ELSEVIER

0144-8617(95)00101-8

Carbohydrate Polymers **28** (1995) 107–112

0144-8617/95/$09.50+.00

Effects of heparin, dextran sulfate, and synthetic (1→6)-α-D-mannopyranan sulfate and acidic fibroblast growth factor on 3T3-L1 fibroblasts

Megumi Kunou & Kenichi Hatanaka*

Department of Biomolecular Engineering, Tokyo Institute of Technology, 4259 Nagatsuta-cho, Midori-ku, Yokohama 226, Japan

(Received 23 May 1995; revised version received 8 August 1995; accepted 16 August 1995)

The influence of sulfated polysaccharides on 3T3-L1 fibroblast proliferation and cellular morphological change stimulated by acidic fibroblast growth factor (aFGF) was investigated. aFGF showed strong mitogenic activity on 3T3-L1 fibroblasts. It exhibited chemotaxis at high concentrations of aFGF (several hundred ng/ml). Sulfated polysaccharides potentiated the motogenic activity of aFGF as well as mitogenic activity. Heparin and dextran sulfate induced cellular morphological change only in the presence of aFGF. Synthetic (1→6)-α-D-mannopyranan sulfate induced the morphological change even in the absence of aFGF. It was suggested that the degree of sulfation and the molecular weight of sulfated polysaccharide must be the important factors in determining the extent of cell proliferation and cellular morphology stimulated by aFGF.

INTRODUCTION

Acidic fibroblast growth factor (aFGF) is the prototype member of a family of heparin-binding growth factors, and exhibits pleiotropic biological activities and an affinity for heparin. aFGF stimulates proliferation, migration and differentiation of cells of mesenchymal and neuroectodermal origin. It is a potent mitogen and angiogenic factor in both normal and malignantly transformed cells (Burgess & Maciag, 1989; Baird & Böhlen, 1990). aFGF participates as an autocrine modulator of cell growth (Katayama & Kan, 1991; Sakaguchi, 1992) and transformation (Jouanneau *et al.*, 1991).

It has been reported that heparin protects aFGF from denaturation and enzymatic degradation (Gospodarowicz & Cheng, 1986; Mueller *et al.*, 1989) and potentiates the mitogenic activity of aFGF (Damon *et al.*, 1989). It has been shown that aFGF binds to cell surface-derived and extracellular matrix-derived heparan sulfate proteoglycans (HSPG) (Yanagishita & Hascall, 1992) as well as heparin. HSPGs also protect aFGF from proteolytic degradation and modulate aFGF activity (Gordon *et al.*, 1989; Ruoslahti & Yamaguchi, 1991). Moreover, HSPGs are essential for both mitogenic activity of aFGF and high affinity binding of aFGF to the receptor on the cell surface (Sakaguchi *et al.*, 1991; Olwin & Rapraeger, 1992).

*Author to whom correspondence should be addressed.

Several studies have demonstrated that the size and degree of sulfation of heparin was potentiation by aFGF was important of size and sulfation of heparin (Sudhalter *et al.*, 1989). Tardieu *et al.* reported that derivatized dextran mimics the effect of heparin on aFGF or bFGF (Tardieu *et al.*, 1992).

In this study, we investigated the effect of synthetic (1→6)-α-D-mannopyranan sulfate at various degrees of sulfation in order to elucidate the structural requirements for the effect of sulfated polysaccharide on aFGF.

