Transforming growth factor- β 1 increases internalization of basic fibroblast growth factor by smooth muscle cells: implication of cell-surface heparan sulphate proteoglycan endocytosis

Eliane BERROU,*[‡] Rozenn QUARCK,* Michaëla FONTENAY-ROUPIE,[†] Sylviane LÉVY-TOLEDANO,* Gérard TOBELEM* and Marijke BRYCKAERT*

*Laboratoire de Physiopathologie cellulaire et moléculaire des cellules du sang et du vaisseau, INSERM, U 348, Hôpital Lariboisière, 75475 Paris Cedex 10, France, and

[†]Laboratoire d'Hématologie, Hôpital Cochin, 75679 Paris Cedex 14, France

Basic fibroblast growth factor (bFGF) was internalized by smooth muscle cells (SMC) from pig aorta. Correlation between heparin inhibition of binding and late internalization (8 h) implicated low-affinity sites in bFGF internalization. Transforming growth factor- β 1 (TGF- β 1) induced a 38 % increase in bFGF internalized between 4 and 8 h. While bFGF and/or TGF- β 1 enhanced cell-surface proteoglycan synthesis, ³⁵S-labelled proteoglycans of the extracellular matrix (ECM) were not affected. This might be explained by the different turnover rates displayed by the two populations of proteoglycans. Although bFGF and/or TGF- β 1 induced a similar stimulation in cell-surface chondroitin sulphate/dermatan sulphate and

heparan sulphate (HS) proteoglycan synthesis, only the turnover of HS proteoglycans was increased. Twice as much HS proteoglycan was internalized in the presence of TGF- β 1 or bFGF. Furthermore, TGF- β 1 induced a 43 ± 12 % increase in HS proteoglycan internalized in the presence of bFGF with a parallel 38 % increase in bFGF internalization. Overall, the results indicated that bFGF bound to two HS proteoglycan populations. bFGF storage (70 % of bFGF bound to SMC) was not affected by TGF- β 1 under our conditions and involved ECM proteoglycans characterized by a low turnover. bFGF internalization up-regulated by TGF- β 1 involved cell-surface HS proteoglycan characterized by a high turnover.

INTRODUCTION

Heparan sulphate (HS) proteoglycans are components of plasma membranes and of the extracellular matrix (ECM) of most mammalian cells. Their HS glycosaminoglycan chains bind a variety of proteins and such interactions implicate HS proteoglycans in many cellular processes including endocytosis of ligands, proliferation and adhesion [1]. HS proteoglycans associate with the plasma membrane by different types of interactions: (i) non-covalent interactions with plasma membrane proteins [2], (ii) intercalation of a glycosylphosphatidylinositol anchor covalently linked to the core protein of a proteoglycan, called 'glypican' [3], and (iii) transmembrane intercalation of a hydrophobic domain of the core protein. Four intercalated HS proteoglycans, collectively called 'syndecans', have been described with homologous transmembrane and cytoplasmic domains but with distinct extracellular domains (for review see [4]). Other HS proteoglycans may also exist as integral components of the ECM. The large HS proteoglycan, 'perlecan', expressed by many cell types, is composed of a 400 kDa protein core [5] containing multiple structural domains.

HS proteoglycans may influence cellular proliferation by interacting with a number of growth factors including fibroblast growth factors, a family of structurally related heparin-binding growth factors which promote cell growth and angiogenesis [6]. Basic fibroblast growth factor (bFGF) interacts with HS proteoglycans of both the ECM and the plasma membrane [7–9]. Interaction occurs via the HS chains and HS-bound bFGF is

protected from proteolysis and thermal denaturation. Several species of HS proteoglycans expressed on the cell surface are involved in bFGF binding [10] and, moreover, interaction of bFGF with its high-affinity receptor and its subsequent biological action is dependent on the presence of cell-surface HS [11–13]. bFGF binding to HS proteoglycans requires specific saccharide sequences with a defined pattern of sulphation. However, this minimal sequence does not promote bFGF receptor binding or activity [10,14,15]. bFGF-mediated signal transduction involves the formation of a ternary complex between HS proteoglycan, bFGF receptor tyrosine kinase transmembrane glycoprotein and bFGF in which HS proteoglycan interacts with both bFGF and bFGF receptor tyrosine kinase [14,16]. In fact, recent study [17] indicates that this active complex is composed of two bFGF receptor tyrosine kinases, one bFGF and at least one HS. Dimerization of high-affinity receptor only occurs in the presence of HS. After binding, bFGF is internalized and accumulates within the cells, in contrast to other growth factors which are rapidly degraded [18]. Recent reports demonstrate that bFGF internalization is mediated by high-affinity receptors and HS proteoglycans [19,20], bFGF associated to HS proteoglycans may be protected from intracellular degradation.

Recent studies emphasize the simultaneous participation of multiple growth factors and cytokines in the pathogenesis of atherosclerosis [21]. In particular, bFGF and transforming growth factor- β 1 (TGF- β 1) are produced by cells present in the atheromatous blood vessel and may be involved in the regulation of the proliferation of smooth muscle cells (SMC). TGF- β 1 is a

Abbreviations used: SMC, smooth muscle cells; bFGF, basic fibroblast growth factor; TGF- β 1, transforming growth factor- β 1; ECM, extracellular matrix; HS, heparan sulphate; CS/DS, chondroitin sulphate/dermatan sulphate.

[‡] To whom correspondence should be addressed.

member of a family of structurally related paracrine effectors which seem to play a physiological role in the control of cell growth, differentiation and angiogenesis. TGF- β 1 inhibits proliferation of most cell types and enhances synthesis of many ECM components including chondroitin sulphate/dermatan sulphate (CS/DS) proteoglycans [22]. So far, the effect of TGF- β 1 on the synthesis of the different HS proteoglycan populations has not been well documented. However, in SMC TGF- β 1 does not stimulate cell layer-derived HS proteoglycan synthesis [23]. The present study was undertaken (i) to examine the effect of TGF- β 1 on the binding and internalization of bFGF by SMC from pig aorta, and (ii) to determine the involvement of ECM- and cell surface-derived HS proteoglycans in these processes by investigating the metabolism of the different populations of HS proteoglycans in the presence of bFGF and/or TGF- β 1.

