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Shedding of Membrane Vesicles Mediates Fibroblast Growth Factor-2 Release from Cells*

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Fibroblast growth factor-2 (FGF-2), a polypeptide with regulatory activity on cell growth and differentiation, lacks a conventional secretory signal sequence, and its mechanism of release from cells remains unclear. We characterized the role of extracellular vesicle shedding in FGF-2 release. Viable cells released membrane vesicles in the presence of serum. However, in serum-free medium vesicle shedding was dramatically down-regulated, and the cells did not release FGF-2 activity into their conditioned medium. Addition of serum to serum-starved cells rapidly induced intracellular FGF-2 clustering under the plasma membrane and into granules that colocalized with patches of the cell membrane with typical features of shed vesicle membranes. Shed vesicles carried three FGF-2 isoforms (18, 22, 24 kDa). Addition of vesicles to endothelial cells stimulated chemotaxis and urokinase plasminogen activator production, which were blocked by anti-FGF-2 antibodies. Treatment of intact vesicles with 2.0 M NaCl or heparinase, which release FGF-2 from membrane-bound proteoglycans, did not abolish their stimulatory effect on endothelial cells, indicating that FGF-2 is carried inside vesicles. The comparison of the stimulatory effects of shed vesicles and vesicle-free conditioned medium showed that vesicles represent a major reservoir of FGF-2. Thus, FGF-2 can be released from cells through vesicle shedding.

Fibroblast growth factor-2 (FGF-2)[1] is the prototype member of a family of structurally related heparin binding growth factors that have mitogenic activity for several cell types and induce mesenchyme formation (1). FGF-2, a potent inducer of blood vessel formation (angiogenesis), stimulates smooth muscle and endothelial cell growth, is involved in wound healing, and plays important roles in the development and differentiation of various organs (2, 3). Elevated levels of FGF-2 have been implicated in the pathogenesis of several diseases characterized by exaggerated neovascularization (4) and in a broad spectrum of cancers (5, 6).

FGF-2 exists in five isoforms with molecular masses of 18, 22, 22.5, 24, and 34 kDa (7). The 18-kDa polypeptide is localized primarily in the cytosol (8), whereas forms with higher molecular mass are found predominantly in the nucleus (7, 9). All FGF-2 forms entail high affinity interactions with tyrosine kinase FGF receptors and low-affinity interactions with proteoglycans (HSPGs) containing heparan sulfate polysaccharides (10, 11).

Although FGF-2 is found associated with the extracellular matrix *in vitro* and *in vivo* and exerts its biological activities by binding to cell membrane receptors, it lacks a conventional secretory peptide and is not released through the classical endoplasmic reticulum (ER)-Golgi pathway. It has been proposed that FGF-2 is released from cells through alternative pathways including cell death, wounding, or sublethal injury (12, 13). However, FGF-2 release does not parallel the release of cytoplasmic markers (14). Inhibitors of protein secretion via the ER-Golgi complex do not block FGF-2 release. In contrast, reagents or treatments that inhibit exo/endocytosis or energy production block externalization of FGF-2 in transfected NIH 3T3 or COS-1 cells. A non-classic secretion pathway has therefore been proposed for FGF-2 release (15–17). Release of 18-kDa FGF-2 from transfected COS-1 cells is inhibited by ouabain, a known inhibitor of Na,K-ATPase (18), and FGF-2 co-immunoprecipitates with the α ATPase subunit (19). It has therefore been proposed that Na,K-ATPase plays an important role in FGF-2 secretion (16, 20).

Other extracellular proteins devoid of signal sequence include FGF-1, interleukin 1α, interleukin 1β, and lectin 14 (L-14). FGF-1 appears to be released in response to stress conditions as a component of multiprotein aggregates (21, 22), and a member of the S100 family of Ca^{2+} binding proteins has been implicated in its release (23, 24). However heat shock does not affect FGF-2 secretion (15). Secretory pathways via extracellular vesicle production have been proposed for both interleukin 1β and L-14. Externalization of L-14, a signaling molecule highly expressed in muscle cells, is developmentally regulated. As myogenic cells differentiate, cytosolic L-14 is concentrated in the cellular ectoplasm beneath regions of the plasma membrane that appear to evaginate and form extracellular vesicles. It has therefore been suggested that shed mem-

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[1] The abbreviations used are: FGF-2, fibroblast growth factor-2; HPSGs, heparan sulfate-containing proteoglycans; ER, endoplasmic reticulum; GFP, green fluorescent protein; FCS, fetal calf serum; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; ERK, extracellular signal-regulated kinase; uPA, urokinase-type plasminogen activator.

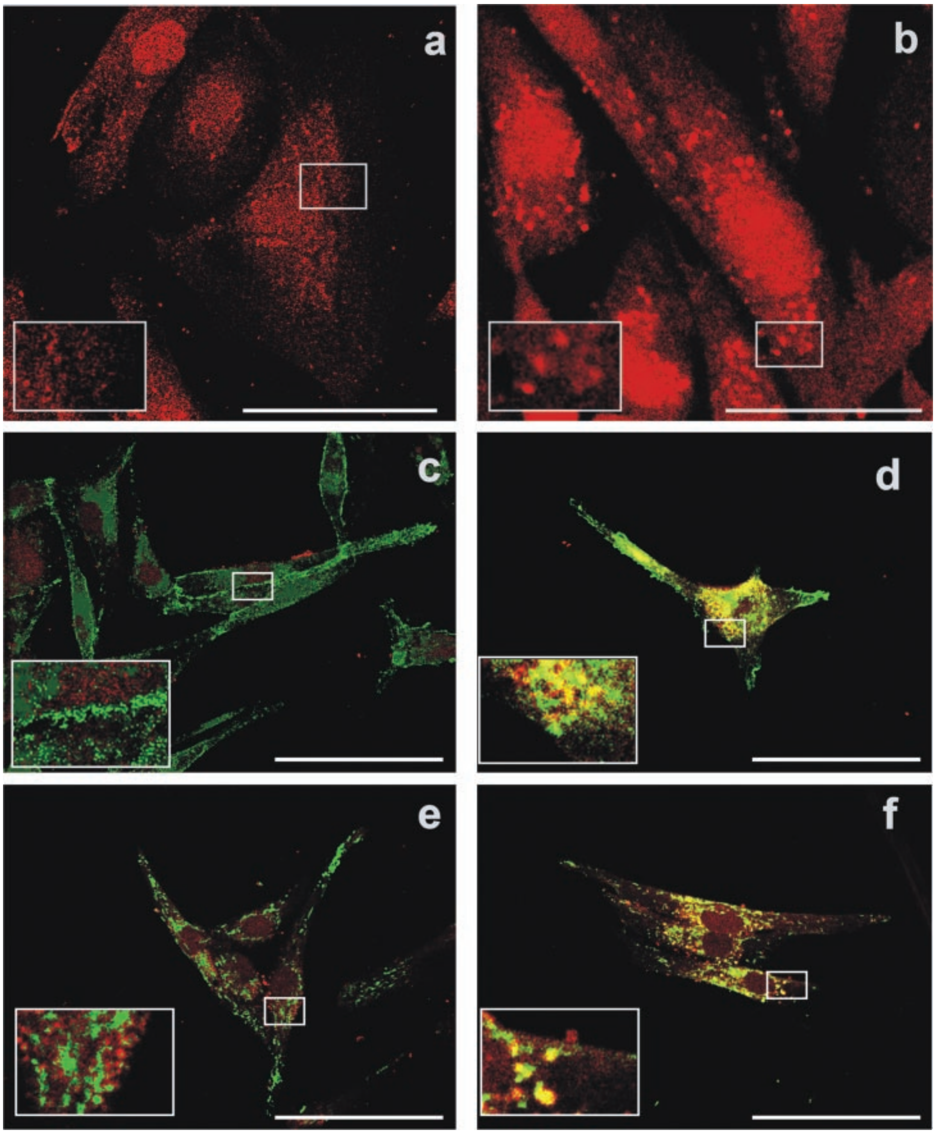

FIG. 1. **Immunolocalization of FGF-2 in SK-Hep1 cells.** Immunolocalization of FGF-2 (*a* and *b*) and double staining of FGF-2/β_1-integrin (*c* and *d*) and of FGF-2/annexin V (*e* and *f*) are shown. Cells were fixed in serum-free media (*a*, *c*, and *e*) and 30 min after serum addition (*b*, *d*, and *f*). *Bar* = 10 μm. *Squares* show selected areas that were magnified on the *left bottom* (×2 in *a* and *b*; ×4 in *c*, *d*, *e*, and *f*). FGF-2 was detected using Texas red-conjugated secondary antibodies. β_1-Integrin was detected using FITC-conjugated secondary antibodies. Biotinylated annexin V added to fixed cells was detected using FITC-conjugated streptavidin.

