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ARTICLE

Synthesis and biological screening of small molecule peptides based on the amino acid sequence of thymosin β4

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Abstract Small molecule peptides based on the amino sequence of thymosin β4 were prepared using solid-phase synthesis, and their bioactivities were investigated. Small molecule peptides are less than ten amino acids in size so can be produced on a large scale and, after modification, can easily penetrate blood vessels or tissues. We studied the ability of the small molecule peptides to promote wound healing, angiogenesis, and hair growth by examining their effects on the proliferation of human dermal fibroblasts, human umbilical vein endothelial cells, and human dermal papilla cells versus those of thymosin β4, vascular endothelial growth factor, and minoxidil, respectively. Several small molecule peptides promoted significantly more cell proliferation than the corresponding positive controls.

Keywords Actin · Bioactivity · Peptide · Proliferation · Sequestering · Thymosin β4

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Introduction

Proteins perform a vast array of functions within living organisms, including catalysis of biochemical reactions. Specific amino acid sequences in a protein usually determine its particular bioactivities. Examples of bioactivities include antibacterial effects, anti-virus activity, anti-tumor activity, insecticidal activity, metabolic activity (immunity, blood-sugar levels, blood pressure, sleep, and memory), and enzyme inhibition.

Thymosin β4 (Tβ4) was initially isolated from the calf thymus in 1981 (Low et al. 1981). Tβ4 contains 43 amino acid residues and has a molecular weight of approximately 4.9 kDa. Tβ4 can be found in most body cells and organs, with high concentrations present in blood platelets, neutrophils, and lymphoid cells (Mora et al. 1997; Huff et al. 2001). Tβ4 induces angiogenesis (Malinda et al. 1997; Philp et al. 2003), accelerates wound healing (Katherine et al. 1999; Philp et al. 2006), and induces hair growth (Philp et al. 2004; Cha et al. 2010) through promotion of cell migration. Furthermore, Tβ4 inhibits apoptosis (Sosne et al. 2004; Lee et al. 2008), accelerates cancer metastasis (Cha et al. 2003; Hsiao et al. 2006; Nummela et al. 2006), and stimulates migration and survival of cardiomyocytes (Bock-Marquette et al. 2004).

Tβ4 is a member of the beta thymosin group, of which there are 15 subtypes reported to date. Beta thymosins all possess the amino acid sequence 17-LKKTETQEK-25, which serves as an actin binding site (Huff et al. 2001). This actin binding site interacts with individual G-actin monomers, thereby preventing formation of F-actin. This peptide sequence was first identified as a regulator of F-actin formation through its induction of a conformational change to the actin cytoskeleton and then it causes cell migration (Cassimeris et al. 1992; Goldstein et al. 2005).

Actin sequestration by Tβ4 accelerates cell migration (Malinda et al. 1997; Kobayashi et al. 2002; Sosne et al. 2002; Cierniewski et al. 2007; Philp et al. 2007) which facilitates many in vivo functions including angiogenesis, wound healing, and hair growth. Furthermore, the peptide Ac-SDKP—which corresponds to the N-terminal fragment of Tβ4—is found in vivo (Lenfant et al. 1989; Aidoudi et al. 1996; Azizi et al. 1997) where it plays pro-angiogenic (Wang et al. 2004; Smart et al. 2007a), anti-inflammatory, and anti-fibrotic (Peng et al. 2001; Yang et al. 2004; Lin et al. 2008) roles.

We prepared small molecule peptides derived from the Tβ4 sequence in an attempt to develop novel drug candidates for cardiovascular diseases and alopecia, as current treatments for these conditions are inadequate. As full-length Tβ4 contains 43 amino acid residues, its high-purity and high-yield large scale production using solid-phase synthesis would be difficult and expensive. Furthermore, adverse events such as protease-mediated degradation and the accumulation and production of antibodies may occur in vivo. Therefore, we produced small molecule peptides to avoid these complications. Peptides were derived from the Tβ4 actin binding site sequence, 17-LKKTETQEK-25 and were six amino acid residues in length. As all beta thymosins have similar amino acid sequences with differences largely concentrated at the C-terminal, we primarily examined 4 or 6 sequenced peptides containing the Tβ4 C-terminal sequence AGES as a basic structure or modified version of it.

