Hyaluronectin blocks the stimulatory effect of hyaluronan-derived fragments on endothelial cells during angiogenesis in vitro

Véronique Trochon[a,c], Christelle Mabilat-Pragnon[a], Philippe Bertrand[b,c], Yves Legrand[a], Jeannette Soria[d], Claudine Soria[a,c], Bertrand Delpech[b,c], He Lu[a,*]

[a] *INSERM U353, Bat. INSERM, Institut d'Hématologie, Hôpital Saint Louis, 1 Ave Claude Vellefaux, F-75475 Paris Cedex 10, France*
[b] *Laboratoire d'Oncologie Moléculaire, Centre Henri Becquerel, Rouen, France*
[c] *Laboratoire DIFEMA, Faculté de Médecine et de Pharmacie de Rouen, Rouen, France*
[d] *Laboratoire Sainte Marie, Hôtel Dieu, Paris, France*

Received 12 September 1997

Abstract Hyaluronic acid (HA) is a glycosaminoglycan of the extracellular matrix. Its fragmentation by the hyaluronidase, secreted by tumor cells, facilitates tumor invasion and the HA degradation products generated stimulate angiogenesis. We report here that the HA-binding protein hyaluronectin (HN) inhibits the stimulatory effect of HA-derived fragments on the proliferation and migration of endothelial cells in vitro, and hampers the organization of endothelial cells into capillary-like structures. Since HN strongly inhibits endothelial cell adhesion to immobilized HA, it is postulated that HN acts by impairing the binding to endothelial cells of HA fragments generated by hyaluronidase, thereby neutralizing the effect of HA degradation products on angiogenesis. Our results reveal a new mechanism by which the angiogenesis induced by HA fragments is modulated by HN.

Key words: Angiogenesis; Endothelial cell; Hyaluronectin; Hyaluronan; Hyaluronidase

1. Introduction

Angiogenesis is essential for many physiological and pathological processes including wound healing, development, tissue repair and tumor growth [1]. In pre-existing microvessels, the endothelial cell sprouting requires coordinated cell proliferation, migration, adhesion to the matrix and the degradation of matrix components [2,3]. For tumor angiogenesis, paracrine stimulation of the endothelial cells is provided by growth factors and matrix proteins. Hyaluronan or hyaluronic acid (HA) is a major glycosaminoglycan of the extracellular matrix. Considerable evidence has indicated that HA plays a role in modulating the growth of new vessels. For example, the degradation of HA by testicular hyaluronidase has been shown to accelerate the wound healing in an animal model and the degradation products of HA had angiogenic activity in vitro and in vivo in a chick chorioallantoic membrane model while degradation products of chondroitin sulfate are devoid of effects [4]. Over-expression of hyaluronidase by tumor cells increased new vessel formation around the tumor implants within the mouse cornea [5]. A great amount of HA was produced by malignant brain tumors and the level of HA positively correlated with the malignancy of the tumor [6]. This angiogenic activity of hyaluronidase could be attributed to the degradation products of HA since semi-purified fragments of 6–20 saccharide units (HA 6–HA 20) of hyaluronidase-degraded HA were the most potent stimulators of proliferation and migration of endothelial cells whereas native high molecular weight HA was inactive [7–9]. In addition, hyaluronan degradation products and vascular endothelial growth factor (VEGF) exerted a synergistic effect on endothelial cell proliferation and capillary formation in vitro [10]. However the molecular mechanism for endothelial cell activation by HA degradation products is still unclear. Previously we have shown that anti-CD44 antibody J 173 inhibits the stimulating effect of HA degradation products on endothelial cells suggesting that the effect of HA fragments on the cells may be partially mediated by the HA receptor CD44 [9].