brane vesicles represent a vehicle for L-14 secretion (25). IL-1β is released from activated immune cells after a secondary stimulus such as extracellular ATP acting on P2X (7) receptors. Mackenzie *et al.* (26) reported that human THP-1 monocytes shed vesicles from their plasma membranes within 2–5 s after the activation of P2X (7) receptors; 2 min later the released vesicles contain IL-1β. The cytokine is then released in a vesicle-free form at later time points.

Membrane vesicles originate from the plasma membrane through a mechanism morphologically similar to that of virus budding. The vesicles are relatively large and heterogeneous in size; their diameters ranging from ~100 to ~1000 nm (27, 28). Vesicle shedding is an active process that requires RNA and protein synthesis (29) and occurs in viable cells with no signs of apoptosis or necrosis. Vesicle membranes carry most surface antigens expressed on the plasma membrane (30); however they originate from domains of the plasma membrane selectively enriched in membrane components including HLA class I molecules, β1 integrins, and membrane-bound matrix metalloproteinase-9 (31). Vesicle shedding has been implicated in cell migration and in tumor progression (32), a function that may be mediated by vesicle membrane-bound proteinases (28, 31, 33–36). Vesicles are also shed by several non-tumor cells (37, 38) and have been reported to vehicle a variety of regulatory factors (25, 26, 29, 30, 36). Vesicles shed by platelets (38) or monocytes (26) are also enriched in phosphatidyl-serine and bind annexin V. They were therefore proposed to possess procoagulant activity.

In addition to membrane vesicles, exosomes, a population of exovesicles released by eukaryotic cells, have also been reported to vehicle secreted proteins. Exosomes are 40–80-nm extracellular vesicles released by exocytosis of multivesicular bodies. These structures are part of the endosomal system and are considered to belong to the category of late endosomes/lysosomes. Exosomes are probably generated by inward budding of the vesicle membrane (39, 40) and are enriched in major histocompatibility complex class I and II molecules, in members of the tetraspan protein superfamily (41–43), and in HSP70 (43, 44). Two cytosolic proteins found in exosomes, gelactin-3 and annexin II, are also found in the extracellular environment. Because these proteins do not posses a signal sequence, it has been suggested that exosomes represent an unconventional secretion pathway for these proteins (45).

Recently, FGF-2 secretion was analyzed in Chinese hamster ovary cells expressing FGF-2-GFP fusion protein under control by the tetracycline resistance transactivator. FGF-2-GFP protein was shown to translocate to the outer surface of the plasma membrane; the secreted FGF-2-GFP fusion protein then accumulated in large HSPG-containing protein clusters on the extracellular surface of the plasma membrane (46). In the present paper, we tested the hypothesis that these large protein clusters are exovesicles and that vesicle shedding may represent a mechanism for FGF-2 release from the cell.

EXPERIMENTAL PROCEDURES

Cells and Culture Media—Human SK-Hep1 hepatoma cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS; Euroclone, Celbio). NIH 3T3 cells transfected with FGF-2 cDNA have been described (15). Transfected and vector-transfected NIH 3T3 cells were grown in Dulbecco's minimum essential medium (Sigma-Aldrich) supplemented with 0.6 g/liter $NaHCO_3$, 500 μg/ml geneticin (G418; Invitrogen) and 10% FCS. Bovine GM7373 fetal aortic endothelial cells (47) were grown in Eagle's minimal essential medium (Sigma) supplemented with 10% FCS, vitamins, and essential and non-essential amino acids. All the cells were negative for mycoplama contamination by the Hoechst 33258 (Sigma) staining assay.

Immunofluorescence—SK-Hep1 cells seeded at low density (2.000 cells/well) onto microscope coverslips in 12-well culture plates (Nunc) were grown overnight in complete medium and then in serum-free medium for 1 week with three medium changes. To study the effect of serum on the intracellular distribution of FGF-2, medium supplemented with 10% FCS was added. After 5, 10, 20, and 30 min of incubation the cells were fixed in 3.7% formaldehyde for 10 min followed by permeabilization with 0.05% Triton X-100 for 5 min. FGF-2 was detected with mouse monoclonal anti-FGF-2 (0.5 mg/ml, 1:200; Upstate Biotechnology type II) antibody and Texas Red-conjugated anti-mouse antibodies (1:200; Amersham Biosciences); biotinylated annexin V was added (2 mg/ml) to fixed cells, washed (five times for 5 min each) with phosphate-buffered saline (PBS), and detected using FITC-streptavidin (Sigma) (1:500); β1-integrins were detected using FITC-conjugated C27 mAb (48). In some experiments isolated vesicles were bound to poly-L-lysine on a coverslip, fixed, permeabilized, and treated with primary and secondary antibodies as described for cells. Immunostained vesicles were analyzed by confocal microscopy (Olympus 1X70 with Melles Griot laser system).

To detect interactions between vesicle-associated FGF-2 and the cell membrane, purified vesicles (50 μg/ml) were added to sparse GM7373 cell cultures pretreated with cycloheximide (10 μg/ml) for 3 h. After 3 h of incubation the cells were fixed with 3.7% formaldehyde for 10 min, followed by permeabilization with 0.01% Triton X-100 for 5 min. FGF-2 was detected with mouse anti-FGF-2 monoclonal antibody (0.5 mg/ml, 1:500; Upstate Biotechnology type II) and FITC-conjugated anti-mouse antibodies (1:500; Amersham Biosciences).

Ultrastructural Analysis—Transmission electron microscopy of vesicle shedding was carried out using a standard technique. Briefly, cells were fixed with 2% glutaraldehyde in culture flasks, scraped, and post-fixed with 1% OsO_4, dehydrated with ethanol, and embedded in Epon 812. Samples were sectioned, post-stained with uranyl acetate and lead citrate, and examined with an electron microscope (Philips CM10, Eindhoven, Netherlands).

For scanning electron microscopy cells were grown on coverslips and fixed with 2% glutaraldehyde in PBS for 30 min. Critical point dried samples were glued onto stubs, coated with gold in a SKD040 Balzer Sputterer, and observed using a Philips 505 scanning electron microscope at 10–30 kV.

Vesicle Purification from Conditioned Medium—Vesicles were purified from conditioned medium as described (28). Briefly, medium conditioned by subconfluent healthy cells for 3, 6, or 24 h were centrifuged at 2000 × *g* and at 4000 × *g* for 15 min. The supernatant was ultracentrifuged at 105,000 × *g* for 90 min. Pelleted vesicles were resuspended in PBS.