EXPERIMENTAL

Cell cultures

Primary cultures of SMC were established from media explants of pig thoracic aorta and were cultured as previously described [24]. Cells in the second to fifth passage were plated into 12-well trays (3×10^4 cells/well) and maintained for 72 h in minimum essential medium with Earle's salts containing 2 mM glutamine, 50 units/ml penicillin, 50 μ g/ml streptomycin, and 10% fetal-calf serum (Gibco). Cultures were made quiescent by incubation in serum-free medium containing 0.2% (w/v) BSA (Gibco) for 96 h with a change 24 h before experiments.

Internalization experiments

Quiescent SMC were incubated in fresh serum-free medium containing 0.2% BSA (control medium) and 10 ng/ml 125 I-labelled bFGF (Amersham, specific radioactivity 400–1200 d.p.m./fmol). TGF- β 1 (R&D Systems) or heparin (Sigma) were added to the control medium at the indicated concentrations and the cells were incubated for 4 or 8 h at 37 °C. At the end of the incubation times, cells were washed twice with phosphate-buffered saline (PBS), twice with 2 M NaCl in 20 mM Hepes, pH 7.5, to remove 125 I-bFGF bound to low-affinity sites, and twice with 2 M NaCl in 20 mM sodium acetate, pH 4.0, to remove 125 I-bFGF bound to high-affinity sites [18]. Then, internalized 125 I-bFGF was recovered by lysing the cells with 0.2 M NaOH and counting in a gamma counter (LKB). Non-specifically associated radioactivity was determined in NaOH extracts of parallel cultures incubated at 4 °C and was subtracted from experimental values. The amount of 125 I-bFGF internalized between 4 and 8 h was calculated by subtracting the amount of 125 I-bFGF internalized over the 4-h incubation period from the amount of 125 I-bFGF internalized over the 8-h incubation period.

Preparation of ECM

Quiescent SMC were incubated for 8 h at 37 °C in the presence or absence of growth factor: 10 ng/ml bFGF (recombinant human, BioTech Trade & Service), and/or 2 ng/ml TGF- β 1. At the end of the incubation time, cells were washed twice with PBS and lysed with 0.5% (w/v) Triton X-100 (Sigma) in PBS containing protease inhibitors (10 mM EDTA, 100 mM 6-amino-hexanoic acid, 5 mM benzamidine hydrochloride) for 30 min at room temperature with gentle shaking. The residual cell layer was washed four times with PBS. The Triton X-100 fraction of control cells was checked for the presence of ECM HS proteoglycan using an antibody raised to the protein core of perlecan isolated from Engelbreth-Holm-Swarm tumour (kindly supplied

by Dr. F. Mascarelli, INSERM U 118, France). Perlecan was detected in the ECM but not in the Triton X-100 fraction (results not shown).

Binding assays

SMC or ECM were incubated with 10 ng/ml 125 I-bFGF and increasing concentrations of heparin (0–1000 ng/ml) for 3 h at 4 °C. The amount of radioactivity associated with low- and high-affinity sites was determined as described in internalization experiments.

Stimulation and metabolic labelling

Quiescent SMC were placed in control medium in the presence or absence of growth factors: 10 ng/ml bFGF and/or 2 ng/ml TGF- β 1. Preliminary experiments indicated that growth factors did not stimulate 35 S-labelled proteoglycan synthesis until 4 h after incubation. So, SMC were incubated for 4 or 7 h at 37 °C with the different growth factors and then 100 μ Ci/ml $\text{Na}_2^{35}\text{SO}_4$ (Amersham, carrier-free, specific radioactivity 1000 Ci/mmol) was added to the medium. After a 4 h-labelling period, the medium was removed, the cells were washed twice with PBS and lysed with 0.5% Triton X-100.

Pulse-chase experiments

After labelling cells 4–8 h after stimulation, the radioactive medium was removed, the cells were washed five times with prewarmed control medium over a 30-min period. Cell cultures were then chased in isotope-free control medium or isotope-free medium containing growth factors. After the indicated chase period, the cells were washed twice with PBS and lysed with 0.5% Triton X-100.

Measurement of [35 S]sulphate incorporation into proteoglycans

Free [35 S]sulphate was eliminated by washes during ECM preparation and [35 S]sulphate incorporation was shown to be specific for the labelling of proteoglycans synthesized by SMC from pig aorta, as described previously [25]. 35 S-labelled proteoglycans from the ECM were recovered by solubilization in 0.2 M NaOH.

[35 S]Sulphate incorporation into Triton X-100-soluble proteoglycans of SMC was determined by separating the 35 S-labelled proteoglycans from residual free [35 S]sulphate and 35 S-labelled glycosaminoglycan free-chains on Zeta-Probe cationic nylon blots (Bio-Rad) [26]. Cationic nylon blots were prepared by soaking for 30 min in TBS (50 mM Tris/HCl, 0.15 M NaCl, pH 8.0) and then sandwiched in a dot blot apparatus (Minifold, Schleicher & Schuell). Samples were applied to the wells and pulled through the dot sandwich by vacuum. Wells were washed three times with TUT (10 mM Tris/HCl, 8 M urea, 0.1% Triton X-100, 1 mM sodium sulphate, pH 8.0), and the blot was removed from the apparatus and washed twice with TBS for 10 min. Glycosaminoglycan free-chains were eliminated by washing the blot, for 1 h on a shaker platform, in TBS containing 0.65 M NaCl. The blot was then washed twice in distilled water and cut into segments, corresponding to the well positions. Radioactivity was measured by a Beckman LS 6000 SC scintillation counter. Under these conditions, the quantity of free [35 S]sulphate that remained on the blot was insignificant compared with that of the 35 S-labelled proteoglycans.

