Immobilization of Bioactive Fibroblast Growth Factor-2 into Cubic Proteinous Microcrystals (Bombyx mori Cypovirus Polyhedra) That Are Insoluble in a Physiological Cellular Environment*

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The supramolecular architecture of the extracellular matrix and the disposition of its specific accessory molecules give rise to variable heterotropic signaling cues for single cells. Here we have described the successful occlusion of human fibroblast growth factor-2 (FGF-2) into the cubic inclusion bodies (FGF-2 polyhedra) of the Bombyx mori cytoplasmic polyhedrosis virus (BmCPV). The polyhedra are proteinous cubic crystals of several microns in size that are insoluble in the extracellular milieu. Purified FGF-2 polyhedra were found to stimulate proliferation and phosphorylation of p44/p42 mitogen-activated protein kinase in cultured fibroblasts. Moreover, cellular responses were blocked by a synthetic inhibitor of the FGF signaling pathway, SU5402, suggesting that FGF-2 polyhedra indeed act through FGF receptors. Furthermore, FGF-2 polyhedra retained potent growth stimulatory properties even after desiccation. We have demonstrated 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.

Hormones and cytokines in circulation represent a typical mode of cell–cell communication via diffusible extracellular signal molecules. Extracellular concentrations and affinities to cell-surface receptors are the major determinants of the potency of signaling proteins. However, with the exception of hematopoietic and/or blood cells, all single cells throughout the various organs receive their signaling cues from the extracellular matrix (ECM).3 The basal lamina underlying the epithelial cell sheets and the specialized ECM in the mesenchyme, such as cartilage and bone, play a crucial role in this regard during the regulation of cellular behavior (1). The ECM provides a supramolecular architecture for the disposition of specific accessory molecules that exist in low abundance, such as growth factors, which are released after proteolytic cleavage to effect a downstream alteration in the behavior of the responding cells. Conversely, cells that regulate remodeling (or degradation under certain conditions) of the ECM do so via the production and secretion of proteases and protease inhibitors (2). Thus, each individual cell sends and receives spatiotemporally restricted signals from its extracellular environment. However, only limited tools are currently available that can generate signaling cues in a spatially restricted manner, at the cellular or subcellular level in vitro, to enable further studies of these events.

Previously, we developed a novel protein expression system that enables us to immobilize foreign proteins on insect virus occlusion bodies of protein crystals, termed polyhedra (3, 4). Polyhedra are the main vectors of virus particles from insects and are also the main agents that facilitate the survival of the virus, as they stabilize the virions allowing them to remain viable for very long periods in the environment (5, 6). As polyhedra are quite stable and are virtually insoluble at physiological pH, infection occurs by the release of the virus particles at high pH in the insect intestine after ingestion. Recently we resolved the 2-Å crystal structure of recombinant and infectious Bombyx mori cytoplasmic polyhedrosis virus (BmCPV) polyhedra, which are the smallest proteinous crystals yet used for de novo x-ray protein crystallography (7). The results suggest that polyhedra can serve as the basis for the development of robust and versatile nanoparticles for biotechnological applications (4).

We previously identified the immobilization signal for the incorporation of foreign proteins into BmCPV cubic polyhedra. In this expression system, the foreign proteins containing the immobilization signal are produced under the control of the polyhedrosis virus; BrdUrd, bromodeoxyuridine; FBS, fetal bovine serum; DME medium, Dulbecco’s modified Eagle’s medium; GFP, green fluorescence protein; PBS, phosphate-buffered saline; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; HSPG, heparan sulfate proteoglycan; MAP, mitogen-activated protein; IT5, insulin, transferrin, and selenite.
Immobilization of FGF-2 in Cubic Protein Crystals

baculovirus polyhedrin promoter and are occluded into BmCPV polyhedra comprising crystallized recombinant BmCPV polyhedrin protein (Fig. 1A) (8, 9). As fibroblast growth factor-2 (FGF-2) is one of the well characterized heparin-binding growth factors and regulates cell proliferation, differentiation, and migration (10), here we attempted to produce polyhedra-immobilized growth factors using this technology, including human FGF-2.

