Immobilization of Bioactive Growth Factors into Cubic Proteinous Microcrystals (Cypovirus Polyhedra) and Control of Cell Proliferation and Differentiation

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ABSTRACT

Certain insect viruses produce stable infectious microcrystals called polyhedra which function to protect the virus after the death of infected larvae. Polyhedra form within infected cells and contain numerous virus particles embedded in a crystalline lattice of the viral protein polyhedrin. We have previously demonstrated that the N-terminal 75 amino acids of the *Bombx mori* cypovirus (BmCPV) turret protein can function as a polyhedrin recognition signal leading to the incorporation of foreign proteins into polyhedra. Foreign proteins tagged with the VP3 polyhedrin recognition signal were incorporated into polyhedra by co-expression with polyhedrin in insect cells. We have also reported that the N-terminal α-helix of polyhedrin directs the incorporation of foreign proteins into polyhedra. Here we show the successful occlusion of human cytokines (FGF-2, FGF-7, EGF, etc) into the BmCPV polyhedra. The polyhedra are proteinous cubic crystals of several microns in size that are insoluble in the extracellular milieu. We demonstrate that BmCPV polyhedra microcrystals that occlude extracellular signaling proteins are a novel and versatile tool that can be employed to analyze cellular behavior at the single cell level.

Keywords: cypovirus, polyhedra, polyhedrin, immobilization, cytokine

1 INTRODUCION

The virus particles, or virions, are protected within these polyhedra and can remain infectious for years outside cells, even in harsh environmental conditions [1, 2]. The polyhedra break down and release the virus only when ingested into the very alkaline environment of the midgut of insect larvae (pH 10–11), resulting in infection of a new host. The genus *Cypovirus* is members of the family *Reoviridae* that infect insect larvae producing polyhedra in the cytoplasm of mid-gut epithelial cells. Viral outer capsid protein 3 (VP3) of the silkworm *Bombx mori* cypovirus (BmCPV) is a turret protein of a virus particle is considered to function as a polyhedrin recognition signal leading to the occlusion of virus particles in polyhedra [3-5]. Recently the atomic structure of BmCPV polyhedra has been determined by using a synchrotron microbeam to collect X-ray diffraction data [6]. It was elucidated that polyhedra are made from trimeric building blocks of the polyhedrin interlocked into a tight scaffold generated by the amino-terminal α-helix (H1). These results have been exploited to encapsulate a wide variety of foreign proteins into polyhedra [7]. I introduce these modified polyhedra or 'nano-containers', which have a variety of applications, including the development of stabilized growth factors for cell-culture. An immobilization signal derived from VP3 or H1 sequence is called VP3 signal or H1 signal (Fig. 1).

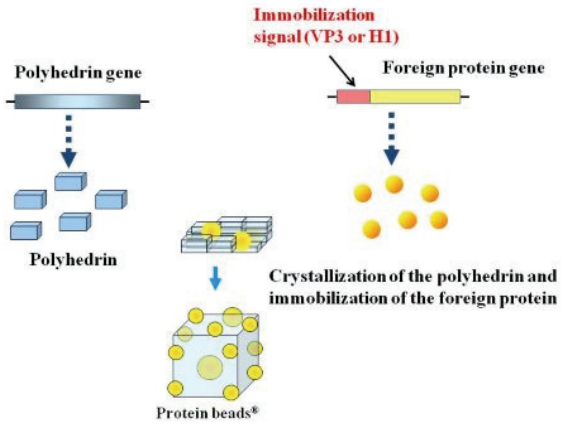

Fig. 1 Scheme of immobilization of foreign proteins into BmCPV polyhedra. Two recombinant baculoviruses are constructed. One recombinant baculovirus expresses BmCPV polyherin and other express a forein protein which is fused with an immobilization signal VP3 or H1. Sf cells are co-infected with these recombinant baculovirses and polyhedra are recovered from the infected cells.

