BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **233,** 182–186 (1997)
ARTICLE NO. RC976422

Integrin β4 Is Involved in Apoptotic Signal Transduction in Endothelial Cells

Jin-Ying Miao, Satohiko Araki, Kazuhiko Kaji,* and Hiroshi Hayashi
*Sugashima Marine Biological Laboratory, School of Science, Nagoya University Toba, Mie 517, Japan; and *Department of Food and Nutritional Sciences University of Shizuoka, Shizuoka 422, Japan*

Received February 28, 1997

To clarify the signal transduction in vascular endothelial cells (VEC) apoptosis induced by deprivation of FGF and serum, we investigated the function of integrin β4 by using the monoclonal antibody (mAb) of this integrin. We added anti-β 4 integrin mAb at the concentration of 5 μg/ml to the cells deprived of FGF and serum, apoptosis of these cells were completely inhibited 24 h after the treatment. Furthermore we plated the cells onto untreated bacterial culture plates on which the cells cannot adhere and spread in MCDB medium without FGF and serum; however, when anti-β 4 integrin mAb was present at 5 μg/ml in the seeding medium, the cells rapidly adhered and spread. Our results first demonstrated that integrin β4 participated in apoptotic signaling in VEC, and our findings indicate that hemidesmosome structures and keratin filament system might be important in regulation of apoptotic signaling.

Integrins are transmembrane receptors implicated in mediating cell-substrate attachment or cell-cell adhesion, and in transducing signals that regulate such diverse processes as growth, differentiation, and migration (1, 2). In contrast to other integrins (integrin β1, 3 etc.) that localize to focal adhesions or otherwise interact with the actin filament system, integrin β4 is found in hemidesmosomes in close proximity to molecules linking to the keratin filament system (3, 4). Although integrin β4 has been implicated in many different cellular processes (5, 6, 7), its functions in the various tissues that express it have not yet been demonstrated.

In rectal carcinoma cells that lack expression of the β4 integrin subunit, when full-length integrin β4 cDNA was expressed, the cells exhibited partial G1 arrest and apoptosis (8). These results suggest that integrin β4 might be involved in apoptosis signal pathway in this carcinoma cell line. In normal cells that express integrin β4, its role in the apoptotic signal transduction is not known. According to the results from Clarke, A. S. et al (8), integrin β4 subunit might be more critical in apoptotic signal transduction than α6 subunit because in these cells integrin α6 subunit is normally expressed. For these reasons, in this study we used the mAb of integrin β4 subunit and studied the function of integrin β4 in apoptosis signaling in vascular endothelial cells that normally express integrin β4, and found that the antibody of integrin β4 promoted attachment and spreading of the cells to culture dishes and by which suppressed apoptosis of these cells. These results indicate that integrin β4 is implicated in apoptotic signal transduction in normal vascular endothelial cells and hemidesmosome structures of these cells might play a critical role in apoptosis.

MATERIALS AND METHODS

Reagents. MCDB-104 medium was purchased from Kyokuto Pharmaceutical Industries, Tokyo, Japan. Fetal bovine serum (FBS) was purchased from Wako Industries, Tokyo, Japan. Fibroblast growth factor (FGF) was extracted from bovine brains by the method of Lobb and Fett (9). Anti-β4 integrin antibody (clone 3E1) was brought from Chemicon International Inc., Temecula, U.S.A. An irrelevant mAb (mouse IgG) was used as a control, and mouse IgG at the same concentration used in this paper did not show any effect on the cells. All other reagents were of ultrapure grade.

Cell culture. Human umbilical vein endothelial cells (HUVEC) were obtained by the method of Jaffe (10). The cells were cultured in gelatin-coated plastic dishes in MCDB-104 medium that was supplemented with 10% fetal bovine serum (FBS) and 70 ng/ml FGF (as well as 100 μg/ml heparin) at 37° C in 5% CO_2 and in air. Throughout our experiments, we used cells with a population doubling level of 15-30.

