BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **238,** 842–846 (1997)
ARTICLE NO. RC977398

Production and Activation of Matrix Metalloprotease-9 (MMP-9) by HL-60 Promyelocytic Leukemia Cells

Laetitia Devy, Agnès Noël,* Eugénia Baramova,* Khalid Bajou,* Chantal Trentesaux, Jean-Claude Jardillier, Jean-Michel Foidart,* and Pierre Jeannesson[1]

*Laboratoire de Biochimie, Faculté de Pharmacie, Université de Reims Champagne-Ardenne, IFR 53, 51 rue Cognacq-Jay, F-51096 Reims Cedex, France; and *Laboratoire de Biologie Générale, Université de Liège, Tour de Pathologie B 23, Sart-Tilman, B-4000 Liège, Belgium*

Received August 29, 1997

Human promyelocytic HL-60 cells have been used as a model of acute leukemia to investigate the expression and the regulation of matrix metalloproteases (MMPs), known to contribute to the degradation of extracellular matrix components. As shown by gelatin zymography, HL-60 cells constitutively released significant amounts of proMMP-9 (92 kDa) and moderate amounts of proMMP-2 (72 kDa). Furthermore, casein zymography confirmed the presence of serine proteases in the form of pro-urokinase. Activation of proMMP-9 was dependent on the plasminogen activator/plasmin (PA/plasmin) system and was inhibited by aprotinin. MMP-9 was only detected in cellular extracts or conditioned media incubated with HL-60 cells, indicating that cells are essential to the activation process. Addition of plasminogen increased by 3-fold the basal invasive rate of these cells across a matrigel layer (2.1% *versus* 0.7% in control cells after 4 h of incubation). Taken together, these results indicate that HL-60 cells exhibit an autocrine activation mechanism of proMMP-9 via the PA/plasmin system and that activation of proMMP-9 increases their invasive potential.

During normal hematopoiesis, the proliferation and differentiation of progenitor cells is strongly dependent on their complex interactions with the bone marrow microenvironment. Such interactions involve intimate contacts between hematopoietic cells, marrow stromal cells and extracellular matrix proteins namely *via* integrins (1).

Malignant transformation of the hematopoietic progenitor cells results in a blockade in their ability to terminally differentiate, causing a rapid accumulation of immature proliferative cells. This abnormal blood cell development may result in a breakdown of these cell-to-stroma interactions leading to the subsequent dissemination of immature blood elements from the bone marrow to the peripheral blood and subsequently to the tissues (2). In this respect, leukemic cells have been described as invasive cells specially *in vitro* for permanent cell lines such as KG-1, K562, HEL, HL-60 and U-937 (3) and *in vivo* in the case of acute leukemia (4). In this study, human promyelocytic HL-60 cells were used as a model of leukemia to investigate the expression and the regulation of certain proteolytic enzymes participating in the degradation of extracellular matrix components. This cell line derived from the peripheral blood of a patient with acute promyelocytic leukemia (5) is shown to constitutively produce significant amounts of the 94 kDa matrix metalloproteinase-9 (MMP-9) or gelatinase B (6) when compared to the highly metastatic human fibrosarcoma HT-1080 cells. In addition, to our knowledge little is known about the activation mechanism of MMP-9 in leukemic cells, although it has been demonstrated that this enzyme was associated with the invasive and metastatic ability of various tumor cells (7). Therefore, we have conducted this study in order to gain insights on the activation mechanisms of proMMP-9 in HL-60 cells.

MATERIALS AND METHODS

Cell culture. Human HL-60 leukemic cells and human HT-1080 fibrosarcoma cells (8) were maintained in RPMI 1640 with 10% fetal calf serum (FCS) (Life Technologies, Cergy Pontoise, France) supplemented with 2 mM L-glutamine in a 5% CO_2. For gelatin zymography assays, serum-free cultures were performed as followed: HL-60 cells were washed twice with PBS, seeded at 3×10^5 cells/well of 24-well plates in 500 μl of UltraCULTURE medium (Boehringer Ingelheim, Gagny, France) supplemented with 2 mM L-glutamine. For HT-1080 cells, subconfluent monolayers of cells in 75 cm^2 culture flasks were washed twice with PBS and incubated with 4 ml of UltraCULTURE medium. They are known to constitutively secrete proMMP-9 (92 kDa) and proMMP-2 (72 kDa) in equal amounts (9). Treatment of

[1] To whom correspondence should be addressed. Fax: 333 26 05 37 30. E-mail: pierre.jeannesson@univ-reims.fr.