2 IMMOBILIZATION OF CYTOKINES

2.1 FGF-2

BmCPV polyhedrin gene was introduced into a baculovirus expression vector and the insect cell line *Spodoptera frugiperda* IPLB-Sf21AE (Sf21 cells) was infected with the recombinant baculovirus designated AcCP-H. The polyhedrin was massively produced and many cubic polyhedra were observed in Sf cells [8].

Fibroblast growth factor-2 (FGF-2) was fused with the VP3 signal at the C-terminal (FGF-2/VP3) or H1 signal at the N-terminal (H1/FGF-2) and co expressed BmCPV polyhedrin. The FGF-2 fused with VP3 or H1 signal was immobilized into the polyhedra. The biological activity of FGF-2/VP3 and H1/FGF-2 polyhedra was assayed using mouse fibroblast NIH3T3 cells. In the presence of both

FGF-2/VP3 polyhedra and H1/FGF-2 polyhedra, NIH3T3 cells proliferated in a dose-dependent manner to an extent that was comparable with the addition of rhFGF-2 (Fig. 2). Effect on proliferation of NIH3T3 by an addition of 1 ng of rhFGF-2 was estimated to correspond with 1.5 x 10^4 cubes of H1/FGF-2 polyhedra and 7.5 x 10^4 cubes of FGF-2/VP3 polyhedra, respectively [9, 10].

It is well known that intracellular signaling events such as phosphorylation of p44/42 mitogen-activated protein kinase (MAPK) are observed after FGF-2 stimulation. It was found that 2.5 x 10^4 cubes of H1/FGF-2 and FGF-2/VP3 polyhedra induced phosphorylation of p44/42 similarly to the phosphorylation induced by addition of rhFGF-2. Moreover, the brighter fluorescence observed for H1/FGF-2 suggests that the dose of the biologically active form of FGF-2 that a single cube of polyhedra can release is higher in H1/FGF-2 form than in FGF-2/VP3 form.

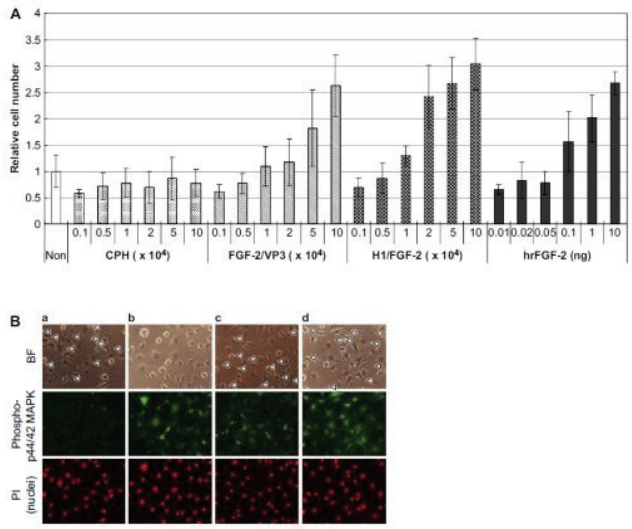

Fig. 2 Effects of FGF-2 polyhedra on the proliferation of NIH/3T3 cells in culture. (A) Proliferation of NIH/3T3 cells by FGF-2 polyhedra. NIH3T3 cells were seeded onto a 96 well cell culture plate at a density of 4,000 cells per well and cultured overnight in DMEM medium containing 10% FCS followed by 6 hours culture in α–MEM medium without FCS to starve the cells. Cells were stimulated with CP-H polyhedra and FGF-2 polyhedra (FGF-2/VP3 and H1/FGF-2) or recombinant human FGF-2 (rhFGF-2). Number of polyhedra or the dose of rhFGF-2 on the horizontal axis. After 5 days, the growth rate of respective cell group was determined by CCK assay using microplate reader. The mean value of triplicate samples and its standard deviation (SD) are shown as a bar and an error bar, respectively. **(B)** Immunocytochemistry of phosphorylated MAPK in NIH3T3 cells. The serum-starved NIH3T3 cells on 96 well culture plate were stimulated with CP-H polyhedra (a), 5 ng of rhFGF-2 (b), FGF-2/VP3 polyhedra (c) or H1/FGF-2 polyhedra (d) (each 2.5 x 10^4 polyhedra) for 15min. The cells were then fixed and its nuclei were stained with propidium iodide (PI). Furthermore, the intracellular phosphorylated MAP kinase was detected by immunocytochemistry using a primary anti-phospho p44/42 MAP kinase antibody and a secondary antibody conjugated with Alexa 488 fluorescent dye. The phosphorylated cells were detected under a fluorescent microscope. The composite image shown indicates phophorylated p44/42 MAP kinase (green) and nuclei (red). Each polyhedra are identified with white triangular shapes.