Determination of viability. Cells were grown until cultures reached sub-confluence and then the medium was replaced with plain MCDB medium (no FGF and FBS) after one wash with the same medium. The cells were then incubated without or with anti-β4 integrin antibody. Trypsinized cells were counted with a Coulter Counter (Coulter Electronics, INC., Hialean, Florida) after 24 h, 48 h and 72 h. Cells that had detached from the dishes were washed away before the treatment with trypsin. The cells that remained

0006-291X/97 $25.00

attached to dishes after washing away of blebs were not stained by erythrosin B (5 mg/ml; Sigma) and, were therefore regarded as living cells.

Nuclear fragmentation assay. Subconfluent cells were deprived of FGF and serum in the absence or presence of mAb of integrin β4 for 24 h, then harvested by rubber policeman and collected by centrifugation. The cells were washed once with PBS^- (phosphate-buffered saline), fixed with 1 % glutaraldehyde solution overnight at room temperature, centrifuged and resuspended in PBS^-, and then stained with Hoechst 33258 at the concentration of 1 mM for 20 min. After three washes with PBS^-, the cells were mounted onto slides for analysis under a fluorescence microscope (20). The percentages of degraded nuclei were determined by averaging the results from three independent experiments in which five fields per slide with an average of ~100 cells per field were counted.

Analysis of DNA fragmentation. Cells (2×10^6) were incubated in a digestion buffer that contained 0.2 mg/ml proteinase K at 50° C for 5 h. The cellular DNA was extracted once with phenol and once with a mixture of phenol, chloroform and 3-methyl-1-butanol (25:24:1, v/v). After digestion with RNase (final concentration, 0.6 mg/ml) at 37° for 30 min, the sample was subjected to electrophoresis on a 2% agarose gel in Tris-acetate buffer (40 mM Tris-acetate, 2 mM EDTA, pH 8.0). The gel was then stained with ethidium bromide and photographed on a UV transilluminator.

Cell adhesion assay. The ability of cell adhesion was quantitated by the method previously described in (11). Briefly, the cells (1×10^4 cells/ml) suspended in MCDB medium without or with mAb of integrin β4 of 5 μg/ml in the absence of FGF and serum were plated onto 24-well untreated bacterial culture plates. After incubation for 2 h at 37°C, wells were washed with serum-free MCDB medium to remove unattached cells. Attached cells were harvested after the treatment with 0.1% trypsin in PBS^- and counted with a Coulter Counter (Coulter Electronics, INC., Hialean, Florida). The concentration of this antibody used here was the sufficient minimum one to observe the effect of the mAb.

RESULTS

Human vascular endothelial cells are normally cultured in MCDB medium with FGF and serum (70 ng/ml and 10 % respectively). When the cells are deprived of FGF and serum, they die by apoptosis (Fig. 1). In order to understand the mechanism by which apoptosis occurs, we investigated the role of integrin β4 in regulation of apoptosis in VEC in which integrin β4 is normally expressed.

When the cells were cultured in MCDB medium without FGF and serum, they gradually detached from the dishes and floated into the medium, and then formed apoptotic bodies, by contrast, when we added monoclonal antibody (mAb) of integrin β4 into this medium these early morphological changes of apoptosis were completely inhibited (Fig. 1). 24 h after deprivation of FGF and serum, about 40 % of cells died in the absence of mAb of integrin β4, while in the presence of this antibody about 98 % of cells remained alive (Fig. 2). 2 days after the start of treatment, 80 % of cells died, but in the presence of mAb of integrin β4, only 20 % of cells died (Fig. 2). The cell death of this 20 % of cells was also apoptosis (data not shown). All obser-

