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Thymopentin (TP5), an immunomodulatory peptide, suppresses proliferation and induces differentiation in HL-60 cells

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Abstract

Thymopentin (Arg–Lys–Asp–Val–Tyr, TP5) has shown immuno-regulatory activities in humans. In the present study, we investigated the effects of TP5 on the proliferation and differentiation of a human promyelocyte leukemia cell line, HL-60. It is noteworthy that TP5 displayed concentration-dependent inhibitory effects on the proliferation and colony formation of HL-60 cells. Furthermore, the decrease or even disappearance of AgNORs from nucleoli was observed in HL-60 cells after the treatment with TP5. The suppression induced by TP5 was accompanied by an accumulation of cell cycle in the G0/G1 phase. Moreover, TP5 significantly increased the NBT-reduction activity of HL-60 cells. Cytofluorometric and morphologic analysis indicated that TP5 had induced differentiation along the granulocytes lineage in HL-60 cells. D-tubocurarine (TUB) significantly antagonized the inhibitory effects induced by TP5, whereas atropine did not exhibit such effect. All the results indicated that TP5 was able to significantly inhibit proliferation and induce differentiation in HL-60 cells. Our observations also implied that TP5 not only acted as an immunomodulatory factor in cancer chemotherapy, but is also a potential chemotherapeutic agent in the human leukemia therapy.

Keywords: Thymopentin; HL-60; Differentiation; Proliferation inhibition; Chemotherapeutic agent

1. Introduction

A common goal of cancer therapy is the restoration of normal growth control in transformed tissues [1]. One area that has been intensively studied in recent years is biological modifiers of cancer growth which are designed to retard proliferation [2], to induce differentiation of these cells to a quiescent, non-dividing stage [3,4], and/or to promote cell death in malignant or pre-malignant cells [5,6]. The immortal human myeloid leukemia cell line, HL-60, serves as an in vitro model which has been extensively used to gain insights into the processes of myeloid cell differentiation and their control mechanisms. These cells are bi-potent, i.e., they can be induced to differentiate either into granulocytes on exposure to retinoic acid (RA) or dimethyl sulphoxide (DMSO) [7,8], or into monocytes/macrophage-like cells with various other agents [9].

Previous observations have demonstrated that some immunomodulatory peptides, like TP5 and thymosin, acted as immunomodulatory agents in cancer chemotherapy [10,11]. Recently, our research group has observed that thymosin α_1 ($T\alpha_1$), a well-known immune system enhancer for the treatment of various diseases, was able to significantly suppress proliferation and induce apoptosis in human leukemia cell lines [12]. TP5 comprises the amino acids (Arg–Lys–Asp–Val–Tyr) and represents residues 32–36 of the nuclear protein thymopoietin (TP) [13]. TP5 has been successfully used in humans to improve immunological parameters in neoplastic, immune deficiency and autoimmune diseases [11,14]. It rectifies imbalances in the immune system without observable side effects, even at very high doses. Furthermore, TP5 also exhibits a particular value in humans with certain

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recurrent viral diseases [15]. However, little if anything is known about the effects of this immunomodulatory peptide on the proliferation and differentiation of human promyelocyte leukemia cells.

In the present study, the effects of TP5 on the proliferation and differentiation of HL-60 were investigated. The anti-proliferation and differentiation effects induced by TP5 were determined by colony formation in soft agar, silver staining and measuring cell viability, cell cycle phase distribution, CD11b and CD14 cell surface antigen (markers of differentiation) expression and NBT-reduction activity. The roles of nicotinic acetylcholine receptors (nAChRs) and muscaric acetylcholine receptors were also investigated.

2. Materials and methods

2.1. Chemicals

TP5 was kindly gifted by Dr. Yang of Hainan Zhonghe Pharmaceutical CO., LTD. (Hainan Province, China). The purity was higher than 99% (HPLC) and the structure was determined by ^{1}H-NMR and TOF-ESI MS. TP5 was dissolved in sterile distilled water, and the solutions were divided into aliquots and stored in 2 mL plastic tubes at −20 °C. The aliquots were thawed and used on the day of the experiment.

2.2. Cells culture

HL-60 cells were cultured in RPMI-1640 medium (Gibco-BRL, USA) supplemented with heat-inactivated 10% fetal bovine serum (Hyclon, USA), 2 mM L-glutamine, penicillin (100 IU/mL) and streptomycin (100 μg/mL). The cells were incubated in a humidified incubator containing 5% CO_2 at 37 °C.

The peripheral blood mononuclear cells (PBMCs) were isolated from human peripheral blood. The peripheral venous blood was drawn from healthy volunteers. Blood samples were collected into sterile bags each containing 25 μL of citrate-phosphate dextrose anticoagulant and diluted to 1:2 with phosphate buffer saline (PBS). The PBMCs were separated by density gradient centrifugation at 1 800 rpm for 20 min on Ficoll-Isopaque (density, 1.077 g/mL). The isolated PBMCs were washed twice, counted by Trypan blue exclusion and suspended in RPMI-1640 growth medium supplied with 15% fetal bovine serum. The cells were incubated in a humidified incubator containing 5% CO_2 at 37 °C.

2.3. Effects of TP5 on the proliferation of HL-60 cells

HL-60 cells were plated at a density of 1×10^5 cells/mL, and cultured for 120h in a medium containing TP5 at various concentrations (100–800 μmol/L). To quantify the number of cells after treatment, cells were counted at different time points of incubation using a hemocytometer under a light microscope (Olympus, Japan). Cell viability was determined by the Trypan blue exclusion assay. Viability was calculated as the ratio of living cells to the total cell population.