The amount of 35 S-labelled HS proteoglycans was determined by deamination cleavage of HS chains with nitrous acid [26]. After elimination of free [35 S]sulphate, the blot was washed twice in distilled water, to remove buffer, followed by two 90-min

treatments on a shaker platform with 50 ml of fresh nitrous acid (0.48 M sodium nitrite combined with 3.6 M acetic acid). The blot was then washed for 1 h with TBS containing 0.65 M NaCl, then twice with distilled water and cut into segments for scintillation counting. The amount of ^{35}S -labelled HS proteoglycans was calculated by subtracting the amount of ^{35}S -labelled CS/DS proteoglycans (acid-treated blot) from the amount of total ^{35}S -labelled proteoglycans (non-acid-treated blot).

Statistics

Most of the data represent the mean of at least four independent experiments and are expressed as the average $\pm$ S.E.M. Statistical significance was assessed using Student's *t*-test for paired comparisons.

RESULTS

TGF- β 1 Increased bFGF Internalization by SMC

The internalization of bFGF is mediated by the high-affinity cell-surface receptor tyrosine kinase but also by its low-affinity HS proteoglycan-binding sites. We investigated the effect of TGF- β 1 on bFGF internalization by quiescent SMC. When SMC were incubated at 37 °C with 10 ng/ml ^{125}I -bFGF, ^{125}I -bFGF continued to accumulate in the cells for up to 8 h. No accumulation of ^{125}I -bFGF was observed in parallel cultures maintained at 4 °C. Addition of 2 ng/ml TGF- β 1 to the incubation medium induced an increase in internalized ^{125}I -bFGF after at least 4 h incubation (Table 1). Between 4 and 8 h, cells incubated in the presence of TGF- β 1 internalized 38 % more ^{125}I -bFGF than control cultures ($0.005 < P \leq 0.01$, $n = 5$). We then investigated the effect of increasing concentrations of TGF- β 1 (0–2 ng/ml) on the amount of ^{125}I -bFGF internalized between 4 and 8 h (Figure 1); 0.2 ng/ml TGF- β 1 enhanced the amount of ^{125}I -bFGF internalized (14.9 ± 1.0 versus 11.7 ± 1.2 fmol/ 10^5 cells, $0.025 < P \leq 0.05$, $n = 3$) and stimulation of internalization reached a plateau (18 fmol/ 10^5 cells) at 1 ng/ml TGF- β 1. All subsequent experiments described in this study were performed with 2 ng/ml TGF- β 1.

Dose-dependence of heparin inhibition of low-affinity binding and internalization

To investigate involvement of low-affinity binding sites in late internalization of bFGF, SMC were incubated for 8 h at 37 °C with 10 ng/ml ^{125}I -bFGF and increasing concentrations of heparin (0–1000 ng/ml) which prevents the binding of ^{125}I -bFGF to

Figure 1 TGF- β 1 dose-dependent stimulation of ^{125}I -bFGF internalization between 4 and 8 h

Quiescent SMC were incubated with 10 ng/ml ^{125}I -bFGF and increasing concentrations of TGF- β 1 (0–2 ng/ml) for 4 or 8 h at 37 °C. At the end of the incubation period, the amount of ^{125}I -bFGF internalized was determined (see the Experimental section). The amount of ^{125}I -bFGF internalized between 4 and 8 h was calculated by subtracting the amount of ^{125}I -bFGF internalized over the 4-h incubation period from the amount of ^{125}I -bFGF internalized over the 8-h incubation period. Data represented the average $\pm$ S.E.M. of three independent experiments done in duplicate.

Figure 2 Binding to low-affinity sites and internalization of ^{125}I -bFGF in the presence of heparin

Quiescent SMC were incubated with 10 ng/ml ^{125}I -bFGF and increasing concentrations of heparin (0–1000 ng/ml). SMC were incubated for 3 h at 4 °C to determine the amount of ^{125}I -bFGF bound to low-affinity sites (○) or were incubated for 8 h at 37 °C to determine the amount of ^{125}I -bFGF internalized (●) (see the Experimental section). The amount of ^{125}I -bFGF bound or internalized in the absence of heparin was defined as 100%. Determinations were done in duplicate and comparable results were obtained in two other independent experiments.

low-affinity sites. Figure 2 shows that heparin at 50 ng/ml inhibited bFGF internalization by 42 % and 0.5 and 1 $\mu\text{g}/\text{ml}$ had maximal effect (75 % inhibition). Moreover, the heparin inhibition of bFGF internalization is equivalent to the heparin inhibition of ^{125}I -bFGF binding to low-affinity sites, determined at 4 °C (Figure 2). Thus, these results showed that late bFGF internalization by SMC was largely mediated by low-affinity sites. The increase in internalized bFGF observed in the presence of TGF- β 1 might imply an increase in the number of low-affinity binding sites and/or an acceleration of their endocytosis.

TGF- β 1 did not have a marked effect on ^{125}I -bFGF binding to low- and high-affinity sites on SMC

In order to determine whether the increase in ^{125}I -bFGF internalization in response to TGF- β 1 was related to an increase in binding of bFGF, the effect of TGF- β 1 on ^{125}I -bFGF binding

Table 1 Effect of TGF- β 1 on ^{125}I -bFGF internalization

Quiescent SMC were incubated with 10 ng/ml ^{125}I -bFGF in the presence or absence of 2 ng/ml TGF- β 1 for 4 or 8 h at 37 °C. At the end of the incubation period, the amount of ^{125}I -bFGF internalized was determined (see the Experimental section). The amount of ^{125}I -bFGF internalized between 4 and 8 h was calculated by subtracting the amount of ^{125}I -bFGF internalized over the 4-h incubation period from the amount of ^{125}I -bFGF internalized over the 8-h incubation period. Data represented the average $\pm$ S.E.M. of five independent experiments done in duplicate.