MATERIALS AND METHODS

Sulfated polysaccharides

Stereoregular (1→6)-α-D-mannopyranan sulfate (MPS_x, x = number of sulfated groups per sugar unit) was prepared by ring-opening polymerization of 1,6-anhydro-2,3,4-tri-*O*-benzyl-β-D-mannopyranose with phosphorus pentafluoride as initiator in dichloromethane at −60°C under high vacuum and subsequent debenzylation of the obtained polymer (Hatanaka *et al.*, 1991). The (1→6)-α-D-mannopyranan was sulfated with piperidin-*N*-sulfonic acid, which was prepared by the reaction of piperidine and chlorosulfonic acid (Nagasawa *et al.*, 1972), in dimethylsulfoxide under conditions described previously (Hatanaka *et al.*, 1991) to give a (1→6)-

Table 1. Characteristics of sulfated polysaccharides

Sulfated polysaccharide	Number of sulfate groups per sugar unit	$M_n \times 10^{-4}$[a]
Heparin	1.5	1.1
Dextran sulfate	2.2–2.6	0.5
MPS_0	0	30.2[b]
$MPS_{0.98}$	0.98	9.3[b]
$MPS_{1.56}$	1.56	16.4[b]
$MPS_{1.99}$	1.99	10.7[b]

[a] Number-average molecular weight.
[b] M_w/M_n of mannopyranan sulfate: 1.2–1.4.

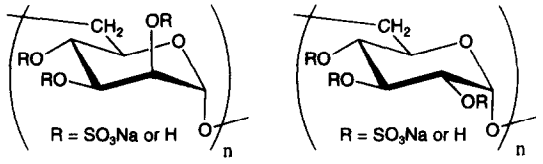

Fig. 1. Structures of (1→6)-α-D-mannopyranan sulfate (MPS) and dextran sulfate.

α-D-mannopyranan sulfate. The product was freeze-dried from water and stored at −20°C.

Heparins and dextran sulfates were purchased from Sigma Chemical Co.(St. Louis, MO, USA). Characterizations of sulfated polysaccharides are summarized in Table 1 and chemical structures of mannopyranan sulfate and dextran sulfate are shown in Fig. 1.

The number of sulfate groups per sugar unit in mannopyranan sulfate was calculated with elemental analysis which was performed by the Toray Research Center, Inc. (Kamakura, Japan). The number-average molecular weight (M_n) of mannopyranan sulfate was determined by gel-permeation chromatography (columns, Tosoh TSK gel (Osaka, Japan); eluent, 66.7 mM phosphate buffer, pH 6.86) using standard dextran as reference.

The reactivity of hydroxyl groups for sulfation was in the order, 3-OH >2-OH ≫4-OH (Hatanaka *et al.*, 1991). Thus, $MPS_{0.98}$ was relatively rich in residues monosubstituted at C-3, $MPS_{1.56}$ contained mainly disubstituted residues (C-2 and C-3). $MPS_{1.99}$ had a sulfate group at C-4.

Cell culture

3T3-L1 fibroblasts were subcultured in tissue culture flasks (75 cm^2; Corning 251 10; Corning Lab. Sci. Co., NY, USA) at subconfluent cell density in Eagle's MEM supplemented with 10% fetal bovine serum (Gibco Lab. Life Tech., Inc., MA, USA), kanamycin and L-glutamine. Cultures were maintained at 37°C in a humidified tissue culture incubator in a 5% CO_2/95% air environment, and were used in experiments between passages 4 and 12.

Cell proliferation assay

Natural aFGF was purchased from Genzyme Co. (Boston, MA, USA). Sulfated polysaccharide and FGF were added to the serum-free mimimal essential medium (MEM) supplemented with GMS-A (insulin 10 μg/ml, transferrin 5.5 μg/ml, sodium pyruvate 0.11 μg/ml, sodium selenite 6.7 ng/ml; Gibco) and bovine serum albumin (0.4 mg/ml). 3T3-L1 fibroblasts were plated at 7000 cells/well on polystyrene 96 well multi-plate (MS-8096F; Sumitomo Bakelite Co. Ltd, Tokyo, Japan) and cultured for 52 h. Cell proliferation was measured by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) activity (Mosmann, 1983). The calorimetric MTT assay is based on the fact that the tetrazolium ring of MTT is cleaved in active mitochondria to formazan product. The amount of formazan generated is directly proportional to the cell number. MTT assay can estimate the vitality of cells or the degree of activation. Cellular morphology was observed by a Olympus CK2 phase-contrast microscope.