Materials and methods

Materials

N-9-Fluorenylmethoxycarbonylamino acid (Fmoc-AA-OH) was purchased from CS Bio Co. (USA). Hydroxybenzotriazole (HOBt), *N,N,N′,N′*-tetramethyl-*O*-(1*H*-benzotriazol-1-yl) uranium hexafluorophosphate (HBTU), *N,N*-diisopropylethylamine (DIEA), piperidine, and trifluoroacetic acid (TFA) were purchased from TCI (Japan). 2-Chlorotrityl (CTC) resin (100–200 mesh) was purchased from GLS (China), and triisopropylsilane (TIS) was purchased from Sigma-Aldrich (USA). Human umbilical vein endothelial cells (HUVECs), human dermal fibroblast (HDF) cells, fibroblast growth medium, HDPCs, and dermal papilla cell growth medium were purchased from PromoCell (Germany). Endothelial cell growth medium was purchased from Lonza (Switzerland), and a Cell Counting Kit-8 (CCK-8) was purchased from Dojindo (Japan).

Peptide synthesis

Using a solid-phase peptide synthesizer (CS136XT, CS Bio Co., USA), Fmoc-amino acid (4 equiv.) and DIEA (5 equiv.) were added to swollen CTC resin (1.92 g, 2 mmol) in dichloromethane (DCM; 20 mL), and the reaction mixture was stirred for 1.5 h. Solvents were then removed, the resin was washed with *N,N*-dimethylformamide (DMF) and DCM, and DCM/Methanol/DIEA (80:15:5, v:v) solution (20 mL) was added to the reaction mixture to conduct capping reactions twice for 20 min each time, followed by washing with DMF. After deprotection of Fmoc with 20 % piperidine in DMF (20 mL) for 30 min, the reaction mixture was washed with DCM and DMF. Next, amino acid-coupling reactions were conducted using a general HBTU-mediated solid-phase peptide synthesis protocol [Fmoc-AA-OH (4 equiv.), HOBt (4 equiv.), HBTU (4 equiv.), DIEA (8 equiv.), DMF (20 mL)] (Bernatowicz et al. 1989).

After deprotection of Fmoc with 20 % piperidine in DMF (20 mL) for 30 min, the reaction mixture was washed with DCM and DMF. The resin was filtered and dried under vacuum. Finally, the resin was treated with deprotection solution [TFA/TIS/water (95:2.5:2.5, v:v)] (20 mL) for 4 h and filtered to remove the resin. The collected filtrate was evaporated and precipitated with cold diethyl ether to obtain a product followed by centrifugation. Finally, solid compounds were obtained. After washing the obtained solid compounds with diethyl ether three times and drying under vacuum, overall peptide yields were 55–92 % (Fig. 1). Purity and molecular weight of the synthesized peptides were analyzed using HPLC (Alliance e2695, Waters, USA) and LC–MS (Xevo TQ MS, Waters, USA), respectively (Copies of original spectra or chromatograms are available as supplementary materials).

Cell culture

HUVECs, HDFs, and HDPCs were cultured on fibronectin-coated dishes in endothelial cell growth medium, fibroblast growth medium, and dermal papilla cell growth medium, respectively, and cells passaged two to six times were used in experiments.

CCK-8 cell proliferation assay

Cells were seeded on 96-well plates and cultured in relevant growth medium for 24 h. For starvation, medium was replaced by supplement-free medium containing 1 % fetal bovine serum. Peptides were added at 2 μM, vascular endothelial growth factor (VEGF) at 1 nM, Tβ4 at 2 μM, and minoxidil at 1 μM, followed by 48–96 h incubation. At the end of the incubation period, cell number was determined

