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In Vivo Involvement of Heparan Sulfate Proteoglycan in the Bioavailability, Internalization, and Catabolism of Exogenous Basic Fibroblast Growth Factor

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ABSTRACT

The in vivo bioavailability of exogenous fibroblast growth factor 2 (FGF2) was studied after i.v. injection of uniformly ^{14}C-labeled FGF2 into young rats. ^{14}C-FGF2 was rapidly accumulated in almost all solid organs within 5 min. After 30 min, more than 65% of FGF2 was retained in liver, 4.5% in kidneys, 1.2% in spleen, 0.15% in adrenal glands, and trace amounts in bone marrow, eyes, lungs, and heart. Suborgan distribution of ^{14}C-FGF2 showed that for kidneys and adrenal glands, the labeling was mainly concentrated in the cortical zone. Incubation of organ sections with 2 M NaCl or heparin eluted all the radioactivity, indicating that labeling was due to FGF2-heparan sulfate proteoglycan (HSPG) interactions. Electrophoretic analysis show only native ^{14}C-FGF2 in the blood and extracellular matrix; however, FGF2 is continuously catabolized in solid organs, indicating that all participate in the clearance of FGF2 by cellular internalization and subsequent catabolism. All FGF2 catabolic fragments bound heparin, demonstrating the preservation of their HSPG-binding site during the in vivo intracellular catabolism of FGF2. Analysis of the high-affinity receptors of FGF2 (FGFR-1 and FGFR-3) and the mitogen-activated protein kinase did not show any increase in either FGFR tyrosine phosphorylation or in mitogen-activated protein kinase activation. This study shows for the first time that exogenous FGF2 is cleared by HSPG cellular internalization and catabolism without inducing the activation of FGFRs within at least five organs in vivo, which strongly suggests that the HSPG-dependent internalization and catabolism pathway may control the in vivo bioavailability of FGF2.

Basic fibroblast growth factor [bFGF, or fibroblast growth factor 2 (FGF2)] is mitogenic for a wide variety of cells derived from the mesoderm and neuroectoderm (Burgess and Maciag, 1989). The angiogenic, neurotrophic, and growth-promoting effects of FGF2 suggest that FGF2 has potential therapeutic applications (Lobb, 1988). Conversely, inappropriate FGF2 expression may contribute to diverse pathologies, including diabetic retinopathy, tumor growth, renal injury, rheumatoid arthritis, and atherosclerosis (Folkman and Klagsbrun, 1987; Klagsbrun and Edelman, 1989; Hicks et al., 1991; Abboud, 1993).

The biological activities of exogenous FGF2 are mediated through specific, high-affinity cell surface receptors (FGFRs) that possess intracellular intrinsic tyrosine-kinase activity (Coughlin et al., 1988). Binding of FGF2 by these receptors induces their transphosphorylation at tyrosine residues and the activation of their tyrosine kinase activity (Coughlin et al., 1988). These receptors are present on a wide variety of cells (Jaye et al., 1992).

Other sites of interaction for FGF2 are the HSPGs, which are widely distributed both in extracellular matrix (ECM) and on cell surfaces (Vlodavsky et al., 1987; Moscatelli, 1988). The HSPGs represent the low-affinity sites for FGF2 (Moscatelli, 1987). FGF2 bound to HSPGs on the cell surface or in the ECM has been described as the reservoir of growth factor that ensures a long-term response by presenting FGF2 to its receptor (Flaumenhalft et al., 1989). Furthermore, the FGF2-HSPG complex protects FGF2 from proteolytic digestion (Saksela et al., 1988; Sommer and Rifkin, 1989). Although no intracellular signal triggered by FGF2 binding to HSPGs has been shown, HSPGs are thought to play a crucial

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ABBREVIATIONS: ABAE, adult bovine aortic endothelial; anti-P(Tyr), anti-phosphotyrosine; ECM, extracellular matrix; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; PBS, phosphate-buffered saline; FGF2, fibroblast growth factor 2; FGFR, fibroblast growth factor receptor; FGF-BP, fibroblast growth factor-binding protein; HSPG, heparan sulfate proteoglycan; mAb, monoclonal antibody; MAPK, mitogen-activated protein kinase; PPIase, peptidyl propyl *cis-trans* isomerase; RPE, retinal pigmented epithelium.

role in the modulation of FGF2 action. Some studies consider that the FGF2-HSPG complex is necessary for FGF2 binding to FGFRs (Yayon et al., 1991), whereas recent studies have shown that this complex is not required for this binding (Roghani et al., 1994), but it increases the affinity of FGF2 for FGFRs. In vitro studies have recently shown that in addition to the FGFR-mediated FGF2 cellular internalization, the cell surface HSPGs might provide a second pathway for FGF2 cellular internalization (Roghani and Moscatelli, 1992; Reiland and Rapraeger, 1993).

The ubiquity of FGF2 and its receptors is impressive. FGF2 probably is the most widely distributed mitogen yet characterized. It has been found without exception in all tissues so far examined and in most, although not all, cells (Lobb, 1988). The family of FGFRs is functionally present in wide variety of cells, if not all (for review, see Jaye et al., 1992). How, then, is the biological activity of FGF2 regulated in vivo?

In a recent study, we used the uniformly ^{14}C-labeled FGF2 and ^{125}I-FGF2 to study the in vivo distribution of FGF2 (Colin et al., 1997). Our results have shown that the use of ^{125}I-FGF2 leads to artifactual labeling of many tissues and organs, probably due to the nature of the labeling agent (^{125}I atoms). These artifacts can be eliminated by the use of the uniformly ^{14}C-labeled FGF2. In the present study, we use this latter to study the in vivo regulation of the bioavailability of exogenous FGF2 and its accessibility to FGFRs. We show that after i.v. injection of ^{14}C-FGF2: (1) FGF2 was rapidly taken up by target solid organs and sequestered in the HSPGs of these organs; (2) although there is no detectable catabolism of FGF2 in the blood and ECM, FGF2 in the organs is subject to cellular internalization and catabolism, and all the resulting catabolic fragments of FGF2 bind heparin; and (3) these cellular internalization and catabolism of FGF2 are not associated with any detectable increase in the tyrosine phosphorylation of FGFRs (FGFR-1 and FGFR-3) or in the mitogen-activated protein kinase (MAPK) activation in the organs, indicating that the bioavailability of FGF2 to FGFRs may be under the control of a dual role of HSPGs (i.e., immobilization and internalization).

