Promotion of stem cell proliferation by vegetable peptone

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Abstract

Objectives: Technical limitations and evolution of therapeutic applications for cell culture-derived products have accelerated elimination of animal-derived constituents from such products to minimize inadvertent introduction of microbial contaminants, such as fungi, bacteria or viruses. The study described here was conducted to investigate the proliferative effect of vegetable peptone on adult stem cells in the absence of serum, and its possible mechanisms of action.

Materials and methods: Cell viability and proliferation were determined using the MTT assay and Click-iT™ EdU flow cytometry, respectively. In addition, changes in expression of cytokine genes were analysed using MILLIPLEX™ human cytokine enzyme-linked immunosorbent assay kit.

Results: Viability of cord blood-derived mesenchymal stem cells (CB-MSC) and adipose tissue-derived stem cells (ADSC) increased significantly when treated with the peptone. In addition, median value of the group treated with peptone shifted to the right when compared to the untreated control group. Furthermore, quantitative analysis of the cytokines revealed that production of vascular endothelial growth factor (VEGF), transforming growth factor-beta1 (TGF-β1), and interleukin-6 (IL-6) increased significantly in response to treatment with our vegetable peptone in both CB-MSCs and ADSCs.

Conclusions: Our findings revealed that the vegetable peptone promotes proliferation of CB-MSCs and ADSCs. In addition, results of this study suggest that induction of stem cell proliferation by vegetable peptone is likely to be related to its induction of VEGF, TGF-β1, and IL-6 expression.

Introduction

Stem cells have the potential to develop into any type of cell or tissue in the human body; there embryonic stem cells and adult stem cells. Mesenchymal stem cells (MSC) are one of the kinds of adult stem cells, which can arise from many sources, including bone marrow and umbilical cord blood (CB), as well as a wide variety of tissues and organs. MSCs are capable of self-renewal and differentiation into different lineages of mesenchymal tissue, including bone, cartilage, fat, tendon, muscle, and haematopoietic supporting stroma (1).

One important type is the umbilical cord blood-derived mesenchymal stem cell (CB-MSC), which contributes to regeneration of mesenchymal tissues (2). Although existence of haematopoietic stem cells in CB has been well documented, studies evaluating MSCs in CB are still in progress. Furthermore, cytokine expression profile of CB-MSCs and influence of cytokines on differentiation of CB-MSCs are not well understood. However, adipose tissue is known to be a dynamic participant in endocrine physiology, serving as the source of secreted cytokines. Adipose tissue expresses haematopoietic growth factors and macrophage-colony stimulating factor (M-CSF), whose expression can lead to expansion of the volume of adipose tissue (3). Adipose tissue-derived stem cells (ADSC) release angiogenic factors, and it has been suggested that these factors contribute to their regenerative capability in ischemic injury models (4).

Comparative studies have demonstrated that MSCs from different sources have comparable characteristics, even though their expression profiles may differ for a large number of genes (5). However, regulatory mechanisms underlying self-renewal and differentiation of MSCs is not well understood, which has limited the potential use of MSCs in practical applications, such as tissue engineering and gene therapy (6). In addition, the cytokine profile of MSCs and influence of cytokines on proliferation of MSCs are still poorly understood.

Utilization of serum-free medium facilitates analysis of actions between growth factors and cytokines, on proliferation and differentiation of MSCs, without complexity of exogenous serum. However, composition of a

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serum varies between donor species, age of the animal serum was obtained from, its feedstock and the season. Such variability can limit applicability of research and development activities. Although supplementing cell culture medium with serum from various animals has several advantages and generally facilitates animal cell technology, use of such serum also presents several disadvantages. Primarily, use of these types of serum results in introduction of animal-derived proteins, which have to be eliminated during downstream processing. In addition, the serum might have the potential to introduce microbial contaminants, such as fungi, bacteria or viruses and in addition in some cases might introduce prions. Finally, they are expensive and widely different in quality. Among these difficulties, potential introduction of contaminants is the most problematic issue, which results in extended use of serum-free medium.

