論　文　内　容　要　旨

Oriented Immobilization of Basic Fibroblast Growth Factor for the Expansion of Mesenchymal Stem Cells

(間葉系幹細胞増幅のための塩基性繊維芽細胞増殖因子の配向固定)

主指導教員：Professor Koichi Kato
(Department of Biomaterials)
副指導教員：Professor Chisa Shukunami
(Department of Molecular Biology and Biochemistry)
副指導教員：Assistant Professor Isao Hirata
(Department of Biomaterials)

AJAY SHAKYA
(Graduate School of Biomedical & Health Sciences)

Introduction

Human mesenchymal stem cells (hMSCs) are highly desirable for their applications in regenerative medicine because of several advantages such as accessibility, ease of isolation, *in vitro* expansion capacity, and multi-lineage differentiation potential. In addition, clinical trials of hMSCs so far have shown no adverse effects. However, given the poor availability of hMSCs and the huge number of cells required for clinical applications, they need to be greatly expanded by *in vitro* culture, which requires a long preparation time. hMSCs have been almost exclusively expanded *in vitro* on a tissue culture plate (TCP) made of polystyrene. However, with this conventional method, there are problems such as slow proliferation of cells and premature senescence occurring in repeated passages. Hence, an efficient technique that addresses these problems is highly required. Basic fibroblast growth factor (bFGF) is a potent mitogen for hMSCs and is routinely added to culture media for the purpose of maintaining stemness and delaying senescence of hMSCs. It was reported that hMSCs expanded in the presence of bFGF exhibited faster proliferation rates.

In our study, we attempted to develop a novel culture technique for the efficient expansion of hMSCs by anchoring bFGF on the culture substrate. Reflecting the role of bFGF in its proliferation and maintenance, we hypothesized that hMSCs should express fibroblast growth factor receptors (FGFRs). Surface-immobilized bFGF will exert its effect by binding to the FGFRs present on the cell membrane of hMSCs, resulting in rapid proliferation of hMSCs, while retaining their stem cell state.

Experimental

Recombinant bFGF with histidine-tag (bFGF-His) at the C-terminus was expressed in *E. coli* and purified by metal chelate chromatography under denaturing conditions. The protein was dialyzed against citrate buffer solution of pH 5 or phosphate buffer solution of pH 7. Alternatively, bFGF-His was dialyzed at pH 5 and further done at pH 7. These samples are referred to as bFGF-His-5, bFGF-His-7, and bFGF-His-5/7, respectively. These proteins were characterized by SDS-PAGE analysis, circular dichroism (CD) spectroscopy, and biological assays using hMSCs.

The three types of bFGF-His were immobilized onto the surface of a glass plate that had been previously functionalized with nitrilotriacetic acid (NTA) and Ni^{2+} ions. The immobilization is expected through the chelate linkage between NTA-Ni^{2+} and the histidine-tag. *In situ* refolding of the surface-immobilized bFGF was attempted by exposing it to citrate buffer solution. The secondary structure of immobilized bFGF-His was analyzed by solid phase CD spectroscopy. hMSCs were cultured on the bFGF-His-immobilized surfaces to examine their proliferation and differentiation potentials.

Results and Discussion

Characterization of bFGF-His

SDS-PAGE analysis revealed that the three types of bFGF-His were successfully purified. It was further shown by CD spectroscopy that bFGF-His-5 and bFGF-His-5/7 were folded into structures similar to that of the native bFGF. pH 5 is distant from the isoelectric point of wild-type bFGF, thus the condition might help enhance the solubility of bFGF-His and the refolding of a bFGF domain to its native form. On the other hand, the spectrum of bFGF-His-7 had negative cotton effects at a wavelength of around 208 and 222 nm, indicating the presence of α-helix. The occurrence of α-helix is suggestive that bFGF-His-7 might not have folded into proper structure.

The biological activity of recombinant proteins was assessed by proliferation assays with hMSCs. Highest cell proliferation rate was observed with bFGF-His-5 followed by bFGF-His-5/7. On the other hand, the addition of bFGF-His-7 had no promotive effect on cell proliferation. These results suggest that the bFGF domain contained in the bFGF-His-5 and bFGF-His-5/7 hold their biological activity, whereas that in bFGF-His-7 do not.

Characterization of immobilized bFGF-His

bFGF-His was anchored in a definite orientation through the coordination of His with a Ni(II) bearing alkanethiol monolayer formed on a gold-evaporated glass plate. The entire process of immobilization was studied in real-time by surface plasmon resonance analysis. The amount of bFGF-His immobilized on the glass surfaces was determined to be 5.3 ± 0.9 ng/mm^2 by the Micro BCA assay.

The surface with immobilized bFGF-His-7 was treated with 20 mM citrate buffer of pH 5 at 4 ^{o}C. It was found the bFGF domain in the protein gradually refolded into the proper structure *in situ* as demonstrated by solid phase CD spectroscopy.

Proliferation of human hMSCs was studied on the surface with immobilized bFGF-His. It was observed that cells proliferated most slowly on the bFGF-His-7-immobilized surface that had not been subjected to citrate buffer treatment. In contrast, rapid cell proliferation was seen on the bFGF-His-7-immobilized surface post-treated with citrate buffer.

Characterization of MSCs expanded on α7-bFGF-His immobilized substrate

The bFGF-His-7-immobilized surface was compared with the surface of polystyrene in terms of cell proliferation and the maintenance of the stem cell state. In the case of cell culture on polystyrene, a culture medium was supplemented with commercial bFGF at 1 ng/ml. The significantly larger number of cells was obtained on the bFGF-His-immobilized surface than polystyrene 4 days after cell seeding. On bFGF-His-immobilized surface, cell number increased approximately 1.4 times on day 4 and 1.5 times on day 5 to 6. This result suggests that, within the same culture duration, a larger number of cells can be obtained on the bFGF-His-immobilized surface than polystyrene. It was further demonstrated that hMSCs proliferated on the bFGF-His-immobilized surface retained their stem cell state as they could successfully differentiate into adipogenic, chondrogenic, and osteogenic lineages under specific differentiation media.

Conclusions

It was shown that bFGF-His is able to refold in citrate buffer of pH 5 during dialysis. Similar to this, the immobilized bFGF-His can also be refolded to be a biologically active form by *in situ* treatment with citrate buffer solution. This was well demonstrated by the cell culture experiments: hMSCs could be rapidly expanded while retaining their stem cell state. These results led us to conclude that the immobilization of bFGF-His on the culture substrate serves to enhance the expansion of hMSCs.