2.2 FGF-7

The epithelium of human skin is mainly composed of keratinocytes (approximately 95%) together with melanocytes and fibroblasts [9]. The proliferation and migration of keratinocytes are pivotal events for re-epithelialization of skin during wound healing [10]. Keratinocytes are known to proliferate in the presence of FGF-7 (also called keratinocyte growth factor) [10]. Hence, we attempted to generate polyhedra containing FGF-7 and examined its proliferative effect on human epidermal keratinocytes (NHEK). FGF-7 polyhedra were generated by fusing VP3 immobilization signal to FGF-7 C-terminal and H1 signal to FGF-7 N-terminal, respectively [7]. FGF-7 polyhedra were deposited on the bottom of culture well and air dried. NHEK were cultured for 5 days in serum free medium on the FGF-7 polyhedra-coated wells. The positive control was cells cultured in medium with a commercial growth supplement specific for cultivation for keratinocyte. This supplement contains FGF-7, EGF and other undisclosed cytokines. The exact quantity of theses cytokines in the growth supplement is unknown. The proliferation of keratinocyte was confirmed in a dose-dependent manner either by addition of H1/FGF-7 polyhedra or FGF-7/VP3 polyhedra. Unlike immobilization of EGFP or that of FGF-2 into polyhedra, there was no significant difference between FGF7/VP3 and H1/FGF7 polyhedra in term of cell proliferation of NHEK (Fig. 3).

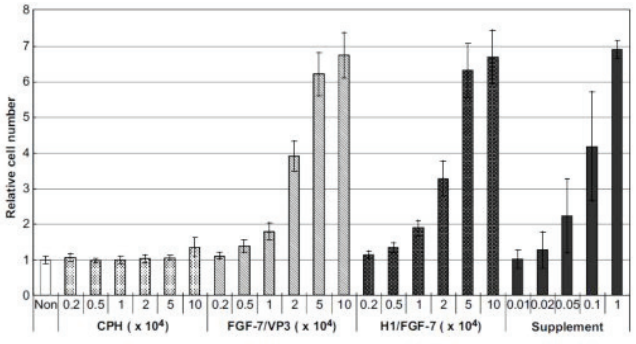

Fig. 3 Culture of keratinocytes by FGF-7 polyhedra. NHEK at passage 3 with DK-SFM containing the growth supplement were seeded at a density of 900 cells / well and

cultured for 5 days at 37°C under 5% CO_2 in air. Comparison of the proliferation of keratinocytes in the presence of various growth supplements. NHEK cells were seeded and grown with or without a commercial growth supplement, with CP-H polyhedra or FGF-7 polyhedra (FGF-7/VP3 and H1/FGF-7) at concentrations of 0.2 to 10 x 10^4 polyhedra per well. The rate of cell proliferation was detected by CCK assay for 4 hour at 37°C under 5% CO_2 in air and measure of absorbance at 450nm. The mean value of triplicate samples and its standard deviation (SD) are shown as a bar and an error bar, respectively.