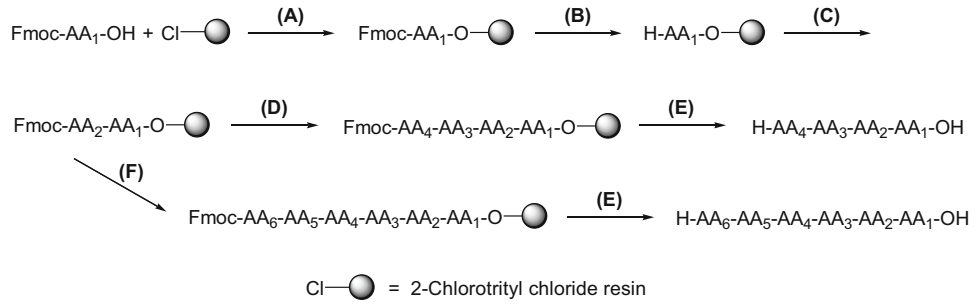

Fig. 1 Solid-phase peptide synthesis. Reagents and condition: (*A*) (1) DIEA, DCM, (2) DCM/MeOH/DIEA (80:15:5); (*B*) 20 % piperidine in DMF; (*C*) Fmoc-amino acid, HOBt, HBTU, DIEA, DMF; (*D*) repeat (*B*) and (*C*) two times with Fmoc-amino acid; (*E*) (1) 20 % piperidine in DMF, (2) TFA/TIS/water (95:2.5:2.5); (*F*) repeat (*B*) and (*C*) four times with Fmoc-amino acid

using the CCK-8 assay kit. Optical density was measured by a microplate reader (Synergy^{TM} 4, BioTek, USA) at a wavelength of 450 nm. The relative cell number was calculated as a percentage of the control wells.

Results and discussion

Peptide synthesis

Peptides can be synthesized using in vitro methods based on solid-phase synthesis using microbeads (Fig. 1), (Merrifield 1973). We used solid-phase synthesis to synthesize small molecule peptides of fewer than ten amino acids in length based upon the Tβ4 sequence. We chose to synthesize these small molecules because they can be produced on a large scale, and are easy to purify and derivatize.

Tβ4 and Tβ10 perform different functions in vivo even though they have similar amino acid sequences (Voisin et al. 1995; Smart et al. 2007b). Furthermore, the Ac-SDKP peptide mediates multiple effects in vivo. Therefore, we compared the sequences of multiple beta thymosin subtypes and identified differences in the C-terminal region. In Tβ4, this region contains the sequence AGES. We designed and synthesized 4 or 6 sequenced peptides that contained this AGES sequence or modified versions of it (Tables 1, 2). We also designed and synthesized peptides of six amino acid residues in length based upon the G-actin binding site which directs many in vivo functions of Tβ4 (Table 2). We achieved reasonable yield and purity for all peptides examined. In particular, we obtained highly pure peptides (over 95 %) from crude peptides by performing recrystallization using diethyl ether without column chromatography.

Biological screening of peptides

We examined the effects of synthesized peptides on three different cell types that perform biological activities requiring proliferation and cell migration: HDF cells, which synthesize structural skin materials and whose dysfunction contributes to wrinkles and drooping and dry skin;

Table 1 List of synthesized peptides of four amino acid residues in length

Sample no.	Sequence	Molecular weight (Da)
1	VGES	390.39
2	DGES	406.35
3	NGES	405.36
4	SGES	378.34
5	HGES	428.40
6	AVES	404.42
7	ARES	461.47
8	ADES	420.37
9	ANES	419.39
10	ASES	392.36

Table 2 List of synthesized peptides of six amino acid residues in length

Sample no.	Sequence	Molecular weight (Da)
11	LQEKQA	715.80
12	LETQEK	746.81
13	LKLKKT	729.95
14	AKLKKT	687.87
15	AQKEQE	731.75
16	KNAGES	604.61
17	SQAGES	577.54
18	HQAGES	627.60
19	AKKLKL	699.93
20	AQKEQK	730.81
21	KETQEK	761.82
22	TETQEQ	734.71
23	HETQEK	770.79
24	LKLKKA	699.93
25	TETQEL	719.74

HUVECs, which have important roles in angiogenesis and are involved in tissue maintenance, vascularization, infection, and thrombosis; and HDPCs, which are involved in hair genesis and growth.