Addition	^{125}I -bFGF internalized (fmol/ 10^5 cells)	
	0–4 h	4–8 h
None	16.4 $\pm$ 3.4	11.7 $\pm$ 1.2
TGF- β 1	16.6 $\pm$ 3.2	16.2 $\pm$ 2.4

Table 2 Validation of determination of the amount of ^{125}I -bFGF bound to high- and low-affinity sites

Quiescent SMC were incubated for 3 h at 4 °C with 10 ng/ml ^{125}I -bFGF. The amount of ^{125}I -bFGF recovered in acidic washes (high-affinity sites) was successively determined before or after prewashing in 2 M NaCl at pH 7.5 (low-affinity sites). Determinations were done in duplicate and comparable results were obtained in another independent experiment.

Washing buffers	^{125}I -bFGF (fmol/ 10^5 cells)
Experimental procedure 1	
0.15 M NaCl, pH 4.0	0.6
2 M NaCl, pH 7.5	41.5
Experimental procedure 2	
2 M NaCl, pH 7.5	39.3
2 M NaCl, pH 4.0	0.7

Table 3 Effect of bFGF and TGF- β 1 on ^{125}I -bFGF binding to low-affinity sites of whole cells and the ECM

Two groups of quiescent SMC were preincubated in the presence or absence of 10 ng/ml bFGF and/or 2 ng/ml TGF- β 1 for 8 h at 37 °C. ECM were obtained from one group of cultures. Binding of ^{125}I -bFGF to low-affinity sites was determined after incubating the cells and the ECM with 10 ng/ml ^{125}I -bFGF for 3 h at 4 °C (see the Experimental section). Data represented the means $\pm$ S.E.M. of three determinations and comparable results were obtained in two other independent experiments.

Preincubation	^{125}I -bFGF bound (fmol/ 10^5 cells)	
	Cell layer	ECM
Control	40.3 $\pm$ 0.8	30.5 $\pm$ 1.2
bFGF	39.6 $\pm$ 0.9	29.0 $\pm$ 1.3
TGF- β 1	41.8 $\pm$ 1.1	27.7 $\pm$ 0.4
bFGF + TGF- β 1	41.0 $\pm$ 1.0	29.7 $\pm$ 2.1

to low- and high-affinity sites was investigated. To validate our methodology for high- and low-affinity site determination and to ensure that the 2 M NaCl wash at pH 7.5 did not release ^{125}I -bFGF bound to high-affinity sites of SMC, we first treated the cells with an acid buffer containing a low concentration of NaCl, to dissociate growth factor from its signalling receptor. A similar amount of ^{125}I -bFGF was recovered under the two experimental procedures (Table 2), indicating that prewashing in 2.0 M NaCl at pH 7.5 did not affect determination of the amount of ^{125}I -bFGF bound to high-affinity sites. In the presence of 10 ng/ml ^{125}I -bFGF, low-affinity sites bound about 60-fold more ^{125}I -bFGF than high-affinity sites. When quiescent SMC were preincubated with 2 ng/ml TGF- β 1 for 8 h, the binding of bFGF to its high- and low-affinity sites was not modified.

bFGF and TGF- β 1 together, or separately, did not affect bFGF binding to low-affinity sites in the ECM

To attempt to discriminate between low-affinity binding sites present in the ECM and on the cell surface, we prepared ECM by lysis of cells with 0.5 % Triton X-100. Binding of ^{125}I -bFGF to low-affinity sites of whole cells and ECM was determined (Table 3). Preincubation of SMC with 10 ng/ml bFGF did not induce modification in binding to low-affinity sites on either the cell layer or in the ECM. TGF- β 1 (2 ng/ml) did not modify binding in the presence or absence of 10 ng/ml bFGF. Moreover, binding of 10 ng/ml ^{125}I -bFGF to the low-affinity sites in the ECM represented about 70 % of total bFGF binding to the low-

Table 4 Effect of bFGF and TGF- β 1 on [^{35}S]sulphate incorporation into proteoglycans of Triton X-100-soluble fractions and ECM

Quiescent SMC were incubated for 4 h at 37 °C in the presence or absence of 10 ng/ml bFGF and/or 2 ng/ml TGF- β 1. Then, 100 $\mu\text{Ci}/\text{ml}$ [^{35}S]sulphate was added to the medium for a further 4 h at 37 °C. SMC were lysed with 0.5 % Triton X-100 and the [^{35}S]sulphate incorporated into proteoglycans of Triton X-100 fractions and ECM was determined (see the Experimental section). The amount of radioactivity determined in control cultures was defined as 100 %. Data represented the means $\pm$ S.E.M. of four independent experiments done in triplicate.

Addition	[^{35}S]Sulphate incorporation (% of control)	
	Triton X-100 fraction	ECM
None	100	100
bFGF	138 $\pm$ 4	107 $\pm$ 5
TGF- β 1	148 $\pm$ 4	102 $\pm$ 6
bFGF + TGF- β 1	188 $\pm$ 11	101 $\pm$ 3

affinity sites on whole cells. Even if growth factors affected bFGF binding to low-affinity sites of the plasma membrane this could not be clearly demonstrated by binding assays performed with whole cells.

bFGF and TGF- β 1 increased the amount of ^{35}S -labelled proteoglycan recovered in Triton X-100-soluble fractions

Studies into the internalization process were also carried out by examining the synthesis and turnover of low-affinity binding sites. We investigated the effect of bFGF and TGF- β 1 on the synthesis and turnover of sulphated proteoglycans present in the ECM or solubilized in Triton X-100.