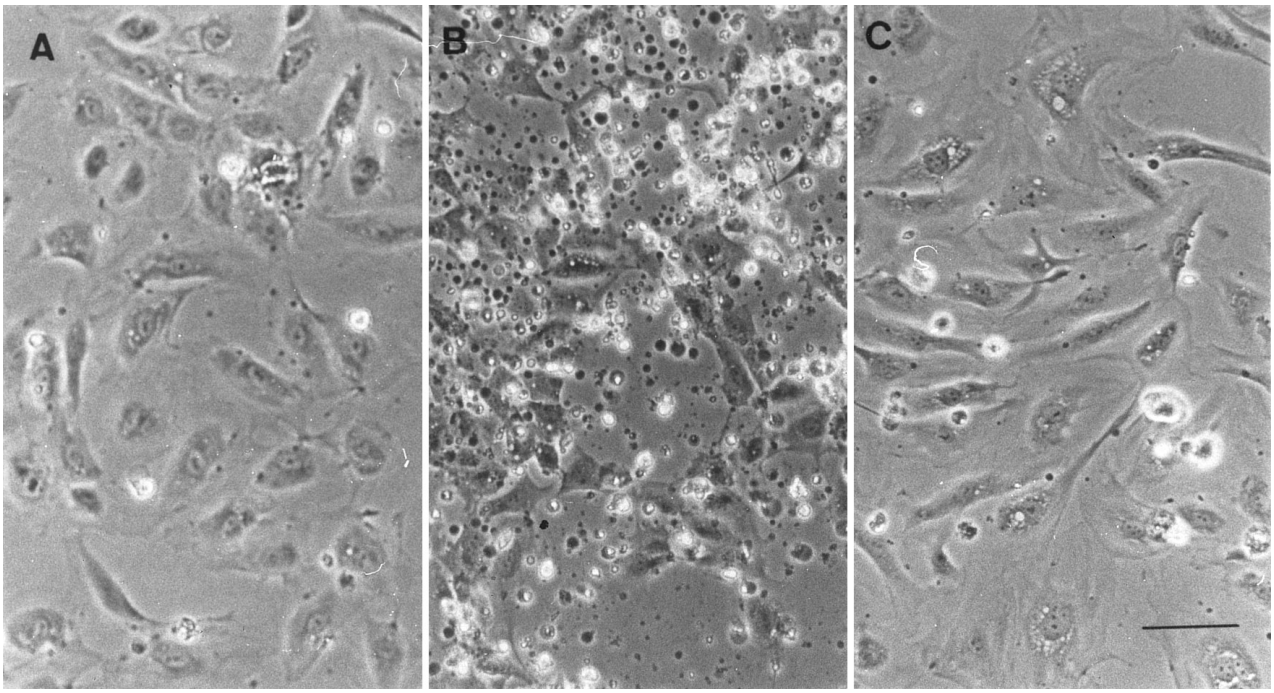

FIG. 1. Inhibition of morphological changes associated with apoptosis in VEC by mAb of integrin β4. Light micrographs of VEC cultured in MCDB medium with (A) or without (B) FGF and serum for 24 h. (C) Cells treated with mAb of integrin β4 (5 μg/ml) for 24 h. Bar, 100 μm.

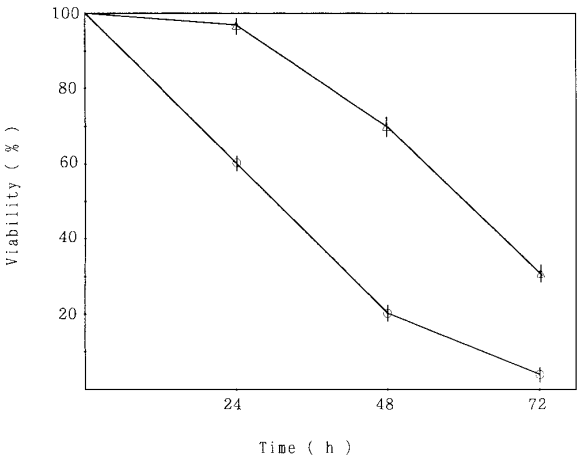

FIG. 2. Effect of mAb of integrin β4 on the viability of VEC deprived of FGF and serum. Cells were cultured in the medium without FGF and serum in the absence (open circles) or presence (open triangles) of anti-β4 integrin antibody (5 μg/ml) for 24 h, 48 h and 72 h, and then viability (%) was determined as described in Materials and Methods. Values are the means and S.D.(bars) of results from five separate experiments.

vations above were specific to the mAb so that an irrelevant mouse IgG did not show such effects.