The effect of 2 μM of each peptide on cell proliferation was compared with the effects of Tβ4, VEGF, and minoxidil to investigate potential biological activities of these small peptides with regard to wound healing, angiogenesis, and hair growth, respectively. To evaluate the wound healing effect of the synthetic peptides in vitro, we assessed their influence on the proliferation of HDFs. Dermal fibroblasts within the dermis layer of the skin are responsible for generating connective tissue and allowing the skin to recover from injury. Tβ4 is a wound recovery agent (Malinda et al. 1999) and was used as a positive control while distilled water served as a negative control. The proliferation rate of the positive control was 150 %, which was lower than that of the majority of synthetic peptides we tested. Peptides 12 and 18 enhanced proliferation relative to the negative control by 197 and 195 %, respectively (Table 3).

The ability of the synthetic peptides to induce neovascularization was assessed by determining their effect on HUVEC proliferation. Blood vessels contain endothelial cells, and these cells function in tissue repair by inducing angiogenesis. For these experiments, the angiogenic factor VEGF was used as a positive control and distilled water as a negative control. The proliferation rate of the positive control was 160 % that of the negative control. Peptides 2, 3, and 14 stimulated proliferation by 199, 197, and 196 % relative to the negative control, respectively (Table 4).

To evaluate whether the synthetic peptides could affect hair growth, we used HDPCs. These cells are present in the hair follicles of the scalp and play a pivotal role in hair formation, growth, and cycling. Minoxidil, known to induce proliferation of hair dermal papilla cells, was used as a positive control, and distilled water was used as a negative control. The proliferation rate of the positive control was 145 %. Peptides 11, 12, 21, and 24 had cell proliferation rates of 175, 176, 175, and 170 % relative to the negative control, respectively (Table 5).

We have identified nine small molecule peptides that stimulated HDF cell proliferation, two of which (12, 18) had a 47 and 45 % greater effect on cell proliferation than the positive control (Tβ4; Fig. 2). Ten peptides stimulated HUVEC proliferation above baseline, and three of these (2, 3, 14) stimulated proliferation to a greater extent than VEGF (39, 37, and 36 % greater proliferation than VEGF-

Table 3 HDF proliferation rate following peptide treatment

Sample	H_2O	Tβ4	1	2	3	4	5
Proliferation rate (%)	100	150	186	115	153	145	120
Sample	6	7	8	9	10	11	12
Proliferation rate (%)	152	178	161	153	165	143	197
Sample	13	14	15	16	17	18	19
Proliferation rate (%)	118	164	160	154	151	195	148
Sample	20	21	22	23	24	25	-
Proliferation rate (%)	138	125	160	115	187	137	-

Table 4 HUVEC proliferation rate following peptide treatment

Sample	H_2O	VEGF	1	2	3	4	5
Proliferation rate (%)	100	160	140	199	197	160	142
Sample	6	7	8	9	10	11	12
Proliferation rate (%)	181	143	151	175	158	152	151
Sample	13	14	15	16	17	18	19
Proliferation rate (%)	175	196	188	184	160	155	168
Sample	20	21	22	23	24	25	-
Proliferation rate (%)	130	175	183	120	125	131	-

Table 5 HDPC proliferation rate following peptide treatment

Sample	H_2O	minoxidil	1	2	3	4	5
Proliferation rate (%)	100	145	135	140	125	160	151
Sample	6	7	8	9	10	11	12
Proliferation rate (%)	136	155	153	154	138	175	176
Sample	13	14	15	16	17	18	19
Proliferation rate (%)	168	149	165	135	164	130	140
Sample	20	21	22	23	24	25	-
Proliferation rate (%)	153	175	163	160	170	147	-