We examined the effect of incubation for 8 or 11 h with bFGF and/or TGF- β 1 on the synthesis of sulphated proteoglycans. Cells were pulsed with [^{35}S]sulphate for the remaining 4 h of the incubation period with growth factors. It was found that 10 ng/ml bFGF and 2 ng/ml TGF- β 1 stimulated [^{35}S]sulphate incorporation into proteoglycans of Triton X-100 fractions after 4 h incubation and gave a maximal effect during the 4–8 h period following stimulation (Table 4). Additive stimulation of [^{35}S]sulphate incorporation was observed when bFGF and TGF- β 1 were simultaneously added. In contrast, incorporation of [^{35}S]sulphate into ECM proteoglycans (Table 4), which represented only 15 % of [^{35}S]sulphate incorporated into proteoglycans of the cell layer during the 4–8 h period, was not modified by the addition of growth factors to the medium. These results correlated with the lack of growth factor effect on ^{125}I -bFGF binding to low-affinity sites of the ECM.

In order to investigate the effect of growth factors on [^{35}S]sulphate incorporation into the different proteoglycans in the Triton X-100 fraction, HS chains of ^{35}S -labelled proteoglycans bound to Zeta-Probe blots were cleaved with nitrous acid and the remaining non-acid-hydrolysed, bound CS/DS proteoglycans were quantified (Table 5). ^{35}S -labelled HS proteoglycans (from 25 % to 30 % of the [^{35}S]sulphate incorporated into proteoglycans in the Triton X-100 fraction) and ^{35}S -labelled CS/DS proteoglycans were similarly increased by incubation with growth factors. Only bFGF stimulated slightly more [^{35}S]sulphate incorporation into HS proteoglycans than into CS/DS proteoglycans ($0.01 < P \leq 0.025$, $n = 4$). Similar stimulations were obtained during the 7–11 h period, indicating stability of growth factor action.

Table 5 Effect of growth factors on [35 S]sulphate incorporation into HS and CS/DS proteoglycans of Triton X-100 fractions

Quiescent SMC were incubated for 4 or 7 h at 37 °C in the presence or absence of 10 ng/ml bFGF and/or 2 ng/ml TGF- β 1. Then, 100 μ Ci/ml [35 S]sulphate was added to the medium for a further 4 h at 37 °C. SMC were lysed with 0.5% Triton X-100 and the [35 S]sulphate incorporated into CS/DS proteoglycans and HS proteoglycans was determined after nitrous acid degradation of HS chains on Zeta-Probe blots (see the Experimental section). The amount of radioactivity determined in control cultures was defined as 100%. Data represented the means $\pm$ S.E.M. of four independent experiments done in triplicate.

Addition	[35 S]sulphate incorporation (% of control)			
	HS proteoglycans		CS/DS proteoglycans	
	4–8 h	7–11 h	4–8 h	7–11 h
None	100	100	100	100
bFGF	153 $\pm$ 8	152 $\pm$ 8	127 $\pm$ 3	133 $\pm$ 3
TGF- β 1	148 $\pm$ 8	149 $\pm$ 10	141 $\pm$ 5	148 $\pm$ 3
bFGF + TGF- β 1	181 $\pm$ 23	186 $\pm$ 21	170 $\pm$ 7	188 $\pm$ 6

Figure 3 Effect of growth factors on the decrease in 35 S-labelled HS proteoglycans in Triton X-100 fractions after a 3-h chase period

Quiescent SMC were incubated for 4 h at 37 °C in the presence or absence of 10 ng/ml bFGF and/or 2 ng/ml TGF- β 1. Then, 100 μ Ci/ml [35 S]sulphate was added to the medium for a further 4 h at 37 °C. After labelling, the cells were chased for various time periods in the same isotope-free medium. After each period, the amount of 35 S-labelled HS proteoglycans in Triton X-100 fractions was determined (see the Experimental section). The amount of radioactivity determined in cultures before the chase period was defined as 100%. Data represented the mean $\pm$ S.E.M. of four independent experiments done in triplicate. (a) Control medium ($r^2 = 0.967$), (b) 10 ng/ml bFGF ($r^2 = 0.978$), (c) 2 ng/ml TGF- β 1 ($r^2 = 0.981$), (d) 10 ng/ml bFGF and 2 ng/ml TGF- β 1 ($r^2 = 0.933$).

35 S-labelled proteoglycan in Triton X-100-soluble fractions and ECM exhibited different turnover rates

Differences in the amounts of [35 S]sulphate incorporated into ECM and Triton X-100-soluble proteoglycans during the 4–8 h labelling period suggest that these proteoglycan populations exhibited different turnover rates. The turnover of proteoglycans in the presence of growth factors was investigated by isotopic chase experiments. SMC were incubated for 4 h in isotope-free medium alone or isotope-free medium containing bFGF and/or TGF- β 1, then proteoglycans were metabolically labelled for an additional 4 h with [35 S]sulphate. An isotopic chase was then performed for 3 h in the presence of growth factors. The amount

Table 6 Effect of growth factors on the half-life of proteoglycans in Triton X-100-soluble fractions

Half-lives of HS proteoglycans were calculated from the data of Figure 3. In these experiments, the decrease in 35 S-labelled CS/DS proteoglycans was also quantified (see the Experimental section) and half-lives were calculated.