We examined the nuclear fragmentation and DNA fragmentation of the cells cultured in FGF- and serum-free medium without or with mAb of integrin β4. As shown in figure 3 A, 24 h after the start of treatment, about 40 % of cellular nuclei became fragmentated in the absence of mAb of integrin β4 or in the presence of an irrelevant mouse IgG, but in the presence of this antibody about 100 % of cellular nuclei remained complete. The analysis of DNA fragmentation also showed that apoptosis induced by deprivation of FGF and serum in VEC was obviously inhibited by anti-integrin β antibody (Fig. 3B).

To address the possibility that integrin β4 participates in regulation of cell-substrate adhesion and spreading, we cultured the cells on untreated bacterial culture plates in the presence or absence of mAb of integrin β4 in MCDB medium without FGF and serum. Under this condition, 2 h after the start of treatment, few cells could attach to the plate in the absence of mAb of integrin β4, while in the presence of this antibody the cells rapidly attached and spread on the plate (Fig. 4 A). At this time, 85 % of cells adhered to the plate in the presence of mAb of integrin β4, while only 20 % of cells attached to the plate when it was absent or control IgG was present (Fig. 4 B). These results, that were specific for the mAb to integrin β4, demonstrated that mAb of integrin β4 promoted cell-substrate adhesion and spreading in the absence of FGF and serum.

DISCUSSION

Human vascular endothelial cells (VEC) are absolutely dependent upon the presence of FGF and serum for *in vitro* proliferation and serial propagation (10, 12, 13). In another hand, adhesion of VEC to matrix is

