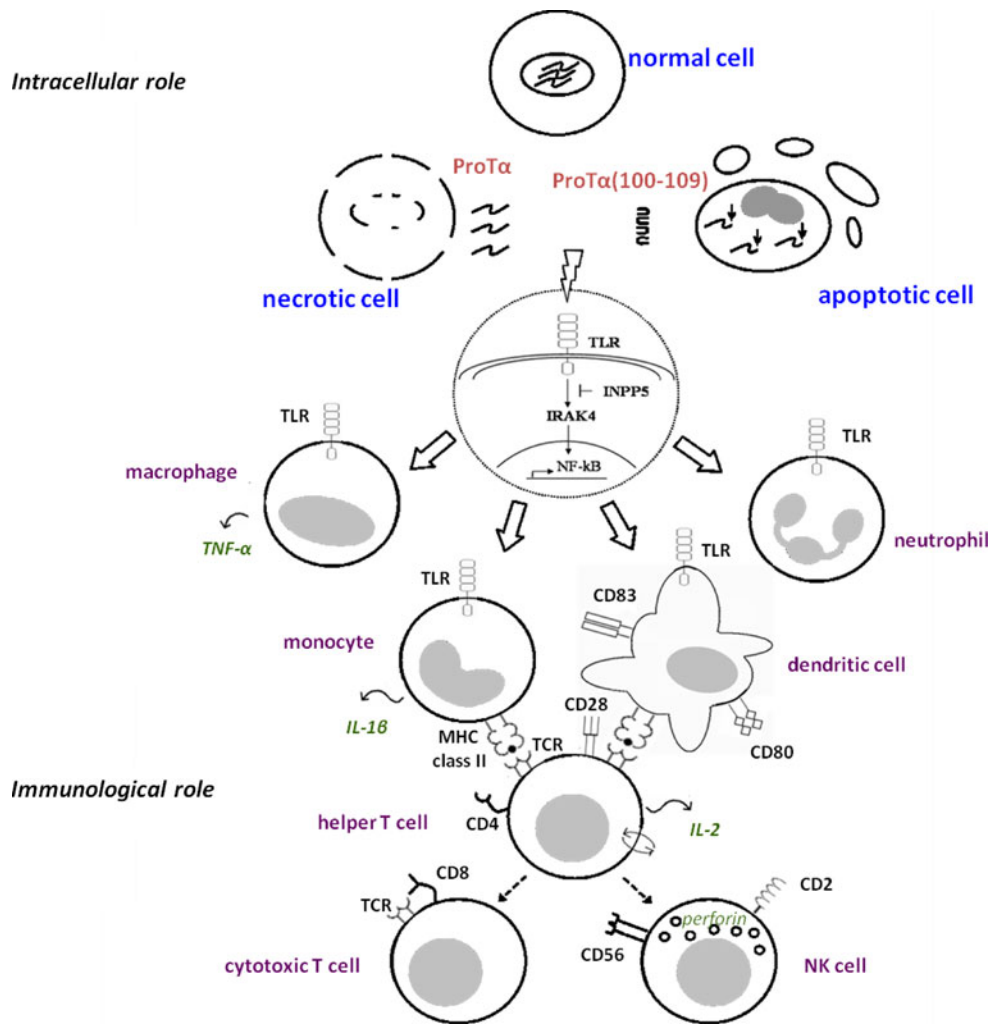
Fig. 1 A proposed scenario explaining how proTα exerts its dual (intra- and extracellular) biologic effect. In a normal cell, proTα is mainly localized in the cell nucleus, where it regulates gene expression and cell proliferation. Under abnormal conditions, cells responding to danger signals die via necrosis or apoptosis. During necrosis, cell components, such as intact proTα, are released extracellularly, due to cell membrane disruption. During apoptosis, proTα is relocalized in the cytoplasm, where its carboxy-terminus is cleaved by caspases. ProTα's truncation generates the immunoactive peptide, proTα(100–109), which polymerizes into β-sheet structures and is excreted from the cell. Extracellularly, both proTα and proTα(100–109) activate innate-immunity cells, for example, macrophages, monocytes, DC and neutrophils via TLR-ligation and signaling. Stimulation of monocytes and DC enhances antigen presentation and strengthens their synapsis with T cells. DC stimulate T cell proliferation and cytokine production, thus providing a favorable environment for enhancing specific and non-specific cytotoxicity. Consequently, effector cells produce lytic molecules (e.g., perforin) and upregulate adhesion-molecule expression (e.g., CD2). In the presence of specific antigens, CD8+ T cell cytotoxicity is enhanced. ∿ secretion of cytokine; ⇨ stimulation of proliferation