Addition	Half-life (min)	
	HS proteoglycans	CS/DS proteoglycans
None	338	154
bFGF	231	147
TGF- β 1	222	143
bFGF + TGF- β 1	193	152

of [35 S]sulphate-labelled proteoglycans in the ECM and Triton X-100 fractions was determined. About 80 % of the initial pre-chase radioactivity was recovered after the 3-h chase period in the ECM of control cultures. Addition of growth factors to the incubation medium did not affect this decrease. In contrast, in the Triton X-100 fraction of control cultures, 48 $\pm$ 4 % of the radioactivity was recovered and significantly decreased with addition of growth factors to the incubation medium: 43 $\pm$ 3 % for bFGF, 42 $\pm$ 3 % for TGF- β 1, 42 $\pm$ 2 % for bFGF and TGF- β 1 ($P < 0.05$, $n = 4$). These results showed that Triton X-100-soluble and Triton X-100-resistant proteoglycans represented two metabolically different populations. Moreover, these results might explain the lack of growth factor effect on ECM-proteoglycan synthesis at 4–8 h following stimulation.

bFGF and TGF- β 1 specifically decreased the half-life of Triton X-100-soluble 35 S-labelled HS proteoglycans

At various chase times, the remaining 35 S-labelled HS and CS/DS proteoglycans in the Triton X-100 fractions were quantified. The time-dependent decrease in radioactivity followed an exponential curve (Figure 3). After a 3-h chase period, 70 $\pm$ 3 % of the initial amount of the 35 S-labelled HS proteoglycans was present in control SMC (Figure 3a). This amount significantly ($P < 0.05$, $n = 4$) decreased to 58 $\pm$ 4 % in bFGF-treated SMC (Figure 3b), to 57 $\pm$ 3 % in TGF- β 1-treated SMC (Figure 3c) and to 50 $\pm$ 4 % in bFGF plus TGF- β 1-treated SMC (Figure 3d). Acceleration, by TGF- β 1, of the decrease in 35 S-labelled HS proteoglycans of bFGF-treated SMC was significant ($0.005 < P \leq 0.01$, $n = 4$). Calculation of the $t_{1/2}$ (Table 6) showed that in control SMC 35 S-labelled HS proteoglycans disappeared from the cell surface with a $t_{1/2} \sim 340$ min. This half-life decreased after addition of bFGF or TGF- β 1 and reached 231 and 222 min respectively. When the two growth factors were present, the half-life decreased to 193 min. In contrast, 35 S-labelled CS/DS proteoglycans disappeared from the cell surface with a $t_{1/2} \sim 140$ min at 150 min which was modified neither by bFGF nor by TGF- β 1.

Stimulation of bFGF internalization by TGF- β 1 correlated with the stimulation of 35 S-labelled HS proteoglycan endocytosis

The loss of HS proteoglycans from the cell surface is accounted for either by release into the medium or by endocytosis followed by different degradation steps. The amount of intact 35 S-labelled HS proteoglycans recovered in the culture medium was below the detection level. The amount of intact 35 S-labelled HS proteoglycans recovered within the cells, after treatment with trypsin, was very low and could not be quantified by the blot technique.

Table 7 Stimulation of ^{35}S -labelled HS proteoglycan endocytosis by growth factors

The amount of ^{35}S -labelled HS proteoglycans internalized during the 3-h chase period was determined by calculating the loss of ^{35}S -labelled HS proteoglycan in Triton X-100 fractions during the chase of SMC incubated in control medium, with 10 ng/ml bFGF, with 2 ng/ml TGF- β 1, or with 10 ng/ml bFGF and 2 ng/ml TGF- β 1. The amount of ^{35}S -labelled HS proteoglycan lost in control cultures was defined as 100%.

Addition	^{35}S -labelled HS proteoglycan endocytosis (% of zero time)
None	100
bFGF	200 $\pm$ 23
TGF- β 1	184 $\pm$ 14
bFGF + TGF- β 1	270 $\pm$ 55

So, we estimated the amount of ^{35}S -labelled HS proteoglycan internalized after a 3-h chase by the loss of ^{35}S -labelled HS proteoglycans in the Triton X-100 fractions during this period. Thirty percent of the initial amount of ^{35}S -labelled HS proteoglycan was lost in control cultures (Figure 3). bFGF or TGF- β 1-treated SMC internalized about twice as much ^{35}S -labelled HS proteoglycans as did control SMC (Table 7). When the two growth factors were present in the medium, a 2.7-fold stimulation was observed. These data indicated that addition of TGF- β 1 to bFGF-treated cells induced an increase ($0.025 < P \leq 0.05$, $n = 4$) in ^{35}S -labelled HS proteoglycan endocytosis ($43 \pm 12\%$) which correlated with the increase in bFGF internalization induced by TGF- β 1 (38%).

DISCUSSION

This study demonstrated that SMC internalize bFGF via a HS proteoglycan pathway, in agreement with studies on other cell types [19,20]. It demonstrates for the first time that this process was regulated by TGF- β 1 which stimulated endocytosis of cell-surface HS proteoglycans without modification of ECM proteoglycan metabolism.

We showed that bFGF and TGF- β 1 stimulated [^{35}S]sulphate incorporation into HS and CS/DS proteoglycans of Triton X-100-soluble fractions to a similar extent, indicating a generalized stimulatory effect on proteoglycan synthesis. Moreover, simultaneous addition of the two growth factors led to an additive stimulatory effect. These data suggested that (i) the two growth factors exhibited identical action on proteoglycan synthesis, and (ii) TGF- β 1 did not affect bFGF action (and vice versa). Several studies demonstrate stimulation of CS/DS proteoglycan synthesis without a TGF- β 1 effect on HS proteoglycan synthesis [23,27]. In contrast, recent reports indicate that TGF- β 1 differentially regulates the synthesis of transmembrane HS proteoglycans depending on the cell type [28,29]. In particular, in SMC the syndecan 1 mRNA level decreased and the syndecan 2 mRNA level increased following TGF- β 1 stimulation [30]. The effect of bFGF on proteoglycan synthesis is less well documented. Stimulation of syndecan 1 synthesis has been described in 3T3 cells [29], while in SMC [30] bFGF had no effect. The latter report did not examine the effect of simultaneous addition of the two growth factors on syndecan expression but a synergistic action of bFGF and TGF- β 1 on syndecan 1 expression by 3T3 cells was observed [29].