In some control experiments, vesicles were purified by affinity binding of the 4000 × *g* supernatant to biotinylated annexin-V (Pierce) and bound to Streptavidin MagneSphere Paramagnetic Particles (SA-PMPs; Promega). To remove components that might bind to the magnetic beads in the absence of annexin-V, conditioned medium was pre-adsorbed with SA-PMPs, in the absence of annexin-V. Vesicles bound to SA-PMPs were solubilized by incubation with Ca^{2+}- and Mg^{2+}-free PBS, 20 mM EDTA for 1 h at room temperature. The amount of isolated vesicles was determined by measuring protein concentration by the Bradford microassay method (Bio-Rad) using bovine serum albumin (Sigma) as a standard.

Heparin-Sepharose Adsorption and SDS-PAGE—Purified vesicles were sonicated three times with 50 pulses for 10 s each and incubated at 4 °C overnight in an end-over-end mixer with 40–80 μl of heparin-Sepharose (Amersham Biosciences) equilibrated in PBS. After four washes with PBS, the beads were resuspended in 30 μl of reducing Laëmmli buffer and electrophoresed in SDS-3–12.5% gradient polyacrylamide gels.

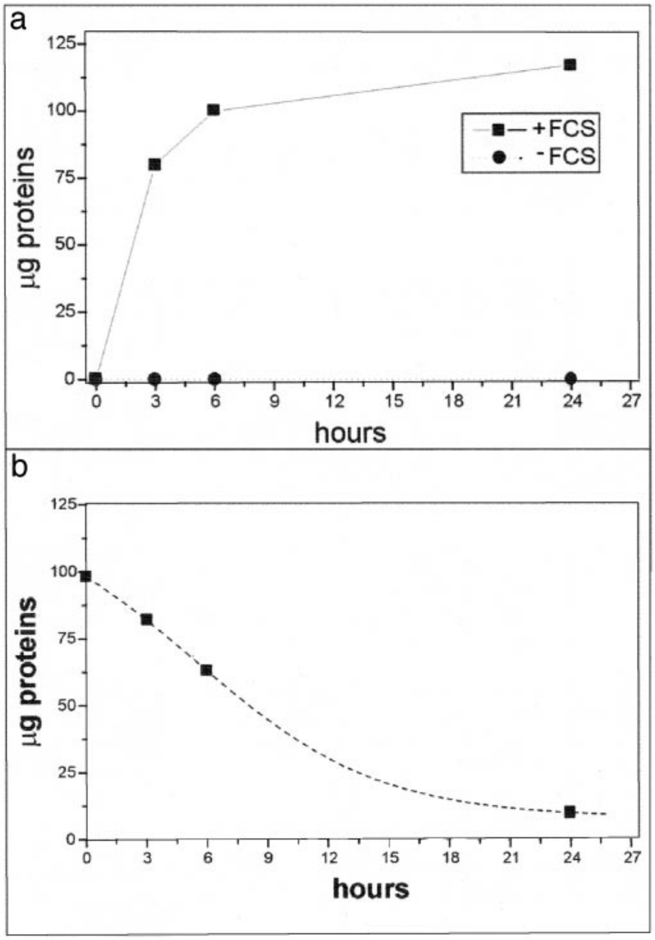

FIG. 2. **Time course of vesicle shedding and shed-vesicle stability in SK-Hep1 cells.** *a*, amount of vesicles recovered from medium conditioned by 4 × 10^7 SK-Hep1 cells. *b*, amount of vesicles recovered from medium conditioned by 24 h of 4 × 10^7 SK-Hep1 cell growth after different incubation periods at 37 °C.

Western Blotting—After electrophoresis in SDS-polyacrylamide gels, the proteins were blotted onto a nitrocellulose membrane (Hybond; Amersham Biosciences) that was saturated with 5% horse serum, 0.1% Tween 20 in PBS for 2 h and then incubated with either monoclonal anti-FGF-2 (2.5 μg/ml; Upstate Biotechnology type II) or anti-HSP70 protein (0.8 μg/ml; Sigma H-5147) or anti CD-44 (10 μg/ml; Calbiochem 217604) antibodies followed by horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibodies (1:7500; Sigma) for 1 h at room temperature. Immunocomplexes were visualized with the ECL Western blotting kit (Amersham Biosciences) using Hyperfilms.

ERK Phosphorylation—To characterize extracellular signal-regulated kinase-1/2 (ERK1/2) phosphorylation, GM7373 cells were seeded in 24-well plates (80,000 cells/cm^2) in Dulbecco's modified Eagle's medium containing 10% FCS. The cells then were starved for 24 h in 0.5% FCS, followed by treatment with either 50 μg/ml SK-Hep1 cell-derived vesicles or 50 ng/ml human recombinant FGF-2 (49) for the indicated time. Cell extracts were analyzed by Western blotting with anti-phospho-ERK1/2 antibody (Santa Cruz Biotechnology, Inc.). Immunocomplexes were visualized by chemiluminescence with the Supersignal® West Pico chemiluminescent substrate (Pierce) according to the manufacturer's instructions. To judge uniform loading, the same membrane was stripped and re-incubated with anti-ERK_2 antibody (Santa Cruz Biotechnology).

Assay for uPA Activity—1.75 × 10^6 GM7373 cells (70,000 cells/cm^2) were grown for 28 h in 5 ml of medium supplemented with different concentrations of vesicles. Cell cultures were washed twice with PBS and incubated for 18–24 h in serum-free medium. After harvesting the culture supernatant, the cells were washed twice with PBS and subsequently scraped with a rubber policeman. Following centrifugation at

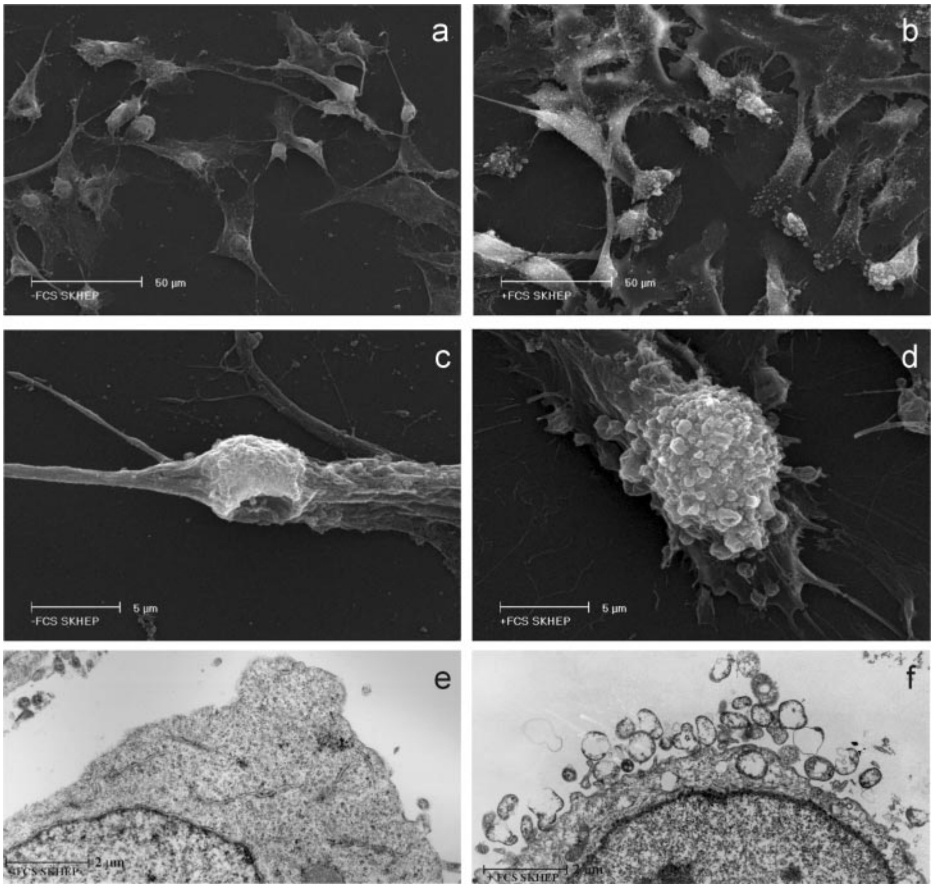

FIG. 3. **Ultrastructural analysis of SK-Hep1 cells.** Scanning (*a–d*) and transmission (*e* and *f*) electron microscopic analysis of SK-Hep1 cells grown in the absence (*a*, *c*, and *e*) or in the presence of 10% FCS (*b*, *d*, and *f*) is shown.