2.4. Effects of TP5 on the clonal proliferation of HL-60 cells in soft agar

The Effects of TP5 on clonal proliferation of HL-60 cells were determined by the inhibition of colony formation in soft agar. The potency of TP5 was measured by extensive concentration–response studies. HL-60 cells were plated into 24-well, flat-bottom plates at a density of 1×10^4 cells/mL using a two-layer soft agar system with a total volume of 400 μL, as described in previous report [1]. The feeder layer was prepared with agar (1%) that had been equilibrated to 42 °C. Prior to addition of this layer to the plate, TP5 at various concentrations (100–800 μmol/L) was pipetted into the wells. 72 h and 96 h after incubation, the rest of TP5 was removed from the wells, respectively. And then, after 4 days' growth in soft agar, the colonies (≥50 cells) were counted with an inverted microscope (Olympus, Japan). All experiments were done at least four times in triplicate dishes per experimental point.

2.5. Effects of TP5 on the proliferation in PBMCs

The effects of TP5 on PBMCs in vitro were determined by the cell density and viability measured by Trypan blue exclusion assay. PBMCs were plated at a density of 20×10^5 cells/mL, and cultured for 120 h in a medium containing TP5 at various concentrations (100–800 μmol/L). After staining with Trypan blue, viable cells were counted in a microscopic counting chamber using a hemocytometer.

2.6. Silver staining

The proliferation activity of HL-60 cells was examined by silver staining. Quick silver staining was carried out according to the method described by Smetana et al. [16]. 50% $AgNO_3$ and 20% gelatin (with 1% formic acid) were evenly mixed in a ratio of 2:1, and were then added to the newly prepared cells. After covered with coverslips, each group of cells was incubated under safelight condition at room temperature for 30 min. The slides were then rinsed thoroughly in distilled water, and observed under the microscope after being dried.

2.7. Effects of TP5 on cell cycle distribution of HL-60 cells

Effects of TP5 on cell cycle distribution were determined by flow cytometry [17]. Exponentially proliferating cells (10^5 cells/well) were placed in 24-well tissue culture plates and cultured for 4 days in RPMI-1640 medium with TP5 (100–800 μmol/L) under the same conditions as mentioned above. Control cells were adjusted to 60–70% confluency at the time of FACS analysis. Each group of cells was collected, washed, resuspended in PBS containing 0.2% Triton-X and 100 μg RNase, and incubated at 37 °C for 1 h. Then the cells were washed with PBS and then incubated with 0.5 mL of DNA-staining solution containing propidium iodide (PI) (50 μg/mL) at 4 °C for 20 min. Cell cycle distribution was determined on a Becton-Dickinson (Braintree, MA) FACScan Flow Cytometer and CellFIT Cell-Cycle Analysis Software. Experiments were repeated at least three times.

2.8. NBT-reduction assays

As a marker of cell differentiation, the reduction of NBT in HL-60 cells was determined by a previously described method [18–20]. HL-60 cells were plated at a density of 1×10^5 cells/mL, and treated with TP5 (100–800 μmol/L) for 72 h. Briefly, after each treatment, cells at a density of 1×10^6/mL were incubated for 1 h in RPMI-1640 growth medium with 60 ng/mL of TPA and 0.5 mg/mL of NBT at 37 °C. Then, 2 mol/L HCl was added to each sample to stop the reaction. After the medium was chilled on ice for 30 min, the cells were collected and suspended in DMSO. The absorbance (OD) of the NBT reduction product (formazane solutions) was measured at 590 nm in a kinetic microplate reader.

2.9. Cell surface antigen expression analysis

Cells (1×10^5 cells/well) were placed in 24-well tissue culture plates, and cultured for 3 days in RPMI-1640 medium with TP5 (200–800 mol/L) under the same conditions as described above. Each group of cells was then collected and washed with PBS. Then, the cells (1×10^6 cells) were re-suspended in 100 mL diluent solution containing 1% bovine serum albumin (BSA) and 1% sodium azide and incubated with 10 μL human monoclonal FITC conjugated CD11b antibody (Sigma, USA) and 10 μL human monoclonal PE conjugated CD14 antibody (Sigma, USA) for 30 min at room temperature without light, respectively. The cells were washed once with diluent solution and then fixed in 300 μL of PBS containing 2% paraformaldehyde. Fluorescence was detected on a Becton Dickinson FACScan™ at the excitation wavelength of 490 nm and emission wavelength of 520 nm. Results were recorded as the mean fluorescence index, which is the product of the % fluorescence at the mean fluorescence intensity, with 1×10^4 cells being counted per treatment.

2.10. Acetylcholine receptors antagonists investigation

TUB chloride (Fluka), a nAChRs antagonist, was tested at a wide range of concentrations from 0.01 to 200 μg/mL. Atropine (The First Drug Company of Dong-ting Lake, Hunan, China), a muscaric acetylcholine receptor antagonist, was also investigated at a range of concentrations from 0.005 to 100 μg/mL. The experimental procedures were consistent with those described in the literature [21].

2.11. Statistical analysis

Results were expressed as the mean±S.E.M. Responses were analyzed with a one-way ANOVA and Scheffe's *F*-test. Individual group comparisons were made using Student's *t*-tests. A $p<0.05$ was used as the criterion for statistical significance.

3. Results

3.1. Effects of TP5 on the proliferation in HL-60 cells

TP5 (100–800 μmol/L) caused a significant suppression of the proliferation in HL-60 cells in a concentration-dependent manner (Fig. 1). ED_{50} (50% of inhibit proliferation) values of TP5 at 48 h, 72 h and 96 h in HL-60 cells were 490 μmol/L, 331 μmol/L and 560 μmol/L, respectively. The anti-proliferation effect of TP5 after 72 h of incubation displayed the most potency among those measured after 48 h, 72 h, and 96 h of incubation (compared with those of after 48 h and 96 h incubation). Furthermore, morphological assessment of HL-60 cultures revealed that there were no significantly visible morphological differences between the HL-60 cells that are treated with TP5 and the ones that are not (Fig. 2).