While bFGF and TGF- β 1 stimulated [^{35}S]sulphate incorporation into proteoglycans of Triton X-100 fractions at 4–8 h following stimulation, ECM-proteoglycan synthesis was not

altered. This observation indicated that the two compartments contained two distinct populations of HS proteoglycan. In reports [31–33] which describe an increase in the synthesis of ECM proteoglycans, the stimulatory effect of TGF- β 1 was observed after a 24 h incubation. Prolonged incubation of SMC might be required for stimulation of ECM-proteoglycan synthesis, such a delay might be explained by the different turnover rates exhibited by ECM and cell-surface proteoglycans.

In order to determine whether HS proteoglycans were involved in a regulatory process of bFGF internalization, we studied the effect of growth factors on the turnover of cell-surface proteoglycan. The half-life of cell-surface HS proteoglycans decreased in the presence of bFGF or TGF- β 1. Yanagishita [34] reports that, in rat ovarian granulosa cells, the half-life of HS proteoglycans on the cell surface is about 4 h in the presence of serum, a value which is in agreement with this study (Table 6). While ^{35}S incorporation into HS and CS/DS proteoglycans of Triton X-100 fractions was enhanced by growth factors to a similar extent, turnover of proteoglycans appeared differentially regulated, only the half-life of HS proteoglycans was decreased by bFGF and TGF- β 1. CS/DS proteoglycans are mainly associated with the plasma membrane, but not via intercalation or via a lipid-anchor, and are processed by receptor-mediated endocytosis [35]. This finding suggested that growth factors might differentially regulate proteoglycan turnover depending on the type of interaction between the protein core and the plasma membrane.

In SMC, HS proteoglycans did not seem to be shed from the cell surface into the medium. This result confirmed previous studies showing that ^{35}S -labelled HS proteoglycans represented only 7% of ^{35}S -labelled proteoglycans secreted into the medium [36,37] and that HS proteoglycans were not released from the cell surface into the medium over a 24-h chase period [38]. On the other hand, recycling of HS proteoglycans was very unlikely, since ^{35}S -labelled HS proteoglycans were not detected within the cells after trypsin treatment (results not shown). Moreover, recycling of HS proteoglycans, has only been described for a parathyroid cell line [39]. We thus estimated the amount of ^{35}S -labelled HS proteoglycan internalized during 3 h by calculating their decrease in the Triton X-100 fractions. Thirty percent of the initial amount of ^{35}S -labelled HS proteoglycans was internalized by control cultures over a 3-h chase period (Figure 3). Stimulation of HS proteoglycan synthesis and increase in their turnover led to stimulation of endocytosis of these proteoglycans. Twice as much HS proteoglycan was internalized in the presence of bFGF or TGF- β 1. Moreover, bFGF plus TGF- β 1-treated cells internalized more HS proteoglycan than bFGF-treated cells. This increase (43%) correlated with the increase in [^{125}I]bFGF internalization induced by TGF- β 1 (38%). This result indicated that TGF- β 1 increased bFGF internalization by stimulating the cell-surface HS proteoglycan endocytosis involved in this process, without modification of the bFGF-binding capacity of HS chains synthesized in the presence of bFGF. Whereas regulation of HS proteoglycan-mediated internalization has not yet been studied, Nugent and Edelman [32] reported that stimulation of [^{125}I]bFGF binding to low-affinity sites correlated with stimulation of ECM-proteoglycan synthesis in TGF- β 1-treated Balb/c3T3 cells.

In this study, the different species of cell-surface HS proteoglycans were not identified. The possibility that a single HS proteoglycan species (target of TGF- β 1) is the major route of bFGF internalization cannot be ruled out. However, recent findings do not support this hypothesis. Aviezer et al. [10] report that different species of cell-surface-derived HS proteoglycans can bind bFGF and thus can mediate bFGF internalization. In addition, Sanderson et al. [40] demonstrate that the fine structure and thus the biological function of HS proteoglycans was not

encoded in the protein core. It must be emphasized that SMC predominantly expressed on their cell surface an HS proteoglycan with a protein core of apparent molecular mass 92 kDa [41]. This proteoglycan might be related to syndecan 2 which is up-regulated by TGF- β 1 in SMC [30].

The HS proteoglycan species involved in bFGF internalization and the effect of bFGF and/or TGF- β 1 on their expression in SMC should be studied in the future. Moreover, controlled variations in patterns of sulphation of cell-surface HS proteoglycans might occur, as recently suggested for developing neural cells [42]. SMC internalized about the same amount of 125 I-bFGF between 4 and 8 h and between 0 and 4 h in spite of stimulation of HS proteoglycan endocytosis by bFGF. Down-regulation of the number of low-affinity binding sites by bFGF has been described in endothelial cells [20] and might explain this discrepancy. The effect of bFGF and/or TGF- β 1 on 125 I-bFGF binding to low-affinity sites of Triton X-100-soluble fractions are currently under investigation.

Nevertheless, this study shows that HS proteoglycan endocytosis is the major pathway of bFGF internalization by SMC. About 10 fmol/ 10^5 cells of bFGF bound to low-affinity sites of the cell surface (30 % of 125 I-bFGF bound to low-affinity sites of SMC, Table 3), this amount represented between one-third and one-half of the amount of bFGF internalized during 8 h, indicating the efficiency of the HS proteoglycan endocytosis pathway. Moreover, this pathway is up-regulated by TGF- β 1. On the other hand, in SMC, 70 % of the 125 I-bFGF bound to low-affinity sites were recovered in the ECM and under our conditions, neither this binding nor the synthesis of 35 S-labelled ECM proteoglycans were affected by TGF- β 1. Taken together, these data indicate that proteoglycans of Triton X-100-soluble fractions and of the ECM exhibit different biological functions. Cell-surface HS proteoglycans with a rapid turnover are involved in processes quickly regulated by growth factors such as bFGF internalization, while ECM HS proteoglycans with a low turnover are involved in processes such as bFGF storage.