RESULTS AND DISCUSSION

aFGF showed high mitogenic activity on 3T3-L1 fibroblasts. The mitogenic activity of aFGF was stimulated by the addition of sulfated polysaccharide to the medium (Fig. 2). MPS with a low degree of substitution (MPS_0 and $MPS_{0.98}$) showed no effect on the cell proliferation stimulated by aFGF. Thus, the degree of sulfation was quite important for the effect of sulfated polysaccharide.

The dose dependency of sulfated polysaccharides is illustrated in Figs 3 and 4. Dextran sulfate in low concentration strongly potentiated the mitogenic activity of aFGF, and potentiation was saturated at high concentrations of dextran sulfate. In the case of heparin, the potentiating effect increased gradually. The potentiated mitogenic activity of aFGF by MPS was dependent on the degree of sulfation of MPS.

Phase-contrast photomicrographs of the fibroblasts are shown in Figs 5 and 6. The cellular morphology was significantly changed to a round shape and aggregated

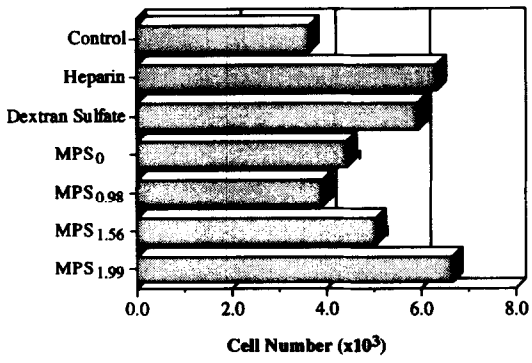

Fig. 2. Effects of sulfated polysaccharide on proliferation of 3T3-L1 fibroblasts stimulated by aFGF (sulfated polysaccharide, 10 μg/ml; aFGF, 10 ng/ml; 37°C, 5% CO_2, 66 h).

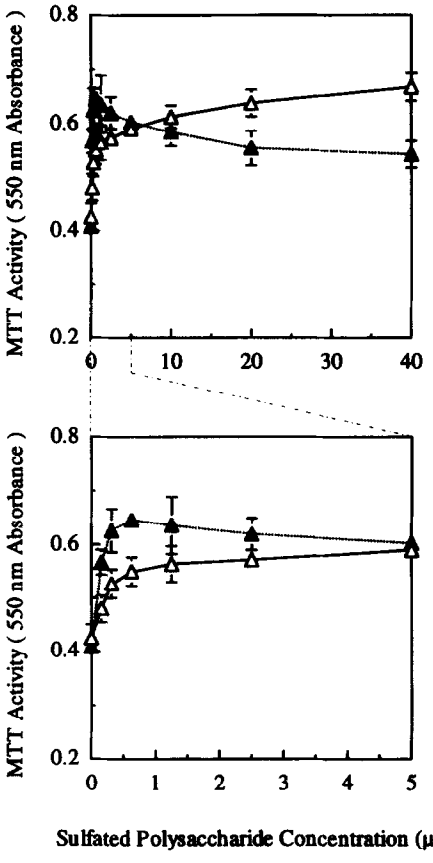

Fig. 3. Effect of sulfated polysaccharide concentration on proliferation of 3T3-L1 fibroblasts stimulated by aFGF (aFGF, 10 ng/ml; 37°C, 5% CO_2, 66 h)(Δ, heparin; ▲, dextran sulfate).

by the addition of both sulfated polysaccharide and aFGF. Heparin and dextran sulfate caused a morphological change only in the presence of aFGF. aFGF had chemotactic activity at high concentrations of several hundred ng/ml. Heparin and dextran sulfate strongly potentiate not only the mitogenic activity of aFGF, but also its chemotactic capacity.