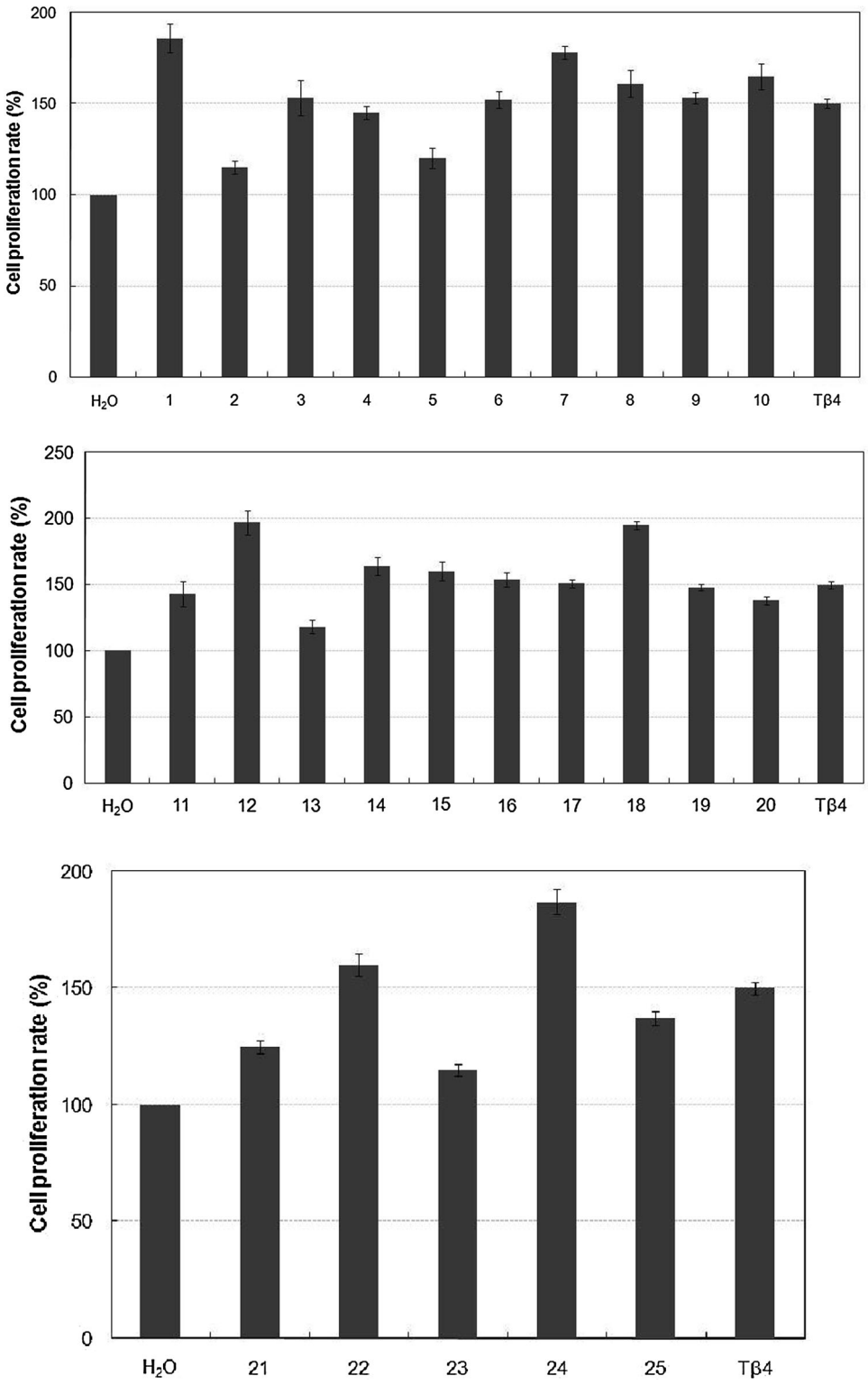

Fig. 2 Comparison of influence of peptides on cell proliferation in HDF cells (H_2O: negative control group, Tβ4: positive control group). Values are the mean ± SD of three independent experiments

treated cells, respectively; Fig. 3). Nine peptides stimulated HDPC proliferation. Four peptides (11, 12, 21, and 24) stimulated proliferation to a greater extent than minoxidil (30, 31, 30, and 25 %, respectively; Fig. 4). These results suggest that the synthetic peptides have the potential to promote wound healing, stimulate angiogenesis, and induce hair growth.