Replacement of all substances of animal origin with synthetic components of plant origin reduces the risk of introducing adventitious agents, yet it is essential that serum-free medium contains all necessary extrinsic proteins to ensure that optimal growth and production levels are attained (7). As a result, total serum replacement can be a challenge given the number of roles it plays in a culture medium. Despite this, it is crucial that as many chemically defined substitutes for serum be found. However, various substitutions may be insufficient to fully replace serum, therefore, alternatives to such chemically defined medium, such as protein-free medium supplemented with protein digests and plant hydrolysates, have also been evaluated (8). Peptones are small polypeptides that are intermediate products in hydrolysis of proteins. Depending on the degree of hydrolysis, peptones may supply nutrients, adhesion components, or growth factor analogues (7). Use of peptones in a serum-free medium is not new, but clear deficiency of most of these was their animal origin. As a result, most of current serum-free media contain some animal-derived peptones and hydrolysates (9,10), or animal-derived purified proteins (11,12).

Vegetable peptones may be safer supplements for animal cell protein-free culture media. Adult stem cells are a potentially powerful candidate cell type for development of regenerative medicine, as well as for study of cell differentiation. Therefore, this study was conducted to evaluate effects of vegetable peptones on proliferation of adult stem cells and the possible mechanisms underlying their action.

Materials and methods

Cells and reagents

Human CB-MSCs were obtained from Cha University (Sungnam, Gyunggi, South Korea). They were cultured in

Dulbecco's modified Eagle's medium (DMEM) containing low glucose levels and supplemented with 15% fetal bovine serum (FBS), penicillin and streptomycin at 37 °C. Medium was changed every 3 days until cells were confluent, at which time they were passaged. Human ADSCs were purchased from Invitrogen (Invitrogen, Carlsbad, CA, USA). Having been cryopreserved, they were thawed at 37 °C and then immediately cultured in MesenPRO RS™ medium (Gibco, Carlsbad, CA, USA). Cells were then expanded using MesenPRO RS™ medium.

Five plant peptones (soy peptone, wheat peptone, broadbean peptone, potato peptone, pea peptone) were obtained from Fluka (Sigma-Aldrich, St. Quentin Fallavier, France) and two (papaic soy peptone, lupin peptone) were obtained from Solabia (Pantin 93698 Cedex, France). Peptones used were prepared as 1% peptone medium in DMEM without serum, by filtration through a 0.2-µm membrane filter. In serum-free conditions, cell culture medium was replaced with serum-free DMEM (cat. no. 11885, Invitrogen). As positive controls, cells were cultured in control medium (DMEM with 15% FBS).

Cell viability

Cell viability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which is based on conversion of the substrate containing a tetrazolium ring into blue formazan, by mitochondrial dehydrogenases. Level of blue formazan was then measured spectrophotometrically and used as an indirect index of cell density. Briefly, cells were exposed to MTT (1 mg/ml) for 3 h at 37 °C. Medium was then removed and cells were solubilized with dimethyl sulphoxide. After complete solubilization, presence of blue formazan was evaluated spectrophotometrically by measuring the absorbance at a wavelength of 570 nm.

Cell proliferation

Methods used to evaluate cell proliferation are generally based on incorporation of thymidine analogues such as ³H thymidine or bromodeoxyuridine (BrdU) during DNA synthesis. The Click-iT™ EdU flow cytometry assay kit is a novel alternative to BrdU assay (Molecular Probes, Eugene, OR, USA). This method replaces antibody-based detection of the nucleoside analogue, BrdU, with EdU (5-ethynyl-2'-deoxyuridine), which is a nucleoside analogue of thymidine that is incorporated into DNA during active DNA synthesis. Briefly, cells were incubated with EdU for 1 h. As negative control, cells from the same population were not treated with EdU. After incubation, samples were analysed using a FACS Vantage flow cytometer (BD Biosciences, San Jose, CA, USA). All

analyses were conducted following the manufacturer's recommended protocols.