**EXPERIMENTAL PROCEDURES**

Reagents—Recombinant human fibroblast growth factor-2 (rhFGF-2) was purchased from R&D Systems. PD98059 and SU5402 were purchased from New England Biolabs and Calbiochem, respectively. Affi-Gel blue gel was purchased from Bio-Rad Laboratories. Bromodeoxyuridine (BrdUrd) was purchased from Roche Applied Science.

**Virus and Cells**—The Spodoptera frugiperda cell line IPLB-Sf21-AE (Sf21) was maintained in tissue culture flasks in TC-100 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS). A recombinant form of BmCPV (AcCP-H), which produces cubic polyhedra (8, 9), was employed in this study. NIH3T3 cells were grown in Dulbecco’s modified Eagle’s (DME) medium (Sigma) supplemented with 10% FBS (Biological Industries) and 50 μg/ml kanamycin (Sigma) at 37 °C under 5% CO2 in air. ATDC5 cells were grown in a 1:1 mixture of DME and Ham’s F-12 (DME/F-12) medium (Asahi Techno Glass Corp.) containing 5% FBS (Biological Industries), 10 μg/ml human insulin (Roche Applied Science), and 10 μg/ml human transferrin (Roche Applied Science), and 3 × 10^-8 M sodium selenite (Sigma) at 37 °C under 5% CO2 in air as described previously (11, 12). The culture medium was replaced three times a week.

Construction of Recombinant Baculoviruses—A cDNA fragment (nucleotides 135–292) derived from BmCPV segment 4 and encoding the N-terminal sequence (amino acids 42–93) of recombinant FGF-2. A “destination vector” was constructed by inserting a cassette consisting of an FGF-2 gene, the ccdB gene, and an attR1 site at the 5’-end, the chloramphenicol resistance gene, the ccdB gene, and an attR2 site at the 3’-end into the Smal site of pVL1393/Vp3. The ORF of the FGF-2 gene was then amplified by PCR, in which the 5’-end, the chloramphenicol resistance gene, the ccdB gene, and an attP1 site at its 5’-end, the open reading frame insert of FGF-2 in the resulting “entry” vector was then transferred to the destination vector via LR clonase reactions. The resulting “expression” vector pAcFGF-2/Vp3 consisted of the N terminus of Vp3 fused in-frame to the open reading frame of FGF-2 in the background of the baculovirus transfer vector pVL1393. The recombinant transfer vector pAcFGF-2/Vp3 was co-transfected into Sf21 cells together with linearized AcNPV DNA (Baculogold baculovirus DNA, Pharmingen). The recombinant AcNPVs produced by rescue of the linearized viral DNA were then harvested and isolated by plaque purification to obtain the recombinant baculovirus AcFGF-2/Vp3.

**Purification of Polyhedra**—Sf21 cells were inoculated with recombinant AcCP-H virus to generate normal polyhedra at a multiplicity of infection of 10 plaque-forming units/cell. For double infections with AcCP-H and AcFGF-2/Vp3 to obtain FGF-2 polyhedra, each virus was added at a multiplicity of infection of 5 plaque-forming units/cell. The infected cells were collected, washed with phosphate-buffered saline (PBS; 20 mM NaH2PO4, 20 mM Na2HPO4, 150 mM NaCl, pH 7.2), and homogenized to remove the cell membrane and nuclear envelope using the ultrasonic homogenizer VP-5S (Taisec). The polyhedra preparations were then washed with sterile water containing penicillin (10,000 unit/ml) and streptomycin (10 mg/ml) by centrifugation at 15,000 rpm for 10 min. The size of each polyhedron was determined using a microscope equipped with a scale bar.