Hyaluronectin (HN) is a matrix protein which binds specifically HA and HA fragments ($K_d = 4.5 \times 10^{-9}$) in vitro [11,12]. HN was observed in the extracellular matrix of nerve tissue tumors and also non-nervous system tumors. Using both immunological studies and Northern blot analysis, HN present in tumors was shown to derive from many different sources including fibroblasts around capillaries, activated monocytes and probably endothelial cells themselves [13,14]. Because HA was localized at the perivascular zone in a tumor, together with HN [6], it was supposed that HA and HN would also interact in vivo. However, the significance of the colocalization of HN and HA in tumor tissue remains unclear. Therefore, it was of obvious interest to elucidate whether HN-HA interaction could modulate the angiogenic properties of HA fragments by competing with HA binding to cells. Thus, this study aims to clarify the effect of HN on the angiogenic activity of purified HA degradation products in an in vitro system.

2. Material and methods

Purified HA from human umbilical cord and hyaluronidase from bovine testes were obtained from Sigma (St. Louis, MO, USA). Sheep brain HN was prepared as previously described [12,15]. Briefly, a sheep brain was homogenized in 0.2 M glycine HCl pH 2.2. After centrifugation, the supernatant was dialysed with PBS and applied to an HA-Sepharose affinity chromatography column and the adsorbent was eluted with 0.2 M glycine HCl, pH 2.8. The elute contains highly purified HN (purity grade > 98%). HA degradation products were obtained by digesting HA with hyaluronidase at 37°C for 6 h followed by an isolation on an Ac A 202 gel chromatography column.

2.1. Cell culture

CPAE (calf pulmonary artery endothelial cells) were provided by Dr. J. Badet (Laboratoire de Biotechnologie des cellules eucaryotes, Université de Créteil, France). These cells were cultured in MEM medium supplemented with 20% FCS, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Gibco, Paisley, UK) and

*Corresponding author. Fax: (33) (1) 53724027.
E-mail: helu@chu-stlouis.fr

PII S0014-5793(97)01337-9

used at passage 12–20. HMEC-1 (human microvascular endothelial cells) was provided by Dr. Ades (Center for Disease Control and Prevention, Atlanta, GA, USA) who established this cell line by transfecting human dermal endothelial cells with SV40 A gene product and large T antigen [16]. HMEC-1 were cultured with MCDB 131 medium (Sigma) supplemented with 10% FCS, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 10 ng/ml EGF (Collaborative Biomedical Products) and 1 mg/ml hydrocortisone streptomycin, 10 ng/ml EGF (Collaborative Biomedical Products) and 1 mg/ml hydrocortisone (Sigma).

2.2. Cell proliferation assay

CPAE were trypsinized and cultured at 1400 cells per well in 96-well plates in complete medium (MEM) containing 20% FCS. After 24 h, the cells were cultured in the same medium with 5% FCS to induce the cells to accumulate at the G0-G1 proliferation stage for another 24 h. For the proliferation assay, the cells were further cultured for 30 h in fresh medium containing 20% FCS, in the presence of various agents such as HN (5 μg/ml or 100 μg/ml), HA (10 μg/ml), its degradation fragments HA-12 (0.5 μg/ml) or hyaluronidase (50 μg/ml). [^{3}H]Thymidine (1 μCi per well) was then added to the cells to allow [^{3}H]thymidine to incorporate for 18 h. For quantification, incorporated [^{3}H]thymidine was absorbed onto a filter paper with a Skatron harvester (Skatron, Lier, Norway) and the radioactivity was determined in a liquid scintillation counter. Triplicate experiments were repeated at least five times. The results are expressed as the percentage of the controls.

2.3. Migration assay of endothelial cells

The migration assay was performed in 24-well culture plates, as previously described [9]: first, 1.2% agarose was dissolved in the culture medium to form a gel in the wells. Half of the agarose cylinder was then cut off from each well. CPAE cells were added into the empty part of the wells and cultured until confluence. Then the remaining agarose gel filling the other half of the well was removed to allow cell migration towards the resulting free space, and various agents including HN, the HA-derived 12 oligosaccharides or hyaluronidase were added at indicated concentrations. Then transparent graph paper was stuck to the bottom of the culture plates to allow the measurement of the cell migration into the half vacant part of the culture well. The results of the migration distance were expressed as the percentage of the migration rate of the control cells. The experiments were repeated at least five times.