3.2. Clonal inhibition of proliferation of HL-60 cells mediated by TP5

We examined the effect of TP5 on the clonal proliferation of HL-60 cells by inhibiting colony formation in soft agar (Fig. 3). TP5 was able to inhibit the clonal proliferation of HL-60 cells at

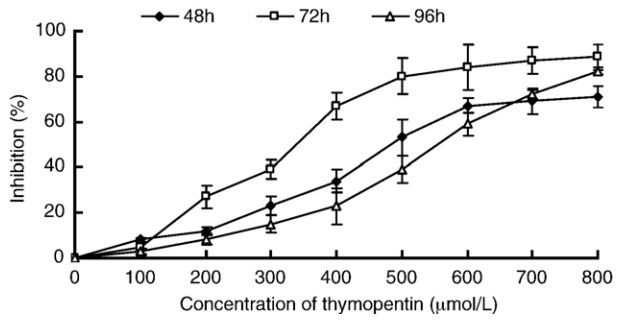

Fig. 1. The inhibitory effect of thymopentin on the proliferation of HL-60 cells. This effect of thymopentin against HL-60 cells in vitro was determined by the cell density and viability using Trypan blue exclusion assay. HL-60 cells were plated at a density of 1×10^5 cells/mL, and cultured for 96 h in medium containing thymopentin at various concentrations (100–800 μmol/L). The cells were counted with a hemocytometer. Values represent the mean±S.E.M. of data from at least three different experiments.

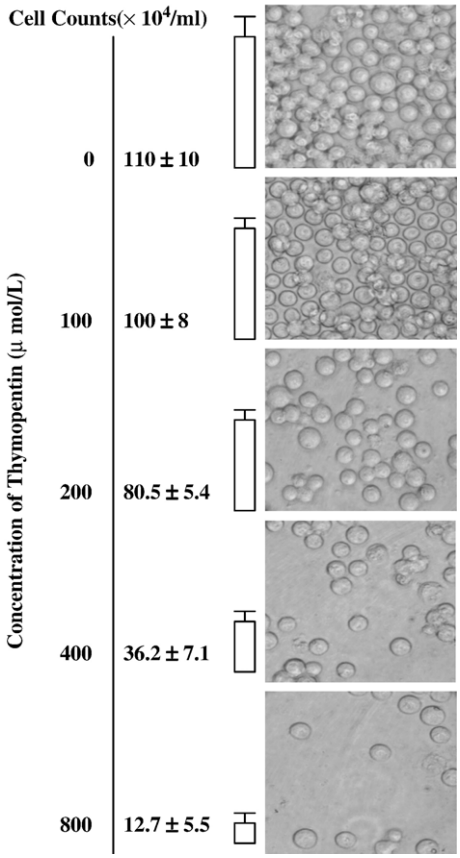

Fig. 2. Photomicrographs of HL-60 cells after 72 h of incubation with thymopentin (100–800 μmol/L) showed the concentration-dependent inhibitory effect of thymopentin on cell proliferation. HL-60 cells were plated at a density of 1×10^5 cells/mL and counted with a hemocytometer. Cell density decreased in a concentration-dependent manner as the concentration of thymopentin is increased from 100 to 800 μmol/L. Values for cell counts are mean±S.E.M. There is no significantly visible change in cell morphology of HL-60 cells treated with thymopentin (magnification ×1920).

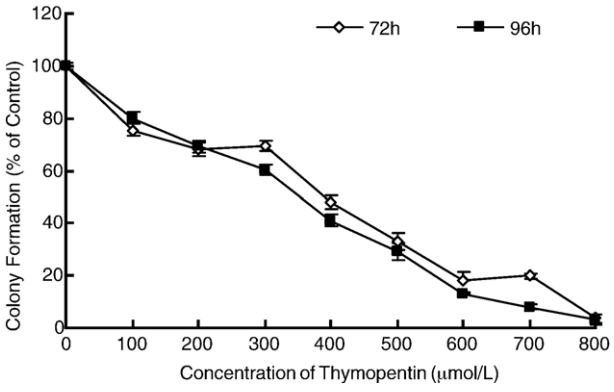

Fig. 3. Concentration–response effects of thymopentin on clonal proliferation of HL-60 cells. The effect of thymopentin on clonal proliferation of HL-60 cells was determined by inhibiting colony formation in soft agar. HL-60 cells were plated at a density of 1×10^4 cells/mL, and cultured in mediums containing thymopentin at various concentrations (100–800 μmol/L). After 3–4 days of growth in soft agar, the colonies (≥50 cells) were counted with an inverted microscope. Results are expressed as the mean±S.E.M. Each point represents a mean of data from at least four experiments with triplicate dishes. Control plates: untreated cell.

72 and 96h in a concentration-dependent manner. ED50 values for TP5 at 72 h and 96 h were 404 μmol/L and 358 μmol/L, respectively.

3.3. Effects of TP5 on the proliferation of PBMCs

We also investigated the effects of TP5 on the proliferation of PBMCs (data not shown). TP5 displayed no inhibitory effect on the proliferation of PBMCs within the concentration range from 100 μmol/L to 800 μmol/L. This suggested that TP5 has no potential side effect on normal blood cells even though it exhibited significant inhibitory effects on the proliferation of HL-60 within the concentration range from 100 μmol/L to 800 μmol/L.