**F-actin Staining**—ATDC5 cells were seeded onto a collagen-coated 8-well Lab-Tek II chamber slide (Nalge Nunc International) at a density of 1 × 10^5 cells/well and cultured to confluence in DME/F-12 medium containing ITS and 5% FBS for 3 days. GFP polyhedra (1 × 10^3) were then added to the confluent cells, which were incubated for a further 10 min. The cells were then washed twice with PBS and fixed in 4% paraformaldehyde for 15 min at room temperature. After fixation, cells were permeabilized with 0.1% Triton X-100 in PBS containing 1% of bovine serum albumin (BSA) and subjected to staining with Alexa Fluor 594 phalloidin (Molecular Probes) and 4’,6-diamidino-2-phenylindole (DAPI; Sigma) for 1 h at room temperature. After washing with PBS three times, the cells were mounted using a ProLong Antifade kit (Molecular Probes). Fluorescent micrographs were captured on a DM IRBE fluorescence microscope (Leica) using a charge-coupled device camera (CCD; Hamamatsu Photonics), and image analysis was performed with IPLabs software (Solution Systems).

**In Vitro BrdUrd Labeling**—ATDC5 cells were seeded onto a collagen-coated 8-well Lab-Tek II chamber slide at a density of 2 × 10^4 cells/well and grown to confluence in DME/F-12 medium containing ITS and 5% FBS. Cells were incubated to quiescence in the culture medium supplemented with 0.5% FBS for 24 h. The cultures were then treated with normal polyhedra (2 × 10^6), FGF-2 polyhedra (2 × 10^6), bovine serum albumin (10 μg/ml), or rhFGF-2 (5 ng/ml) for a further 15 h. BrdUrd (10 μM) was added to the cultures and incubated for another hour at 37 °C under 5% CO2 in air. The cultures were washed three times with PBS and fixed with 70% ethanol containing 50 mM glycine-HCl buffer (pH 2.0) at room temperature. The fixed cells were washed with 10% FBS containing DME/F-12 medium...
Immobilization of FGF-2 in Cubic Protein Crystals

The virion outer capsid protein VP3 of BmCPV encoding the immobilization signal was fused to the 18-kDa form of FGF-2 at the C terminus of FGF-2. The fusion protein is then co-expressed with BmCPV polyhedrin and incorporated into the resulting polyhedra. The immobilization signal derived from the VP3 region of BmCPV was introduced respectively.

**FIGURE 1. Preparation and characterization of FGF-2 polyhedra.** A, schematic representation of the preparation of FGF-2 polyhedra. The immobilization signal derived from the VP3 region of BmCPV was introduced at the C terminus of FGF-2. The fusion protein is then co-expressed with BmCPV polyhedrin and incorporated into the resulting polyhedra. B, immobilization of FGF-2 into polyhedra was confirmed by both SDS-PAGE (left panel) and Western blot analysis (right panel). Lanes 1 and 2 show normal polyhedra and FGF-2 polyhedra, respectively. C, the size distributions of the normal polyhedra (white bar) and FGF-2 polyhedra (hatched bar) are indicated. D, ATDC5 cells were seeded onto a collagen-coated 8-well Lab-Tek II chamber slide at a density of $1 \times 10^7$ cells/well and cultured to confluence in DME/F-12 medium containing ITS and 5% FBS. Normal polyhedra ($0.5 \times 10^5$) or FGF-2 polyhedra ($0.5 \times 10^5$) were added to a well of medium was collected. After centrifugation at 15,000 rpm for 5 min, 100 µl of SDS sample buffer containing 50 mM Tris-HCl (pH 6.8), 2% SDS, 50 mM dithiothreitol, 0.1% bromphenol blue, and 10% glycerol. The resulting cell lysates were then harvested, sonicated, and cleaned by centrifugation. Samples were electrophoresed on 12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad). After preincubation with blocking buffer containing 5% non-fat milk, 0.1% Tween 20, 25 mM Tris-HCl (pH 7.6), 150 mM NaCl, the membranes were incubated with either p44/42 MAP kinase or phospho-p44/42 MAP kinase (Thr-202/Tyr-204) antibodies, followed by horseradish peroxidase-conjugated anti-rabbit IgG antibody (New England Biolabs). Peroxidase activity was detected and visualized by ECL Western blotting detection reagents (Amersham Biosciences), according to the manufacturer’s instructions.