Cytokine profile

For quantitative analysis of cytokines, supernatants of stem cells cultured with peptone in serum-free medium were analysed using MILLIPLEX™ human cytokine enzyme-linked immunosorbent assay (ELISA) kit following the manufacturer's recommended protocols (Millipore, St. Charles, MO, USA).

Results

Effects of peptone on proliferation of MSCs

In this study, we classified seven peptones based on their origin, amino acid content, and oligopeptide molecular weight distribution. To accomplish this, we first evaluated using the MTT assay involvement of peptones in proliferation of the adult stem cells. Results of this study revealed that only two peptones (pea peptone and wheat peptone) induced proliferation of adult stem cells. Table 1 represents amino acid distribution (mg/g) of these two peptones.

Specifically, pea peptone and wheat peptone increased cell proliferation rate significantly, by 25% and 20% in

CB-MSCs, respectively (Fig. 1a). Similarly, pea peptone and wheat peptone induced a 20% and 25% increase in the cell proliferation rate of ADSCs, respectively (Fig. 1b). These effects were further confirmed by results of the Click-iT™ EdU flow cytometry assay of CB-MSCs. As shown in Fig. 2, median value of the group treated with pea peptone or wheat peptone was shifted to the right when compared to serum-free group (7.23 and 7.30, respectively vs. 3.08). Taken together, these findings suggest that these vegetable peptones could contribute effectively to proliferation of adult stem cells cultured in media free from animal-derived components.

Cytokine profile analysis of conditioned medium of MSCs treated with peptone

Although the aforementioned results demonstrated that pea peptone and wheat peptone induced proliferation of the adult stem cells, they did not elucidate the mechanisms by which the effects of these peptones occurred. Thus, we evaluated changes in cytokine expression profiles in the cells, in response to treatment with the peptones, using a human cytokine ELISA kit. Specifically, we measured levels of epidermal growth factor (EGF), fibroblast growth factor-2 (FGF-2), Flt-3 ligand, granulocyte-colony stimulating factor (G-CSF), granulocyte/monocyte-colony stimulating factor (GM-CSF), transforming growth

Table 1. Amino acid distribution (mg/g) of peptones

Pea peptone	Total amino acids (T)	Free amino acids (F)	(F/T) × 100		Total amino acids (T)	Free amino acids (F)	(F/T) × 100
Aspartic acid	103.37	2.31	2.23	Methionine	0.00	2.25	0.00
Threonine	32.28	7.56	23.42	Isoleucine	38.68	8.25	21.33
Serine	48.71	6.12	12.56	Leucine	60.10	19.49	32.43
Glutamic acid	169.25	4.84	2.86	Tyrosine	20.93	9.84	47.01
Proline	38.83	0.93	2.40	Phenylalanine	42.01	14.54	34.61
Glycine	37.93	4.19	11.05	Histidine	19.64	4.11	20.93
Alanine	37.08	4.94	13.32	Lysine	68.50	14.46	21.11
Cysteine	49.77	3.22	6.47	Arginine	80.56	25.72	31.93
Valine	40.96	9.30	22.72	Tryptophan	12.69	3.39	26.71
Wheat peptone	Total amino acids (T)	Free amino acids (F)	(F/T) × 100		Total amino acids (T)	Free amino acids (F)	(F/T) × 100
Aspartic acid	17.52	2.11	12.04	Methionine	5.83	2.64	45.28
Threonine	15.27	2.88	18.86	Isoleucine	24.45	3.17	12.97
Serine	31.51	5.12	16.25	Leucine	44.07	10.38	23.55
Glutamic acid	245.63	3.46	1.41	Tyrosine	11.72	4.04	34.47
Proline	82.54	0.35	0.42	Phenylalanine	33.66	3.56	10.58
Glycine	19.50	1.95	10.00	Histidine	11.19	1.63	14.57
Alanine	15.50	2.46	15.87	Lysine	12.60	2.74	21.74
Cysteine	32.69	1.67	5.11	Arginine	18.30	5.11	27.92
Valine	22.84	4.76	20.84	Tryptophan	4.49	5.57	124.05

Figure 1. Effects of peptones on the viability of cord blood-derived mesenchymal stem cells (CB-MSC) or adipose tissue-derived stem cells (ADSC). Adult stem cells were grown for 3 days in a combination culture medium that contained pea peptone or wheat peptone. In serum-free condition, cell culture medium was replaced with serum-free Dulbecco's modified Eagle's medium (DMEM). For positive control, cells were cultured in control medium (DMEM with 15% fetal bovine serum). Cell viability was then determined by MTT assay, as described in the Materials and methods section. Values are expressed as percentage of the control, which was defined as 100%. Mean values for each group were obtained from four samples ($n = 4$). (a) CB-MSCs, (b) ADSCs.