2.4. Formation of capillaries in two models of angiogenesis in fibrin gels

The first model using aggregates of CPAE was devised according to the method of Pepper et al. [17] and details of experiment were described by Trochon et al. [9]. The second model with microcarrier beads was devised according to the method of Nehl et al. [18]. Briefly HMEC-1 were allowed to attach to the Cytodex-3 microcarrier beads (Sigma) by incubating cells with beads in complete medium with 10% FCS during 4 h at 37°C. The microcarrier beads were then suspended in a larger volume of the same medium and then agitated for 5 min every 30 min at 30 rpm/min during the first 12 h, followed by culture with continuous stirring for 4 days. When the whole surface of the microcarrier beads was covered with endothelial cells, they were concentrated by centrifugation for 5 min at $800 \times g$ at room temperature and embedded in a fibrin matrix. Fibrin matrix was prepared as follows: purified fibrinogen at 3 mg/ml (Kabi, Stockholm, Sweden) was dialyzed against MCDB 131 and then mixed with FCS (10%), glutamine (1%), penicillin/streptomycin (1%) and aprotinin (2 μM). Human thrombin at 1 IU/ml was added to the fibrinogen solution. Once a fibrin gel had formed, 500 μl of complete culture medium containing 2 μM aprotinin was added on the surface of the fibrin matrix and this was changed every 3 days. Formation of capillary tubes arising from the periphery of microcarrier beads could be observed from the beginning of 4 days culture. These capillaries were photographed with a camera on a reverse microscope and their lengths were measured on the pictures. Statistical analysis (Mann-Whitney method) was performed to test if the length of capillaries was different.

2.5. Adhesion to hyaluronic acid

96-well plates (Greiner, D. Dutcher) were coated with 100 μl HA (1 mg/ml in distilled water; Sigma) overnight at room temperature. Negative controls consisted of wells coated with 2% bovine serum albumin in PBS for 2 h at 37°C. Plates were washed three times with 100 μl PBS containing 2% BSA and then three times with 100 μl PBS. HMEC-1 were detached from culture flasks by incubation with 1.5 mM EDTA and resuspended to a final concentration of 5×10^5 cells/ml in an adhesion buffer: NaCl 140 mM, HEPES 10 mM, glucose 5.56 mM, KCl 5.4 mM, $CaCl_2$ 2 mM, $MgCl_2$ 1 mM, $MnCl_2$ 1 mM, 1% BSA, pH 7.4. For inhibition studies, the HA-coated wells were preincubated with 60 μl HN (2 mg/ml) for 10 min at room temperature. Then, 100 μl HMEC-1 suspension were added to the wells and incubated for 20 min at 37°C. Non-adherent cells were removed by washing the wells three times with 200 μl adhesion buffer plus 1% BSA. The measurement of cell phosphatase activity was used to quantify adherent cells. Briefly, 100 μl of paranitrophenylphosphate (Sigma) at 3 mg/ml in acetate buffer pH 5.5 containing 0.1% Triton X-100, were added to the wells and incubated for 2 h at 37°C. The reaction was then stopped by 1 N NaOH. Released paranitrophenol, that indicates the adherent cell number, was measured by reading the adsorbance at 405 nm in an ELISA reader (Titertek Twinreader). Non-specific binding on BSA-coated plates was subtracted from the data. Results are expressed as the percentage of cells adherent to HA-coated wells in the absence of HN. All experiments were performed in triplicate.