Preclinical studies with Tα1: the first step toward clinical trials

An early sign that Tα1 might be active against tumors and of clinical value was first suggested about 25 years ago, when Favalli and coworkers revealed that Tα1-treatment for 4 days, followed by a single injection of IFN-α/β, restored lymphocyte activity in cyclophosphamide (CY)-suppressed tumor-bearing mice [93]. Tα1 stimulated the tumor-specific T and NK cell-mediated cytotoxic activity of lymphocytes, whereas mice bearing Lewis lung (3LL) carcinoma administered CY and subsequently treated with Tα1+ IFN-α/β [94] or IL-2 [95], showed an increase of tumor-infiltrating lymphoid cells and consequently a dramatic and rapid tumor regression. In the same mouse model, Tα1 was shown to synergize with the cytokine cocktail IRX-2 (pooled supernatant of phytohemagglutinin-stimulated normal donors' PBL), leading to significant improvement in the survival of the animals [96].

Tα1 was further shown to be beneficial in rodents inoculated with other types of tumors (Table 5). When accompanied by low-dose IL-2 or IFN-α/β, CY-treated mice challenged with Friend's leukemia cells experienced complete tumor regression and a significant percentage of them

Table 5 Thymosin α1 as a therapeutic agent for in vivo cancer treatment

Cancer/Species	Treatment	Effect	Ref.
Preclinical/animal studies			
Colorectal/mouse	Tα1 + IL-2 + 5-FU	Increase of NK cell activity; increase of CD4+ and CD8+ cells in the periphery; prevention of extra-hepatic tumor spread; reduction in liver metastases; increase of survival	[98, 99]
Leukemia/mouse	Tα1 + (IL-2 or IFN-α/β) + CY	Complete tumor regression; cure	[97]
Lung/mouse	Tα1 + IFN-α/β	Increase of tumor-infiltrating lymphoid cells	[94–96]
	Tα1 + IL-2	Rapid tumor regression	
	Tα1 + IRX-2	Increase of survival	
Lymphoma/mouse	Tα1	TAM activation; increase of TAM-derived IL-1, TNF-α, nitric oxide, reactive oxygen intermediates; increase of pino/phagocytosis, antigen presentation and cytotoxicity of PMN	[100]
Melanoma/mouse	Tα1 + IFN-α/β +CY	Restoration of lymphocytic activity; stimulation of tumor-specific and NK cell cytotoxicity	[93]
Clinical studies			
NSCL/human	Tα1	Maintenance of normal CD4+ T cell counts; increase of relapse-free and overall survival	[101]
	Tα1 + IFN-α-2α + CDDP + V16	Complete/partial response in some patients; increase of median survival	[103]
	Tα1 + IFN-α + ifosfamide	Maintenance of normal CD4+, CD8+ T cell and NK cell counts; decrease of hematologic toxicity; increase of average response; increase of time-to-progression	[104]
	Tα1 + IFN-α + DTIC	Increase of NK cell cytotoxicity; increase of CD4+ T cell counts	[105]
Melanoma/human	Tα1 + IL-2 + DTIC	Complete response/stable disease in some patients; increase of median survival	[102]
	Tα1 + IFN-α + DTIC	Complete/partial response in some patients; increase of median survival; increase of median time-to-progression	[105]
	Tα1 + DTIC	Increase of patients experiencing tumor response; increase of duration of response; increase of median survival; increase of progression-free survival	[106]
Liver/human	Tα1 + TACE	Increase of CD3+, CD8+, CD16/56+ cell counts; increase of time to recurrence; increase of median and overall survival; full protection from bacterial infections	[107–109]

were even cured [97]. Similarly, in a colorectal cancer model in rats, administration of 5-fluorouracil (5-FU) followed by Tα1 and low-dose IL-2 increased peripheral blood NK cell activity, leading to considerable reduction in liver metastases and prevention of extra-hepatic tumor spreading [98]. When the same research team optimized the aforementioned triple chemo-immunotherapeutic combination, an increase in the number of CD4+ and CD8+ T cells in the periphery was observed, which prevented extra-hepatic metastases, and thus considerably increased the average survival time of the animals [99].