3.4. Silver reaction for proteins characteristic for interphase AgNORs

In untreated control cells, nucleoli in silver stained cells appeared as clusters of black particles–silver stained particles (SSPs) in less distinct 'nucleolar matrix' (Fig. 4A). Numerous silver stained particles representing silver stained nucleolus organizer regions (AgNORs) (large arrows, Fig. 4A) were observed in large nucleoli of nuclei in control cells. It is noteworthy that the number of AgNORs (large arrows, Fig. 4B) in a distinct nucleolar 'matrix' was reduced after treatment with 400 μmol/L TP5. Furthermore, some cells with a single SSPs (small arrows, Fig. 4B) were also observed in cells treated with 400 μmol/L TP5. After treatment with TP5 of a relatively high concentration (600 μmol/L) for 72 h, no visible AgNORs and only some single SSPs (small arrows, Fig. 4C) were observed in nucleoli of HL-60 cells. Moreover, visible AgNORs and SSPs almost disappeared in nucleoli of HL-60 cells (large thick arrow, Fig. 4D) treated with 800 μmol/L TP5. AgNORs decreased or disappeared from nucleoli in a concentration-dependent manner (data not shown).

3.5. Effects of TP5 on the cell-cycle distribution of HL-60 cells

We also studied the cell-cycle distribution of HL-60 cells after treatment with TP5 (100–700 μmol/L) for 72 h or 96 h (Fig. 5). The percentage of HL-60 cells in the G0/G1 phase increased from 32% (vehicle-treated cells, control group) to 44.4%, 58.7% and 60.8% after treatment with 200, 500 and 700 μmol/L TP5 for 72 h, respectively. However, the percentage of the S phase decreased from 62.8% (control cells) to 51%, 37.8% and 36.4% after treatment with 200, 500 and 700 μmol/L TP5 for 72 h, respectively (Fig. 5A). Similar results were also observed after 96 h (Fig. 5B). These results indicated that TP5 could promote cell-cycle accumulation in the G0/G1 phase of HL-60 cells. These results are consistent with the results of cell numbers and viability assays. Therefore, the HL-60 cell growth inhibition exerted by TP5 is probably due to the inhibition of cellular progression to G0/G1 phases.

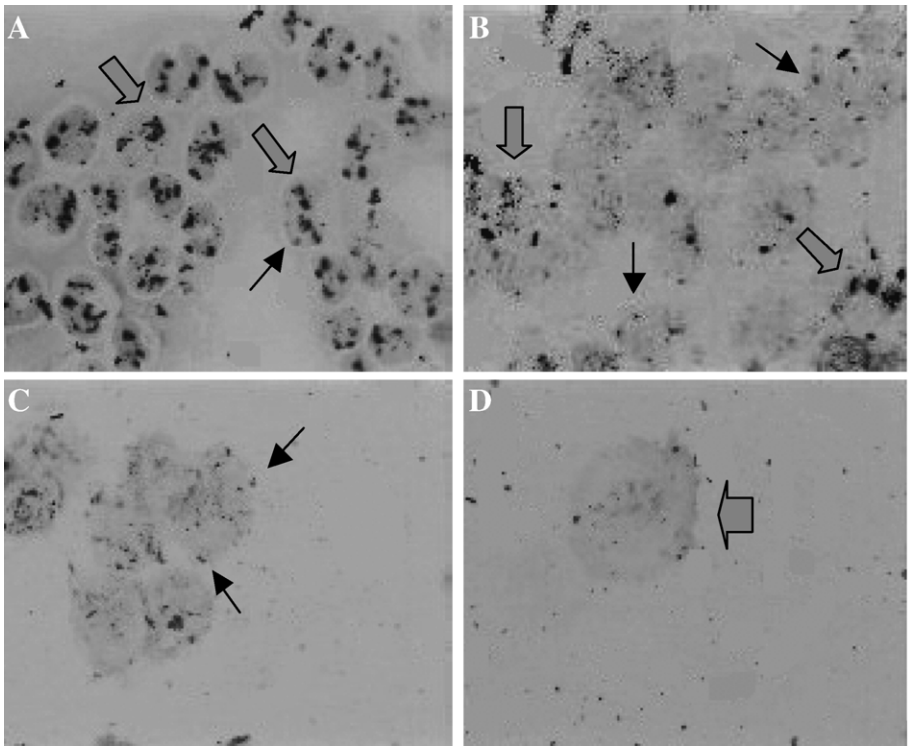

Fig. 4. Photomicrographs of silver stained HL-60 cells. After HL-60 cells were incubated with thymopentin for 72 h, their proliferation activity was determined by silver staining. (A) control cells (untreated with thymopentin); (B) cells treated with 400 μmol/L thymopentin; (C) cells treated with 600 μmol/L thymopentin; (D) cells treated with 800 μmol/L thymopentin. The silver stained nucleolus organizer regions (AgNORs) in nucleoli of cells were observed under the microscope. Large arrows: numbers of AgNORs; small arrows: small single black-silver stained particles (SSPs); large thick arrow: the absence of AgNORs in nucleoli (magnification × 1600).