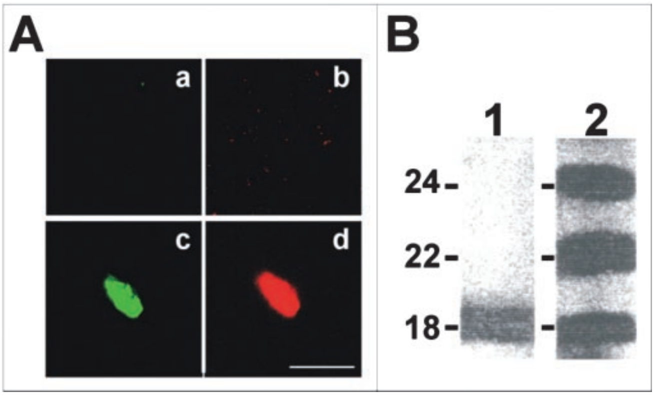

FIG. 4. **Presence of FGF-2 in SK-Hep1 shed membrane vesicles**. *A*, immunostaining of purified vesicles with biotinylated annexin V and anti-FGF-2 antibodies. Biotinylated annexin V, added to fixed vesicles, was detected using FITC-conjugated streptavidin. FGF-2 was detected using Texas red-conjugated secondary antibodies. *Bar* = 10 µm. *a* and *b*, negative controls (*a*, SK-Hep1 vesicles plus FITC streptavidin; *b*, SK-Hep1 vesicles plus Texas red-conjugated secondary antibodies); *c*, immunostaining for annexin V; *d*, immunostaining for FGF-2. *B*, Western blot analysis of vesicles for FGF-2. *Lane 1*, human recombinant FGF-2 (100 ng); *lane 2*, SK-Hep1 vesicles (recovered from 500 µg of vesicle proteins submitted to heparin-Sepharose chromatography).

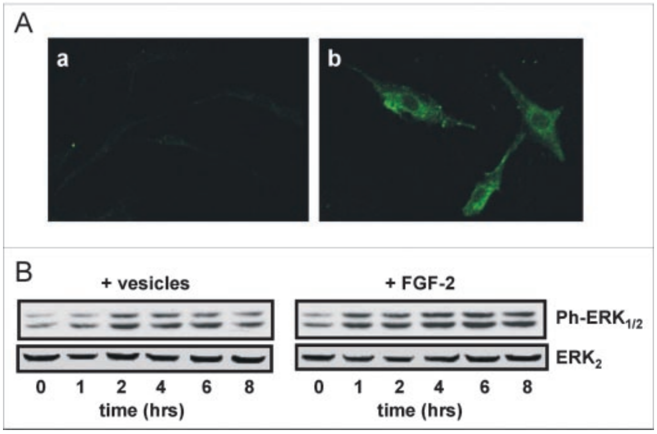

FIG. 5. **Interaction of vesicle-released FGF-2 with target-cell plasma membranes and stimulation of ERK1/2 phosphorylation.** *A*, immunolocalization of released FGF-2. *a*, untreated cells; *b*, cells treated with 50 µg/ml Sk-Hep1 vesicles. *B*, stimulation of ERK1/2 phosphorylation by shed vesicles. Western blot analysis of GM7373 cells extracts by anti-phospho-ERK1/2 antibody (*Ph-ERK$_{1/2}$*) and by ERK$_2$ antibody (*ERK$_2$*) is shown. + *Vesicles*, GM7373 cells treated with 50 µg/ml SK-Hep1 vesicles for the indicated time; + *FGF-2*, GM7373 cells treated with 50 ng/ml FGF-2 for the indicated time.

800 × *g* for 10 min at 4 °C, protein concentration was measured by the Bradford method.

Cell extracts (30 µg of proteins) were loaded onto SDS 7.5% polyacrylamide gels. Zymography was performed as described (50) with overlay gels containing 3% non-fat dry milk and 40 µg/ml bovine plasminogen (Sigma). Densitometric analysis of the lysis bands was performed using Eastman Kodak Co. Science 1D Image Analyzer software. White bands of caseynolysis of images were converted to dark bands to better display the activity.

Cell Migration Assay—Falcon cell culture inserts with 8-µm pore polyethylene terephthalate membranes were coated with gelatin (50 µg/ml). Five hundred microliters of Dulbecco's minimum essential medium containing 10^5 GM7373 cells was added to the upper chamber, whereas the bottom chamber received 500 µl of Dulbecco's minimum essential medium with or without the indicated concentrations of vesicles, vesicle-free supernatants, and anti-FGF-2 neutralizing antibodies (type I; Upstate Biotechnology). After 4 h of incubation at 37 °C, the inserts (16 mm diameter) were removed and fixed in methanol. The cells on the upper surface of the filter were removed with a cotton swab, and the cells migrated across the membrane were stained with 1% crystal violet and counted.

Heparinase and NaCl Treatments—Conditioned media were incubated either with heparinase III 4 milliunits/ml for 2 h at 37 °C or with