0006-291X/97 $25.00

HT-1080 cells by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) for 24 h increases the constitutive proMMP-9 secretion and activates proMMP-2. Conditioned media were obtained after 24 or 48 h of culture, centrifuged at 1500 g for 10 min and frozen at −20°C until use. Cellular extracts were prepared in 100 μl of 0.1 M Tris-HCl buffer pH 8.1 containing 0.4% Triton X-100 at 4°C (10). Treatment with native human plasminogen (11) was done under serum-free conditions.

Gelatin zymography. Gelatinolytic activities in conditioned media and cell extracts were performed according to Heussen and Dowdle (12). Briefly, 20 μl of conditioned media or aqueous cellular extracts (corresponding to 10^4 cells) were applied to 10% SDS-polyacrylamide gels containing 1 mg/ml gelatin (Sigma). After electrophoresis, SDS was removed from the gel by two incubations in 2% Triton X-100 for 30 min. The gels were incubated overnight at 37°C in 50 mM Tris-HCl (pH 7.4), 0.2 M NaCl and 5 mM $CaCl_2$ (TCS buffer). The gels were stained for 90 min with Coomassie Blue and destained for another 30 min. Proteolytic activities were evidenced as clear bands against the blue background of stained gelatin. In some experiments, 10 mM EDTA or 100 mM ϵ-aminocaproic acid (ϵ-ACA) were added to the TCS buffer in order to prove the MMP or serine protease nature of the gelatinolytic activities.

Casein zymography. This assay previously described by Marshall *et al.* (13) was used to determine the types and molecular weights of plasminogen activators (PAs). Briefly, aliquots of conditioned media were subjected to substrate gel electrophoresis in 10% polyacrylamide gel impregnated with 1 mg/ml casein and 8 μg/ml plasminogen (Sigma). After electrophoresis, the gels were washed twice (30 min each) in a buffer containing 100 mM glycine and 2.5% Triton X-100 and developped overnight at 37°C in 100 mM glycine. Lysis zones became visible at positions corresponding to 55 kDa and 33 kDa. Samples run on casein gels without plasminogen served as controls.

Invasion assay. Costar Transwell units (Costar) were used for invasion assays. Briefly, 6.5 mm polycarbonate filters with 8 μm pores size were coated with 100 μl of a matrigel solution (500 μg/ml) (3, 14). After an overnight incubation at 37°C in an humidified atmosphere, the bottom compartment of the chambers was filled with 0.6 ml of UltraCULTURE medium, in absence of chemoattractant and FCS. Cells (5×10^5) were suspended in 0.2 ml medium, and added to the upper compartment. The chambers were incubated for 2 to 24 h at 37°C, in 5% CO_2 and then the contents of the lower compartments were collected. The rate of invasion was expressed in percentages, as a fraction of the total number of cells placed in the chamber. To assess the effect of plasminogen on the HL-60 cells invasive behavior, cells were preincubated 1 h with plasminogen (80 μg/ml) before plating on the matrigel-covered filter.

RESULTS

Gelatinolytic Activities of HL-60 Cells

Gelatinolytic activities of HL-60 cells were assessed by gelatin zymography and compared to that of HT-1080 fibrosarcoma cells treated with 10 ng/ml of TPA (Fig. 1A, *lanes 1* and *2*). Aliquots of 48-h conditioned media of HL-60 cells grown in suspension exhibited 2 bands, which can be attributed to MMPs since these enzymatic activities were completely abolished by the chelating agent EDTA 10 mM, but not by ϵ-ACA 100 mM (data not shown). The highest molecular weight which is the major one can be attributed to proMMP-9 and the lowest to proMMP-2 as assessed by western blotting (data not shown).

Activation of proMMP-9 by Plasminogen

The activation of pro-MMPs has been suspected to be dependent on the PA/plasmin system (15). Addition of a purified preparation of native human plasminogen to HL-60 cells cultured for 48 h in serum free medium led to the activation of proMMP-9 (Fig. 1A, *lanes 3-8*). This activation was detectable as soon as 24 h (data not shown). The proportion of the activated form increased as a function of plasminogen concentration ranging from 20 to 180 μg/ml. In order to confirm that the observed plasminogen effects on proMMP-9 activation could be ascribed to plasmin generated by cells, we treated HL-60 cells with increasing concentrations of plasmin (10-100 μg/ml). Under these conditions, the activation of proMMP-9 was similarly observed (Fig. 1B).