2.3 EGF

The biological activity of epidermal growth factor (EGF) immobilized into polyhedra by VP3 and H1 immobilization signal was also examined (Fig. 4). H1/EGF polyhedra cubes or EGF/VP3 polyhedra cubes were spotted on culture wells and desiccated. Proliferation of keratinocyte was observed with no difference between EGF/VP3 polyhedra and H1/EGF polyhedra. However a double dose of EGF polyhedra was required to attain the same rate of keratinocyte proliferation compared to FGF-7 polyhedra [7].

Furthermore, the synergic effect of FGF-7 and EGF polyhedra on the proliferation of keratinocyte was assayed. While low numbers of H1/FGF-7-polyhedra (0.1 x 10^4) or H1-EGF polyhedra (5 x 10^4) failed to induce a strong growth of keratinocyte (< 2-fold), the combination of both immobilized growth factors at the same concentrations induced a 3-fold proliferation of keratinocytes. A proliferative effect of 75% of the commercial supplement was reached for either 10 x 10^4 H1/FGF-7, 10 x 10^4 H1/EGF or a combination of 1 x 10^4 H1/FGF-7 and 5 x 10^4 H1/EGF [7].

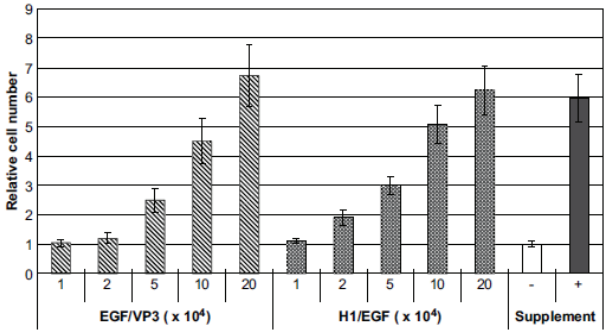

Fig. 4. Synergistic effect of growth factors by the mixture of FGF-7 and EGF polyhedra. Culture of keratinocytes by EGF polyhedra. EGF polyhedra (EGF/VP3 and H1/EGF) were mounted on a 96 well culture plate. The number of EGF polyhedra was 1 to 20 x 10^4. NHEK at passage 3 with DK-SFM containing the growth supplement were seeded without its supplement at a density of 900 cells / well and cultured for 5 days at 37°C under 5% CO_2 in air. The cells were also incubated with or without the growth supplement. The rate of cell proliferation was detected by CCK assay.

2.4 Application of polyhedra as protein nanocontainers

Mouse chondrogenic ATDC5 cells efficiently differentiate into chondrocytes to form cartilage nodules in the presence of insulin and FBS. However, the nodule formation is strongly inhibited by the application of growth stimuli such as the FGF-2 treatment during induction of chondrogenesis. The nodule formation was assayed by the formation of alcian blue positive-cartilage nodules in ATDC5 culture (Fig. 5). Empty-polyhedra do not affect the formation of nodules, whereas a single addition of FGF-2/VP3 polyhedra blocked chondrogenic differentiation in a dose dependent manner. The formation of cartilage nodules was partially inhibited by a single addition of FGF-2 (1 ng/ml) and almost completely blocked when FGF-2 was added to the culture on every occasion of medium change [11].

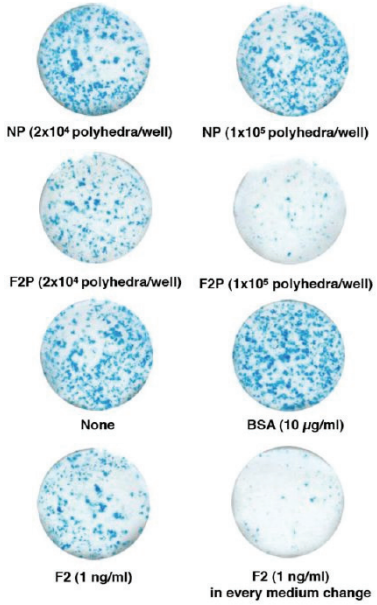

Fig. 5 Effects of FGF-2 polyhedra on the differentiation of ATDC5cells (Alcian blue staining). ATDC5 cells were incubated with or without empty-polyhedra (NP), FGF-2/VP3 polyhedra (F2P), BSA (10μg/ml), or FGF-2 (F2, 1 ng/ml) and maintained in DME/F-12 medium containing ITS and 5% FBS for another 2 weeks to induce chondrogenic differentiation; the culture medium was replaced twice a week. Polyhedra or FGF-2 were added to the confluent cells only once except for the different experimental setting in which cells were treated with FGF-2 (1 ng/ml) at every change of medium. Alcian blue staining of each culture is shown.