MPS with high degrees of sulfation potentiated the mitogenic activity. In contrast to heparin and dextran

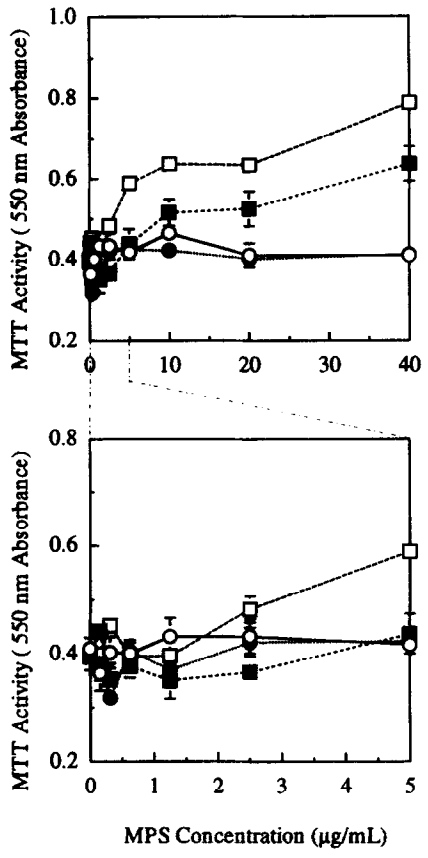

Fig. 4. Dose dependence of effects of MPS on proliferation of 3T3-L1 fibroblasts stimulated by aFGF (aFGF, 10 ng/ml; 37°C, 5% CO_2, 66 h) (○, MPS_0; ●, $MPS_{0.98}$; ■, $MPS_{1.56}$; □, $MPS_{1.99}$).

sulfate, cellular morphological change to the round shape was observed also in the absence of aFGF. MPS_0 and $MPS_{0.98}$ did not affect mitogenic and chemotactic activities.

Heparin and dextran sulfate induced cellular morphological changes only in the presence of aFGF. However, $MPS_{1.56}$ and $MPS_{1.99}$ induced cellular morphological changes also in the absence of aFGF. It seems that the difference in morphological effects between heparin or dextran sulfate and mannopyranan sulfate ($MPS_{1.56}$ or $MPS_{1.99}$) was due to the difference in molecular weight, rather than chemical structure. Since $MPS_{0.98}$ had no effects on aFGF activity and cellular morphology, it is suggested that the degree of sulfation is quite important for the effects of MPS.

aFGF and basic fibroblast growth factor (bFGF) do not contain the recognizable signal sequences for secretion. Therefore, FGFs are primarily present in the cytoplasm and the nucleus (Renko *et al.*, 1990; Imamura *et al.*, 1992) in contrast to trace amount of FGFs in the conditioned medium of most cells (Schweigerer *et al.*, 1987; Rogelj *et al.*, 1988). The definitive mechanism of release of FGFs has not been described, although it has been proposed that FGFs are