**Quantification of FGF-2 by Enzyme-linked Immunosorbent Assay (ELISA)—**ATDC5 cells were plated into a 24-well at a density of $2 \times 10^4$ cells/well and grown to confluence in DME/F-12 medium containing ITS and 5% FBS. Normal polyhedra ($0.5 \times 10^5$) or FGF-2 polyhedra ($0.5 \times 10^5$) were added to a well with or without cells and incubated for 24 h in DME/F-12 medium containing ITS and 5% FBS. Five hundred µl of conditioned medium or medium without cells was collected. After centrifugation at 15,000 rpm for 5 min, 100 µl of medium was used to determine the quantity of FGF-2 using a Quantikine human FGF basic immunoassay kit (R&D Systems). Absorbance at 450 and 570 nm was determined using a model 450 microplate reader (Bio-Rad).

**RESULTS**

The virion outer capsid protein VP3 of BmCPV encoding the immobilization signal was fused to the 18-kDa form of FGF-2 at either the N or C terminus for the production of polyhedra containing immobilized FGF-2. This chimeric protein was then blotted electrophoretically onto a nitrocellulose membrane. The membrane was saturated with washing buffer (20 mM Tris-HCl (pH 7.5), 500 mM NaCl) containing 1% gelatin and incubated overnight at 25 °C with monoclonal antibody against FGF-2 bFM-2 (100 ng/ml). After several washes, the membrane was then incubated for 1 h at 25 °C with a 1:3000 dilution of goat anti-mouse IgG conjugated with horse-radish peroxidase (Bio-Rad). NIH3T3 cells and ATDC5 cells were washed twice with PBS twice dissolved in 100 µl of SDS sample buffer containing 50 mM Tris-HCl (pH 6.8), 2% SDS, 50 mM dithiothreitol, 0.1% bromphenol blue, and 10% glycerol. The resulting cell lysates were then harvested, sonicated, and cleaned by centrifugation. Samples were electrophoresed on 12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad). After preincubation with blocking buffer containing 5% non-fat milk, 0.1% Tween 20, 25 mM Tris-HCl (pH 7.6), 150 mM NaCl, the membranes were incubated with either p44/42 MAP kinase or phospho-p44/42 MAP kinase (Thr-202/Tyr-204) antibodies, followed by horseradish peroxidase-conjugated anti-rabbit IgG antibody (New England Biolabs). Peroxidase activity was detected and visualized by ECL Western blotting detection reagents (Amersham Biosciences), according to the manufacturer’s instructions.

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co-expressed with a BmCPV polyhedrin using a baculovirus expression vector system (Fig. 1A). The polyhedra were then purified from the co-infected host cells. A band of ~35 kDa was detectable for both BmCPV polyhedra that did not harbor any foreign proteins and for the polyhedra containing FGF-2 fused to VP3 at the C terminus (Fig. 1B, left panel). Although we could not readily distinguish the FGF-2 fusion protein (with an estimated size of ~30 kDa) from the large quantities of co-existing polyhedra by SDS-PAGE, the expression of FGF-2 could be detected as an ~30 kDa band by Western blotting using a specific monoclonal antibody (Fig. 1B, right panel) (13). However, only degradation products of FGF-2 were found in polyhedra where FGF-2 was fused with VP3 at the N terminus (data not shown). We therefore used the C-terminally fused FGF-2 polyhedra in our subsequent analyses and employed the non-fused polyhedra as negative controls.