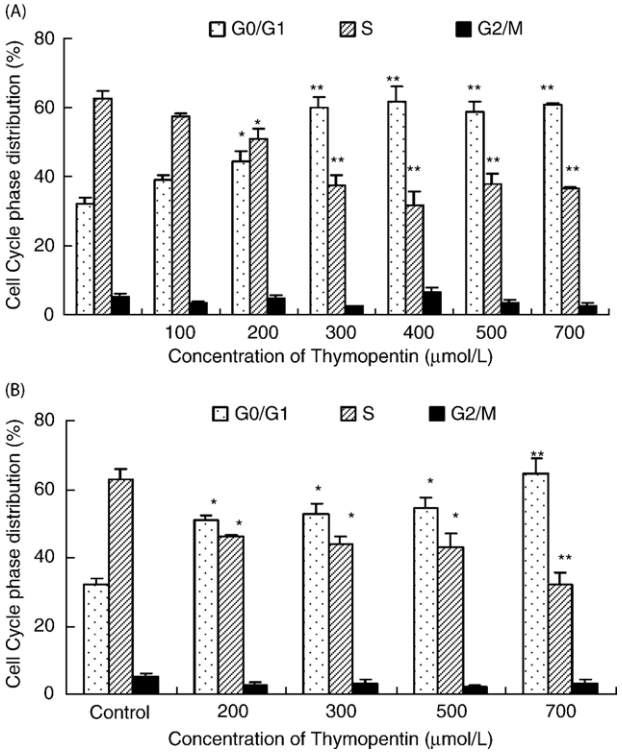

Fig. 5. Changes of cell cycle distribution of HL-60 cells after treatment with thymopentin. HL-60 cells were plated at a density of 1×10^5 cells/mL, and cultured in mediums containing thymopentin at various concentrations (200–800 μmol/L). After incubation, the cell cycle distribution was measured by flow cytometry. (A) Cells treated with thymopentin for 72 h, (B) Cells treated with thymopentin for 96 h. Control: untreated cells. Values represent the mean±S.E.M. of data from three different experiments. *$p<0.05$ vs. control, **$p<0.01$ vs. control.

3.6. Induction of NBT-reduction activity of HL-60 by TP5

We further assessed NBT-reduction in the HL-60 cells treated with TP5 to determine whether it induced the differentiation of the cells (Fig. 6). Rarely were NBT positive cells observed throughout the 72 h incubation with the control cells. TP5 (≥500 μmol/L) significantly induced the generation of NBT-reducing activity, and the absorbance of the NBT reduction product ($OD_{590nm}/1\times10^6$ cells) increased from 0.14 (control) to 0.58 (700 μmol/L) at 72 h. More and more cells became NBT positive as the concentration of TP5 increased.

3.7. Effects of TP5 on the CD11b and CD14 antigens on cell-surface

Expression of cell-surface CD11b and CD14 antigens is one of the major differentiation markers of HL-60 cells. CD11b is a cell surface marker for differentiation into either monocytes or granulocytes [22]. As shown in Fig. 7, TP5 concentration-dependently increased the number of CD11b antigen expressing cells within a concentration range from 200 to 800 μmol/L. However, TP5 had weak or virtually no such potency in increasing monocytic surface CD14 antigen expression even at a concentration up to 800 μmol/L. These findings clearly

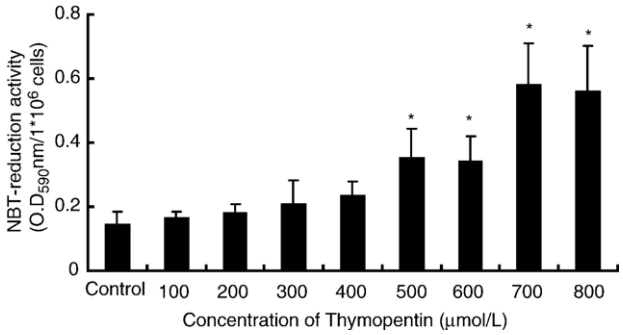

Fig. 6. Effects of thymopentin on the differentiation of HL-60 cells were determined by NBT-reduction. HL-60 cells were plated at a density of 1×10^5 cells/mL, and were treated with thymopentin (100–800 μmol/L) for 72 h. Thymopentin significantly induced the generation of NBT-reduction activity in a concentration-dependent manner. Values represent the mean±S.E.M. of data from five different experiments.

indicated that TP5 could induce differentiation of HL-60 cells along the granulocytes lineage.

3.8. Effects of acetylcholine receptors antagonists

TUB, a nAChRs antagonist, concentration-dependently reduced the inhibitory effects of TP5 on the proliferation of HL-60 (Fig. 8A), whereas atropine, a muscaric acetylcholine receptor antagonist, did not exhibit such effects (Fig. 8B). Furthermore, cell survival (% of control) displayed no significant changes before and after only TUB or atropine administration (data not shown). TUB could significantly reduce the effects of TP5 at a high concentration (200 μg/mL). However, TUB could only partially antagonize the effects at relatively low concentrations (<100 μg/mL).

4. Discussion

The present investigation confirmed the effects of TP5 on the proliferation and differentiation of human promyelocytic cell

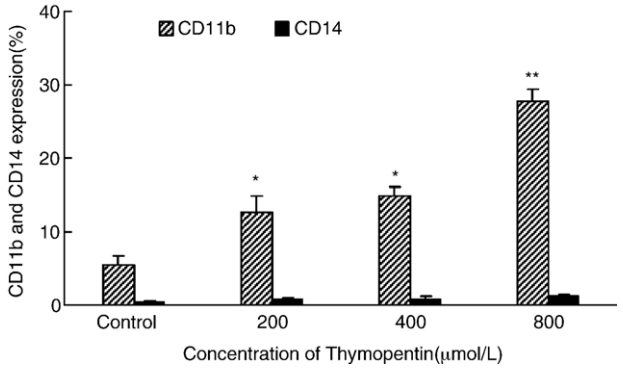

Fig. 7. Cell surface antigen CD11b and CD14 expression analysis. HL-60 cells were plated at a density of 1×10^5 cells/mL, and cultured for 72 h in mediums containing thymopentin at various concentrations (200–800 μmol/L). Thymopentin (200–800 μmol/L) significantly increased the number of CD11b antigen expressing cells in a concentration-dependent manner. However, thymopentin had weak or virtually no such potency in increasing CD14 antigen-expression even at a concentration up to 800 μmol/L. Control: untreated cells. Values represent the mean±S.E.M. of data from three different experiments.