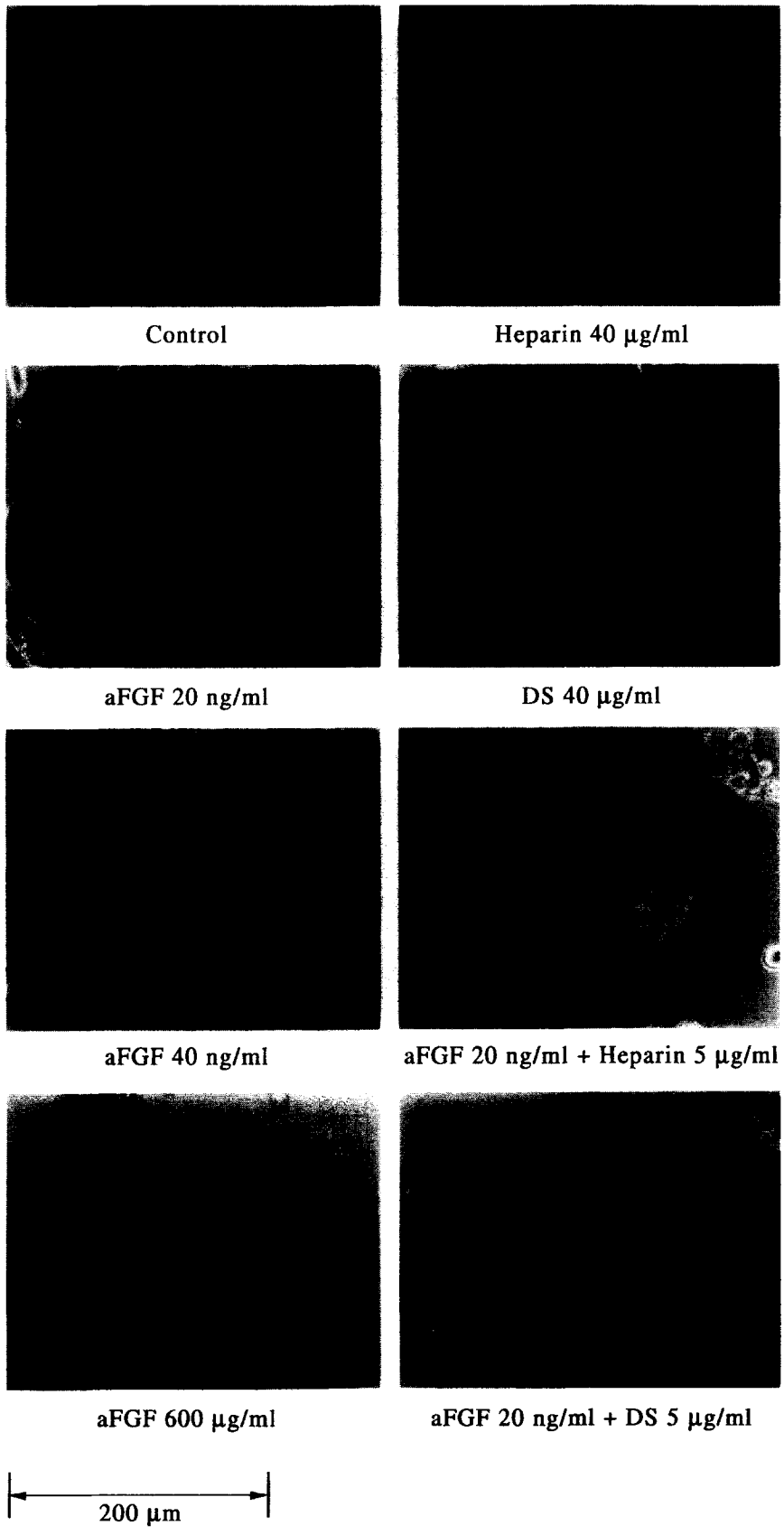

Fig. 5. Phase-contrast photomicrographs of 3T3-L1 fibroblasts (37°C, 5% CO_2, 66 h).