Fig. 1C indicates the size distribution of purified normal and FGF-2 polyhedra determined by microscopy. Most of these complexes were found to be within the 2–7 μm size range. To further visualize these dimensions, polyhedra containing immobilized enhanced GFP were added to confluent cultures of mouse chondrogenic ATDC5 cells (Fig. 1D) (11, 12). The nuclei and actin filaments of these cells were then stained by phalloidin and 4',6-diamino-2-phenylindole, respectively, and each of the enhanced GFP polyhedra was detectable as green fluorescent cubes of less than 10 μm in size (Fig. 1D). Additionally, these polyhedra showed an affinity for cell surfaces, an association that was not disrupted by either medium changes or extensive rinsing with PBS prior to fixation and staining. This adhesive characteristic of polyhedra is a favorable property that enabled us to target these complexes to a specific site in cultured cells and thereby examine the dynamic cellular behavior at the single cell level.

To evaluate the biological activity of FGF-2, we studied the effects of FGF-2 polyhedra on the proliferation of mouse chondrogenic ATDC5 cells in culture. Undifferentiated ATDC5 cells are known to express FGFR1 and FGFR2, and the expression of FGFR3 is induced during chondrogenic differentiation (14). When FGF-2 polyhedra were added to the undifferentiated quiescent ATDC5 cells at confluence, proliferating cells were detected by in situ BrdUrd labeling (Fig. 2B). In contrast to quiescent flat cells making close contacts with one another (Fig. 2E), BrdUrd-labeled growing cells lost their contacts and took on spindle-like morphology (Fig. 2F). In contrast, normal polyhedra did not show any growth stimulatory activity (Fig. 2A). Recombinant human FGF-2 in solution similarly stimulated the growth of ATDC5 cells (Fig. 2D) and induced morphological change (Fig. 2H) as compared with those treated with BSA only (Fig. 2C). Next, we studied the effects of FGF-2 polyhedra on DNA synthesis of mouse fibroblasts in culture. FGF-2 polyhedra were found to stimulate DNA synthesis of NIH3T3 cells in a dose-dependent manner to an extent that was comparable with 10% FBS supplementation, whereas normal polyhedra did not exert any such growth effects (Fig. 3A). We found in our current experiments that dissolved rhFGF-2 at a dose of 1 ng/ml similarly stimulates DNA synthesis in ATDC5 cells, whereas SU5402, a synthetic inhibitor of the FGF signaling, efficiently blocks this effect (Fig. 3B). Moreover, when we added 5 × 10³ and 1 × 10⁴ FGF-2 polyhedra to serum-starved ATDC5 cells, BrdUrd incorporation was found to be 3.6 and 4.6 times higher, respectively, than in the nonstimulated cells (Fig. 3B). SU5402 exposure also clearly inhibited this FGF-2 polyhedra stimulation of DNA synthesis in these experiments (Fig. 3B). These results suggest that FGF-2 polyhedra stimulate the growth of ATDC5 cells through FGF receptor-mediated signaling, which triggers downstream signal transduction cascades including the RAS-MAP kinase, phosphatidylinositol 3-kinase, and STAT (signal transducers and activators of transcription) pathways (15, 16).

ATDC5 cells efficiently differentiate into chondrocytes to form cartilage nodules in the presence of insulin (10 μg/ml) and 5% FBS (11). However, the nodule formation is strongly inhibited by the application of growth stimuli such as FGF-2 treatment during induction of chondrogenesis (17). As the nodule formation was easily visualized by Alcian blue staining, we assessed the bioactivity of FGF-2 polyhedra in terms of the formation of Alcian blue-positive cartilage nodules in ATDC5 cul-