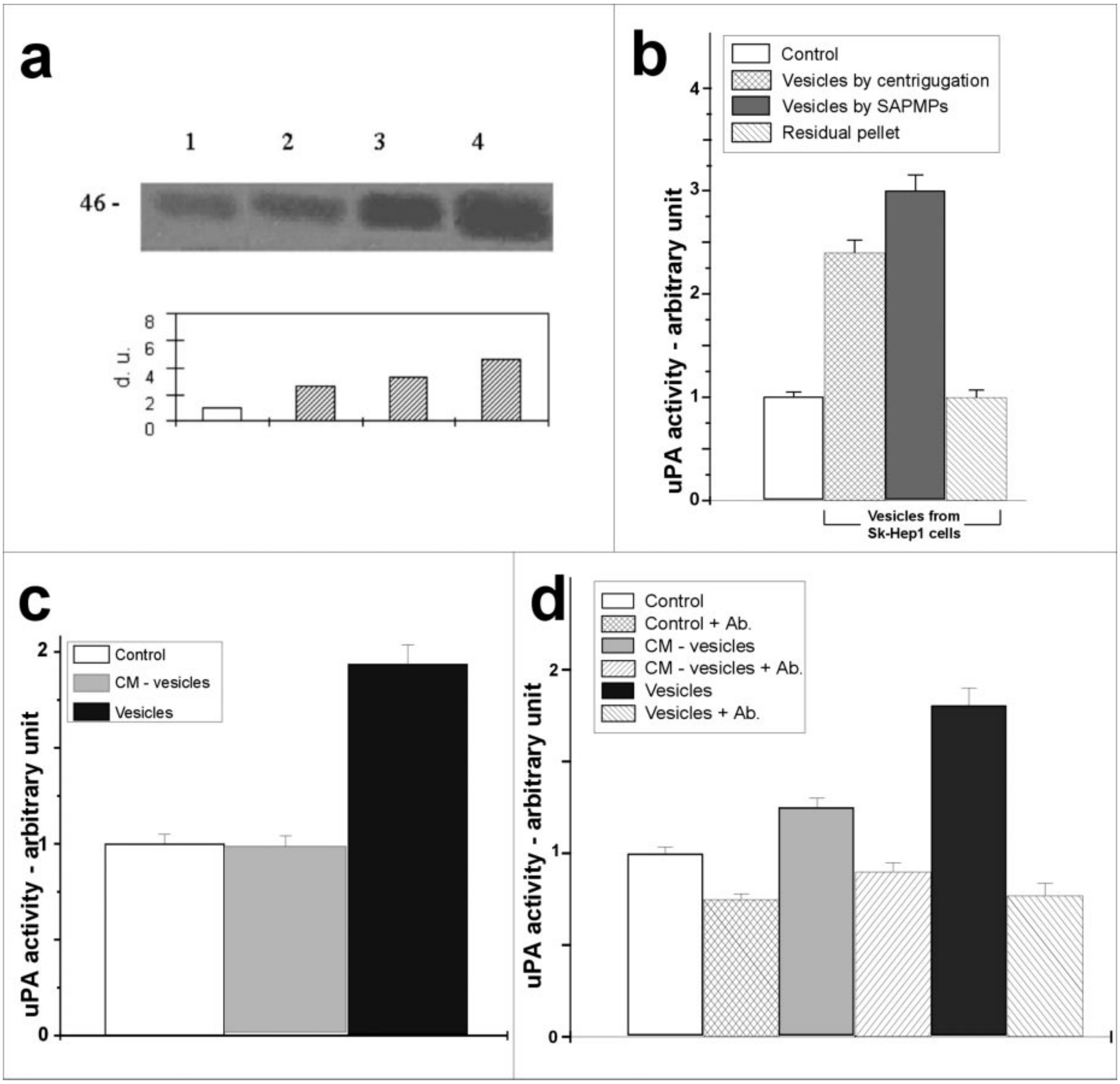

FIG. 6. **Induction of uPA activity in GM7373 cells by SK-Hep1 vesicles and vesicle-free conditioned medium.** GM7373 cells were incubated for 18 h with different concentration of vesicles. Endothelial cells were lysed and processed for uPA activity as described. *a*, uPA activity of GM7373 cells incubated without vesicles (*lane 1*) or with 25, 50, and 100 μg/ml SK-Hep1 vesicles (*lanes 2–4*). *b*, effects of differently purified vesicles. *Control*, uPA activity of untreated GM7373 cells; *Vesicles by centrifugation*, uPA activity of cells treated with 6 μg/ml SK-Hep1 vesicles obtained by ultracentrifugation; *Vesicles by SAPMPs*, uPA activity of cells treated with 6 μg/ml SK-Hep1 vesicles obtained by affinity binding to annexin-V, which had been biotinylated previously and bound to Streptavidin MagneSphere Paramagnetic Particles (*SAPMPs*; Promega); *Residual pellet*, uPA activity of cells treated with 6 μg/ml pellet material obtained from conditioned media after vesicles absorption with SAPMPs. *c*, comparison between vesicles and vesicle-free media, conditioned by 1 h of Sk-Hep1 cell growth. *Control*, uPA activity of untreated GM7373 cells; *CM-vesicles*, uPA activity of GM7373 cells growing in the presence of vesicle-free conditioned media obtained from 5 ml of SK-Hep1 cultures. *Vesicles*, uPA activity of GM7373 cells growing in the presence of vesicles purified from 5 ml of SK-Hep1 conditioned media (3 μg/ml). *d*, comparison between vesicles and vesicle-free media, conditioned by 3 h of Sk-Hep1 cell growth. *Control*, uPA activity of untreated GM7373 cells; *control + Ab*, uPA activity of GM7373 cells growing in the presence of 2.5 μg of inhibitory anti-FGF-2 monoclonal antibodies in 5 ml; *CM-vesicles*, uPA activity of GM7373 cells growing in the presence of vesicle-free conditioned media obtained from 5 ml of SK-Hep1 cultures; *CM-vesicles + Ab*, uPA activity of GM7373 cells growing in the presence of vesicle-free conditioned media obtained from 5 ml of SK-Hep1 cultures + 2.5 μg of inhibitory anti-FGF-2 antibodies in 5 ml; *Vesicles*, uPA activity of GM7373 cells growing in the presence of vesicles purified from 5 ml of SK-Hep1 conditioned media (3 μg/ml); *Vesicles + Ab*, uPA activity of GM7373 cells growing in the presence of vesicle-free conditioned media obtained from 5 ml of SK-Hep1 cultures + 2.5 μg of inhibitory anti-FGF-2 antibodies in 5 ml. *d.u.* represents densitometric values of uPA activity, expressed in arbitrary units, considering 1.0 the basal uPA activity of GM7373 cells.

2 M NaCl (final concentration) for 30 min at 37 °C without or after sonication (three times with 80 pulses for 30 s each). After centrifugation at 100,000 × *g*, pelleted vesicles were resuspended in PBS and assayed for their ability to stimulate uPA expression in GM7373 cells.

RESULTS

FGF-2 Is Associated with Membrane Vesicles Shed from SK-Hep1 Cells—SK-Hep1 cells produce high levels of FGF-2 (51). Previous observations (52) have shown that FGF-2 cannot be detected in serum-free medium conditioned by SK-Hep1 cells, consistent with the inhibitory effect of serum deprivation on FGF-2 externalization (15). Similarly, serum deprivation inhibits vesicle shedding (28, 53) raising the hypothesis that FGF-2 externalization and vesicle shedding may represent related processes. We therefore characterized the effect of serum on the expression and intracellular localization of FGF-2 in SK-Hep1 cells. FGF-2 expression was analyzed by Western blotting of starved cells or cells collected 10 min, 2 h, and 24 h after serum addition. FGF-2 levels were not affected by serum