3. Results

3.1. Stimulation of endothelial cell proliferation by hyaluronidase and HA degradation fragments

Hyaluronidase (50 μg/ml) caused an approximately twofold increase in the proliferation of CPAE. This could be due to the production of fragments from HA contained in the FCS and constitutively secreted by the endothelial cells. To further verify this, we used the purified HA degradation products (HA-12) to stimulate endothelial cells. As shown in Fig. 1, HA-12 induced proliferation of the endothelial cells (maximal effect was reached at 0.5 μg/ml, with a 46% increase). In contrast, undegraded HA had no effect even at a concentration as high as 10 μg/ml. Addition of exogenous HA (5–15 μg/ml) together with 50 μg/ml hyaluronidase did not enhance the effect of hyaluronidase on the endothelial cell response. These results suggested that the endogenous HA concentration in culture medium (0.15 μg/ml) is high enough for the generation of HA fragments with the maximal effect. In addition, hyaluronidase treatment may destroy the cell surface HA coat, thereby exposing of HA receptors. Consequently, hyaluronidase induced a more pronounced cell proliferation than that induced by HA fragments.

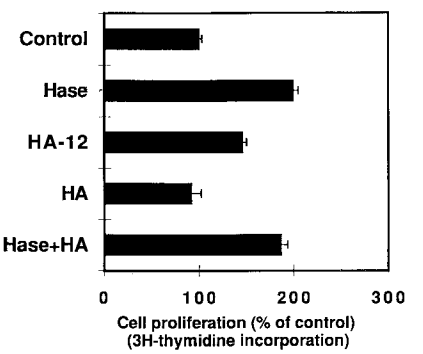

Fig. 1. Effects of hyaluronidase and HA-12 on CPAE proliferation. CPAE were cultured for 24 h with 5% FCS and further cultured for 30 h in the same medium containing 20% FCS in the absence or presence of hyaluronidase (50 μg/ml) or HA-12 (0.5 μg/ml) or undegraded HA (10 μg/ml)+hyaluronidase (50 μg/ml). Then [^{3}H]thymidine was added to the cells for a further 18 h incubation and incorporated [^{3}H]thymidine was measured. Data from five experiments. Means ± S.E.M., $n = 6$.

Table 1
Effects of HN on hyaluronidase and HA-12-induced endothelial cell proliferation and migration

	Cell proliferation (% of control) [^{3}H]thymidine incorporation	Cell migration rate (% of control)
Control	100	100
HA-12 (0.5 μg/ml)	146.1 ± 4.3	119 ± 2
HA-12 (0.5 μg/ml)+HN (5 μg/ml)	110.0 ± 3.5	99 ± 3
Hase (50 μg/ml)	200.2 ± 5.0	124 ± 2
Hase (50 μg/ml)+HN (100 μg/ml)	96.8 ± 8.2	100
Hase (50 μg/ml)+HA (10 μg/ml)	186.7 ± 7.0	ND
Hase (50 μg/ml)+HA (10 μg/ml)+Hn (100 μg/ml)	91.0 ± 8.1	ND

The migration and proliferation assays were performed for CPAE cells as described in Section 2. The data were from at least five experiments. Means ± S.E.M.

3.2. Neutralization by HN of the stimulation of endothelial cell proliferation and migration induced by hyaluronidase and HA-12

Next we studied the effect of HN on endothelial cell proliferation and migration, HN (5 μg/ml) neutralized the stimulating effect of 0.5 μg/ml HA-12 both on endothelial cell proliferation and migration (Table 1). This concentration of HN used was chosen because it was previously shown that a 10-fold excess (w/w) was necessary to saturate free HA [19,20]. In the same manner, HN (100 μg/ml) abolished the stimulating effect induced by 50 μg/ml hyaluronidase. Indeed, lower concentrations of HN did not show any significant effect. Moreover, 100 μg/ml neutralized the stimulating effect induced by the addition of hyaluronidase (50 μg/ml) plus HA (10 μg/ml) on cell proliferation. Thus, a 20-fold higher concentration of HN was required to neutralize the effect of hyaluronidase in comparison with the concentration needed to neutralize the effect of HA fragments. It may imply that during the degradation of cell-associated HA by hyaluronidase, the HA fragments produced immediately bound to cell receptor(s) with a high efficiency, therefore reducing the efficiency of HN in these neutralizing assays.