Figure 2. Effects of peptone on proliferation of cord blood-derived mesenchymal stem cells (CB-MSC). CB-MSCs were grown for 3 days in a combination culture medium containing pea peptone or wheat peptone. Cell proliferation was then determined using Click-iT™ EdU flow cytometry assay, as described in the Materials and methods section. Analysis of the samples was conducted on a FACS Vantage flow cytometer. (a) serum-free, (b) pea peptone in serum-free, and (c) wheat peptone in serum-free.

factor-beta1 (TGF- β 1), interleukin-3 (IL-3), interleukin-6 (IL-6), interleukin-8 (IL-8), tumour necrosis factor-alpha (TNF- α), and vascular endothelial growth factor (VEGF) in both CB-MSCs and ADSCs treated with pea peptone or wheat peptone. We found that production of VEGF, TGF- β 1, and IL-6 were significantly increased by treatment with these peptones (Fig. 3).

Although treatment with pea peptone induced production of 678 pg/ml (increase of 8.83 ± 1.689 fold compared to untreated controls), 485 pg/ml (an increase of 3.79 ± 0.195 fold), and 332 pg/ml (an increase of 2.55 ± 0.217 fold) of VEGF, TGF- β 1, and IL-6, respectively, wheat peptone induced production of 1019 pg/ml (increase of 13.27 ± 1.447 fold) and 387 pg/ml (increase of 3.02 ± 0.234 fold) of VEGF and TGF- β 1, respectively, in CB-MSCs. In addition, production of IL-6 by CB-MSCs was not affected

by treatment with wheat peptone (Fig. 3). Similarly, pea peptone induced production of 122 pg/ml (increase of 6.70 ± 0.280 fold), 194 pg/ml (increase of 5.78 ± 0.445 fold), and 166 pg/ml (increase of 1.11 ± 0.080 fold) of VEGF, TGF- β 1, and IL-6, respectively, whereas wheat peptone induced production of 172 pg/ml (increase of 9.49 ± 0.896 fold), 170 pg/ml (increase of 5.07 ± 0.356 fold), and 192 pg/ml (increase of 1.28 ± 0.324 fold), of VEGF, TGF- β 1, and IL-6, respectively, in ADSCs (Fig. 4). However, no significant changes in other cytokines were observed in response to treatment with pea peptone or wheat peptone in either adult stem cell type (Figs 3–4). Taken together, these findings suggest that the stimulatory effect of vegetable peptone on proliferation of these adult stem cells may be mediated by VEGF, TGF- β 1 and IL-6.

(b)

Relative expression	serum (-)	pea/serum (-)	wheat/serum (-)
VEGF	1 ± 0.132	8.83 ± 1.689	13.27 ± 1.447
EGF	NM	NM	NM
FGF-2	NM	NM	NM
Flt-3 ligand	NM	NM	NM
G-CSF	NM	NM	NM
GM-CSF	NM	NM	NM
TGF-β1	1 ± 0.079	3.79 ± 0.195	3.02 ± 0.234
TNF-α	NM	NM	NM
IL-3	NM	NM	NM
IL-6	1 ± 0.167	2.55 ± 0.217	0.88 ± 0.166
IL-8	NM	NM	NM

*, NM < 30 pg/ml.