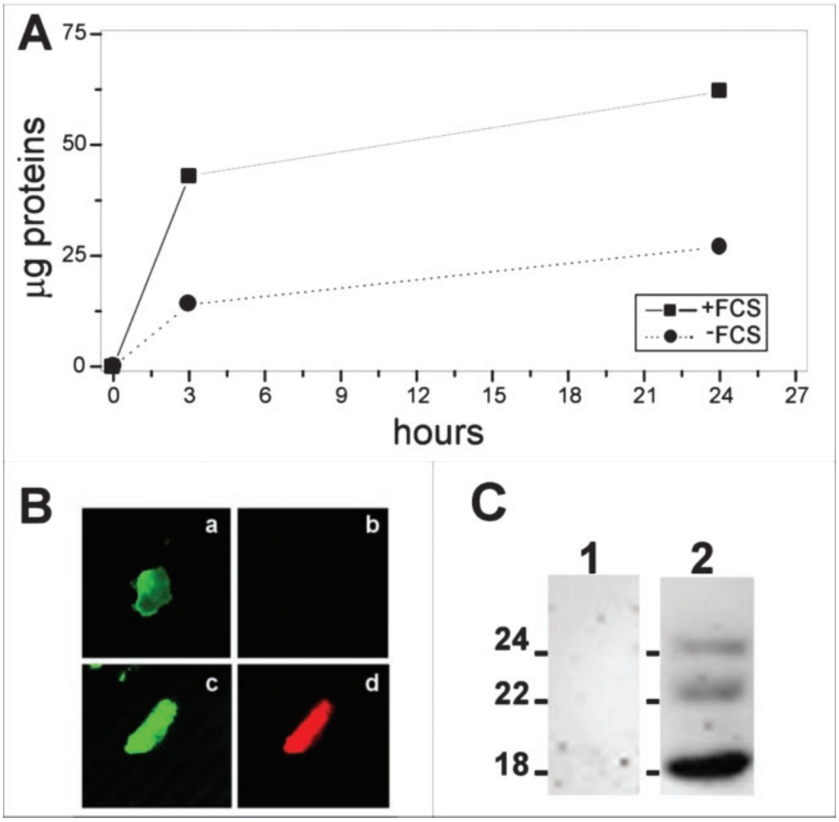

FIG. 7. **FGF-2 is released by FGF-2-transfected NIH-3T3 cells in shed vesicles.** *A*, time course of vesicle shedding in by FGF-2-transfected NIH-3T3 cells in the absence or in the presence of 10% FCS. *B*, immunostaining of purified vesicles with biotinylated annexin V and anti FGF-2 antibodies. *a* and *b*, vesicles shed by parental NIH 3T3 cells; *c* and *d*, vesicles shed by FGF-2 transfected NIH 3T3 cells; *a* and *c*, immunostaining for annexin V; *b* and *d*, immunostaining for FGF-2. Biotinylated annexin V, added to fixed vesicles, was detected using FITC-conjugated streptavidin. FGF-2 was detected using Texas red-conjugated secondary antibodies. *Bar* = 10 μm. *C*, Western blot analysis of vesicles for FGF-2. *Lane 1*, NIH 3T3 vesicles (50 μg); *lane 2*, vesicles from FGF-2-transfected NIH 3T3 cells (50 μg).

(data not shown). Under serum-free conditions, immunofluorescence staining with monoclonal anti-FGF-2 antibody followed by confocal microscopy showed that FGF-2 localized in the nucleus and in the cell cytoplasm, where it formed small aggregates (Fig. 1*a*). Addition of serum caused rapid clustering of FGF-2 in larger patches (Fig. 1*b*). Under these conditions, FGF-2 immunoreactive material was found to colocalize with areas of the cell plasma membranes enriched in β1 integrins (Fig. 1*d*) and characterized by an increased capability to bind annexin V (Fig. 1*f*), in keeping with a localization of the growth factor in plasma membrane regions involved in vesicle formation (26, 38). This colocalization was not observed when cells were grown under serum-free conditions (Fig. 1, *c* and *e*).

In agreement with these and previous observations (28), SK-Hep1 cells did not release vesicles in the absence of serum. However, in the presence of FCS SK-Hep1 cells shed a large amount of vesicles (Fig. 2). Vesicle accumulation in the conditioned medium occurred rapidly, reaching a plateau ~3–6 h after serum addition. Vesicles were produced by viable cell cultures in which the number of apoptotic or necrotic cells, evaluated by acridine orange and trypan-blue staining, was found to be negligible (less than 3%). To analyze vesicle stability conditioned media were incubated at 37 °C for different times before vesicle purification. As shown in Fig. 2*b*, vesicles appear to have a short half-life, probably because of their content in proteolytic enzymes.

Scanning and transmission electron microscopic analysis of SK-Hep1 cells confirmed that vesicle shedding is induced by serum addition (Fig. 3). Indeed, serum-starved cells showed a smooth plasma membrane (*a*, *c*, and *e*), whereas cells grown in the presence of FCS were characterized by a rough membrane in the process of shedding membrane vesicles (*b*, *d*, and *f*). Vesicles appeared to bud from the cell membrane and to enclose a small amount of cytoplasmic material (*f*). As shown in Fig. 3*b*, vesicle shedding was a generalized phenomenon that was not limited to isolated, damaged cells. The morphology and size (300–900 nm diameter) of the vesicles shed by SK-Hep1 cells were similar to those shed by 8701-BC or HT-1080 tumor cells (28, 34) and clearly distinct from the significantly smaller exosomes (40).

In another set of experiments, purified vesicles bound to a poly-L-lysine-coated coverslip were fixed, permeabilized, and immunostained with anti-FGF-2 antibodies. In parallel, vesicles were assessed for their capacity to bind annexin V. As shown in Fig. 4*a*, annexin V-binding vesicles stained intensively with anti-FGF-2 antibodies. Accordingly, Western blotting of isolated vesicles showed FGF-2 immunoreactive bands of 18, 22, and 24 kDa, consistent with the low and high molecular weight forms of FGF-2 (Fig. 4*b*). In keeping with previous observations with different tumor cell types (28, 31, 34, 35),[2] SK-Hep1 cell-derived vesicles carried matrix metalloproteinase-2 and matrix metalloproteinase-9 and the hyaluronic acid receptor, CD-44. In addition, the lack of HSP70 (data not shown), further distinguished these vesicles from typical exosomes (44).

FGF-2 Released from SK-Hep1 Vesicles Binds to the GM7373 Cell Membrane and Generates Intracellular Signaling—To assess the biological significance of vesicle-associated FGF-2, we tested purified vesicles for their ability to deliver FGF-2 to the endothelial cell surface and activate downstream signaling. After incubation of GM7373 endothelial cells with vesicles purified from SK-Hep1 cell-conditioned medium, the cells, which do not produce FGF-2, stained positively with antibodies to this growth factor (Fig. 5*A*). Downstream signaling triggered by the binding of FGF-2 to its tyrosine-kinase receptors encompasses the activation of mitogen-activated protein kinase kinase, with consequent phosphorylation of ERKs (54). Accordingly, vesicle-treated GM7373 cells showed a long lasting increase in ERK1/2 phosphorylation (Fig. 5*B*). Vesicle-mediated ERK1/2 phospho-

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[2] D. Cassarà and M. L. Vittorelli, unpublished data.