Cell Involvement in proMMP-9 Activation

In order to precise whether such an activation could take place at the cellular level, cellular extracts were prepared from cells cultured for 24 and 48 h in presence of plasminogen 20, 40 and 80 μg/ml. As shown in Figure 2A, zymographic analysis of cellular extracts demonstrated the presence of latent and mature forms of MMP-9 after 48 h of culture. By contrast, no activation has been observed when plasmin (10 to 40 μg/ml) was directly added to HL-60 conditioned medium (Fig. 2B). All together, these data indicate that proMMP-9 activation occurs at the cellular level and that plasmin activity is not generated in the medium but probably results from membrane activation of plasminogen.

Activation of pro-uPA by Plasminogen

The conversion of inactive plasminogen into plasmin is known to be mediated by PAs, specially urokinase-type plasminogen activator (uPA) (16). As shown by casein zymography (Fig. 3), HL-60 cells secrete a 55 kDa form of uPA. Addition of plasminogen 10 μg/ml for 48 h led to the appearance of the 33 kDa active form of uPA. The band migrating in the vicinity of 100 kDa could be attributed to the complex between uPA and plasminogen activator inhibitors (PAIs). These findings strongly suggest that in HL-60 cells, the processing of pro-uPA may participate to proMMP-9 activation.

Inhibitory Effects of Aprotinin on proMMP-9 Activation in HL-60 Cells

The effects of aprotinin, an inhibitor known to directly inhibit plasmin (17) was studied on the activation of proMMP-9. HL-60 cells were preincubated with aprotinin for 1 h and then cultured in the presence of plasminogen (40 μg/ml) for 48 h. Zymographic analysis of conditioned media (Fig. 4) showed that aprotinin led to the disappearance of the band corresponding to

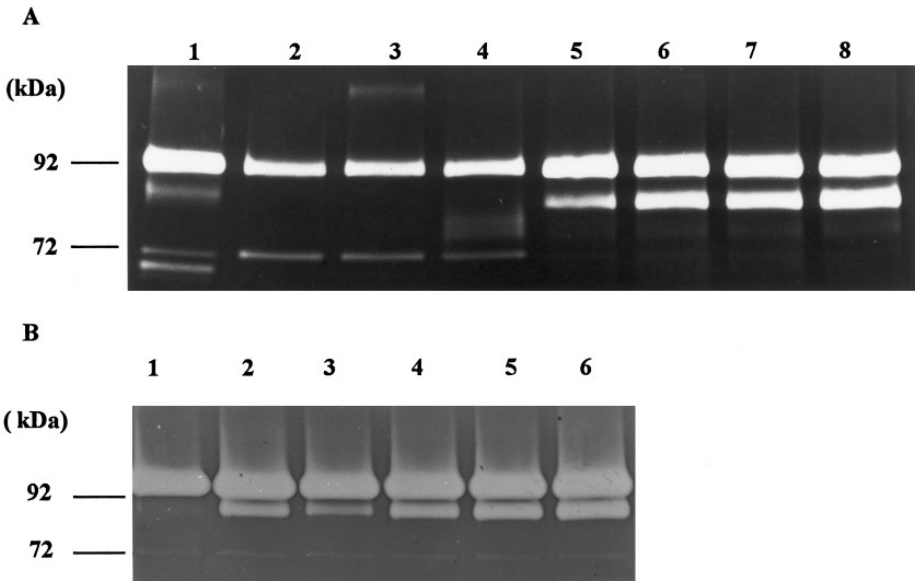

FIG. 1. Expression of gelatinases in HL-60 cells and activating effect of plasminogen and plasmin. (A) TPA-treated HT1080 cells were used as control (*lane 1*). HL-60 cells were cultured for 48 h in the absence (*lane 2*) or presence of plasminogen 1 μg/ml (*lane 3*), 10 μg/ml (*lane 4*), 20 μg/ml (*lane 5*), 40 μg/ml (*lane 6*), 80 μg/ml (*lane 7*) and 180 μg/ml (*lane 8*). (B) Cells were cultured in the absence (*lane 1*) or presence of plasmin 10 μg/ml (*lane 2*), 20 μg/ml (*lane 3*), 40 μg/ml (*lane 4*), 80 μg/ml (*lane 5*) and 100 μg/ml (*lane 6*). Zymographic analysis was performed on conditioned media as described under Materials and Methods.