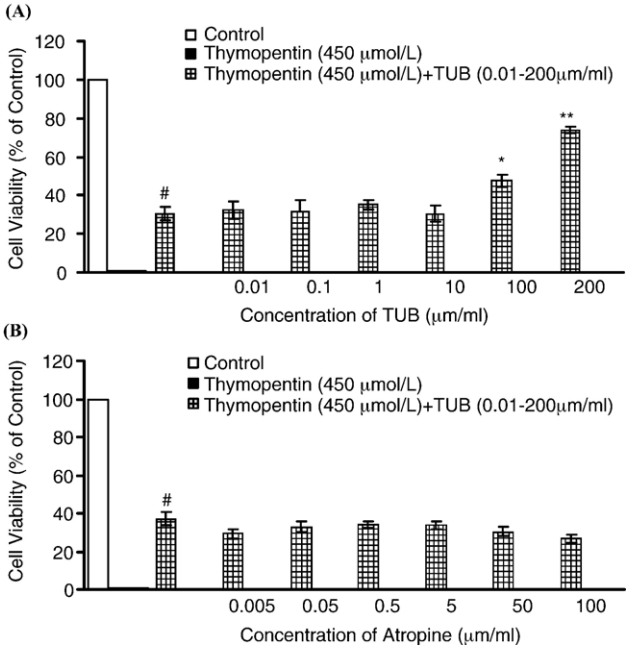

Fig. 8. The blocking effects of TUB (A) and atropine (B) on the inhibitory effects of TP5 on the proliferation of HL-60 cells. The cell viability was determined by Trypan blue exclusion assays. Results are expressed as the mean ± S.E.M. of 3–5 measurements. Control: cells were cultured in mediums without TP5; $^{\#}p<0.01$ vs. control; $^{*}p<0.05$ vs. TP5-treatment groups; $^{**}p<0.01$ vs. TP5-treatment groups.

line HL-60. A combination of retinoic acid (RA) with the crude thymus extract Thymex-L can increase the sensitivity of HL-60 cells for RA [23]. Gonser S et al. demonstrated that TP5 treatment of irradiated HL-60 cells increased the number of cells undergoing apoptosis [24]. Recently, our research group has observed that $T\alpha_1$ was able to significantly suppress proliferation and induce apoptosis in human leukemia cell lines [12]. All the observations mentioned above indicated that these immunomodulatory peptides could act on the human leukemia cells. Our present observation confirmed the effects of TP5 on the proliferation of HL-60 cells. The results of NBT-reducing activity and CD11b and CD14 cell surface antigen expression also suggested the role of TP5 on the differentiation of HL-60 cells. These results further provided direct evidence that immunomodulatory peptides were able to significantly affect the proliferation and differentiation of human promyelocytic cells.

In our present investigation, TP5 caused a significant suppression of the proliferation in HL-60 cells within a concentration range from 100 to 800 μmol/L, but not at relatively low concentrations (≤50 μmol/L), which was in agreement with a previous report [23]. It has been observed that the number of SSPs, representing AgNORs, reflects not only the nucleolar biosynthetic activity but also the cell proliferation [25]. In our findings, the decrease or disappearance of AgNORs from nucleoli was observed in HL-60 cells after treatment with TP5. The decrease or disappearance of AgNORs represented further morphological signs of TP5 on the proliferation of HL-60 cells. In the present study, the suppression induced by TP5 (100–700 μmol/L, after 72 h or 96 h of incubation) was accompanied by an accumulation of cell cycle in the G0/G1 phase. However, Gonser S et al. suggested that TP5 had no effect on the cell cycle distribution of non-irradiated HL-60 cells at concentrations of 1 mmol/L, 3 mmol/L and 10 mmol/L after 48 h of incubation, but increased accumulation of cells in the G2/M phase of the cell cycle after irradiation [24]. The discrepancy between our data and these data may be induced by differences in drug concentration, cell density, incubation time (72 h or 96 h versus 48h) and the situation of cells (like non-irradiated or irradiated cells). Many factors can lead to a cell cycle accumulation in the G0/G1 phase, like cyclin D, cyclin E1, bcl-2, c-myc and the cyclin-dependent kinase inhibitors (CDKIs) [1,26,27]. These cell cycle related mechanisms may take effect in the HL-60 cell cycle accumulation induced by TP5, however, the detailed mechanisms are largely unknown.

A common goal of cancer therapy is to restore normal growth control in transformed tissues [1]. One area that has been intensively studied in recent years is biological modifiers of cancer growth which are designed to retard proliferation [2], to induce differentiation of these cells to a quiescent, non-dividing stage [3,4], and/or to promote cell death in malignant or pre-malignant cells [5,6]. Cell differentiation is disrupted in the acute leukaemia and many solid tumours. Studies of cell differentiation in vitro have led to the postulate that this is a consequence of uncoupling of controls that normally integrate proliferation and cell maturation [28,29]. Whilst such imbalances may provide a growth advantage for leukaemia cells, cell survival is also important. Proliferation at the expense of maturation may also allow cells to avoid apoptosis, so that inappropriately high numbers of undifferentiated cells are maintained [30]. In the present study, TP5 was able to induce the differentiation of HL-60 cells along the granulocytes lineage. This may provided important implications that TP5 could act as a valuable human leukaemia therapy agent because of its potent effects on the differentiation of human leukaemia cells.

Recent studies have revealed that some neuronal nAChRs mRNAs and/or proteins could be detected in non-neuronal cells such as leukocytes. One model is based on the use of a radiomimetic agent bleomycin on the HL-60 cell line, which is known to be from the hematological origin and likely to express nAChRs [31,32] together with the *bcl-2* anti-apoptotic gene. Our previous study demonstrated that TUB was able to reduce the inhibitory effects of $T\alpha_1$ on the proliferation of HL-60, K562 and K562/ADM, but that atropine did not, indicating that nAChRs were involved [12]. In the present study, TUB also significantly attenuated the TP5-induced inhibitory effects on the proliferation of HL-60, but atropine did not, indicating that TP5 suppressed the proliferation of HL-60 via a nAChRs mediated mechanism.