Materials and Methods

Reagents. Bovine FGF2 (146-amino-acid form) expression plasmid pRFC6 was a gift from H. Prats (INSERM, U 397, France). *Escherichia coli* peptidyl prolyl *cis-trans* isomerase (PPIase) expression plasmid pJLEC-2B was a gift from C. T. Walsh (Harvard Medical School, Boston, MA). Lysis buffer consisted of phosphate-buffered saline (PBS) containing 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml trypsin inhibitor, 1 mM tosyl-L-phenylalanine chloromethyl ketone, 10 μg/ml leupeptin, and 10 μg/ml pepstatin A. RIPA buffer consisted of PBS containing 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml trypsin inhibitor, 1 mM tosyl-L-phenylalanine chloromethyl ketone, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μM sodium fluoride, 1 mM sodium orthovanadate, and 1% Triton X-100. Mouse anti-FGFR (clone VBS-7) monoclonal antibody (mAb), which recognizes FGFR-1 and FGFR-3, was purchased from CliniSciences (Montrouge, France). Mouse anti-phosphotyrosine (clone 4G10) mAb [anti-P(Tyr) mAb] was purchased from Euromedex (Strasbourg, France). Rabbit anti-activated MAPK polyclonal antibody, which recognizes the dually phosphorylated p44 (Erk-1)/p42 (Erk-2) MAPK (threonine 202/tyrosine 204), was from New England Biolabs, Inc. (Beverly, MA). The enhanced chemiluminescence (ECL) system was purchased from Amersham Corp (Les Ulis, France). Protein A-agarose, heparinase I (EC 4.2.2.7), and BIOMAX MR film were obtained from Sigma (St. Quentin Fallavier, France).

^{14}C-Uniform Labeling of Recombinant FGF2. The recombinant FGF2 was produced, biosynthetically labeled with ^{14}C, and purified as described previously (Colin et al., 1997). The uniform labeling of the growth factor leads to a specific activity of 2.4 μCi/μg. The growth-promoting activity was controlled in adult bovine aortic endothelial (ABAE) and retinal pigmented epithelial (RPE) cells (Colin et al., 1997).

^{14}C-Uniform Labeling of Recombinant PPIase. The *E. coli* strain DH5a was transformed with the plasmid pJLEC-2B encoding *E. coli* periplasmic PPIase (Liu and Walsh, 1990). The uniform ^{14}C labeling of PPIase was obtained by the method described by Colin et al. (1997). Briefly, *E. coli* strain overproducing PPIase was fed with ^{14}C-glucose as the sole source of carbon. The ^{14}C-PPIase was then purified as described by Liu and Walsh (1990). The specific radioactivity of the recombinant ^{14}C-PPIase was 2.5 μCi/μg.

Intravenous Injection and Tissue Distribution of FGF2. All studies on animals comply with the Decret sur l'Experimentation Animale [French law on rules for animal experimentation; decret 87-848, 19 octobre (1987)] and conformed to ARVO Resolution of the Use of Animals in Research and to the guidelines of the INSERM Committee on Animal Research. Rats were obtained from IFFA CREDO (les Oncins, France). Four-week-old male Sprague-Dawley rats (average weight, 100 g) were anesthetized by inhalation of a mixture of fluothan/O_2/nitrogen oxide and injected with 4×10^6 cpm (0.8 μg in 100 μl) of ^{14}C-FGF2 as a bolus through the saphenous vein. After injection, animals were anesthetized at different times with the same mixture and sacrificed, and 20-μm-thick sagittal sections were performed as described previously (Colin et al., 1997). The FGF2 distribution was observed by autoradiography of tissue sections using Hyperfilm-β Max and quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). In a series of experiments, rats were sacrificed at 30 min or 24 h after ^{14}C-FGF2 administration. The organs were excised, their mass were determined and homogenized in lysis buffer (50% w/v), and the radioactivity was measured in a LKB-Pharmacia 1211 mini-β-counter. The results represent the mean value of three separate experiments. Variation between assays was typically less than 10%.

Autoradiography of Tissue Sections. Eight $\times 10^6$ cpm (1.6 μg in 100 μl) of ^{14}C-FGF2 were injected i.v.ly into rats. Then, 30 min or 24 h later, rats were perfused, after anesthesia, with saline buffer and subsequently fixed in situ with 4% glutaraldehyde in the same buffer. Tissues were dehydrated in a graded series of ethanol and embedded in paraffin. Sections 7-μm-thick were cut from tissue blocks, placed on glass slides and deparaffinized with toluene twice for 15 min, and dehydrated with absolute ethanol twice for 15 min. The slides were dipped in nuclear emulsion K2 diluted in water 1:1 v/v (Ilford-Anitec, St. Priest, France), exposed for 2 weeks at 4°C, developed in Kodak D19, and fixed in Hypam Ilford fix. After washing, the slides were stained with hemalun/eosin. Some slides before coating with nuclear emulsion were washed twice for 15 min each in PBS alone (control), with 10 μg/ml heparin (Colin et al., 1997), or with 2 M NaCl in PBS to selectively remove the FGF2 from HSPGs of tissues as described previously (Gonzalez et al., 1990) and then dehydrated as described above. Before coating with the nuclear emulsion, some slides were also treated with heparinase I (EC 4.2.2.7) by incubation in 20 mM Tris·HCl, pH 7.5, 50 mM NaCl, 4 mM $CaCl_2$, 0.01% bovine serum albumin, and 2.5 U of heparinase I/ml at 37°C for 18 h; washed; and then dehydrated as described above.

Analysis of the Radioactive Degradation Products of ^{14}C-FGF2. Rats were injected with 8×10^6 cpm (1.6 μg in 100 μl) of ^{14}C-FGF2 and sacrificed after 30 min or 24 h, and different organs were excised and quickly homogenized in ice-cold lysis buffer (50% w/v). The insoluble materials (ECM and cellular debris), obtained by centrifugation at 12,000*g* for 45 min at 4°C, were resolved in SDS-polyacrylamide gel electrophoresis (PAGE) (16% acrylamide). The

supernatants were collected. 0.5 ml of supernatant of each organ was precleared by incubation with 100 μl of Sepharose beads (50% v/v in PBS) for 30 min at 4°C. Suspensions were centrifuged, and the supernatants were collected and incubated with 100 μl of heparin-Sepharose beads (50% v/v in PBS) for 30 min at 4°C. The suspensions were centrifuged, the supernatants were discarded, and the beads were washed with 1 ml of 1 M NaCl in PBS, suspended in 100 μl of 2× Laemmli's sample buffer, and resolved in SDS-PAGE (16% acrylamide). Gels were fixed and dried, and the ^{14}C products were visualized after autoradiography (BIOMax MR film; Kodak).

In a series of experiments, to compare the in vivo FGF2 catabolic fragments with those resulting from the in vitro catabolism of FGF2, samples of ^{14}C-FGF2 internalized in RPE cells obtained as previously described (Colin et al., 1997) and samples obtained from main radiolabeled organs (kidney and liver) were analyzed in SDS-PAGE.