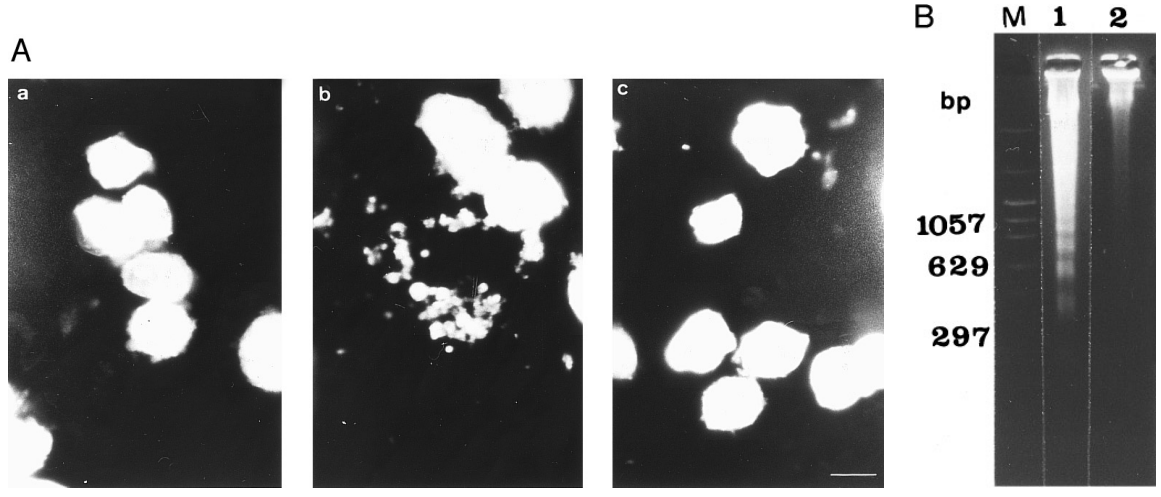

FIG. 3. Effect of mAb of integrin β4 on nuclear fragmentation and DNA degradation in VEC induced by deprivation of FGF and serum. (A) Quantitation of nuclear fragmentation in VEC. The cells were cultured under the same condition with Fig. 1 for 24 h, cell nuclei were stained with the fluorescent dye Hoechst 33258 and viewed using fluorescence microscopy as described in Materials and Methods. a, nuclei of cells cultured in MCDB medium with FGF and serum. b and c, nulei of cells cultured in the medium without FGF and serum in the absence and presence of mAb of integrin β4 (5 μg/ml) respectively. Bar, 10 μm. (B) DNA degradation. Cells were lysed and cellular DNA was isolated and subjected to gel electrophoresis on a 2 % agarose gel. Lane 1, DNA isolated from the cells cultured in MCDB medium without FGF and serum for 24 h, clear DNA ladders are shown. Lane 2, DNA isolated from the cells cultured in the FGF- and serum-free medium in the presence of mAb of integrin β4 (5 μg/ml) for 24 h, DNA fragmentation was inhibited. DNA markers are shown in lane M.