We thank Dr. M. C. Brahimi-Horn and Dr. E. Deudon for critical reading of the manuscript. This work was supported by grants from the 'Institut National de la Santé et de la Recherche Médicale' (INSERM, U 348), the 'Fondation de France' and the 'Association pour la Recherche contre le Cancer' (ARC)

REFERENCES

- Kjellén, L. and Lindahl, U. (1991) *Annu. Rev. Biochem.* **60**, 443–475
- Kjellén, L., Oldberg, Å. and Höök, M. (1980) *J. Biol. Chem.* **255**, 10407–10413
- David, G., Lories, V., Decock, B., Marynen, P., Cassiman, J. J. and Van den Berghe, H. (1990) *J. Cell Biol.* **111**, 3165–3176
- Bernfield, M., Kokenyesi, R., Kato, M., Hinkes, M. T., Spring, J., Gallo, R. L. and Løse, E. J. (1992) *Annu. Rev. Cell Biol.* **8**, 365–393
- Noonan, D. M., Fulle, A., Valente, P., Cai, S., Horigan, E., Sasaki, M., Yamada, Y. and Hassell, J. R. (1991) *J. Biol. Chem.* **266**, 22939–22947
- Basilico, C. and Moscatelli, D. (1992) *Adv. Cancer Res.* **59**, 115–165
- Vlodavsky, I., Folkman, J., Sullivan, R., Fridman, R., Ishai-Michaeli, R., Sasse, J. and Klagsbrun, M. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 2292–2296
- Saksela, O., Moscatelli, D., Sommer, A. and Rifkin, D. B. (1988) *J. Cell Biol.* **107**, 743–751
- Kiefer, M. C., Stephans, J. C., Crawford, K., Okino, K. and Barr, P. J. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 6985–6989
- Aviezer, D., Levy, E., Safran, M., et al. (1994) *J. Biol. Chem.* **269**, 114–121
- Yayon, A., Klagsbrun, M., Esko, J. D., Leder, P. and Ornitz, D. M. (1991) *Cell* **64**, 841–848
- Rapraeger, A. C., Krufka, A. and Olwin, B. B. (1991) *Science* **252**, 1705–1708
- Olwin, B. B. and Rapraeger, A. (1992) *J. Cell Biol.* **118**, 631–639
- Guimond, S., Maccarana, M., Olwin, B. B., Lindhal, U. and Rapraeger, A. C. (1993) *J. Biol. Chem.* **268**, 23906–23914
- Walker, A., Turnbull, J. E. and Gallagher, J. T. (1994) *J. Biol. Chem.* **269**, 931–935
- Kan, M., Wang, F., Xu, J., Crabb, J. W., Hou, J. and McKeehan, W. L. (1993) *Science* **259**, 1918–1921
- Pantoliano, M. W., Horlick, R. A., Springer, B. A., et al. (1994) *Biochemistry* **33**, 10229–10248
- Moscatelli, D. (1988) *J. Cell Biol.* **107**, 753–759
- Roghani, M. and Moscatelli, D. (1992) *J. Biol. Chem.* **267**, 22156–22162
- Rusnati, M., Urbinati, C. and Presta, M. (1993) *J. Cell. Physiol.* **154**, 152–161
- Ross, R. (1993) *Nature (London)* **362**, 801–809
- Massagué, J. (1990) *Annu. Rev. Cell Biol.* **6**, 597–641
- Chen, J. K., Hoshi, H. and McKeehan, W. L. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 5287–5291
- Berrou, E., Breton, M., Deudon, E. and Picard, J. (1988) *J. Cell. Physiol.* **137**, 430–438
- Breton, M., Berrou, E., Brahimi-Horn, M. C., Deudon, E. and Picard, J. (1986) *Exp. Cell Res.* **166**, 416–426
- Rapraeger, A. and Yeaman, C. (1989) *Anal. Biochem.* **179**, 361–365
- Bassols, A. and Massagué, J. (1988) *J. Biol. Chem.* **263**, 3039–3045
- Rapraeger, A. (1989) *J. Cell Biol.* **109**, 2509–2518
- Elenius, K., Määttä, A., Salmivirta, M. and Jalkanen, M. (1992) *J. Biol. Chem.* **267**, 6435–6441
- Cizmeci-Smith, G., Stahl, R. C., Showalter, L. J. and Carey, D. J. (1993) *J. Biol. Chem.* **268**, 18740–18747
- Dodge, G. R., Kovalsky, I., Hassell, J. R. and Iozzo, R. V. (1990) *J. Biol. Chem.* **265**, 18023–18029
- Nugent, M. A. and Edelman, E. R. (1992) *J. Biol. Chem.* **267**, 21256–21264
- Westergren-Thorsson, G., Schmidtchen, A., Särnstrand, B., Fransson, L. Å. and Malmström, A. (1992) *Eur. J. Biochem.* **205**, 277–286
- Yanagishita, M. (1992) *J. Biol. Chem.* **267**, 9505–9511
- Hausser, H., Hoppe, W., Rauch, U. and Kresse, H. (1989) *Biochem. J.* **263**, 137–142
- Deudon, E., Berrou, E., Breton, M. and Picard, J. (1992) *Int. J. Biochem.* **24**, 465–470
- Wight, T. N. (1989) *Arteriosclerosis* **9**, 1–20
- Campbell, J. H., Rennick, R. E., Kalevitch, S. G. and Campbell, G. R. (1992) *Exp. Cell Res.* **200**, 156–167
- Takeuchi, Y., Yanagishita, M. and Hascall, V. C. (1992) *J. Biol. Chem.* **267**, 14677–14684
- Sanderson, R. D., Turnbull, J. E., Gallagher, J. T. and Lander, A. D. (1994) *J. Biol. Chem.* **269**, 13100–13106
- Schmidt, A. and Buddecke, E. (1990) *Exp. Cell Res.* **189**, 269–275
- Nurcombe, V., Ford, M. D., Wildschut, J. A. and Bartlett, P. F. (1993) *Science* **260**, 103–106