Immunoprecipitation and Immunoblotting. Rats were injected with ^{14}C-FGF2 (8×10^6 cpm, 1.6 μg in 100 μl) and sacrificed at the indicated times, and labeled organs were dissected and homogenized in ice-cold RIPA buffer (50% w/v). Organ lysates were cleared at 12,000*g* for 45 min at 4°C, and the protein concentration in each lysate was quantified by the Bradford method (1976). For each organ, the protein content of the clarified lysates was adjusted by adding ice-cold RIPA buffer, and equal volumes (100 μl) were immunoprecipitated overnight at 4°C with 4 μg of the indicated antibody; 40 μl of protein A-agarose conjugate was then added, and the reaction mixture was incubated for 2 h at 4°C. Agarose beads were collected, washed three times with RIPA buffer, resuspended in 2× Laemmli's sample buffer, and boiled, and the immunoprecipitated proteins were resolved in SDS-PAGE (10–15% gradient gels) under reducing conditions and transferred onto polyvinylidene difluoride (PVDF) membranes. The PVDF membranes were blocked by incubation with 5% nonfat dry milk in PBS/0.05% Tween 20, washed with PBS/0.05% Tween , and incubated with the indicated primary antibody for 1 h at room temperature. After washing, the PVDF membranes were incubated with the appropriate secondary antibody, followed by ECL detection.

MAPK Activation. For Western blotting analysis, 50 μg of lysate protein from organs issued from FGF2-injected and noninjected rats was electrophoresed in SDS-PAGE (10–15% gradient gels) under reducing conditions. After transfer, the PVDF membranes were blocked by incubation with 5% nonfat dry milk in PBS/0.05% Tween 20, washed with PBS/Tween 0.05%, and incubated with an anti-activated MAPK polyclonal antibody for 1 h at room temperature. After washing, the PVDF membranes were incubated with the appropriate secondary antibody, followed by ECL detection according to the manufacturer's suggested protocol. The protein bands detected on the autoradiography were quantified by using LKB Ultrascan XL laser densitometer (Pharmacia).

Results

Fate of ^{14}C-FGF2 After a Single i.v. Bolus Dose. To study the time course distribution of FGF2, rats were sacrificed at 5 min, 30 min, 4 h, 24 h, or 120 h after ^{14}C-FGF2 administration. Whole-body sections showing all labeled tissues for each time are presented in Fig. 1. After 5 min, the radioactivity was essentially localized in solid organs (Fig. 1, A and B), but ^{14}C-FGF2 was still detectable in the blood. The liver, spleen, kidneys, and adrenal glands were strongly labeled; bone marrow, heart, and lung accumulated less radioactivity. We also noted a discrete labeling at the periphery of the retina. No labeling was detected in brain. After 30 min, the labeling almost disappeared from the blood and diminished in heart and lung (Fig. 1, C and D). In contrast, the labeling was strongly retained for 4 h in kidneys, adrenal glands, spleen, and bone marrow (Fig. 1, E and F) and for 24 h in liver (Fig. 1, G and H). Thereafter, the labeling gradually decreased in several organs, including kidneys, adrenal glands, bone marrow, and eye (Fig. 1, I and J). Throughout the kinetic study, autoradiograms did not reveal any labeling of the stomach, intestine, or bladder. After 120 h, the loss in organ labeling was associated with significant increase in the background of radioactivity in the whole body, indicating a recycling of ^{14}C-labeled amino acids (Fig. 1, I and J). This time course study illustrates that after a rapid disappearance from the blood, the labeling appeared in specific solid organs and remained for long time.

A differential tissue labeling within some organs such as adrenal glands or kidneys was also observed. Adrenal glands were labeled in the cortex but not in the medulla until 24 h (Fig. 1, B, C, F, and H), whereas the labeling within the kidneys changed with time. At 5 min after injection, only the cortical zone of kidney was labeled, and it continued to accumulate ^{14}C-FGF2 until 30 min associated with an extension

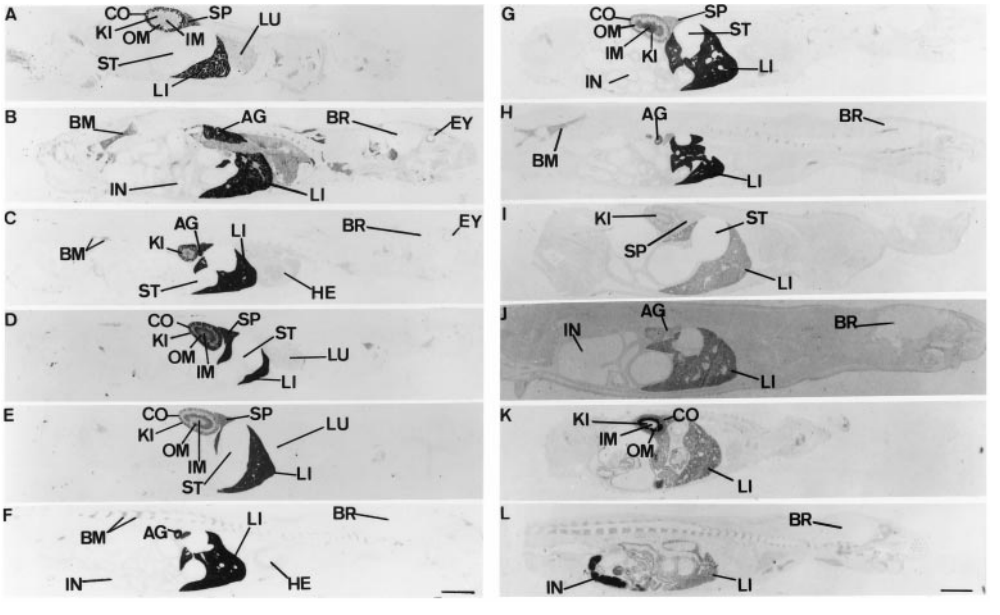

Fig. 1. Whole-body autoradiography of Sprague-Dawley male rats after i.v. injection of ^{14}C-FGF2. Rats were injected (2 μCi/100 g) and sacrificed after 5 min (A and B), 30 min (C and D), 4 h (E and F), 24 h (G and H), and 120 h (I and J). Control rats were injected with ^{14}C-PPIase (0.5 μCi/100 g) and sacrificed after 4 h (K and L). Body sections were exposed for 15 days with BIOMAX film. AG, adrenal gland; BL, bladder; BM, bone marrow; BR, brain; CO, cortex; EY, eye; HE, heart; IN, intestine; KI, kidney: OM, outer medulla; IM, inner medulla; LI, liver; LU, lung; SP, spleen; ST, stomach. Bar: 1 cm.

of the labeling to the outer medulla (Fig. 1, A and D). At 4 h, an important fraction of the radioactivity of the cortical zone was found in the outer medullary zone of kidney; however, the inner medullary zone was very weakly labeled (Fig. 1E). At 24 h, the labeling of cortex decreased, and that of medulla was increased (Fig. 1G).