To elucidate the mode of action of Tα1, Shrivastava and colleagues extensively studied murine macrophages selectively activated by the peptide [100]. When mice bearing spontaneous T cell lymphoma (Dalton's lymphoma (DL)) were treated with Tα1, their tumor-associated macrophages (TAM) were directly responsive to the peptide, were highly activated, produced high levels of IL-1, TNF-α, reactive

oxygen intermediates, nitric oxide and exhibited increased pinocytosis, phagocytosis, antigen presentation and tumor cytotoxicity. When tested ex vivo, these TAM efficiently differentiated to DC with enhanced antitumor activity. Most importantly, adoptive transfer of Tα1-treated peritoneal macrophages in DL-bearing mice delayed tumor growth and prolonged their survival [100]. The outcome of this study demonstrated for the first time that, similarly to proTα, Tα1 acts upstream lymphocyte activation, by stimulating macrophages, rendering them capable of directly destroying cancer cells, and by indirectly activating other immune cells via cytokine secretion.

Clinical studies with Tα1

These animal studies indicated that Tα1 could be effective in humans, in principle for immune restoration following chemotherapy, and several trials to evaluate its efficacy

were licensed. The first was conducted in 1985 by the group of Alan Goldstein [101], where in post-irradiated non-small cell lung (NSCL) cancer patients (42 individuals), administration of Tα1 (900 μg/m^2/day) for 14 days showed normalization or maintenance of normal CD4+ T cell percentages and improvement of relapse-free and overall survival. Several years later in 1994 [102], patients with metastatic melanoma (46 in total) were treated with dacarbazine (DTIC), followed by 2 s.c. doses of Tα1 (2 mg/dose) and IL-2. Complete response was recorded in 2 patients and stable disease in 5, median survival was 11 months, and the toxicity of the regimen was acceptable. Further, a phase II study was conducted in 1995 to evaluate the efficacy of cisplatin (CDDP) and etoposide (VP-16) combined with Tα1 (1 mg/day; 11 doses s.c.) and low-dose IFN-α-2α for the treatment of 56 patients with advanced NSCL cancer [103]. Two patients experienced complete and 22 a partial response to treatment, which, as a whole, was well tolerated; median survival was 12.6 months. Although the chemotherapeutic drugs depressed NK cell activity and lymphocyte number, their adverse effects were less prominent in patients also receiving Tα1 + IFN-α-2α. A subsequent phase II randomized trial was conducted by Salvati and colleagues [104]. Twenty-two patients with advanced NSCL cancer were randomized to receive only ifosfamide (chemotherapy) or ifosfamide followed by Tα1 (same dose as in [103]) + low-dose IFN-α (chemo-immunotherapy). The latter enhanced the average response rate to 33% compared to 10% of chemotherapy alone. Although these percentages were not significant, the difference in time-to-progression was statistically significant, and patients receiving chemo-immunotherapy showed reduced hematologic toxicity and no decrease in CD4+, CD8+ T and NK cell counts [104]. Following the initial study of Lopez and colleagues [102], the evaluation of the effect of Tα1 was extended in a phase II open trial for advanced metastatic melanoma [105]. Out of 20 stage III/IV patients treated with DTIC + Tα1 (1 mg/day; s.c.; 8 doses at chemotherapy intervals for 3–9 cycles) + low-dose IFN-α, 5 patients experienced complete and 5 more a partial response. Median survival and median time-to-progression were 11.5 and 5.5 months, respectively, and no toxicity was recorded. As in NSCL cancer, patients treated with the triple combination showed improved NK cell activity and higher CD4+ T cell counts compared to those receiving only DTIC. Taken altogether, melanoma and NSCL cancer patients who were administered Tα1 did not benefit dramatically in terms of complete response or survival time, but incorporation of the peptide to standard chemotherapy regimes improved the functionality and counts of some lymphocyte subsets (NK cell cytotoxicity; CD4+/CD8+ T cell ratio).