FIGURE 2. Effects of FGF-2 polyhedra on the proliferation of ATDC5 cells in culture. ATDC5 cells were seeded onto a collagen-coated 8-well Lab-Tek II chamber slide at a density of 2 × 10⁶ cells/well and cultured to confluence in DME/F-12 medium containing ITS and 5% FBS. Cells were then incubated to be quiescent in 0.5% FBS-containing DME/F-12 medium for 24 h and further cultured in the presence of normal polyhedra (NP, 2 × 10⁴ polyhedra), FGF-2 polyhedra (F2P, 2 × 10⁴ polyhedra), BSA (10 μg/ml), or FGF-2 (F2, 5 ng/ml) for 15 h. For detection of proliferating cells, BrdUrd solution was added to each well at a final concentration of 10 μm, and cells were incubated for an additional hour at 37°C under 5% CO₂ in air. BrdUrd-labeled DNA was then detected by using a primary anti-BrdUrd antibody and a secondary antibody conjugated with Alexa 488 fluorescent dye. The labeled cells were analyzed under a fluorescent microscope (A–D) and a phase-contrast microscope (E–H). Scale bar, 100 μm.
Normal polyhedra do not affect the formation of nodules, whereas FGF-2 polyhedra blocked chondrogenic differentiation in a dose-dependent manner. A single addition of rhFGF-2 (1 ng/ml) to the confluent ATDC5 cells resulted in a partial inhibition of differentiation. In contrast, the formation of cartilage nodules was almost completely blocked when rhFGF-2 was added to the culture on every occasion of medium change.

To further examine the downstream signaling pathways activated by FGF-2 polyhedra, the phosphorylation of p44/p42 MAP kinases was analyzed in both NIH3T3 cells and ATDC5 cells.

FIGURE 3. Effects of FGF-2 polyhedra on the proliferation of NIH3T3 and ATDC5 cells in culture. The rate of DNA synthesis was evaluated by BrdUrd incorporation (A and B). A, NIH3T3 cells were plated into a 96-well plate at a density of $1 \times 10^4$ cells/well and cultured to confluence in DME medium containing 10% FBS. Cells were serum-starved in 0.5% FBS-containing DME medium for 40 h and then incubated with either 10% FBS or the indicated amounts of normal polyhedra (NP) or FGF-2 polyhedra (F2P). B, ATDC5 cells were plated into a 96-well plate at a density of $1 \times 10^4$ cells/well and cultured to confluence in DME/F-12 medium containing ITS and 5% FBS. Cells were then serum-starved in 0.5% FBS-containing DME/F-12 medium for 22 h and incubated with BSA (10 μg/ml), FGF-2 (F2, 1 ng/ml), SU5402 (20 μg/ml), or various concentrations of normal or FGF-2 polyhedra. Data are representative of the means ± S.D. of triplicate assays.

FIGURE 4. Effects of FGF-2 polyhedra on the differentiation of ATDC5 cells (Alcian blue staining). ATDC5 cells were plated into a 12-well plate at a density of $2 \times 10^4$ cells/well and cultured to confluence in DME/F-12 medium containing ITS and 5% FBS. Cells were then incubated with or without normal polyhedra (NP), FGF-2 polyhedra (F2P), BSA (10 μg/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.

MAP kinases were detectable in NIH3T3 cells that had been incubated with 5 ng/ml rhFGF-2 or FGF-2 polyhedra (Fig. 5A). Likewise, phosphorylation of p44/p42 MAP kinases was observed in ATDC5 cells incubated with 5% FBS, 1 ng/ml rhFGF-2, or FGF-2 polyhedra for 30 min (Fig. 5B). In contrast, these phosphorylation events were not evident in cells incubated with normal polyhedra. Moreover, when ATDC5 cells were pretreated with SU5402, the phosphorylation of p44/p42 was found to be efficiently inhibited (Fig. 5B), which is concomitant with the blocked increase in DNA synthesis following exposure of these cells to this agent (Fig. 3B). Interestingly, phosphorylation of p44/p42 in ATDC5 cells incubated with FGF-2 polyhedra could be detected at a higher level even at 12 h
after treatment, whereas only low levels of p44/p42 phosphorylation were evident in ATDC5 cells treated with 1 ng/ml FGF-2 (Fig. 5C).