To examine the correlation of ^{14}C-FGF2 distribution with its molecular structure, we studied the distribution of another cationic polypeptide that has molecular mass and charge similar to those of FGF2. The PPIase, with a molecular mass of 18.5 kDa and pHi 8.6, was chosen as a control. Rats were injected with 0.5 μCi of uniformly ^{14}C-labeled PPIase and sacrificed after 4 h; body sections can be seen in Fig. 1, K and L. In comparison with ^{14}C-FGF2 (Fig. 1, E and F), the labeling with ^{14}C-PPIase was rapidly concentrated in kidney (Fig. 1K) and feces (Fig. 1L), accompanied by a weak labeling intensity in liver. Furthermore, the labeling with this latter within the kidney was not in the cortex but rather in the outer medullary zone, indicating clearly that the clearance of ^{14}C-PPIase is different from that of ^{14}C-FGF2 and that the distribution, as well as the relatively long duration of this latter in all organs, is directly related to its structure and not to its molecular mass or charge (Fig. 1K).

Quantification of the radioactivity was at first determined per area unit (pixel) for each organ using a PhosphorImager (Fig. 2, A and B). At 5 min, the higher labeling intensities were detected in liver, adrenal glands, spleen, and kidneys. The labeling intensity of liver was 2-fold higher than that of adrenal glands, spleen, or kidneys (Fig. 2A). At 30 min, the labeling of heart, lung, and eye began to diminish (Fig. 2B), whereas that of the other organs increased, indicating that the distribution of ^{14}C-FGF2 was not complete before this time. The radioactivity in liver continued to accumulate until 4 h, whereas the radioactivity in the other organs was diminished, except in bone marrow, where it remained stable until 4 h (Fig. 2, A and B).

We have also determined the total radioactivity contained in liver, kidneys, spleen, and adrenal glands at 30 min and 24 h postinjection of ^{14}C-FGF2 by counting the radioactivity present in the homogenates of the dissected organs (Fig. 2C). After 30 min, more than 65% of injected radioactivity was retained in liver, whereas that found in kidneys, spleen, and adrenal glands represented only 4.5%, 1.2%, and 0.15% of the injected radioactivity, respectively. Relative to the radioactivity in each organ detected at 30 min, 60% of the radioactivity was maintained in liver, and more than 50% was eliminated from the other organs after 24 h (Fig. 2C).

Distribution of ^{14}C-FGF2 in Organs. Autoradiography of liver and spleen sections revealed strong labeling with silver grains throughout the tissue sections (data not shown). However, the labeling in kidneys and adrenal glands was localized to more specific zones. It was concentrated in the cortex of both organs, and especially in the glomeruli of the kidney at 30 min (Fig. 3,A and B). At 24 h, this labeling was still detectable in the cortex, glomerulosa and fasciculata zones of adrenal glands (Fig. 3C), and kidney cortex, where the labeling of the glomeruli was always stronger that than in proximal tubules (Fig. 3D).

FGF2 binding to FGFRs is resistant to 2 M NaCl wash at neutral pH, whereas its binding to HSPGs is sensitive to this treatment (Moscatelli, 1987). To identify the nature of the binding sites, some tissue sections were primarily incubated with 2 M NaCl in PBS. The results presented in Fig. 4 show kidney and adrenal gland sections of rats injected with ^{14}C-FGF2. No detectable radioactivity was observed after the incubation of the organ sections with 2 M NaCl in comparison with the respective controls incubated with PBS alone (Fig. 4, A–D). The displacement of FGF2 binding to HSPGs was also performed by using heparin. Consistent with the results obtained with 2 M NaCl, the incubation of ^{14}C-FGF2-labeled tissue sections with 10 μg/ml heparin led to a drastic elimination of the labeling of these tissue sections, which confirms the binding of FGF2 onto HSPGs within the labeled organs (Fig. 4, E and F). Heparinase I (EC 4.2.2.7) cleaves the linkage between *N*-sulfated glucosamine and 2-*O*-sulfated iduronic acid (Linhardt et al., 1990), destroying the highly sulfated FGF2-binding sites on heparan sulfate chains. Inversely, FGF2 protects HSPG against heparinase I (Tumova and Bame, 1997). Likewise, we treated tissue sections with heparinase I. The results showed that FGF2 was partially released after this treatment (Fig. 4, G and H), further indicating the immobilization of FGF2 onto HSPGs within the labeled organs and corroborating our results obtained with 2 M NaCl and heparin treatments.

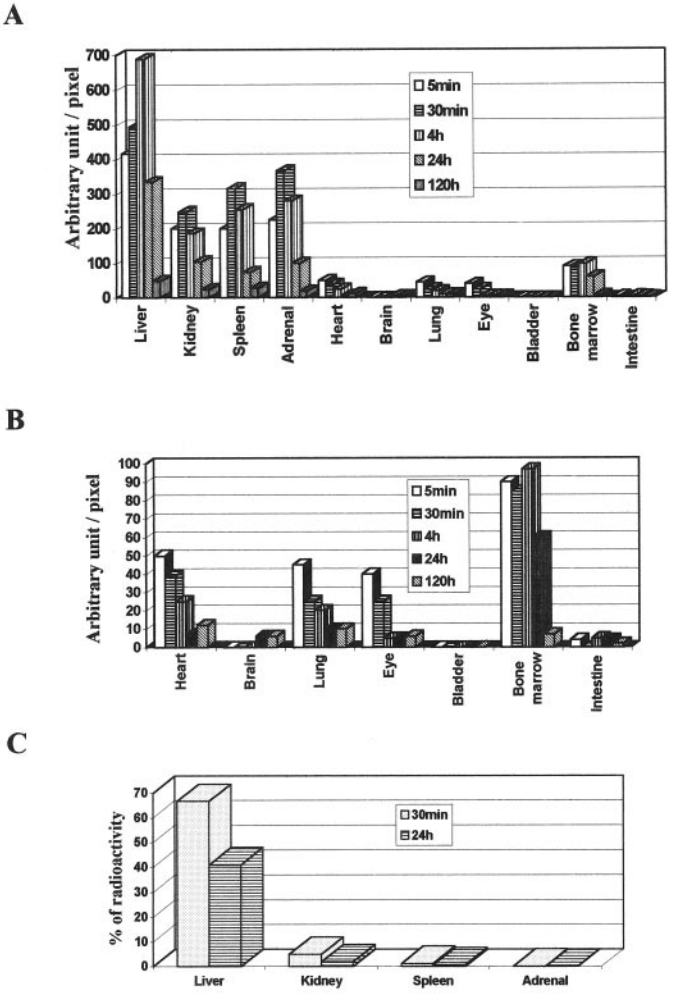

Fig. 2. Quantitative distribution of radioactivity in different organs after i.v. injection in male rat of uniformly ^{14}C-labeled FGF2. The radioactivity was directly quantified from sections using PhosphorImaging technology (A, for all organs; B, for weakly labeled organs) and by counting tissue extracts (C). Data for each organ were expressed as the mean value of three sagittal sections derived from three separate experiments.