Of the 25 small molecule peptides we synthesized, several induced greater cell proliferation than the positive control compounds. Given that small molecule peptides typically

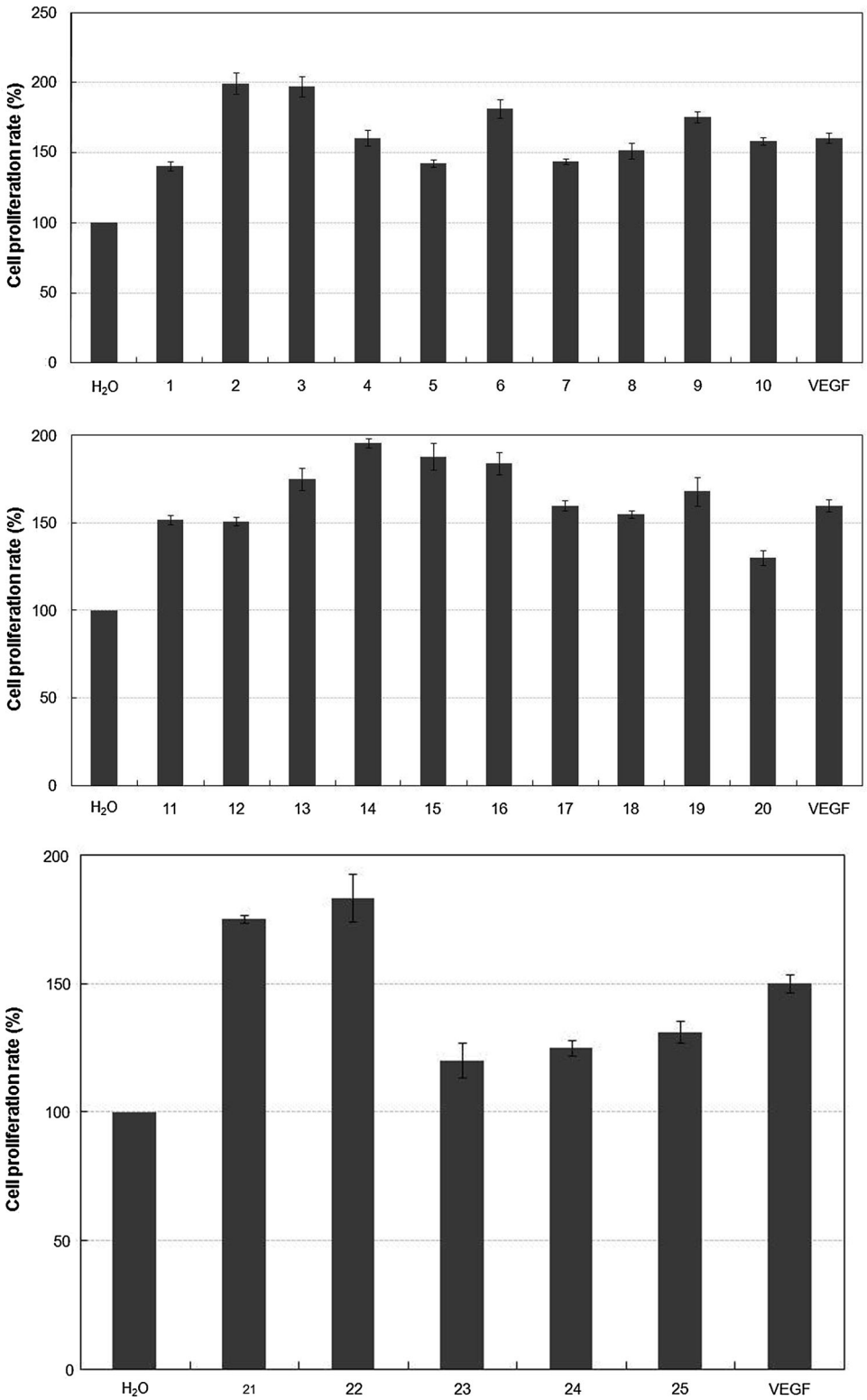

Fig. 3 Comparison of influence of peptides on cell proliferation in HUVECs (H_2O: negative control group, VEGF: positive control group). Values are the mean ± SD of three independent experiments

penetrate tissues with relative ease and cause fewer side effects than larger molecules, peptides may have great potential as new bioactive medicines. Furthermore, it is scheduled to build the library of small molecule peptides and to conduct the in vivo related studies for candidate molecules which show potential to be developed as medication. And also, mechanism-related studies will be performed regarding to activity relation of structure and efficacy.