Most recently, the largest, thus far, multicenter open randomized trial in metastatic melanoma patients evaluating Tα1 administration was completed. Maio and coworkers [106] recruited 488 stage IV patients from 64 centers and treated them with DTIC + IFN-α, DTIC + Tα1 or DTIC + IFN-α + Tα1 (1.6–6.4 mg; s.c.; 8 doses at chemotherapy intervals for 6 cycles). After a 24-month follow-up, the findings of this study were that adding Tα1 to chemotherapy (a) increased the number of patients experiencing tumor response (11.2 vs 4.1%); (b) increased their median overall survival by 2.8 months (9.4 vs 6.6); (c) improved the duration of response from 1.9 to 23.2 months; (d) increased 6-month progression-free survival by 12% (21.1 vs 9.1%); and (e) was safe and non-toxic. In agreement with the initial study of Schulof et al. [101], Tα1 could potentiate the efficacy of DTIC with or without the concomitant addition of IFN-α.

Three more trials, set on a different basis, evaluated the therapeutic potential of Tα1 in patients post-hepatectomy or with unresectable hepatocellular carcinoma treated with transarterial chemoembolization (TACE). In the first [107], Tα1 (900 μg/m^2; s.c.; twice weekly for 6 months) prolonged patients' survival and increased significantly the percentages of CD3+, CD8+, CD16/56+ cells after 1 month of treatment. In the second [108], Tα1 increased patients' time to tumor recurrence by 3 months and median survival by 2 months. Finally, in the most recent phase II randomized trial of Gish and colleagues [109], the same dose of peptide, but administered to the patients almost daily (1.6 mg; s.c.; 5-times weekly for 6 months), conferred full protection from bacterial infections and increased overall survival for over 13 months compared to Tα1-untreated patients.

As with any novel agent introduced in the clinic, an important issue that should be discussed at this point with respect to the use of Tα1 in humans is toxicity and safety. In the most recent methodical review of Wolf and colleagues [110], these parameters were extensively analyzed among other thymic peptides also for Tα1. According to the aforementioned clinical studies, Tα1 doses up to 6.4 mg were safe, did not induce toxicity and lacked severe side effects. When given in combination with chemotherapy, no adjunctive toxicity or interference between chemo- and immunotherapy was recorded; on the contrary in many cases, Tα1 allowed a general reduction in intrinsic toxicity of chemotherapeutic drugs. Immunological monitoring of some of the recruited patients showed a cyclic variation in immune responses, with a dramatic decline after chemotherapy and a boosting after Tα1 treatment, which occurred repeatedly during each cycle of therapy. Unfortunately, as this, unique for the moment, overall evaluation concludes, only weak evidence supports the clinical effectiveness of Tα1 on the management of cancer, although some patients undergoing chemotherapy might benefit via Tα1-induced reduction in the risk of infectious complications.

Discussing our current view of proTα and its potential clinical implications

All the aforementioned in vitro and in vivo studies with proTα, as well as the experience gained from the clinical trials conducted for Tα1, have brought to light some interesting observations. *Firstly,* the levels of proTα in cancer tissues, maybe also in biologic fluids such as urine, should be appropriately validated in order for the polypeptide to be used as a potential biomarker for cancer prognosis and progression. In the case of a heterogeneous disease like cancer, such a study will require samples from a cohort of many cancer patients, classified on the basis of their tumors' histological characteristics, stage of the disease, treatment approach and long-term follow-up, in conjunction with clinical responses and the values of already established biomarkers. *Secondly,* due to its elevated expression in cancer cells, targeting intracellular proTα, consequently inhibiting its activity, is predicted to cause the inability of cancer cells to proliferate and the induction of cancer cell apoptosis. The eventual development and in vivo testing of proTα-specific inhibitors could provide a future option for anticancer treatment. *Thirdly,* administration of proTα can provide, via a variety of mechanisms, some as yet unknown, the appropriate environment (i.e., a stimulating cytokine milieu) that enhances the efficacy of tumor-specific and non-specific effector cells, as already shown in vitro and in animals in vivo. As current approaches for cancer patient treatment and management encourage adjuvant therapy, proTα could be an excellent candidate that could act cooperatively with other cancer therapies, for example, chemotherapy and/or irradiation. *Finally,* from experience to date, administration of relatively high concentrations of proTα in animals and of Tα1 in animals and humans lacks toxicity and severe adverse effects.