Processing of ^{14}C-FGF2 in Organs. Homogenization, followed by centrifugation, leads to two fractions for each organ: an insoluble fraction corresponding to the ECM and cellular debris and a soluble fraction. The radioactivity contained in the soluble fraction (supernatants) from the homogenates of the labeled organs was concentrated by an affinity step on heparin-Sepharose. The nature of the radioactivity retained on this support was analyzed by SDS-PAGE. The results presented in Fig. 5A showed that the native ^{14}C-FGF2 (18 kDa) was cleaved to give 16 kDa in all studied organs as early as 30 min, except in the blood and bone marrow (Fig. 5A). At 24 h, in liver, the native ^{14}C-FGF2 had totally disappeared, and only two small fragments of 8 and 5.5 kDa were detected. These two fragments were also present in spleen, kidneys, and adrenal glands but were accompanied by varying amounts of the native 18-kDa form (Fig. 5A). Electrophoresis of the insoluble extract obtained at 30 min and 24 h from the strongly labeled organs (liver, kidneys, and spleen) showed only intact FGF2 (Fig. 5B). The fragments resulting from the in vivo catabolism of ^{14}C-FGF2 were then compared with those previously described for the in vitro catabolism of this growth factor by RPE cells (Malecaze et al., 1993; Colin et al., 1997). It seems that the in vivo and in vitro catabolism of this growth factor produces similar catabolic fragments (i.e., of 16, 8, and 5.5 kDa) (Fig. 5, A and C).

Effect of Bolus Injection of ^{14}C-FGF2 on FGFRs Phosphorylation in Organs. It has been shown that the biological activities of exogenous FGF2 are mediated through FGFRs, which closely correlates with FGFR tyrosine transphosphorylation (Coughlin et al., 1988). Therefore, we examined the tyrosine phosphorylation status of FGFR-1 and FGFR-3 in organs after 5, 15, 30, 90, and 180 min of ^{14}C-FGF2 injection. FGFR-1 and FGFR-3 were immunoprecipitated from organ homogenates from ^{14}C-FGF2 injected or noninjected rats using an anti-FGFR mAb that recognizes both forms. The immunoblotting with anti-P(Tyr) mAb revealed one band of 110 kDa corresponding to FGFR-1 (Fig. 6A), indicating that only FGFR-1 could be found under its activated state. The levels of phosphorylated FGFR-1 in ^{14}C-FGF2-labeled organs are similar to those in controls (organs from noninjected rats) (Fig. 6A), indicating that despite the

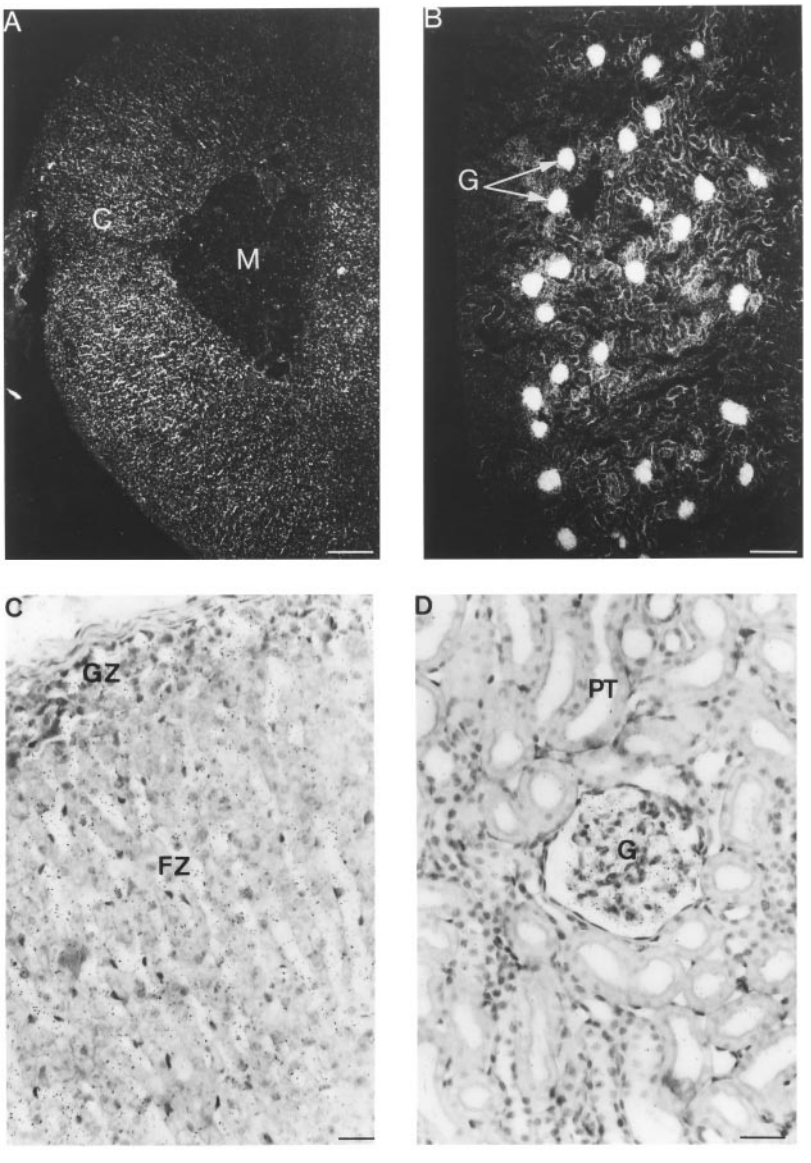

Fig. 3. Autoradiographs of adrenal gland and kidney cortex tissue sections in rats injected with ^{14}C-FGF2. Dark-field micrographs, rats were sacrificed after 30 min: A, adrenal gland; B, kidney. Bright-field micrographs, rats were sacrificed after 24 h: C, adrenal gland; D, kidney. C, cortex; FZ, fasciculata zone; G, glomerulus; GZ, glomerulosa zone; M, medulla; PT, proximal tubule. Bars, 105 μm (A and B) and 30 μm (C and D).

massive deposition of this growth factor and its catabolism within these organs, there is neither a detectable increase in the tyrosine phosphorylation of FGFR-1 nor a detectable activation of FGFR-3. Likewise, immunoprecipitation of the phosphotyrosine-containing proteins from the different organs with anti-P(Tyr) mAb and immunoblotting with the anti-FGFR mAb also showed that only FGFR-1 could be found under its activated state and that there is no detectable activation of FGFR-3 and there are no changes in the tyrosine phosphorylation status (further activation) of FGFR-1 despite the presence of exogenous FGF2 in these organs (Fig. 6B).

Effect of Bolus Injection of ^{14}C-FGF2 on MAPK Activation in Organs. FGF2 have four type of FGFRs: FGFR-1, FGFR-2, FGFR-3, and FGFR-4 (Jaye et al., 1992). Because we analyzed only the activation states of FGFR-1 and FGFR-3, there is the possibility that FGF2 could activate an FGFR form other than those we have analyzed. Thus to confirm the nonactivation of FGFR-1 and FGFR-3 in the labeled organs and to provide insight into the possibility of the activation of other types of FGFRs, we analyzed the activation state of MAPK in the different FGF2-labeled organs, as it is well established that the activation of FGFRs leads to the activation of MAPK (Cobb et al., 1991). Equivalent amounts of lysate proteins from FGF2-labeled organs and controls were resolved in SDS-PAGE followed by immunoblotting with an anti-activated MAPK antibody. The results have shown that there is not a significant increase in the activation of MAPK (p44, Erk1; p42, Erk2) in the ^{14}C-FGF2-labeled organs (liver, kidneys, spleen, and adrenal glands) relative to the same organs taken from noninjected rats (Fig. 7). These observations confirm our previous results showing the nonactivation of FGFR-1 and FGFR-3 and could give insight into the nonactivation of the other types of FGFRs (FGFR-2 and FGFR-4).