MMP-9 confirming that this process was dependent on the serine protease activity.

Increase in HL-60 Cells Invasiveness by Plasminogen

Boyden chambers (18) were used to quantify the invasive potential of HL-60 cells after MMP-9 activation by plasminogen. The invasive rate varies according to the incubation period (Fig. 5). Indeed, after a 4 h-incubation time, 0.7% of cells were able to cross the matrigel layer. This rate reached a plateau phase (1.2%) between 8 and 24 h. Preincubation with plasminogen (80 μg/ml for 1 h) increased by 6-fold the number of invasive cells in the first 2 h (1.8% *versus* 0.3% in the control cells). The maximum of invasiveness was reached between 4 and 8 h (2.1% *versus* 0.7 % in control cells).

DISCUSSION

Human promyelocytic HL-60 cells have been demonstrated to represent a convenient model of invasive leukemic cells (3). They were reported to constitutively release minimal amounts of the latent form of the MMP-9 or gelatinase B which increased markedly after monocytic differentiation using TPA (19, 20). This MMP belongs to the gelatinase subfamily and is thought to play a central role in cancer cell invasion and metastasis since it degrades type IV collagen which is a major component of the extracellular matrix (21). In our experimental conditions, significant amounts of proMMP-9 and minute amounts of proMMP-2 are detected as soon as 24 h of incubation in serum-free medium, in the absence of any inducer. Recently, Ries *et al* (6) have described the presence of small amounts of an additional gelatinolytic activity which was identified as a 63 kDa fragment of MMP-9. It is likely that experimental differences could explain the apparent discrepancy between our results and those reported by Ries *et al*. Indeed, the 63 kDa form was detected in significant amount when HL-60 cells were cultured for 3 to 7 days in the presence of TPA-induced monocytic differentiation, while it was weakly produced in unstimulated cells (6).

The activation of purified proMMP-9 can be achieved by incubation with various enzymes such as trypsin, cathepsin G, kallikrein, but not by plasmin (22). This was confirmed by our results showing that proMMP-9 present in medium conditioned by HL-60 cells could not be activated by incubation with plasmin for 48 h at 37°C. However, we provided evidence of the importance of the cells for proMMP-9 activation in presence of plasminogen or plasmin since the mature form was only observed either in cellular extracts or in conditioned media incubated with cells.

As assessed by casein zymography, HL-60 cells produced pro-uPA which was converted into active uPA in presence of plasminogen. The mechanism by which this cascade is initiated is still largely unclear and does not exclude that minimal amounts of active uPA are present on HL-60 cells to initiate plasmin production necessary to pro-uPA activation (23, 24).