We found that the conditioned medium of ATDC5 cells incubated with FGF-2 polyhedra for 24 h had the activity to stimulate the proliferation of ATDC5 cells (data not shown). We then analyzed the quantity of FGF-2 in medium incubated with or without the confluent ATDC5 cells for 24 h using ELISA. Expectedly, FGF-2 was detected in medium incubated with FGF-2 polyhedra under both conditions (Fig. 6A). In the absence of cells, 1 × 10^4 FGF-2 polyhedra released 19.33 ± 1.15 pg of FGF-2 into 100 μl of the medium. In contrast, in the presence of cells, only 3.63 ± 0.15 pg of FGF-2 was released into 100 μl of the medium from 1 × 10^4 FGF-2 polyhedra, suggesting that released FGF-2 might be trapped in the extracellular matrix components such as heparan sulfate proteoglycans. We then analyzed the quantity of FGF-2 released from FGF-2 polyhedra incubated with PBS, serum-free medium, or 5% FBS containing the nonconditioned or the conditioned medium with or without heparin (Fig. 6B). Significant amounts of FGF-2 were released from FGF-2 polyhedra within 15 min except when incubated with PBS. Even after 24 h, a very low level of FGF-2 was detected in PBS (data not shown). The conditioned medium containing 5% FBS was more effective for the FGF-2 release than the nonconditioned medium containing 5% FBS. However, the FGF-2 release in the nonconditioned medium containing 5% FBS was facilitated by the presence of heparin. Interestingly, increasing amounts of released FGF-2 were observed only when heparin was added in either the nonconditioned or the conditioned medium. Similar amounts of released FGF-2 were still detected in both the media incubated with FGF-2 polyhedra for 24 h (data not shown). Probably because of instability and degradation of released FGF-2, a decrease of released FGF-2 was detected in the absence of heparin. When FGF-2 polyhedra were incubated with serum-free medium or nonconditioned medium containing 5% FBS, the FGF-2 release reached maximal levels at 1 h and declined thereafter. The con-
ditioned medium containing 5% FBS reached maximal levels at 5 h and declined thereafter, suggesting that heparan sulfate proteoglycans secreted from cells interact with released FGF-2 to stabilize it.

Our previous study had additionally shown that polyhedra containing immobilized foreign proteins are highly resistant to a loss of potency from dehydration (9). Desiccated FGF-2 polyhedra placed on coverslips retain the ability to stimulate the growth of ATDC5 cells when compared with normal polyhedra, as evidenced by the appearance of dividing cells (data not shown). To explore the application of FGF-2 polyhedra acting as a signaling cue in a spatially restricted manner in vitro, we seeded ATDC5 cells on and around desiccated FGF-2 polyhedra and examined how these FGF-2 polyhedra affected cellular proliferation (Fig. 7A). As shown in Fig. 6, FGF-2 was released from polyhedra into the culture medium, but most of the released FGF-2 seemed to be trapped in the pericellular or extracellular spaces possibly via heparan sulfate proteoglycans. Indeed, cellular proliferation was significantly stimulated only in the vicinity of FGF-2 polyhedra (Fig. 7C). Because of decreased adhesiveness, proliferating cells could be recognizable as refractile cells (above the dotted line in Fig. 7C) under phase-contrast microscopy, whereas quiescent cells with normal polyhedra remained flat and monolayer (Fig. 7B). For comparison, we spotted Affi-Gel blue beads presoaked with FGF-2 on the culture plate and inoculated ATDC5 cells (Fig. 7E). FGF-2-bound beads markedly stimulated the proliferation of ATDC5 cells, which took on refractile morphology. However, unlike micrometer-sized FGF-2 polyhedra, Affi-Gel beads blocked the view of cells under a microscope because of the size of beads (Fig. 7, D and E). Rapid diffusion of released FGF-2 from Affi-Gel beads resulted in activation of cells irrespective of cell location. Moreover, Affi-Gel blue beads were prone to detach from the culture plate especially in the plate spotted with BSA-bound beads. In contrast, FGF-2 polyhedra adhered to cells (Fig. 7C) and acted as an effective means of release onto cultured cells.