3.3. Inhibition of capillary tube formation by hyaluronectin in two models using fibrin matrix with CPAE or HMEC-1

One of models used was established as described by Pepper et al. [17]. Embedding the aggregates of CPAE in the fibrin gel gave rise to the formation of endothelial-lined sacs with capillary-like tubes after 2 days. As shown in Fig. 2, addition of HN at 20 μg/ml to the fibrin gel resulted in a reduction of the length of tubes around the CPAE aggregates by 41.2% decrease after 4 days incubation ($P < 0.05$, data from 10 aggregates in each groups).

The second model used was described by Nehls et al. [18]. Microcarrier beads seeded with HMEC-1 were embedded in the fibrin gel. After 4 days culture, the formation of small, endothelial cell-linked, hollow tube-like structures was observed. HN at 20 μg/ml incorporated into the fibrin gel reduced the length of tubes around the microcarrier beads (Fig. 3). The difference was more obvious after 20 days incubation. Addition of HN induced 34.6% reduction of the mean tube length ($P < 0.05$, data from 15 microcarrier beads for each condition). Higher concentrations of HN had not shown more pronounced effects in two models described (results not shown). Finally, this inhibition of angiogenesis induced by HN is not due to an artefact related to a modification of the fibrin gel as we have checked that thrombin clotting time and fibrin polymerization curves showed no difference in the presence or absence of HN (data not shown).

3.4. Inhibition of endothelial cell adhesion to HA-coated plates by hyaluronectin

The possibility that HN neutralizes the stimulatory effect of HA fragments by interrupting the binding of HA fragments to cells was finally tested in a cell adhesion assay. In our conditions, HN (120 μg/ml) significantly reduced cell adhesion to HA-coated wells by 45 ± 4.9%. Further increase in concentrations of HN did not increase the effect.

4. Discussion

Hyaluronidase secreted by tumor cells is believed to be an important factor in tumor growth and metastasis. Recently, it was shown that hyaluronidase PH-20, similar to the one present on human sperm (testicular hyaluronidase), expressed on tumor cells may participate in the induction of angiogenesis [5]. Here, we present evidence that testicular hyaluronidase induces the proliferation and migration of vascular endothelial cells, and this could explain the angiogenic effect of hyaluronidase in vivo. A similar action was seen with fragments of HA while undegraded HA was devoid of effect. HN, which exhibits a strong and specific affinity for HA (K_d

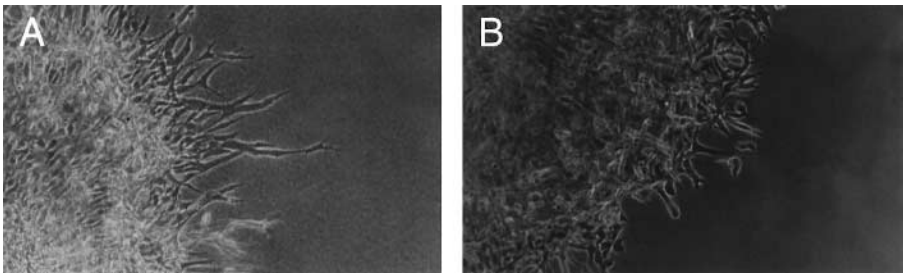

Fig. 2. Effect of HN on endothelial cell capillary formation in a fibrin matrix using CPAE. Phase contrast views of CPAE aggragates in fibrin gels, 4 days after embedding in the absence (A) or in the presence (B) of HN at 20 μg/ml.