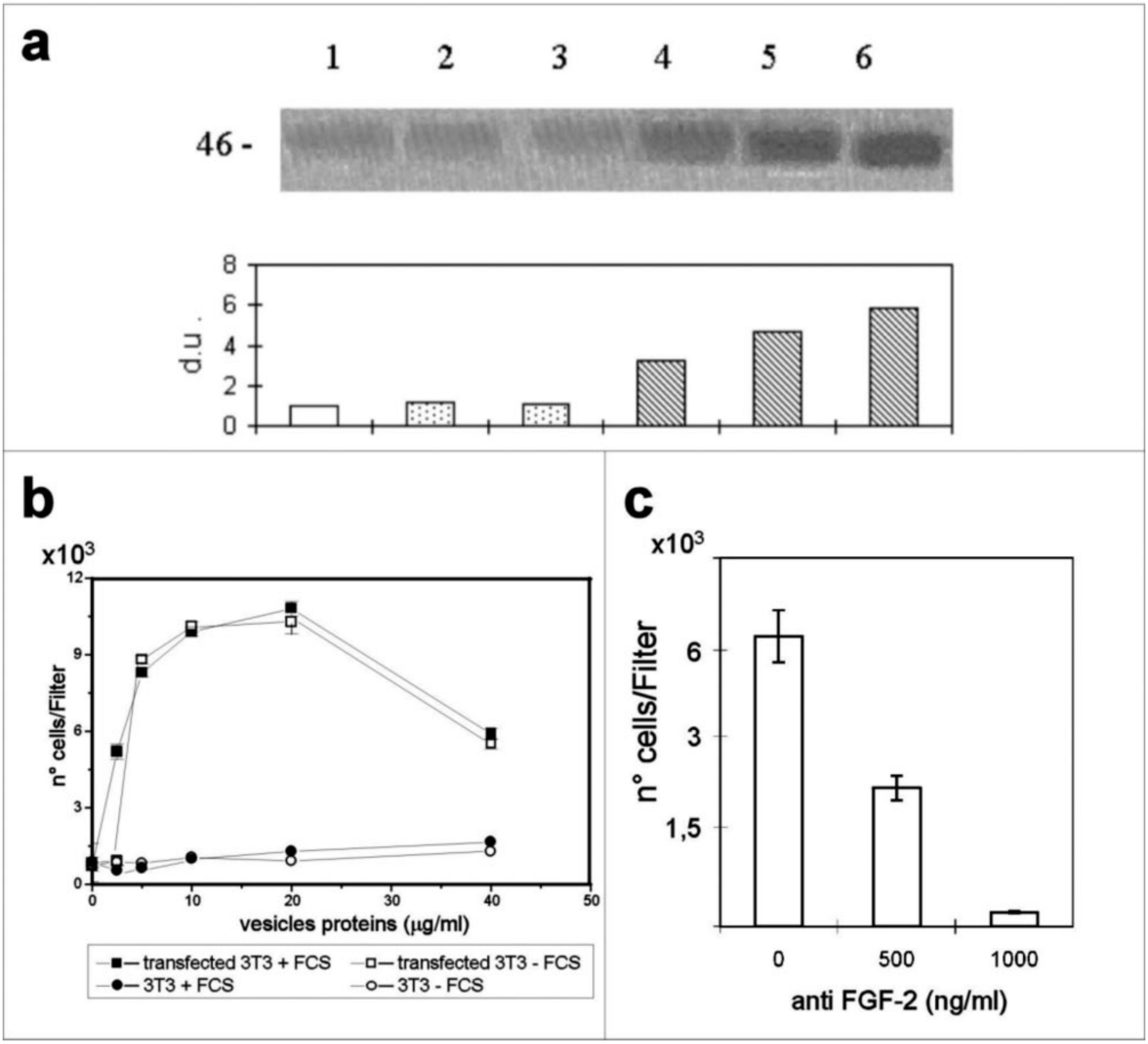

FIG. 8. **Vesicles shed by FGF-2-transfected NIH 3T3 cells induce uPA activity and have chemotactic effects on GM7373 cells.** *a*, induction of uPA activity in GM7373 cells. GM7373 cells were incubated for 18 h with different concentration of vesicles. Endothelial cells were lysed and processed for uPA activity as described. uPA activity of GM7373 cells incubated without vesicles (*lane 1*), with 25 or 50 μg/ml non-transfected NIH 3T3 vesicles (*lanes 2* and *3*), with 25, 50, or 100 μg/ml transfected NIH 3T3 vesicles (*lanes 4*, *5*, and *6*) is shown. *b*, chemiotactic effects of vesicles shed by FGF-2-producing and non-producing cells. GM7373 endothelial cells were assayed for cell migration in the presence of indicated concentrations of vesicles shed by non-transfected and FGF-2-transfected NIH 3T3. Vesicles were recovered from media of cells grown in serum-free (−*FCS*) or in complete medium (+*FCS*). *c*, inhibition of vesicle-mediated chemotaxis by anti-FGF-2 antibody. GM7373 cells were incubated with vesicles shed by FGF-2-transfected NIH 3T3 cells (20 μg of vesicle protein/ml) with the indicated concentrations of neutralizing anti-FGF-2 antibody and processed as above. *b* and *c*, mean and experimental variability are shown.

rylation appeared to be delayed compared with activation triggered by the recombinant growth factor, probably because of the slow release of FGF-2 from disrupted vesicles.

Another typical response elicited by FGF-2 is uPA production (51, 55). The uPA stimulatory activity of vesicle-associated FGF-2 was measured by casein/plasminogen zymography of endothelial cell extracts. As shown in Fig. 6*a*, vesicles induced a dose-dependent increase in cell-associated uPA activity. Both control and vesicle-treated GM7373 cells showed a caseinolytic band with an apparent molecular mass of 46 kDa, corresponding to bovine uPA and distinct from human uPA (55 kDa). This observation ruled out the possibility that the increase in uPA activity was mediated by vesicle-associated human uPA remaining bound to the endothelial cell surface.

To confirm that the uPA-inducing activity was associated with shed vesicles and was not mediated by co-sedimented material in the vesicle preparation, vesicles were bound to biotinylated annexin V and recovered by affinity chromatography on streptavidin-bound magnetic beads. As shown in Fig. 6*b*, vesicles purified by this technique had a uPA-inducing activity similar to that of vesicles isolated by ultracentrifugation.

To compare the stimulatory effects of vesicles and of vesicle-free supernatants, vesicles, recovered from a fixed volume of SK-Hep1 conditioned medium, were assessed for their capacity to induce uPA up-regulation in GM7373 cells. In parallel, the same volume of vesicle-free supernatant was tested under the same experimental conditions. As shown by Fig. 6*c* when this comparison was made using media collected after 1 h of cell growth, only vesicles had a clearly detectable stimulatory effect on GM7373 cells, whereas vesicle-free supernatant was ineffective. In media collected after 3 h of cell growth (Fig. 6*d*) both vesicles and vesicle-free supernatants had some stimulatory

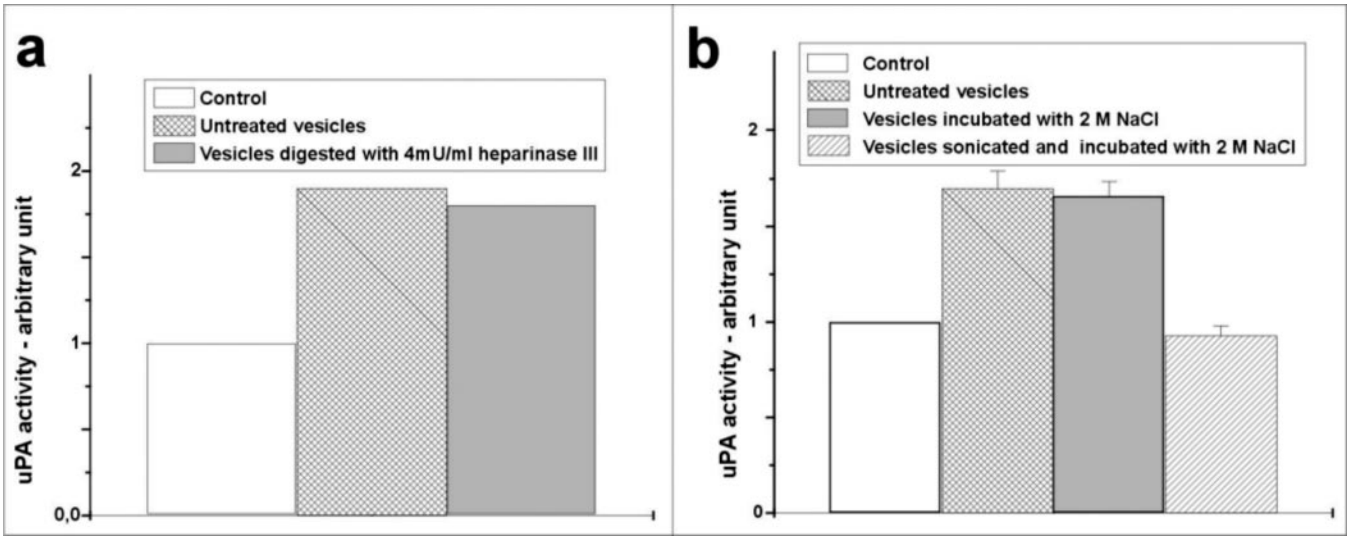

FIG. 9. **Effects of HSPG removal on the uPA stimulatory effect of vesicles on GM7373 cells.** *a*, effects of heparinase III on uPA stimulatory effect of vesicles shed by transfected 3T3 cells. *Control*, uPA activity of untreated GM7373 cells; *Untreated vesicles*, uPA activity of cells treated with 20 μg/ml of vesicles. Vesicles digested with 4 milliunits/ml of heparinase III, uPA activity of cells treated with 20 μg/ml of vesicles digested with heparinase for 2 h at 37 °C. *b*, effects of 2 M NaCl incubation on uPA stimulatory effect of vesicles shed by Sk-Hep1 cells. *Control*, uPA activity of untreated GM7373 cells; *Untreated vesicles*, uPA activity of cells treated with 30 μg/ml of vesicles. *Vesicles incubated with 2 M NaCl*, uPA activity of cells treated with 30 μg/ml vesicles incubated for 30 min at 37 °C. *Vesicles sonicated and incubated with 2 M NaCl*, uPA activity of cells treated with 30 μg/ml vesicles purified from sonicated media and incubated as described.