Discussion

Several lines of evidence presented in this study strongly suggest that the in vivo bioavailability of exogenous FGF2 could be under the control of HSPGs and the FGF2-cellular internalization pathway mediated by these complex macromolecules. Our results presented here have shown that 1) labeling of organs is due to the binding of ^{14}C-FGF2 to HSPGs and 2) for the first time the in vivo intracellular catabolism of FGF2 in different organs, including liver,

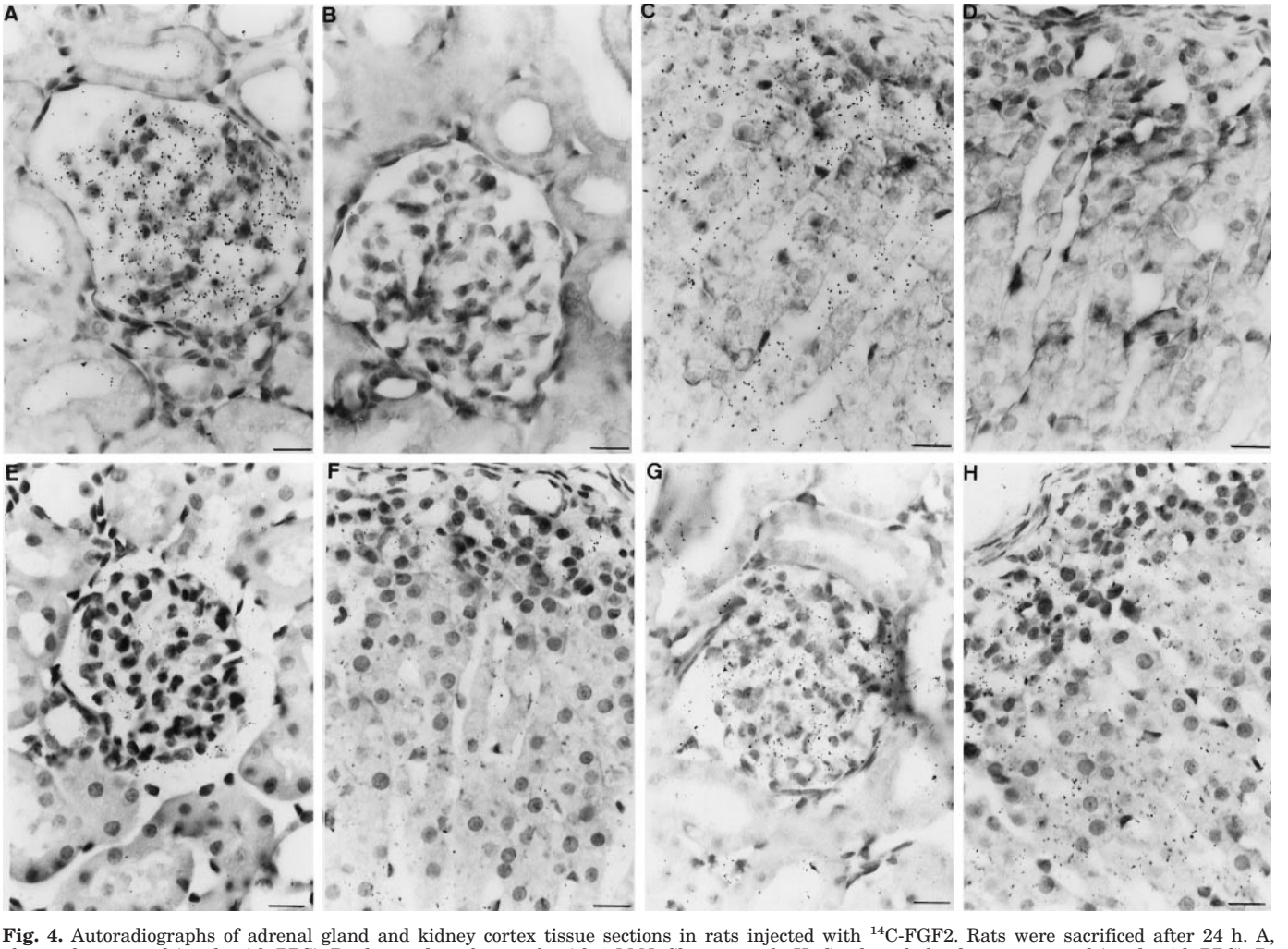

Fig. 4. Autoradiographs of adrenal gland and kidney cortex tissue sections in rats injected with ^{14}C-FGF2. Rats were sacrificed after 24 h. A, glomerulus, control (wash with PBS). B, glomerulus after wash with 2 M NaCl at neutral pH. C, adrenal gland cortex, control (wash with PBS). D, adrenal gland cortex after wash with 2 M NaCl at neutral pH. E and F, glomerulus and adrenal gland cortex, respectively, after incubation with heparin (10 μg/ml). G and H, glomerulus and adrenal gland cortex, respectively, after treatment with heparinase I (2.5 U/ml). Bar: 14 μm. For B and D, the radioactivity inside the sections was indistinguishable from the background radioactivity observed outside the sections.

spleen, kidneys, adrenal glands, and bone marrow produces the same catabolic fragments as those already reported in vitro. 3) All of the fragments resulting from the in vivo catabolism of ^{14}C-FGF2 keep bind heparin. 4) There is no detectable increase in either the tyrosine phosphorylation of FGFR-1 and FGFR-3 or in the activation of MAPK, despite the presence of ^{14}C-FGF2, and its catabolism in all labeled organs.