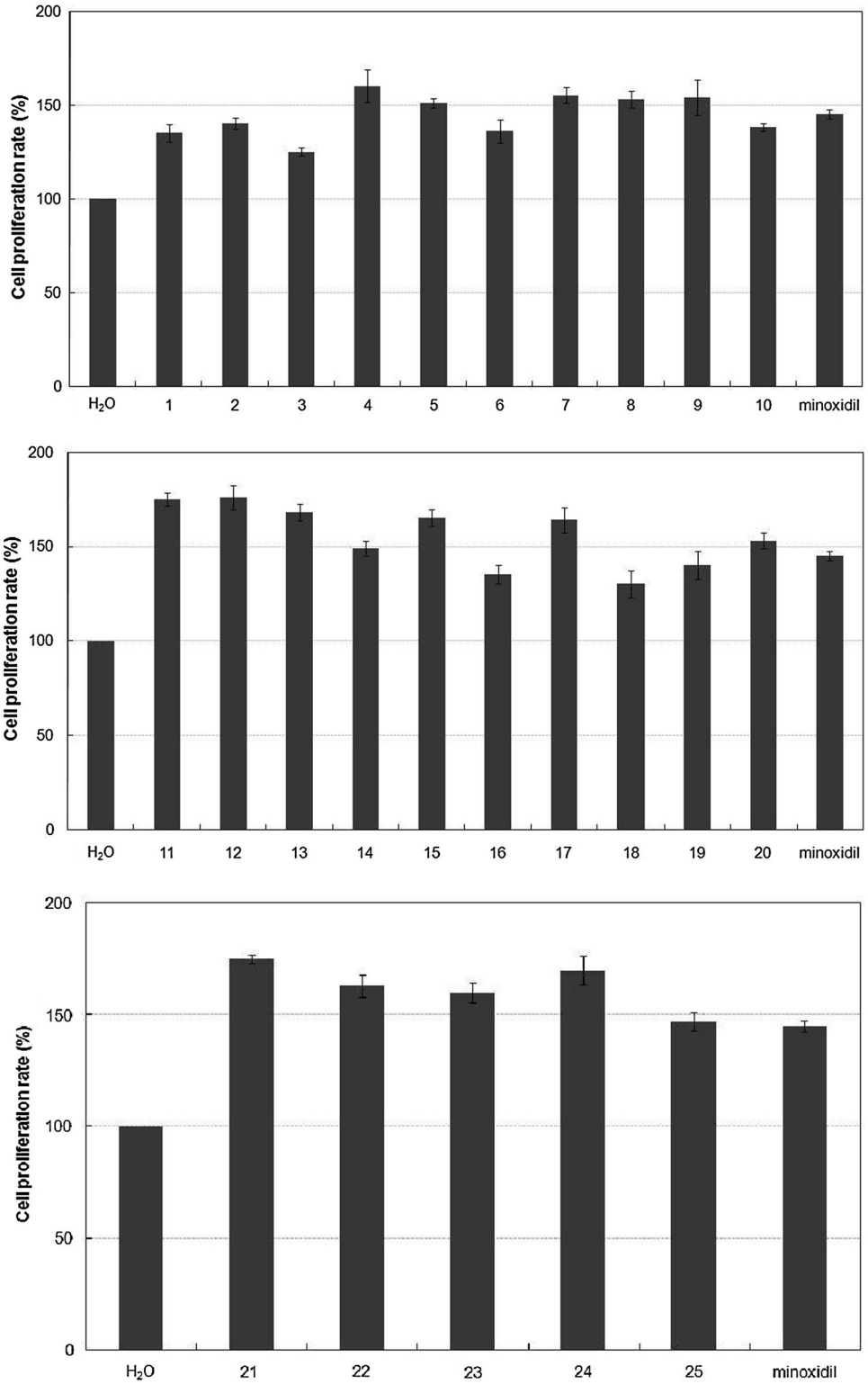

Fig. 4 Comparison of influence of peptides on cell proliferation in HDPCs (H_2O: negative control group, minoxidil: positive control group). Values are the mean ± SD of three independent experiments

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