TP5 is a synthetic pentapeptide corresponding to the active structure of the natural 49 amino acids containing thymic hormone which has shown immunoregulatory activity in many animal model systems and human in vitro tests [15]. A multitude of in vivo studies have shown efficacy of TP5 treatment in the therapy of various diseases including primary

and secondary immune deficiencies, autoimmunities and infections etc [33]. Combined chemo-immunotherapeutical anti-cancer treatment seems to be more efficacious than chemotherapy alone, and the significant hematopoietic toxicity associated with most chemotherapeutical clinical trials can be reduced significantly by the addition of immunotherapy [10]. TP5 has recently been evaluated as an immunotherapeutic agent for the treatment of cancer. Tumour-induced immuno-suppression leads to an imbalance within the immune system, but an effective response is needed to eliminate residual tumour cells after removal of the major tumour tissue by cytostatic chemotherapy, radiation, or surgery. On the other hand, pathogens must be eliminated in concomitant infectious diseases. TP5-treatment led to encouraging results in malignant diseases [11,33]. Furthermore, many observations have demonstrated that administrations of TP5 induced positive clinical responses in cancer patients or animal models without relevant side effects and evident toxicity even at very high doses [10,34–36]. Interestingly, in the present study, our results suggested that TP5 was able to suppress the proliferation and induce differentiation in HL-60. Furthermore, TP5 exhibited no potential side effect on normal blood cells (like PBMCs) even though it exhibited significant inhibitory effects on the proliferation of HL-60. These observations implied that TP5 not only acted as an immunomodulatory factor in cancer chemotherapy, but also had potential as a chemotherapeutic agent in human leukemia therapy.

In conclusion, all the results indicated that TP5 was able to significantly inhibit proliferation and induce differentiation in HL-60 cells via a nAChRs mediated mechanism. Our observations also implied that TP5 not only acted as an immunomodulatory factor in cancer chemotherapy, but also had potential as a chemotherapeutic agent in human leukemia therapy.

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References

[1] M.J. Campbell, G.S. Reddy, H.P. Koeffler, Vitamin D3 analogs and their 24-Oxo metabolites equally inhibit clonal proliferation of a variety of cancer cells but have differing molecular effects, J. Cell. Biochem. 66 (1997) 413–425.

[2] N. Novichenko, S. Konno, Y. Nakajima, T.C. Hsieh, W. Xu, K. Turo, T. Ahmed, J.W. Chiao, Growth attenuation in a human prostate cell line mediated by a phorbol ester, Proc. Soc. Exp. Biol. Med. 209 (1995) 152–156.

[3] L. Liu, S. Shack, W.G. Stetler-Stevenson, W.R. Hudgins, D. Samid, Differentiation of cultured human melanoma cells induced by the aromatic fatty acids phenylacetate and phenylbutyrate, J. Invest. Dermatol. 103 (1994) 335–340.

[4] D. Samid, S. Shack, C.E. Myers, Selective growth arrest and phenotypic reversion of prostate cancer cells in vitro by nontoxic pharmacological concentrations of phenylacetate, J. Clin. Invest. 91 (1993) 2288–2295.

[5] J. Welsh, Induction of apoptosis in breast cancer cells in response to vitamin D and antiestrogens, Biochem. Cell. Biol. 72 (1994) 537–545.

[6] C.J. Li, C. Wang, A.B. Pardee, Induction of apoptosis by beta-lapachone in human prostate cancer cells, Cancer Res. 55 (1995) 3712–3715.

[7] T.R. Breitman, S.E. Selonick, S.J. Collins, Induction of differentiation of the human promyelocytic leukemia cell line HL-60 by retinoic acids, Proc. Natl. Acad. Sci. U. S. A. 77 (1980) 2936–2940.

[8] S.J. Collins, F.W. Ruscetti, R.E. Gallagher, R.C. Gallo, Terminal differentiation of human promyelocytic leukemia cells induced by dimethyl sulfoxide and other polar compounds, Proc. Natl. Acad. Sci. U. S. A. 75 (1978) 2458–2462.

[9] S.J. Collins, The HL-60 promyelocytic leukemia cell line: proliferation, differentiation, and cellular oncogene expression, Blood 70 (1987) 1233–1244.

[10] B. Bodey, B. Bodey Jr., S.E. Siegel, H.E. Kaiser, Review of thymic hormones in cancer diagnosis and treatment, Int. J. Immunopharmacol. 22 (2000) 261–273.

[11] V.K. Singh, S. Biswas, K.B. Mathur, W. Haq, S.K. Garg, S.S. Agarwal, Thymopentin and splenopentin as immunomodulators, current status, Immunol. Res. 17 (1998) 345–368.

[12] Y.Z. Fan, H. Chang, Y. Yu, J. Liu, R. Wang, Thymosin α1 suppresses proliferation and induces apoptosis in human leukemia cell lines, Peptides 27 (2006) 2165–2173.

[13] T. Solmajer, Design of a novel thymopoietin analogue based on conformational analyses, Drugs Des. Deliv. 6 (1990) 213–221.

[14] G. Goldstein, M.P. Scheid, E.A. Boyse, D.H. Schlesinger, J. Van Wauwe, Synthetic pentapeptide with biological activity characteristic of the thymic hormone thymopoietin, Science 204 (1979) 1309–1310.

[15] E. Sundal, D. Bertelletti, Management of viral infections with thymopentin, Arzneimittelforschung 44 (1994) 866–871.

[16] K. Smetana, I. Jiraskova, L. Perlaky, H. Busch, The silver reaction of nucleolar proteins in the main structural compartments of ring-shaped nucleoli in smear preparations, Acta. Histochem. 101 (1999) 167–183.