Our data suggest the implication of the PA/plasmin

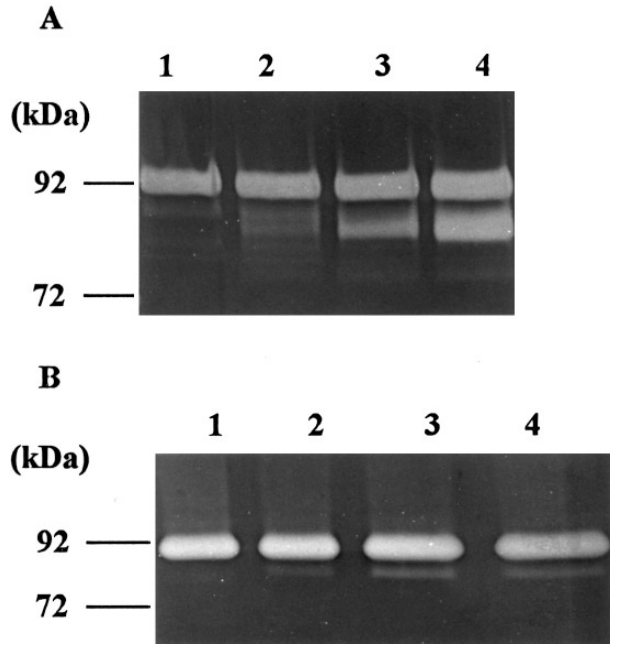

FIG. 2. Involvement of HL-60 cells in proMMP-9 activation. (A) Cells were incubated for 48 h in the absence (*lane 1*) or presence of plasminogen 20 μg/ml (*lane 2*), 40 μg/ml (*lane 3*) and 80 μg/ml (*lane 4*). Then, zymographic analysis was performed on cellular extracts as described under Materials and Methods. (B) Cells were cultured for 48 h and then conditioned medium was incubated in the absence (*lane 1*) or presence of plasmin 10 μg/ml (*lane 2*), 20 μg/ml (*lane 3*) and 40 μg/ml (*lane 4*).

system for proMMP-9 activation. Such assumption is based on the observation that increasing concentrations of plasminogen or plasmin induce a dose-dependent proMMP-9 activation whereas aprotinin inhibits this maturation process. The PA/plasmin system has already been described for MMPs like stromelysin, and collagenases, in other tumor cells, mainly originating from solid tumors (2) in which the cycle of activation generally occurs by a paracrine pathway involving the participation of neighboring cells such as stromal cells which produced uPA. On the contrary, HL-60 cells, like HT-1080 cells (25, 26) are able to produce both proMMP-9 and uPA which is required for plasminogen conversion into plasmin. Such coexpression of MMPs and PAs seems to be a common characteristic of invasive cancer cells (27, 28). Finally, the fact that proteolytic activity of plasmin only occured in presence of cells strongly suggests that besides uPA, additional factors are required for proMMP-9 activation. As described for proMMP-2 activation (29, 30), it could involved membrane proteases such as membrane type-MMPs (MT-MMPs).

A strong correlation between the production of metalloproteases and metastasis has been established for

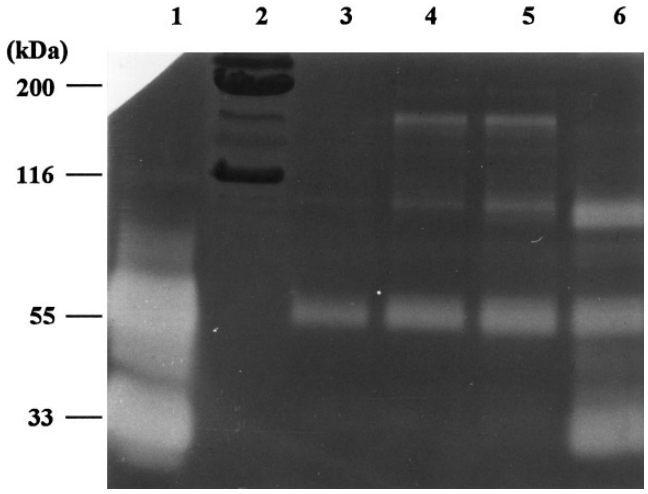

FIG. 3. Zymographic analysis of the PAs secreted by HL-60 cells. Human uPA 1U/ml (*lane 1*) and high range molecular weight markers (*lane 2*) were used as controls. Cells were cultured in the absence (*lane 3*) or presence of plasminogen 1 μg/ml (*lane 4*), 2 μg/ml (*lane 5*) and 10 μg/ml (*lane 6*) for 48 h and conditioned media were subjected to casein zymography as described under Materials and Methods.

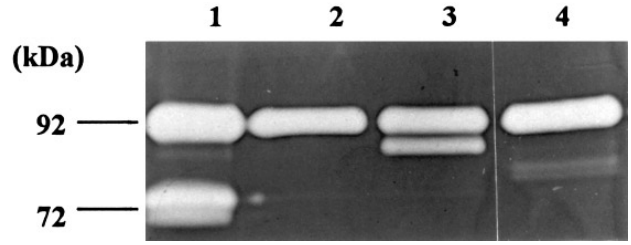

FIG. 4. Effect of aprotinin on the activation of proMMP-9 produced by HL-60 cells. TPA-treated HT1080 cells were used as control (*lane 1*). Cells were preincubated in the absence (*lanes 2 and 3*) or presence of aprotinin 0.5 μg/ml (*lane 4*) and then cultured in the presence of plasminogen 20 μg/ml (*lanes 2–4*) for 48 h. Zymographic analysis was performed on conditioned media as described under Materials and Methods.