DISCUSSION

Under physiological conditions, normal polyhedra by themselves are inert and insoluble entities (5, 6), which allowed us to employ these complexes as versatile micron-sized carriers of growth factors, receptor binding domains, and cell binding domains of the ECM. We have successfully demonstrated in our current study that FGF-2 polyhedra efficiently stimulate the proliferation of mesenchymal cell lines such as NIH3T3 fibroblasts and ATDC5 chondrogenic cells (Figs. 2 and 3). This mitogenic activity was mediated by FGFR as evidenced by inhibition of cell proliferation with SU5402, a specific inhibitor of tyrosine kinase activity of FGFR (Figs. 3B and 5B). A single treatment of ATDC5 cells with FGF-2 polyhedra also efficiently inhibited chondrogenic differentiation. Thus, polyhedra immobilizing FGF-2 with VP3 are micron-sized bioactive substances.

Using a quantitative ELISA, we found that FGF-2 was released from FGF-2 polyhedra into the medium. However, FGF-2 was almost undetectable in PBS incubated with FGF-2 polyhedra for 10 h (Fig. 6B). Similarly, we failed to detect FGF-2 in water containing FGF-2 polyhedra stored at 4 °C for more than 2 years (data not shown). The release of immobilized FGF-2 from polyhedra was facilitated by the addition of 5% FBS to the culture medium, but the stability of released FGF-2 was unchanged (Fig. 6B). It is noteworthy that the presence of heparin markedly promoted the release of immobilized FGF-2 from polyhedra in the culture medium. Moreover, released FGF-2 was stably retained in the culture medium by the presence of heparin (Fig. 6B). These observations are consistent
Immobilization of FGF-2 in Cubic Protein Crystals

with the previous findings that the binding of FGF-2 to heparin increases its stability against thermal denaturation and protease digestion under physiological conditions (18, 19). As shown in Fig. 5C, we observed the persistent phosphorylation of MAP kinases stimulated by FGF-2 polyhedra. Taking into consideration the fact that FGF-2 was released from polyhedra even after 10 h of incubation (Fig. 6B), the FGF signaling pathway was kept active in ATDC5 cells by the sustained release of FGF-2 from polyhedra.

Heparin, or heparan sulfate proteoglycans (HSPGs), directly interacts with FGF ligands and play an important role for dimerization and activation of FGF receptors (20, 21). ATDC5 cells express several HSPGs including syndecans.4 Fig. 6A suggests that the confluent culture of ATDC5 cells could bind over 80% of FGF-2 released from polyhedra, probably through interactions with cell-surface HSPGs. Then bound FGF-2 may activate the FGF receptor signaling pathway with the aid of HSPGs.

We further show that the growth-stimulatory activities of FGF-2 polyhedra are retained even in a desiccated form and remain very stable in contrast to solubilized rhFGF-2. BmCPV polyhedra are smaller in dimension than most mammalian cells (Fig. 1, C and D), but because of their adhesive properties they can be spotted easily or micro-manipulated using laser devices onto a particular site of a culture plate (22). Significantly, a combination of polyhedra immobilizing a variety of biologically active substances enabled us to devise artificial extracellular microenvironments in culture that can mimic actions of growth factors associated with the ECM. Recent x-ray protein crystallography clearly demonstrates that polyhedra are made of trimers of the polyhedrin protein. Polyhedrin has a new fold and evolved to assemble into unique three-dimensional cubic crystals. The polyhedrin trimers are extensively cross-linked in polyhedra by noncovalent interactions and pack with an exquisite molecular complexity similar to that of antigen-antibody complexes (7). Because of the selectivity of the VP3 immobilization signal and the insolubility of polyhedra, just a simple rinsing and straining out of polyhedra can attain purification of the expressed proteins without any sophisticated purification step in solution (3, 4). Unlike existing virus-like nanoparticles, polyhedra can be versatile nanocontainers that accommodate a wide range of cargos. We conclude therefore that biologically active polyhedra will prove to be a versatile source of extracellular signals in the micron size range and will facilitate the future analysis of cellular and tissue dynamics during the directed migration and induction of cellular polarity at the single cell level.

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