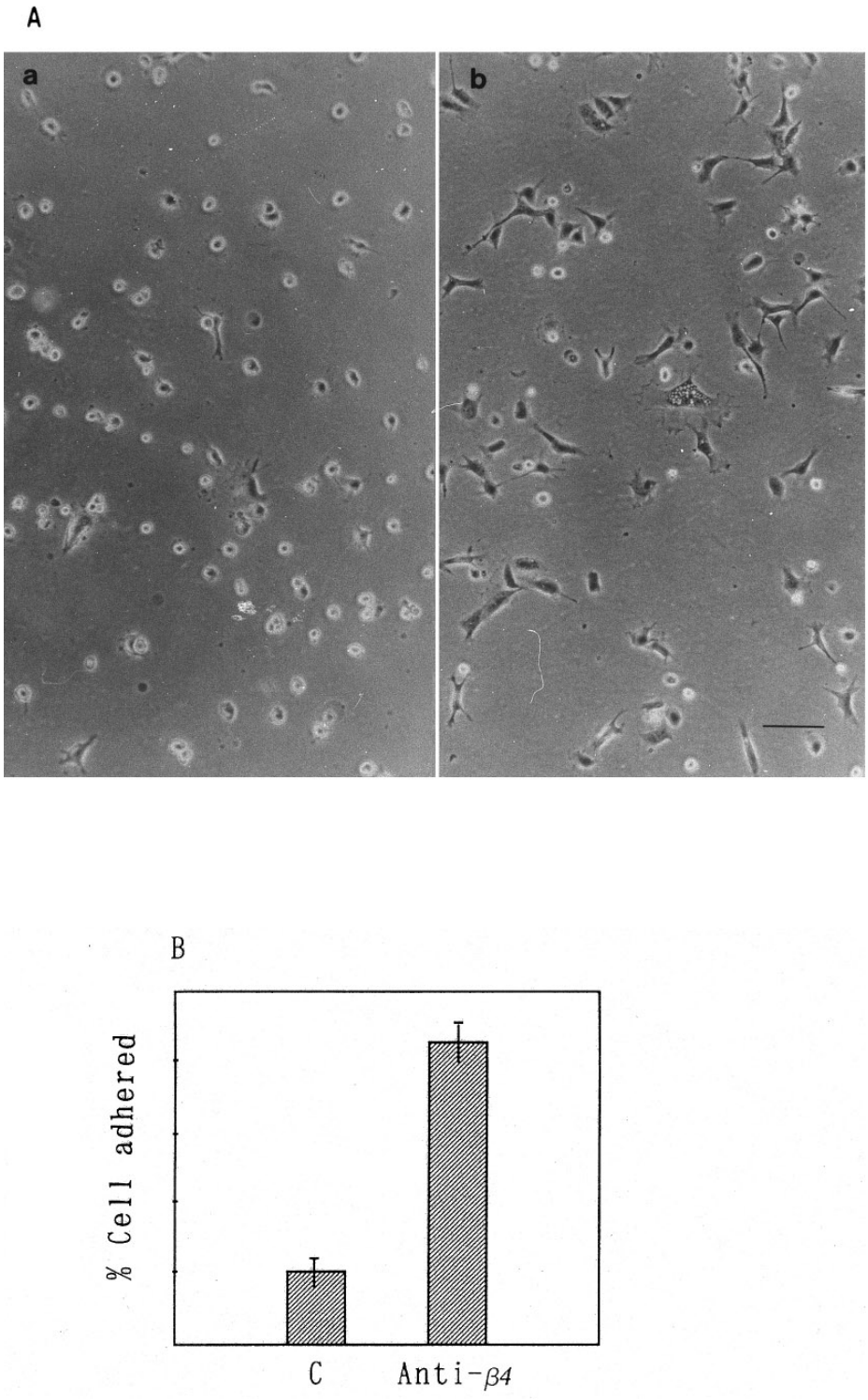

FIG. 4. Anti-β4 integrin antibody promoted cell-substrate adhesion and spreading in VEC. (A) Light micrographs of VEC cultured on untreated bacterial culture plates in the medium without FGF and serum in the absence (a) or presence (b) of mAb of integrin β4 (5 μg/ml) for 2 h. Bar, 100 μm. (B) Percentages of cells adhered to the substrate. C, percent of cells adhered in the absence of mAb against integrin β4; Anti-β4, percent of cells adhered in the presence of this antibody (5 μg/ml). Means and S.D.from five independent experiments are shown.

absolutely required for survival and proliferation in response to growth factors (14). VEC proliferate more rapidly in growth factor containing medium as they adhered and spread on matrix (14). When these cells are deprived of FGF and serum, which induces detachment of the cells from culture dishes, or cultured under conditions that prevent adhesion and spreading, they stop growing, lose viability and finally undergo apoptosis (14, 15, 16, 17, 18). However, the mechanism by which VEC undergo apoptosis under these conditions is not clear although it was shown that integrin $\beta 1$ and $\beta 3$ might be involved in these apoptotic signal pathways (18, 19). The major finding of this work is that mAb of integrin $\beta 4$ suppresses apoptosis that was induced by deprivation of FGF and serum, as well as by prevention of cell adhesion on substratum. Our results first demonstrated that integrin $\beta 4$ participated in apoptotic signal transduction in VEC which normally express this integrin.

The mAb of integrin $\beta 4$ used in this study is specific to human $\beta 4$ integrin (21). In VEC, integrin $\beta 4$ subunit and $\alpha 6$ subunit pair to form $\alpha 6\beta 4$ heterodimer that binds to various isoforms of the basement membrane component laminin (22,23,24). Our results suggest that the interaction between integrin $\alpha 6\beta 4$ and laminins might play an important role in apoptotic signal transduction in VEC.

Our previous reports showed that FGF and the enhancer of protein kinase C inhibited apoptosis of VEC (25,26), and the experimental results from Stepp et al showed that the expression of integrin $\alpha 6\beta 4$ was dose-dependently suppressed by FGF and protein kinase C enhancer (PMA) in VEC (27). It has been shown that *ras*-transformed endothelial cells are more resistant to apoptosis (28), and in our experiment it was shown that the expression of *c-H-ras* gene declined after FGF and serum deprivation that induces apoptosis of VEC (unpublished observation). Taken together, these results indicate that protein kinase C, p21 and integrin $\beta 4$ are implicated in signaling pathway of VEC apoptosis that is induced by deprivation of FGF and serum although the cascade of these element is not clear.

Unlike other integrins that localized to focal adhesions or otherwise interact with the actin filament system, integrin $\beta 4$ is found in hemidesmosomes in close proximity to molecules linking to the keratin filament system (5). Our findings herein indicate that hemidesmosome structures and keratin filament system might be very critical in regulation of apoptotic signaling in VEC.