The short residence of FGF2 in blood we detected is in good agreement with the half-life of FGF2 in serum (Whalen et al., 1989; Hondermarck et al., 1990). Additionally, our study provides new information on the distribution of exogenous FGF2 in that it was not simply restricted to the organs with the greatest clearance function (liver, kidneys, and spleen), but it was also deposited in bone marrow, eye, and adrenal glands that are known as target organs (Hicks et al., 1991; Savona et al., 1991; Wilson et al., 1991). Furthermore, the ^{14}C-FGF2 labeling was localized to specific zones such as the cortex of the kidneys and adrenal glands, well known to contain target cells and to be rich in HSPGs (Kanwar and Farquar, 1979; Savona et al., 1991; Floege et al., 1992). The expected behavior for small positively charged polypeptides (18 kDa), especially in the kidneys, is to be cleared easily (Kanwar et al., 1991). Consistently, the injected cationic polypeptide ^{14}C-PPIase was rapidly localized in the medullary zone of kidneys. In contrast, ^{14}C-FGF2 was persistently retained in the cortex of kidneys, suggesting an important role for FGF2 affinity for heparin in its retention within the cortex as was the heparin-binding hepatocyte growth factor (Zioncheck et al., 1994). We detected minor labeling of the proximal tubules within kidneys after ^{14}C-FGF2 injection, whereas strong labeling of these entities by ^{125}I-FGF2 was reported by Whalen et al. (1989). This could be caused by the high concentrations of ^{125}I-FGF2 administrated and/or the

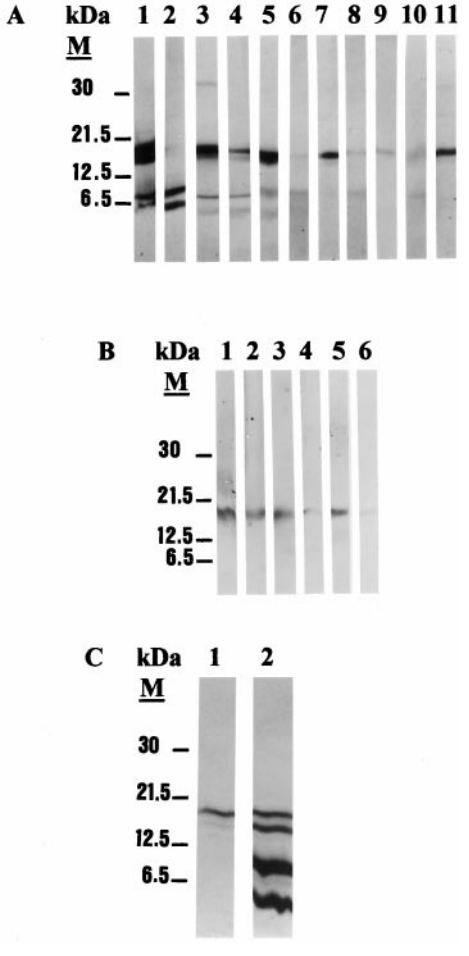

Fig. 5. Analysis of ^{14}C-FGF2 processing in vivo. A, autoradiography of SDS-PAGE of supernatants obtained from different organs extracts retained on heparin-Sepharose beads. Lane 1: liver, 30 min; lane 2: liver, 24 h; lane 3: spleen, 30 min; lane 4: spleen, 24 h; lane 5: kidney, 30 min; lane 6: kidney, 24 h; lane 7: adrenal gland, 30 min; lane 8: adrenal gland, 24 h; lane 9: bone marrow, 30 min; lane 10: bone marrow, 24 h; and lane 11: blood, 30 min. At 24 h, neither native FGF2 nor its catabolic fragments are detectable in blood (data not shown). B, autoradiography of SDS-PAGE of insoluble fractions (ECM). Lane 1: liver, 30 min; lane 2: liver, 24 h; lane 3: spleen, 30 min; lane 4: spleen, 24 h; lane 5: kidney, 30 min; lane 6: kidney, 24 h. C, Autoradiography of SDS-PAGE of the catabolic fragments obtained from the in vitro catabolism of ^{14}C-FGF2 by RPE Cells. Lane 1, 30 min; lane 2, 24 h.

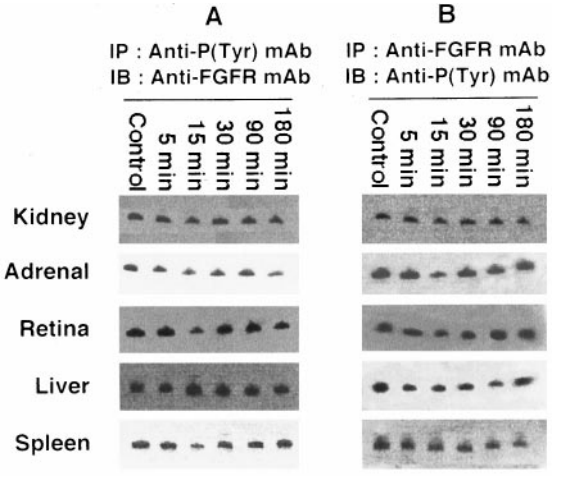

Fig. 6. Analysis of FGFR tyrosine phosphorylation status. A, phosphotyrosine-containing proteins were immunoprecipitated with anti-P(Tyr) mAb from different organs excised from rats injected with ^{14}C-FGF2 and sacrificed at 5, 15, 30, 90, and 180 min after injection or from noninjected rats as control. The immunoprecipitates were resolved in SDS-PAGE and blotted with anti-FGFR mAb. A and B, representative immunoblots are shown of experiments repeated at least tree times. B, FGFR was immunoprecipitated with anti-FGFR mAb from the different organs as in A, and the immunoprecipitates were resolved in SDS-PAGE and blotted with anti-P(Tyr) mAb, followed by ECL detection. IP, immunoprecipitated; IB, immunoblotted.

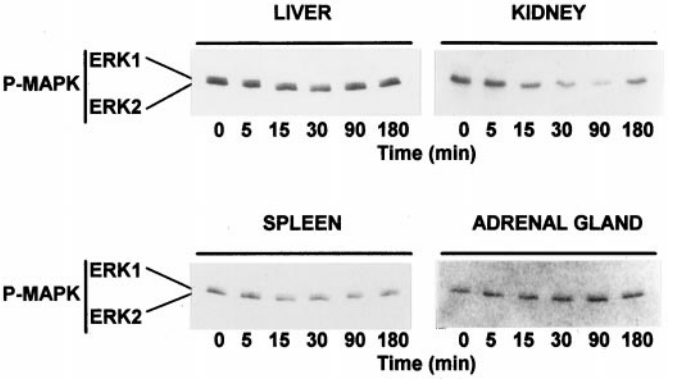

Fig. 7.: Analysis of MAPK activation status. Equivalent amounts of Lysate protein from different organs excised from rats injected with ^{14}C-FGF2 and sacrificed at 5, 30, 90, and 180 min after injection or from noninjected rats as control (0) were resolved in SDS-PAGE and blotted with anti-active MAPK polyclonal antibody followed by ECL detection. Representative immunoblots are shown from experiments repeated at least three times. P-MAPK, dually phosphorylated MAPK.

use of ^{125}I for FGF2 labeling, which leads to artifacts in FGF2 distribution (Colin et al., 1997).