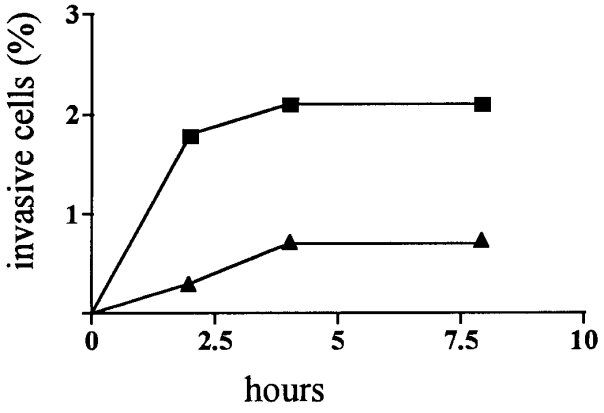

FIG. 5. Effect of plasminogen on HL-60 cells invasion through the matrigel-coated filters. The assay was performed in Costar Transwell units as described under Materials and Methods. Cells were pretreated (■) or not (▲) with plasminogen 80 μg/ml for 1 h.

various tumor cell lines (7). The invasive behavior of HL-60 cells, activated or not by plasminogen has been evaluated by a matrigel-based *in vitro* model (3, 14, 31). Although it is not an universal model (32), it allows a rapid and quantitative assessment of invasiveness of most tumor cell lines (33). Without plasminogen, HL-60 cells exhibited a basal invasive potential of 0.7% after 4 h of incubation. Such results, in accordance with those of Janiack *et al* (3), indicate that leukemic cells are able to cross a matrigel barrier more rapidly than solid tumor cells (6 to 72 h) without requiring chemotactic factors. Pretreatment of cells with plasminogen increased by 3-fold their invasion process after 4 h of incubation. In addition, the basal gelatinolytic activity detected in absence of plasminogen could be attributed to the slow release of plasminogen from the matrigel since this tumor membrane extract can be considered as a reservoir for plasminogen and uPA (34).

In conclusion, our data demonstrate that human leukemic HL-60 cells are able to produce large amounts of activable proMMP-9 *via* the PA/plasmin system. The uPA cycle can be supported by a single cell and the activation pathway of proMMP-9 probably takes place at the cell surface. The cooperation between MMPs and PA/plasmin in the activation of proMMP-9 contributes to increase the invasive potential of these cells. These results could also explain why in acute leukemia, immature hematopoietic cells egress from the bone marrow microenvironment and infiltrate peripheral tissues.

ACKNOWLEDGMENTS

This work was supported by the Ligue Nationale contre le Cancer, Comités de l'Aisne et des Ardennes, the Commission of European Communities (BIOTECH N° CT960464), and the CGER 1996/1999. L. D. is a recipient of a fellowship from Région Champagne-Ardenne. A. N. is an associated researcher of the National Found for Scientific Research (FNRS-Brussels, Belgium). We thank Dr N. Gueddari for kindly providing HL-60 cells, and Dr H. Bobichon for the photographs.