REFERENCES

1. Hynes, R. O. (1992) *Cell* **69,** 11–25.
2. Watt, F. M., Kubler, M. D., Hotchin, N. A., Nicholson, L. J., and Adams, J. C. (1993) *J. Cell Sci.* **106,** 175–182.
3. Carter, W. G., Kaur, P., Gil, S. G., Gahr, P. J., and Wayner, E. A. (1990) *J. Cell Biol.* **111,** 3141–3154.
4. Stepp, A. M., Spurr-Michaud, S., Tisdale, A., Elwell, J., and Gipson, I. K. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 8970–8974.
5. Dowling, J., Yu, Q. C., and Fuchs, E. (1996) *J. Cell Biol.* **134,** 559–572.
6. Mainiero, F., Pepe, A., Yeon, M., Ren, Y., and Giancotti, F. G. (1996) *J. Cell Biol.* **134,** 241–253.
7. Xia, Y. P., Gil, S. G., and Carter, W. G. (1996) *J. Cell Biol.* **132,** 727–740.
8. Clarke, A. S., Lotz, M. M., Chao, C., and Mercurio, A. M. (1995) *J. Biol. Chem.* **270,** 22673–22676.
9. Lobb, R. R., and Fett, J. W. (1984) *Biochemistry* **23,** 6295–6296.
10. Jaffe, E. A., Nachman, R. L., Becker, C. G., and Minick, R. C. (1973) *J. Clin. Invest.* **52,** 2745–2756.
11. Mazaki, Y., Mochii, M., Kodama, R., and Eguchi, G. (1996) *Develop. Growth Differ.* **38,** 429–437.
12. Garfinkel, S., Hu, X., Prudovsky, I. A., Mcmahon, G. A., Kapnik, E. M., Mcdowell, S. D., and Maciag, T. (1996) *J. Cell Biol.* **134,** 783–791.
13. Maciag, T., Hoover, G. A., Stemerman, M. B., and Weinstein, R. (1981) *J. Cell Biol.* **91,** 420–426.
14. Re, F., Zanetti, A., Sironi, M., Polentarutti, N., Lanfrancone, L., Dejana, E., and Colotta, F. (1994) *J. Cell Biol.* **127,** 537–546.
15. Araki, S., Shimada, Y., Kaji, K., and Hayashi, H. (1990) *Biochem. Biophys. Res. Comm.* **168,** 1194–1200.
16. Ingber, D. E. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 3579–3583.
17. Ingber, D. E. (1992) *Cancer Biol.* **3,** 57–63.
18. Meredith, J. E., Fazeli, Jr. B., and Schwartz, M. A. (1993) *Mol. Biol. Cell* **4,** 953–961.
19. Brooks, P. C., Montgomery, A. M. P., Rosenfeld, M., Reisfed, R. A., Hu, T. H., Klier, G., and Cheresh, D. A. (1994) *Cell* **79,** 1157–1164.
20. Schwartz, L. M., and Osborne, B. A. (1995) *Methods in Cell Biology* **46,** pp. 268.
21. Hauptmann, S. (1995) *Int. J. Cancer* **61,** 819–825.
22. Lee, E. C., Lotz, M. M., Steele, G. D., and Mercurio, A. M. (1992) *J. Cell Biol.* **117,** 671–678.
23. Niessen, C. M., Hogervorst, F., Jaspars, L. H., De Melker, A. A., Delwel, G. O., Hulsman, E. H. M., Kuikman, I., and Sonnenberg, A. (1994) *Exp. Cell Res.* **2111,** 360–367.
24. Spinardi, L., Einheber, S., Cullen, T., Milner, T. A., and Giancotti, F. G. (1995) *J. Cell Biol.* **129,** 473–487.
25. Araki, S., Shimada, Y., Kaji, K., and Hayashi, H. (1990) *Biochem. Biophys. Res. Commun.* **172,** 1081–1085.
26. Hase, M., Araki, S., Kaji, K., and Hayashi, H. (1994) *J. Biochem.* **116,** 905–909.
27. Stepp, N. T., Cornelius, L. A., and Romani, N. (1995) *J. Invest. Dermatol.* **104,** 266–270.
28. Lu, X., Hee, Park, S., Thompso, T. C., and Lane, D. P. (1992) *Cell* **70,** 153–161.