Our study provides several lines of evidence indicating that ^{14}C-FGF2 deposition in all investigated labeled organs is mainly due to the binding of FGF2 by HSPGs because (1) a wash of organ sections with 2 M NaCl eluted all the radioactivity, (2) heparin displaced FGF2 from labeled organ sections, and (3) the treatment of labeled organ sections with heparinase I releases partial amount of FGF2. Furthermore, our in vivo pharmacokinetic study has not shown any detectable catabolic form of ^{14}C-FGF2 in either blood or ECM, illustrating the protection of FGF2 against proteolysis by the HSPGs of ECM (Saksela et al., 1988; Sommer and Rifkin, 1989). Our data have also shown that the labeling gradually decreased and almost completely disappeared in some organs 5 days postinjection of ^{14}C-FGF2, suggesting that FGF2 is under continuous cellular internalization, intracellular catabolism, and recycling of the resulting amino acids. The catabolic fragments of FGF2 that we have demonstrated in our study were similar in all organs and to those seen in FGF2 internalization and catabolism in vitro (Malecaze et al., 1993; Colin et al., 1997), reflecting similar intracellular processing of FGF2 in the different organs. In a recent in vitro study, we found two novel transitory catabolic fragments of the internalized FGF2 of 14 and 7 kDa (Colin et al., 1997), which could be the results of a very specific event or events in the intracellular processing of FGF2. In our present in vivo study, these two fragments are not detectable, probably due to either their transitory characteristics or that the intracellular event or events generating them are not functioning. One of the striking findings reported in this study is that all the catabolic forms of ^{14}C-FGF2 (16, 8, and 5.5 kDa) maintain the ability to bind heparin as they were immobilized on heparin-Sepharose beads. The heparin-binding sites are thus protected against proteolysis during the in vivo cellular internalization and catabolism of exogenous FGF2, supporting our hypothesis that FGF2 is internalized and then catabolized under FGF2-HSPG complex forms. This gives insight into a novel role for HSPGs in the in vivo intracellular catabolism of FGF2.

Recently, two cellular internalization pathways have been identified for FGF2 (Roghani and Moscatelli, 1992; Reiland and Rapraeger, 1993). Binding of FGF2 to FGFRs stimulates the transphosphorylation of FGFRs on tyrosine residues, resulting in the internalization of the FGF2-FGFR complex. The second pathway seems to be directly mediated by HSPGs on the cell surface. Despite the presence and the catabolism of ^{14}C-FGF2 in solid organs, we were not able to detect significant increases in either the tyrosine phosphorylation of FGFR-1 and FGFR-3 or in the activation of MAPK within all investigated organs, relative to uninjected rats. These findings strongly suggest that the exogenous FGF2 does not bind to FGFRs, and consequently does not activate their intrinsic tyrosine kinase activity, which implies that FGF2 was not internalized by cells through FGFRs. This could also give insight into the absence of the two novel transitory catabolic fragments (14 and 7 kDa) of FGF2, as the latter are only detectable in vitro (Colin et al., 1997). Alternatively, there is a possibility that minute amounts of FGFR kinase may be activated by the exogenous ^{14}C-FGF2. Although we were unable to detect any increase in the tyrosine phosphorylation of FGFRs, this possibility cannot be completely ruled out as a minute amount of activated FGFR kinase could be below the lower detection limit of the monoclonal antibodies used in this study.

Recently, three soluble FGF2-binding proteins (FGF-BPs) corresponding to the extracellular domains of FGFRs were reported to circulate in blood and to be present in the ECM even after endogenous FGF2 has been removed with 2 M NaCl (Hanneken et al., 1994, 1995). There is the possibility that the injected ^{14}C-FGF2 might bind to FGF-BPs in the blood and be rapidly targeted to the ECM. This is consistent with our results in the matter of the short residence of ^{14}C-FGF2 in blood, the localization of ^{14}C-FGF2 to the ECM, and the elution of the ^{14}C-FGF2 from ECM using 2 M NaCl. FGF-BPs could also provide a potentially new regulatory mechanism for controlling FGF2 bioavailability by competing with the FGFRs for the binding of FGF2 in vitro (Hanneken et al., 1995). Our in vivo results not only are consistent with these observations, because there is no detectable activation of neither FGFR-1 and FGFR-3 nor MAPK within all investigated organs, but also provide novel information into the successive steps. Indeed, the immobilization step of FGF2 into the ECM, whether by FGF-BPs or HSPGs or by both, is followed by HSPG-mediated cellular internalization and catabolism of FGF2, thereby clearing the excess of FGF2 susceptible to trigger cellular FGFRs activation. In controls (uninjected rats), we detected a basal level of tyrosine phosphorylation of FGFRs, indicating that there is a basal activation of the latter by the endogenous extracellular FGF2. The inability of the exogenous FGF2 to produce a significant activation of FGFRs could be due to the targeting of the injected exogenous FGF2 to an extracellular compartment restricting its interaction with FGFRs. HSPGs, dependent on their local concentration, may either promote or restrict FGF2–FGFRs interaction and activity, and the balance between these two actions of HSPGs (inhibiting and promoting) will determine the degree and extent of FGF2-induced cellular responses (Givol and Yayon, 1992).

Endogenous FGF2 can stimulate cell growth through a mechanism that is independent of the cell surface FGFRs (Bikfalvi et al., 1995). Exogenous FGF2 also induces cell proliferation, correlating with an induced tyrosine phosphorylation of FGFRs (Coughlin et al. 1988). Our results demonstrate that the in vivo bioavailability of the exogenous FGF2, and hence its biological activity, could be under the control of HSPGs, including the corollary role of HSPGs in the immobilization and cellular internalization of FGF2. This is in good agreement with the ubiquitous presence of FGF2 in normal tissues, the low proliferation of endothelial and other target cell types with turnover time measured in years (Denekamp, 1984), and with the fact that i.v. administration of FGF2 in experimental animals does not increase [^{3}H] thymidine incorporation in endothelial or vascular smooth muscle cells (Whalen et al., 1989).

FGF2 is involved in a great number of pathologies, including diabetic retinopathy, renal injury, tumor growth, and degenerative diseases (Folkman and Klagsbrun, 1987; Klagsbrun and Edelman, 1989; Hicks et al., 1991; Abboud, 1993). Thus, the identification of the pathways regulating the in vivo activities of FGF2 in both healthy and pathological tissues presents a central issue for better comprehension of these pathologies and consequently better therapeutics. Precedent data have shown that HSPGs could positively modu-

late the in vivo activities of FGF2 1) by protecting FGF2 from acid, heat, or proteolytic degradations; 2) by limiting its diffusibility and providing a reservoir from which the molecule is readily released; and 3) by mediating its binding to the FGFRs. More recently, however, a dual role of HSPGs in restricting/permitting FGF2 binding to the FGFRs has been proposed as a FGF2 activity-regulating factor (Givol and Yayon, 1992). Furthermore, we have shown that the changes in the expression of the HSPGs of ECM or the cell surface perlecan participate in the regulation of FGF2 mitogenic activity in vitro (Guillonneau et al., 1996). Our present in vivo study demonstrates that FGF2 bioavailability, and consequently its activities, could be regulated by a clearance mechanism provided by HSPGs in healthy tissues. It remains to be elucidated whether the pathologies linked to an abnormal biological activity of FGF2 may be related to a dysfunction of the novel in vivo FGF2 activity-regulating pathway we report in this study for many normal tissues.

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