Figure 3. Cytokine profile analysis of conditioned medium of cord blood-derived mesenchymal stem cells (CB-MSC) treated with peptone. Adult stem cells were grown for 3 days in a combination culture medium containing pea peptone or wheat peptone. Quantitative analysis of the cytokines was then conducted by analysing supernatants of CB-MSCs that were cultured with or without peptone under serum-free conditions. Mean values for each group were obtained from four samples ($n = 4$). * indicates that treatment with peptone induced the production of < 30 pg/ml of cytokines.

Discussion

In this study, we evaluated the proliferation-inducing effect and possible mechanisms by which peptones elicit their activating effects on proliferation of human adult stem cells. Our data demonstrated that our peptones induced proliferation of the cells, which suggests that they may be a possible alternative for use of animal serum in culture systems of adult stem cells. Among the various pathways that are known to be involved in cell proliferation, we suggest that the peptones prompted adult stem cell proliferation by inducing production of VEGF, IL-6, and TGF-β1. Of these proteins, VEGF is known to exert its proliferation-promoting functions *via* two tyrosine kinase receptors: VEGF-1 (Flt-1) and VEGF-2 (Flk-1/KDR) (13–16). It has also been shown that VEGF is involved in proliferation of endothelial progenitor cells and human stromal-vascular fraction cells (17–19). Furthermore,

Gerber *et al.* reported that VEGF regulates haematopoietic stem cell survival *via* an apparent autocrine loop mechanism (20).

IL-6, which was also induced in cells that were treated with peptones, is reportedly involved in cell proliferation *via* activation of Janus-activated kinases and signal transducers and activators of transcription (STAT) (21). Activated STAT3 and mitogen-activated protein kinase induced by IL-6 are known to play a crucial role in cell proliferation (22,23). Collectively, these findings indicate that VEGF and IL-6 are involved in proliferation of the cells, induced by peptones.

Recent studies have revealed that production of VEGF and IL-6 can be induced by TGF-β, which regulates a variety of cellular responses. For example, Jeon *et al.* have reported that TGF-β induces VEGF at the transcriptional and protein levels in mouse macrophages (24). In addition, several studies have shown that TGF-β is involved in

(b)

Relative expression	serum (-)	pea/serum (-)	wheat/serum (-)
VEGF	1 ± 1.093	6.70 ± 0.280	9.49 ± 0.896
EGF	NM	NM	NM
FGF-2	NM	NM	NM
Flt-3 ligand	NM	NM	NM
G-CSF	NM	NM	NM
GM-CSF	NM	NM	NM
TGF-β1	1 ± 0.166	5.78 ± 0.445	5.07 ± 0.356
TNF-α	NM	NM	NM
IL-3	NM	NM	NM
IL-6	1 ± 0.082	1.11 ± 0.080	1.28 ± 0.324
IL-8	NM	NM	NM

*, NM < 30 pg/ml.

Figure 4. Cytokine profile analysis of conditioned medium of adipose-derived stem cells (ADSC) treated with peptone. Adult stem cells were grown for 3 days in a combination culture medium containing pea peptone or wheat peptone. Quantitative analysis of the cytokines was then conducted by analysing the supernatants of ADSCs cultured with or without peptone under serum-free conditions. Mean values for each group were obtained from four samples ($n = 4$). * indicates that treatment with peptone induced the production of < 30 pg/ml of cytokines.

angiogenesis through the induction of VEGF in human tumour cells (25), mouse fibroblasts (26), and epithelial cells (27). Furthermore, Peláiz *et al.* have demonstrated that increase in IL-6 secretion elicited by TGF-β1 may play an important role in its proliferative effect (28). In the light of these reports, our results suggest that stimulation of proliferation induced by peptones is mediated through induction of TGF-β1 gene expression. Specifically, these findings indicate that the peptones induced expression of TGF-β1, which sequentially upregulates expression of VEGF and IL-6, consequently promoting cell proliferation.

Taken together, data from this study demonstrate that peptones promote cell proliferation by upregulating expression of VEGF, IL-6, and TGF-β1. When combined with results of one of our previous studies, that revealed that TGF-β1 induces expression of VEGF and IL-6, the findings here indicate that TGF-β1 plays a crucial role in peptone-induced proliferation of adult stem cells.

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