FGF-2/VP3 polyhedra mounted and desiccated on culture dish retain the biological ability of FGF-2 (Fig. 6).

To explore application of FGF-2/VP3 polyhedra acting as a signaling cue in a spatially restricted manner *in vitro*, ATDC5 cells were seeded on and around FGF-2/VP3 polyhedra. Cellular proliferation was significantly stimulated only in the vicinity of FGF-2/VP3 polyhedra (within the area denoted by the dotted line) (Fig. 6C). For comparison, Affi-Gel blue beads presoaked with FGF-2 were spotted on the culture plate. The FGF-2 bound beads also markedly stimulated the proliferation of ATDC5 cells, however, unlike FGF-2/VP3 polyhedron coverslips, a rapid diffusion of released FGF-2 from Affi-Gel beads stimulated the proliferation of cells irrespective of cell location (Fig. 6E). These results showed that FGF-2/VP3 polyhedra adhered to cells and acted as effective releasers onto cultured cells [11].

Under physiological conditions, polyhedra are inert and insoluble entities and this allows to use them as versatile micron sized carriers of cell growth factors, receptor binding domains, and cell binding domains of the ECM. The modified cypovirus polyhedra containing foreign proteins can function as nanocontainers stabilizing incorporated proteins in a functional form. Cytokine chips where polyhedra contain various combinations of cytokines regulate cellular signaling, proliferation and differentiation at the single cell level. Such perspectives will certainly drive active research on the application of polyhedra in stem cell research, tissue engineering and regenerative medicine. Another application is as a stable delivery vehicle for cargos such as vaccine components, fluorescent probes or drugs [11].

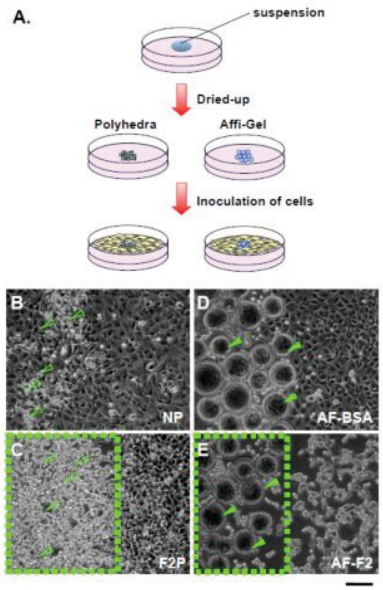

Fig. 6 Effects of desiccated FGF-2/VP3 polyhedra or FGF-2-bound Affi-Gel blue gel on the proliferation of ATDC5 cells. Affi-Gel blue gel (AF) was incubated with FGF-2 (10 μg/ml) or vehicle containing BSA (10 μg/ml). (A) A 12-well plate was spotted with a suspension of polyhedra or Affi-Gel blue gels and desiccated. (B) Empty-polyhedra (NP, 2 x 10^4), (C) FGF-2/VP3 polyhedra (F2P, 2 x 10^4), (D) Affi-Gel blue gel treated with BSA (AF-BSA, 1 x 10^3), (E) Affi-Gel blue gel treated with FGF-2 (AF-F2, 1 x 10^3). ATDC5 cells were seeded into the plate and maintained in DME/F-12 medium containing 5% FBS. Open and closed arrowheads indicate polyhedra and Affi-Gel blue gel, respectively. Scale bar, 100 μm.

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