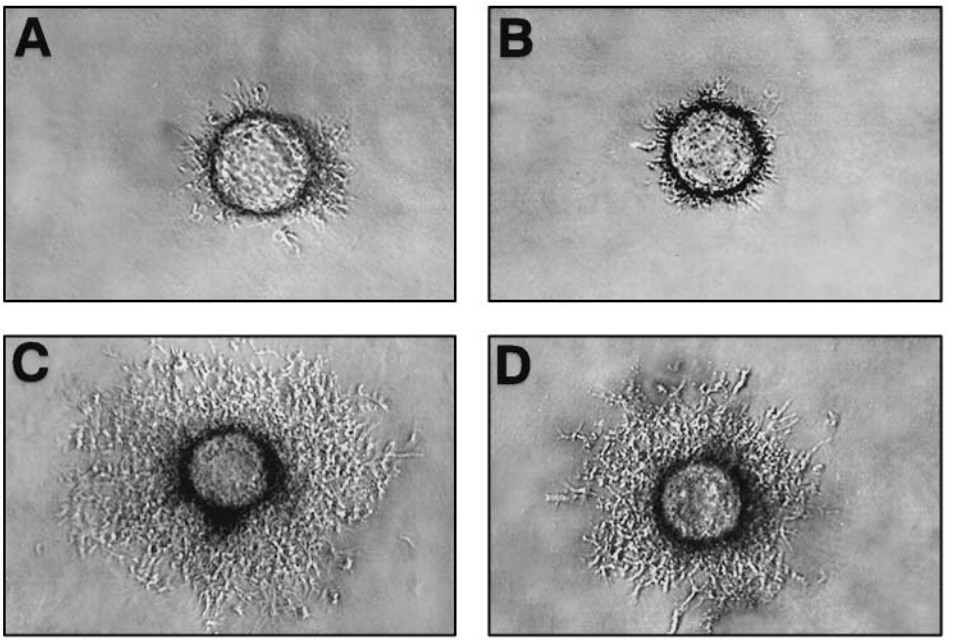

Fig. 3. Effect of HN on endothelial cell capillary formation using HMEC-1. Microcarrier beads covered with endothelial cells were embedded in a fibrin matrix in the absence or presence of HN (20 μg/ml). A: Control (4 days); B: HN (4 days); C: control (20 days); D: HN (20 days).

5.4×10^{-9} M) and HA fragments [12,19], is a glycoprotein synthesized by the tumors stromal cells and secreted into the extracellular matrix. This association between HA and HN in vitro may occur also in vivo because a similar histological colocalization of HA and HN in brain tumors was observed [6]. Therefore, it was logical to test whether HN may modulate the effect of HA fragments on angiogenesis.

This effect of HN was studied on proliferation and migration of endothelial cells and on capillary tube formation, three steps essential for angiogenesis. HN inhibited the effect of HA-12 on endothelial cell proliferation and to a lesser extent on endothelial cell migration; it also inhibited HA-12-induced capillary tube formation (not shown). HN also inhibited the combined effect of HA+hyaluronidase on endothelial cell proliferation. Until now, precise concentrations of HA fragments, HN and hyaluronidase have not been available for normal complete culture medium. However, we deduced from our results that HA is usually in excess over HN, thus a large number of HA fragments could be produced by exogenously added hyaluronidase and a trace amount of HN present in culture medium is not sufficient to counterbalance this effect. A 10-fold excess of HN (w/w) was required to neutralize the action of HA fragments as previously indicated [20]. However, higher concentrations of HN were needed for neutralizing the action of hyaluronidase than that of HA-12. This difference may not be surprising because in our experimental conditions, the action of added HA fragments could be hampered by endogenous HA coating the cells. In contrast, the addition of hyaluronidase, by degrading HA, could result in the exposure of free HA binding receptor(s) on the cell surface and concomitantly, in the production of available HA fragments in close vicinity. The high concentration of HN needed for angiogenesis inhibition does not exclude a physiological significance of HN because of its accumulation in the matrix around the vessels causing local high level of HN, as shown by histological studies [6].

Since HN does not inhibit the degradation of HA by hyaluronidase (not shown), it is postulated that HN acts by inhibiting the binding to endothelial cells of HA fragments generated by hyaluronidase, so that their effect on angiogenesis would be blocked. The inhibition of HA binding to endothelial cells receptors by HN is also suggested by our results showing that HN strongly inhibits endothelial cell adhesion to immobilized HA.