REFERENCES

1. Wilson, J. G. (1997) *Acta Haematol.* **97,** 6–12.
2. Tapiovaara, H., Alitalo, R., and Vaheri, A. (1996) *Adv. Cancer Res.* **69,** 101–133.
3. Janiak, M., Hashmi, H. R., and Janowska-Wieczorek, A. (1994) *Exp. Hematol.* **22,** 559–565.
4. Freireich, E. J. (1984) *Cancer* **53,** 2026–2032.
5. Collins, S. J., Ruscetti, F. W., Gallagher, R. E., and Gallo, R. C. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 2458–2462.
6. Ries, C., Lottspeich, F., Dittmann, K. H., and Petrides, P. E. (1996) *Leukemia* **10,** 1520–1526.
7. Bernhard, E. J., Gruber, S. B., and Muschel, R. J. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 4293–4297.
8. Rasheed, S., Nelson-Rees, W. A., Toth, E. M., Arnstein, P., and Gardner, M. B. (1974) *Cancer* **33,** 1027–1033.
9. Moll, U. M., Youngleib, G. L., Rosinski, K. B., and Quigley, J. P. (1990) *Cancer Res.* **50,** 6162–6170.
10. Baramova, E. N., Coucke, P., Leprince, P., De Pauw-Gillet, M. C., Bassleer, R., and Foidart, J. M. (1994) *Anticancer Res.* **14,** 841–846.
11. Chibber, B. A. K., Deutsch, D. G., and Mertz, E. T. (1974) *in* Methods in Enzymology (Jakoby, W. B., and Wilchek, M., Eds.), pp. 424–432, Academic Press, San Diego, CA.
12. Heussen, C., and Dowdle, E. B. (1980) *Anal. Biochem.* **102,** 196–202.
13. Marshall, B. C., Sageser, D. S., Rao, N. V., Emi, M., and Hoidal, J. R. (1990) *J. Biol. Chem.* **265,** 8198–8204.
14. Emonard, H., Callé, A., Grimaud, J. A., Peyrol, S., Castronovo, V., Noël, A., Lapière, C. M., and Foidart, J. M. (1987) *J. Invest. Dermatol.* **89,** 156–163.
15. Vassali, J. D., and Pepper, M. S. (1994) *Nature* **370,** 14–15.
16. Blasi, F. (1993) *BioEssays* **15,** 105–111.
17. Lu, H., Mabilat, C., Yeh, P., Guitton, J. D., Li, H., Pouchelet, M., Shoevaert, D., Legrand, Y., Soria, J., and Soria, C. (1996) *FEBS Lett.* **380,** 21–24.
18. Albini, A., Iwamoto, Y., Kleinman, H. K., Martin, G. R., Aaronson, S. A., Kozlowski, J. M., and McEwan, R. N. (1987) *Cancer Res.* **47,** 3239–3245.
19. Davis, G. E., and Martin, B. M. (1990) *Cancer Res.* **50,** 1113–1120.
20. Ries, C., Kolb, H., and Petrides, P. E. (1994) *Blood* **83,** 3638–3646.
21. Mackay, A. R., Hartzler, J. L., Pelina, M. D., and Thorgeirsson, U. P. (1990) *J. Biol. Chem.* **265,** 21929–21934.
22. Tschesche, H., Knäuper, V., Krämer, S., Michaelis, J., Oberhoff, R., and Reinke, H. (1992) *Matrix* **Suppl. 1,** 245–255.
23. Pöllänen, J., Stephens, R. W., and Vaheri, A. (1991) *Adv. Cancer Res.* **37,** 273–328.
24. Alving, B. M., Krishnamurti, C., Liu, Y. P., Lucas, D. L., and Wright, D. G. (1988) *Thromb. Res.* **51,** 175–185.
25. Baramova, E. N., Bajou, K., Remacle, A., L'Hoir, C., Krell, H. W., Weidle, U. H., Noël, A., and Foidart, J. M. (1997) *FEBS Lett.* **405,** 157–162.
26. Mazzieri, R., Masiero, L., Zanetta, L., Monea, S., Onisto, M., Garbisa, S., and Mignatti, P. (1997) *EMBO J.* **16,** 2319–2332.
27. O'Grady, R. L., Upfold, L. I., and Stephens, R. W. (1981) *Int. J. Cancer* **28,** 509–515.
28. Salo, T., Liotta, L. A., Keshi-Oja, J., Turpeenniemi-Hujanen, T., and Tryggvason, K. (1982) *Int. J. Cancer* **30,** 669–673.
29. Corcoran, M. L., Hewitt, R. E., Kleiner, D. E., Jr., and Stetler-Stevenson, W. G. (1996) *Enzyme Protein.* **49,** 7–19.
30. Kinoshita, T., Sato, H., Takino, T., Itoh, M., Akizawa, T., and Seiki, M. (1996) *Cancer Res.* **56,** 2535–2538.
31. Reich, R., Thompson, E. W., Iwamoto, Y., Martin, G. R., Deason, J. R., Fuller, G. C., and Miskin, R. (1988) *Cancer Res.* **48,** 3307–3312.
32. Noël, A. C., Callé, A., Emonard, H. P., Nusgens, B. V., Simar, L., Foidart, J., Lapiere, C. M., and Foidart, J. M. (1991) *Cancer Res.* **51,** 405–414.
33. Simon, N., Noël, A., and Foidart, J. M. (1992) *Invas. Metast.* **12,** 156–167.
34. Farina, A. R., Tiberio, A., Tacconelli, A., Cappabianca, L., Gulino, A., and Mackay, A. R. (1996) *Biotechniques* **21,** 904–909.