activity, nevertheless vesicles still exerted a stimulatory effect stronger than the corresponding vesicle-free supernatants. To confirm that the uPA-inducing activity of SK-Hep1 cell-derived vesicles was indeed mediated by vesicle-associated FGF-2, experiments were also performed in the presence of neutralizing anti-FGF-2 antibodies. As shown by Fig. 6*d* the stimulatory effects of both vesicles and vesicle-free supernatants were fully neutralized by anti-FGF-2 monoclonal antibodies (05117; Upstate Biotechnology). Unrelated monoclonal antibodies belonging to the same isotype (Sigma monoclonal antibody T9026, against tubulin), tested at the same concentration, did not modify the stimulatory effects of vesicles and vesicle-free conditioned media (data not shown). These data indicated that vesicle-associated FGF-2 retains the capacity to up-regulate uPA expression in endothelial cells and that most, if not all of the uPA-inducing activity present in the conditioned medium of SK-Hep1 cells is mediated by FGF-2 initially associated with vesicles.

FGF-2-transfected NIH 3T3 Cells Release Biologically Active FGF-2 by Shedding Membrane Vesicles—FGF-2-transfected NIH 3T3 cells have been shown to release limited but significant amounts of this growth factor (15). Therefore, we tested whether vesicle shedding was also responsible for FGF-2 release in this cell type. As shown in Fig. 7*A*, vesicle shedding was stimulated by serum in both parental and FGF-2-overexpressing NIH 3T3 cells. These cells, however, released vesicles also in serum-free medium. Immunolocalization experiments and Western blotting analysis showed the presence of low and high molecular weight FGF-2 isoforms in annexin V-binding vesicles shed by FGF-2-trasfected cells but not by parental cells (Fig. 7, *B* and *C*).

In keeping with the data obtained with SK-Hep1 cells, vesicles purified from the conditioned medium of FGF-2 transfectants showed the capacity to up-regulate uPA expression in endothelial GM7373 cells (Fig. 8*a*). In addition, vesicles shed by FGF-2-producing NIH 3T3 cells induced a chemotactic response in endothelial cells, and their effect was abolished by neutralizing anti-FGF-2 antibody (Fig. 8, *b* and *c*). The specificity of these effects was further demonstrated by the lack of activity of vesicles released by vector-transfected NIH 3T3 cells.

FGF-2 Is Localized Inside Shed Vesicles—To assess whether FGF-2 is present inside the vesicles or associated with HSPGs on their surface, purified vesicles were digested with heparinase III or washed with a high ionic strength solution (2.0 M NaCl). Both treatments are known to release FGF-2 from HSPGs (57). The vesicles were then tested for uPA-inducing activity on GM7373 cells. As shown in Fig. 9, *a* and *b*, neither the heparinase III treatment nor the NaCl wash abolished the ability of vesicles to up-regulate uPA expression in endothelial cells. When, however, incubation with NaCl was performed after sonication, the vesicle stimulatory effect was completely lost (Fig. 9*b*). These results are consistent with an intravesicular localization of the growth factor.

DISCUSSION

FGF-2 is found associated with the extracellular matrix *in vitro* and *in vivo* and interacts with cell membrane receptors. However, it lacks a hydrophobic signal sequence for secretion through the ER/Golgi system, and the mechanism(s) of its release are not understood. Because reagents or treatments that block protein secretion via the ER-Golgi complex do not affect FGF-2 release (15) (19), three routes have been proposed for FGF-2 externalization: cell death (12), sublethal cell injury (13), or exocytosis via ER/Golgi-independent pathway(s) (15, 19, 46).

In this study we tested the hypothesis that FGF-2 release from cells occurs by vesicle shedding. Membrane vesicles bud from the plasma membrane of viable cells and can be purified from cell-conditioned medium (for a review see Ref. 58). Both FGF-2 secretion and vesicle shedding are energy-dependent phenomena (15, 29), and they are not inhibited by reagents that block protein secretion via the ER-Golgi complex. Therefore, the two processes appear to be modulated by the same mechanisms.

The data reported show that serum addition to SK-Hep1 cells that constitutively express high levels of FGF-2 rapidly results in vesicle shedding and that the intracellular localization of FGF-2 is also strongly affected by these culture conditions. Shortly after serum addition to SK-Hep1 cells, FGF-2 appears in granules under the cell membrane and colocalizes with patches of the cell membranes that show increased concentration of $\beta 1$ integrins and annexin V-binding activity. Vesicle membranes were shown to be enriched in $\beta 1$ integrins (31) and to have annexin V-binding capacity (26, 38). These observations indicate that FGF-2 clusters within areas of the cell membrane where vesicle budding occurs, and FGF-2 is subsequently externalized with shed vesicles. Consistent with this hypothesis, we found that the 18-, 22-, and 24-kDa FGF-2 forms are associated with shed membrane vesicles.

The same FGF-2 isoforms are also present in vesicles shed by NIH 3T3 cells transfected with FGF-2 cDNA. Based on morphology, size, presence of gelatinolytic enzymes, and annexin V-binding activity, vesicles shed by SK-Hep1 and by transfected NIH 3T3 cells are similar to membrane vesicles shed by several tumor (28, 31, 34, 53) or normal cells (25, 26, 38). Their diameters are much larger than those reported for exosomes (40). Moreover, exosomes are enriched in HSP70 (43, 44) whereas vesicles shed by SK-Hep1 or NIH 3T3 cells do not contain this protein.

Vesicles shed by SK-Hep1 cells or NIH 3T3 cells transfected with FGF-2 cDNA induce increased uPA expression and migration (chemotaxis) in vascular endothelial cells, and both these effects are blocked by neutralizing anti-FGF-2 antibody. Our data demonstrate that most of the uPA-inducing activity present in the conditioned medium of SK-Hep1 cells is associated with shed vesicles. Because of the short half-life of shed vesicles, the capability of vesicle-associated FGF-2 to interact and activate FGF receptors and the neutralizing capability of anti-FGF-2 antibodies are explained by vesicle disruption. The possibility that FGF-2 released into the culture medium is bound by vesicle-associated, low affinity HSPG binding sites is ruled out by our finding that vesicles treated with heparinase or 2.0 M NaCl completely retained their uPA-inducing activity on endothelial cells, whereas their stimulatory activity was completely lost when they were sonicated before NaCl treatment.

Direct observations of immunolabeled vesicle-associated FGF-2 on endothelial cell plasma membranes, and signal transduction kinetic, indicates that FGF-2, present inside the vesicles, is delivered to target cells following vesicle breakdown, which occurs spontaneously. Contact of the phospholipid vesicle membrane with the cell membrane may also result in increased vesicle permeability and release of the encapsulated material. This hypothesis is supported by the finding that liposome-encapsulated FGF-2 retains the ability to up-regulate endothelial cell expression of uPA (11). The slow release of FGF-2 induced by contact between vesicle and plasma membranes may represent a mechanism for continuous delivery of limited amounts of FGF-2 in close vicinity to the cell membrane. The presence of FGF-2 in vesicle-free conditioned media, which is not observed when media are recovered after very short conditioning periods, could be also explained by vesicle disruption; however, at present, we cannot exclude that other release mechanisms are also involved. In conclusion, vesicle shedding appears to represent the mechanism, or at least an important mechanism, for the release of biologically active FGF-2 from viable cells.

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