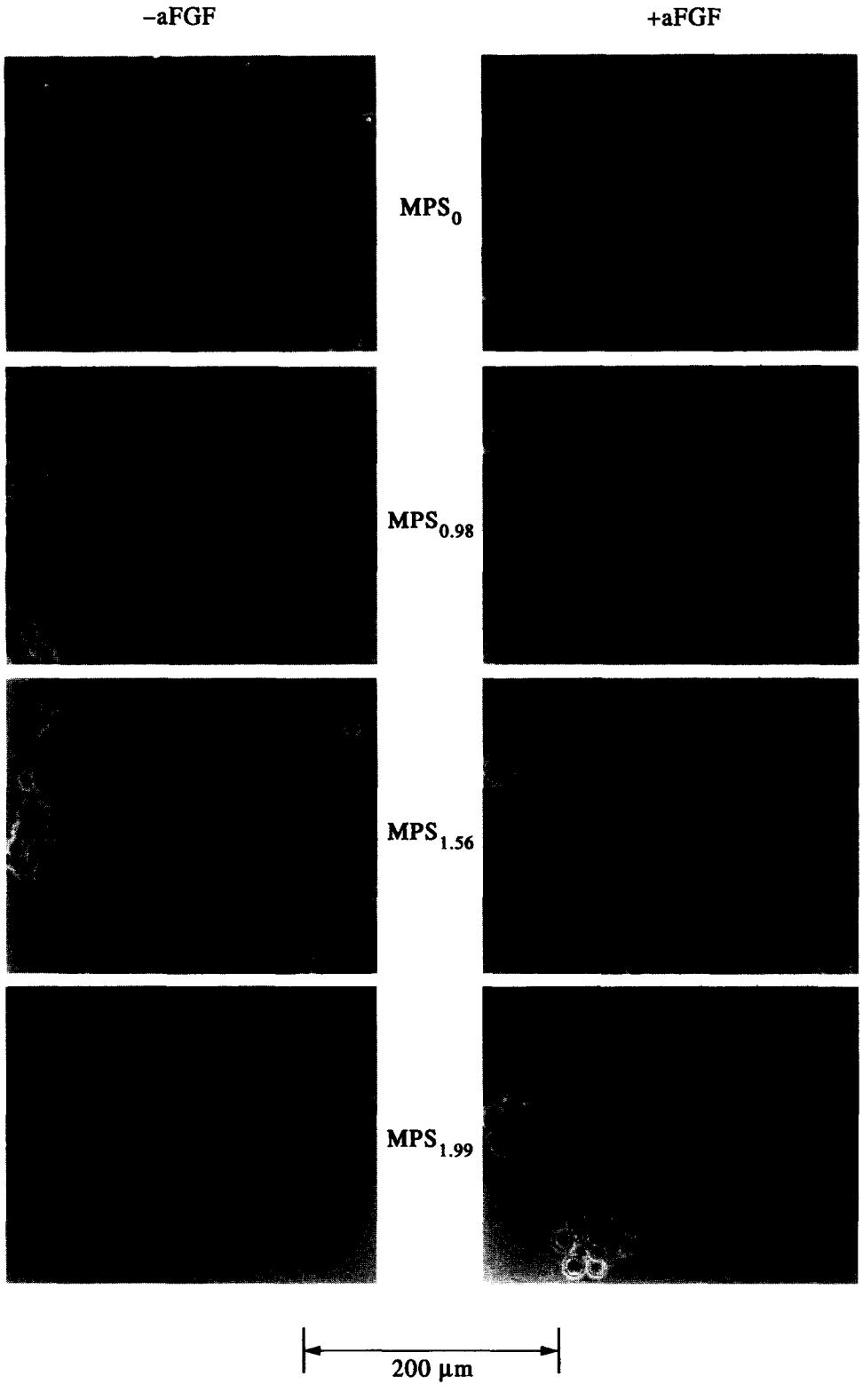

Fig. 6. Phase-contrast photomicrographs of 3T3-L1 fibroblasts in the presence or absence of 10 ng/ml aFGF (MPS concentration, 20 μg/ml: 37°C, 5% CO_2, 66 h).

released from dead and damaged cells (McNail *et al.*, 1989; Haimovitz-Friedman *et al.*, 1991). FGFs really act as survival factors (Tamm *et al.*, 1991). Sulfated polysaccharide which had more than one sulfate group per sugar unit exhibited cytotoxicity (data not shown). If cellular morphological change caused by $MPS_{1.56}$ and $MPS_{1.99}$ is initiated by FGFs released from dead and damaged cells, the different effects of MPS and heparin

cannot be explained. Results indicate that sulfated polysaccharide accelerates the chemotactic activity of aFGF. However, in the absence of aFGF, it is suggested that MPS acts on cells directly.

Our results show that the effects of sulfated polysaccharide on 3T3-L1 fibroblasts activated by aFGF depend on the degree of sulfation and molecular weight of the sulfated polysaccharide. It has also been suggested that sulfated polysaccharides interact not only with aFGF, but also with cells.