[17] T. Okano, K. Nakagawa, N. Kubodera, K. Ozono, A. Isaka, A. Osawa, M. Terada, K. Mikami, Catalytic asymmetric syntheses and biological activities of singly dehydroxylated 19-nor-1a,25-dihydroxyvitamin D3 A-ring analogs in cancer cell differentiation and apoptosis, Chem. Biol. 7 (2000) 173–184.

[18] M. Eugenia Riveiro, C. Shayo, F. Monczor, N. Fernandez, A. Baldi, N. De Kimpe, J. Rossi, S. Debenedetti, C. Davio, Induction of cell differentiation in human leukemia U-937 cells by 5-oxygenated-6,7-methylenedioxycoumarins from *Pterocaulon polystachyum*, Cancer Lett. 210 (2004) 179–188.

[19] R.L. Baehner, D.G. Nathan, Quantitative nitroblue tetrazolium test in chronic granulomatous disease, N. Engl. J. Med. 278 (1968) 971–976.

[20] D.L. Lucas, S. Tanuma, P.J. Davies, D.G. Wright, G.S. Johnson, Maturation of human promyelocytic leukemia cells induced by nicotinamide: evidence of a regulatory role for ADP-ribosylation of chromosomal proteins, J. Cell. Physiol. 121 (1984) 334–340.

[21] D. Gimonet, R. Grailhe, P. Coninx, F. Antonicelli, B. Haye, F. Liautaud-Roger, Functional role of nicotinic acetylcholine receptors in apoptosis in HL-60 cell line, Eur. J. Pharmacol. 482 (2003) 25–29.

[22] G.S. Kansas, M.J. Muirhead, M.O. Dailey, Expression of the CD11/CD18, leukocyte adhesion molecule 1, and CD44 adhesion molecules during normal myeloid and erythroid differentiation in humans, Blood 76 (1990) 2483–2492.

[23] K. Schulze-Forster, K. Eckert, H.R. Maurer, The thymus extract thymex-L potentiates the retinoic acid-induced differentiation of the human myeloid leukemia cell line HL-60, Biochim. Biophys. Acta 1265 (1995) 110–116.

[24] S. Gonser, N.E. Crompton, G. Folkers, E. Weber, Increased radiation toxicity by enhanced apoptotic clearance of HL-60 cells in the presence of the pentapeptide thymopentin, which selectively binds to apoptotic cells, Mutat. Res. 558 (2004) 19–26.

[25] D. Trere, A. Pession, M. Derenzini, The silver stained proteins of

interphasic nucleolar organizer regions as a parameter of cell duplication rate, Exp. Cell Res. 184 (1989) 131–157.
[26] M. Koda, T. Murate, S. Wang, K. Ohguchi, S. Sobue, M. Ikeda, K. Tamiya-Koizumi, Y. Igarashi, Y. Nozawa, Y. Banno, Sphingosine kinase 1 is involved in dibutyryl cyclic AMP-induced granulocytic differentiation through the upregulation of extracellular signal-regulated kinase, but not p38 MAP kinase, in HL60 cells, Biochim. Biophys. Acta 1733 (2005) 101–110.
[27] T. Nishinaka, Y.H. Fu, L.I. Chen, K. Yokoyama, R. Chiu, A unique cathepsin-like protease isolated from CV-1 cells is involved in rapid degradation of retinoblastoma susceptibility gene product, RB, and transcription factor SP1, Biochim. Biophys. Acta 1351 (1997) 274–286.
[28] J. Bartek, J. Bartkova, J. Lukas, The retinoblastoma protein pathway in cell cycle control and cancer, Exp. Cell Res. 237 (1997) 1–6.
[29] L. Sachs, Control of normal differentiation and the phenotypic reversion of malignancy in myeloid leukemia cells, Nature. 274 (1978) 535–539.
[30] M.J. Campbell, M.T. Drayson, J. Durham, M.L. L.Wallington, G.S. Siu-Caldera, G. Reddy, Metabolism of 1a, 25 $(OH)_2$D3 and its 20-epi analog integrates clonal expansion, maturation and apoptosis during HL-60 cell differentiation, Mol. Cell. Endocrinol. 149 (1999) 169–183.
[31] D. Gimonet, R. Grailhe, P. Coninx, F. Antonicelli, B. Haye, F. Liautaud-Roger, Functional role of nicotinic acetylcholine receptors in apoptosis in HL-60 cell line, Eur. J. Pharmacol. 482 (2003) 25–29.
[32] Y. Villiger, I. Szanto, S. Jaconi, C. Blanchet, B. Buisson, K.H. Krause, D. Bertrand, J.A. Romand, Expression of an alpha7 duplicate nicotinic acetylcholine receptor-related protein in human leukocytes, J. Neuroimmunol. 126 (2002) 86–98.
[33] S. Gonser, E. Weber, G. Folkers, Peptides and polypeptides as modulators of the immune response: thymopentin—An example with unknown mode of action, Pharm. Acta Helv. 73 (1999) 265–273.
[34] N. Cascinelli, F. Belli, L. Mascheroni, L. Lenisa, C. Clemente, Evaluation of clinical efficacy and tolerability of intravenous high dose thymopentin in advanced melanoma patients, Melanoma Res. 8 (1998) 83–89.
[35] F. Recchia, G. Sica, S. De Filippis, M. Rosselli, P. Pompili, G. Saggio, S. Rea, Chemo-immunotherapy in advanced head and neck cancer, Anticancer Res. 19 (1999) 773–777.
[36] F. Gallo, M.C. Morale, D. Sambataro, Z. Farinella, U. Scapagnini, B. Marchetti, The immune system response during development and progression of carcinogen-induced rat mammary tumors: prevention of tumor growth and restoration of immune system responsiveness by thymopentin, Breast Cancer Res. Treat. 27 (1993) 221–237.