The apparent question that arises is how can the same molecule, proTα, adopt different properties and carry out discrete and different functions depending on whether it is localized inside or outside the cell. This paradox is not unique to proTα. It has already been described for a series of proteins, prominent examples being heat-shock protein (HSP) 90 and high mobility group protein B1 (HMGB1) [111]. Both HSP90 and HMGB1 have well-characterized intracellular functions (as a molecular chaperone and a regulator of transcription, respectively), but when excreted, by non-classical ER-Golgi-independent secretory pathways, have documented extracellular roles, related to immunomodulation (as proinflammatory cytokine-like mediators) [112, 113]. HSP90 and HMGB1 interact with TLR, possibly also with other pattern recognition receptors (PRR) and sensitize innate immune cells (e.g., DC), thus promoting adaptive immune responses. These endogenous molecules are now classified in the diverse group of factors termed "alarmins," to which more molecules are constantly being added. In fact, thymosins are now considered as putative alarmins, as they share several common characteristics and modes of action with HSP and HMGB [40]. In the case of proTα, these shared properties were further complemented by the recent finding that in human macrophages, proTα signals via ligating TLR4, the most promiscuous of the TLR, binding also HSP90 and HMBG1 [39]. This data confirmed our previous observation that proTα-stimulated human monocytes upregulated the expression of IRAK4 [85].

From a practical viewpoint and in relevance to anticancer treatment, HSP90 and HMGB1 have recently been proposed as target molecules for developing novel anticancer strategies, and clinical trials have already been scheduled [114, 115]. Thus, our suggestion that proTα *per se* can be targeted to inhibit cancer cell development is not promisorial and could provide a novel means of anticancer treatment. Furthermore, taking as paradigms HSP90 and HMGB1, proTα could act as an adjuvant stimulating immune responses to specific peptides or tumor antigens, without causing severe adverse effects. Unpublished data from our laboratory show that in the presence of specific HER-2/neu epitopes, DC matured with proTα or its immunoactive decapeptide, proTα (100–109) [89], not only exhibit the appropriate phenotype, but are functionally competent to induce HER-2/neu-specific CD4+ and CD8+ T cells responses in vitro (Ioannou et al. manuscript in preparation). It is of interest to note that a smaller peptide deriving from HMGB1 has been reported to possess similar immune functionalities as the parental molecule [116].

The use of TLR ligands as adjuvants is a matter of intense investigation, as they are critical compounds for the success of antitumor vaccination. Notable examples are CpG oligodeoxynucleotides signaling through TLR9, single- or double (poly-I:C)-stranded RNA, ligating TLR7/8 and 3, imiquimod, a TLR7 agonist and monophosphoryl lipid A (MPLA), a TLR4 agonist, the latter being the only adjuvant approved for human use since alum [117]. The recent phase I/II trial in prostate cancer patients vaccinated with synthetic peptides combined with imiquimod proved that TLR7 coactivation was beneficial for the induction of strong antitumor immunity and the generation of clinical responses [118]. Therefore, at this stage, it would be quite challenging to propose proTα or its C-terminal fragment as new adjuvant candidates to enhance the efficacy of cancer vaccines.

Conclusion

Considering the distinct properties of proTα, the next step should be research aimed primarily at independently

evaluating the potency of proTα's combination with chemo-, radiotherapy and/or antitumor vaccination and maybe, its potential use in predicting cancer outcome. Using the appropriate control groups, randomized trials on cancer patients may offer the opportunity making advances in cancer treatment in the near future.

Accrued data point to the design of clinical studies integrating either the full length polypeptide or, preferably, its immunoactive fragment(s) (Table 4), as adjuvants to currently applied vaccination regimes for treating cancer patients. The lessons learned so far suggest that in order to verify the immunostimulating efficacy of proTα, administration of the polypeptide, or of its immunoactive fragment(s), at chemotherapy intervals, for definitely more than one chemotherapy cycle and possibly for a prolonged period of treatment, could be a therapeutic choice.

At this point, and albeit intensive research followed by published studies/trials on the ways of using this polypeptide in humans needs to be supplied, it is worth mentioning that proTα is available in the form of a "drug" [119], and indications, dosage and administration instructions for its use in patients are provided in the above mentioned website.

After almost three decades of research, the initial notion that the thymic peptide proTα might be useful for treating human diseases remains promising. In the case of cancer where immune system integrity is of the utmost importance, the use of proTα, most likely in combination with other immune modulators, still appears to offer potential.

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