Hitherto, angiogenesis was believed to depend on the balance between positive and negative factors in the tumor [21]. Our results reveal a new mechanism by which the angiogenesis induced by HA fragments is modulated by HN. Angiogenesis could therefore proceed by either an increase in the production of HA degradation products due to a hyaluronidase excess and/or a decrease in HN production. This view is supported by the observations that HN levels are particularly low in the more malignant brain tumors (glioblastoma grade III and IV) compared to normal brain or astrocytoma that exhibit relatively low invasive and angiogenic properties [6].

Acknowledgements: We thank Drs. Lawley and Ades for providing HMEC-1 cell line. This work was supported by the Institut National de la Santé et de la Recherche Médicale (INSERM), the Ligue Nationale Contre le Cancer, the Association pour la Recherche sur le Cancer (ARC). V. Trochon is funded by the Ligue Nationale Contre le Cancer.

References

[1] Folkman, J. (1995) Nature Med. 1, 27–31.
[2] Sattar, A. (1992) Semin. Arthr. Rheumatol. 22, 37–43.
[3] West, D.C. and Kumar, S. (1988) Lancet 1, 715–716.
[4] West, D.C., Hampson, I.N., Arnold, F. and Kumar, S. (1985) Science 228, 1324–1326.
[5] Liu, D.C., Pearlman, E., Diaconu, E., Guo, K., Mori, H., Haqqi, T., Markowitz, S., Willson, J. and Sy, M.S. (1996) Proc. Natl. Acad. Sci. USA 93, 7832–7837.
[6] Delpech, B., Maingonnat, C., Girard, N., Chauzy, C., Maunoury, R., Olivier, A., Tayot, J. and Creissard, P. (1993) Eur. J. Cancer 7, 1012–1017.
[7] West, D.C. and Kumar, S. (1989) Exp. Cell. Res. 183, 179–196.

[8] Lees, V.C., Fan, T.P.D. and West, D.C. (1995) Lab. Invest. 73, 259–266.
[9] Trochon, V., Mabilat, C., Bertrand, P., Legrand, Y., Smadja-Joffe, F., Soria, C., Delpech, B. and Lu, H. (1996) Int. J. Cancer 66, 664–668.
[10] Montesano, R., Kumar, S., Orci, L. and Pepper, M.S. (1996) Lab. Invest. 75, 249–262.
[11] Delpech, B. and Havalent, C. (1981) J. Neurochem. 36, 855–859.
[12] Bertrand, P. and Delpech, B. (1985) J. Neurochem. 45, 434–439.
[13] Ponting, J.M. and Kumar, S. (1995) J. Anat. 187, 331–346.
[14] Delpech, B., Girard, N., Olivier, A., Maingonnat, C., Van Driessche, G., Van Beeumen, J., Bertrand, P., Duval, C., Delpech, A. and Bourguignon, J. (1997) Int. J. Cancer 72, (in press).
[15] Delpech, B. (1982) J. Neurochem. 38, 978–984.
[16] Xu, Y.L., Swerlick, R.A., Sepp, N., Bosse, D., Ades, E.W. and Lawley, T.L. (1994) J. Invest. Dermatol. 102, 833–837.
[17] Pepper, M.S., Montesano, R., Vassalli, J.D. and Orli, L. (1991) J. Cell. Physiol. 146, 170–179.
[18] Nehls, V. and Drenckhahn, D. (1995) Microvasc. Res. 50, 311–322.
[19] Delpech, B., Delpech, A., Brückner, G., Girard, N. and Maingonnat, C. (1989) In: The Biologie of Hyaluronan, Ciba foundation Symposium, Vol. 143, pp. 208–232, John Wiley, Chichester.
[20] Delpech, B., Maingonnat, C., Delpech, A., Maes, P., Girard, N. and Bertrand, P. (1991) Int. J. Biochem. 23, 329–337.
[21] Holmgren, L., O'Reilly, M.S. and Folkman, J. (1995) Nature Med. 2, 149–153.