REFERENCES

Baird, A. & Böhlen, P. (1990). In *Peptide Growth Factors and Their Receptors*, **Vol. I,** eds M.B. Sporn & A.B. Roberts. Springer, Berlin and Heidelberg, pp. 369–418.

Burgess, W.H. & Maciag, T. (1989). *Ann. Rev. Biochem.*, **58**, 575–606.

Damon, D.H., Lobb, R., D'Amore, P.A. & Wagner, J.A. (1989). *J. Cell. Physiol.*, **138,** 221–226.

Gordon, B.R., Choi, H.U., Conn, G., Ahmed, A., Ehrmann, B., Rosenberg, L. & Hatcher, V.B. (1989). *J. Cell. Physiol.*, **140,** 584–592.

Gospodarowicz, D. & Cheng, J. (1986). *J. Cell. Physiol.*, **128,** 475–484.

Haimovitz-Friedman, A., Vlodavski, L., Chanduri, A., Witte, L. & Fuks, Z. (1991). *Cancer Res.*, **51,** 2552–2558.

Hatanaka, K., Kurihara, Y., Uryu, T., Yoshida, O., Yamamoto, N., Mimura, T. & Kaneko, Y. (1991). *Carbohydr. Res.*, **214,** 147–154.

Imamura, T., Tokita, Y. & Mitui, Y. (1992). *J. Biol. Chem.*, **267**, 5676–5679.

Jouanneau, L., Gavrilovic, J., Caruelle, D., Jaye, M., Moens, G., Caruelle, J.-P. & Thiery, J.-P. (1991). *Proc. Natl. Acad. Sci. USA*, **88,** 2893–2897.

Katayama, M. & Kan, M. (1991). In Vitro *Cell. Dev. Biol.*, **27A,** 533–541.

McNail, P.L., Muthukrishnan, L., Warder, E. & D'Amore, P.A. (1989). *J. Cell. Biol.*, **109**, 811–822.

Mosmann, T. (1983). *J. Immunol. Methods*, **65,** 55–63.

Mueller, N.S., Thomas, K.A., Sakvo, J.D. & Levine, E.M. (1989). *J. Cell. Physiol.*, **140,** 439–448.

Nagasawa, K., Harada, H., Hayashi, S. & Misawa, T. (1972). *Carbohydr. Res.*, **21,** 420–426.

Olwin, B.B. & Rapraeger, A. (1992). *J. Cell. Biol.*, **118,** 631–639.

Renko, M., Quarto, N., Morimoto, T. & Rifkin, D.B. (1990). *J. Cell. Physiol.*, **144,** 108–114.

Rogelj, S., Weinberg, R.A., Ganning, P. & Klagsbrun, M. (1988). *Nature*, **331,** 173–175.

Ruoslahti, E. & Yamaguchi, T. (1991). *Cell*, **64,** 867–869.

Sakaguchi, K. (1992). *J. Biol. Chem.*, **267,** 24554–24562.

Sakaguchi, K., Yanagishita, M, Takeuchi, Y. & Aurbach, D.B. (1991). *J. Biol. Chem.*, **266,** 7270–7278.

Schweigerer, L., Neufeld, G., Friedman, J., Abragam, J.A., Fiddes, J. C. & Gospodarowicz, D. (1987). *Nature*, **326,** 197–200.

Sudhalter, J., Folkman, J., Svahn, C.M., Bergenda, K. & D'Amore, P.A. (1989). *J. Biol. Chem.*, **264,** 6892–6897.

Tamm, I., Kikuchi, T. & Zychlinsky, A. (1991). *Proc. Natl. Acad. Sci. USA*, **88,** 3372–3376.

Tardieu, M., Gamby, C., Avramoglou, T., Jozefonvicz, J. & Barritault, D. (1992). *J. Cell. Physiol.*, **151,** 192–203.

Yanagishita, M. & Hascall, V.C. (1992). *J. Biol. Chem.*, **267,** 9451–9454.