

troponin T in embryonic skeletal muscle in vitro is under neurogenic control (20). It will be interesting to determine if the neurogenic regulation of cardiac troponin T occurs at the level of transcription.

THOMAS A. COOPER  
CHARLES P. ORDAHL\*

University of California,  
San Francisco 94143

#### References and Notes

1. J. Vanderkerckhove and K. Weber, *FEBS Lett.* **102**, 219 (1979); *J. Mol. Biol.* **126**, 783 (1978); *Differentiation* **14**, 123 (1979).
2. G. F. Gauthier, S. Lowey, P. Benfield, A. W. Hobbs, *J. Cell Biol.* **92**, 471 (1982); M. T. Crow, P. S. Olson, F. E. Stockdale, *ibid.* **96**, 736 (1983); S. Lowey, P. A. Benfield, D. D. LeBlanc, G. S. Waller, *J. Muscle Res. Cell Motility* **4**, 695 (1983).
3. R. G. Whalen, S. M. Sell, G. S. Butler-Browne, K. Schwartz, P. Bouveret, I. Pinset-Harstrom, *Nature (London)* **292**, 830 (1981); E. Bandman, R. Matsuda, R. C. Stochman, *Dev. Biol.* **93**, 508 (1982); P. A. Benfield, S. Lowey, D. D. LeBlanc, G. S. Waller, *J. Muscle Res. Cell Motility* **4**, 717 (1983).
4. T. Obinata *et al.*, *Dev. Biol.* **101**, 116 (1984).
5. J. E. Dennis, T. Shimizu, F. C. Reinach, D. A. Fischman, *J. Cell Biol.* **98**, 1514 (1984).
6. A. I. Caplan, M. Y. Fiszman, H. M. Eppenberger, *Science* **221**, 921 (1983).
7. Reviewed by D. A. Fischman, in *Muscle Development: Molecular and Cellular Control*, M. L. Pearson and H. F. Epstein, Eds. (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1982), p. 397.
8. J. J. Lee, R. J. Shott, S. J. Rose, T. L. Thomas, R. J. Britten, E. H. Davidson, *J. Mol. Biol.* **172**, 149 (1984) and references therein.
9. R. G. Whalen, S. M. Sell, A. Eriksson, Lars-Eric Thornell, *Dev. Biol.* **91**, 478 (1982).
10. T. Masaki and C. Yoshizaki, *J. Biochem. (Tokyo)* **76**, 123 (1974); L. Gorza, S. Sartore, C. Triban, S. Schiaffino, *Exp. Cell Res.* **143**, 395 (1983); L. J. Sweeney, W. A. Clark, P. K. Umeda, R. Zak, F. J. Manasek, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 797 (1984).
11. N. Toyota and Y. Shimada, *J. Cell Biol.* **91**, 497 (1981).
12. The cardiac actin gene was shown to be expressed in early skeletal muscle development [A. J. Minty, S. Alonso, M. Caravatti, M. E. Buckingham, *Cell* **30**, 185 (1982)]; these authors initially thought that it acted as an embryo-specific isoform in this tissue. Subsequent work indicates that the cardiac and skeletal-actin genes are coexpressed in both tissues, probably throughout ontogeny, albeit at varying ratios [M. E. Buckingham *et al.*, *Exp. Biol. Med.* **9**, 228 (1984); U. Nudel *et al.*, *ibid.*, p. 219; C. P. Ordahl, I. Peng, T. A. Cooper, *ibid.*, p. 211; P. Gunning, P. Ponte, H. Blau, L. Keddes, *Mol. Cell. Biol.* **3**, 1994 (1983)]. It is likely that the cardiac and skeletal actins are expressed as pairs, which would be similar to the expression of cytoskeletal and smooth muscle actins [see Gunning *et al.* (above) for a discussion of this]. This situation probably differs, therefore, from the transient expression of cardiac myosin heavy and light chains and troponin T in embryonic skeletal muscle.
13. C. P. Ordahl, D. Kiousus, S. M. Tilghman, C. Ovitt, J. Fornwald, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 4519 (1980); C. P. Ordahl, T. Cooper, C. Ovitt, J. Fornwald, A. F. Calman, A. I. Caplan, in *Muscle Development: Molecular and Cellular Control*, M. L. Pearson and H. F. Epstein, Eds. (Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y., 1982), p. 225.
14. P. S. Thomas, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 5201 (1980); *Methods Enzymol.* **100**, 255 (1983).
15. E. Southern, *J. Mol. Biol.* **98**, 503 (1975).
16. Since the restriction enzymes used cut well outside the coding region of the cDNA (except in the case of one Hind III site; T.A.C. and C.P.O., unpublished) two genes would have to be highly conserved within their intron and flanking regions to give identical patterns of hybridization in this type of experiment. In addition, overlapping fragments have been isolated from a chicken genomic library and the composite restriction map is in agreement with the fragment pattern shown in Fig. 3.
17. B. C. Orcutt, D. G. George, J. A. Fredrickson, M. O. Dayhoff, *Nucleic Acids Res.* **10**, 157 (1982).
18. J. R. Pearlstone, P. Johnson, M. R. Carpenter, L. B. Smillie, *J. Biol. Chem.* **252**, 983 (1977).
19. This is based on the fact that the 106A4 mRNA is both abundant and persistent throughout cardiac ontogeny. We cannot, of course, rule out the existence of additional cardiac troponin T isogenes not detected by our probes.
20. N. Toyota and Y. Shimada, *Cell* **33**, 297 (1983).
21. J. Messing, *Methods Enzymol.* **101**, 20 (1983).
22. We thank J. R. Coleman for valuable discussions and critical reading of the manuscript. This work is supported by grants from NIH (R01-GM32018) (C.P.O.), a postdoctoral fellowship from NICHD (T.A.C.), and a research career development award from NICHD (C.P.O.).

\* To whom correspondence should be addressed.

23 April 1984; accepted 27 July 1984

reduced by culture in vitro and reexpress themselves when tumor cells are transferred to a host. Such findings indicate that the metastatic phenotype of tumor cells is genetically constituted but subject to modulation by as yet unidentified factors encountered in the host and in culture.

Histopathological studies indicate that metastatic cells are able to traverse basement membranes (5). This is not unexpected, since basement membranes represent the major physical barrier blocking passage of cells across blood vessel walls and movement of cells from one tissue compartment to another. Basement membranes are continuous extracellular matrices that separate epithelial cells from the underlying stroma. They are composed of several unique components, including type IV collagen; glycoproteins such as fibronectin, laminin, and entactin; and a heparan sulfate proteoglycan. Tumor cells will attach to a variety of substrates. However, metastatic cells show a higher affinity for type IV collagen than for other collagens using laminin as an attachment factor (6, 7). Highly malignant murine fibrosarcoma cells have increased amounts of laminin on their cell surface, whereas far less laminin is found on cells of low malignancy (8). This would favor binding of the cells to basement membranes. Furthermore, the metastatic tumor cells produce a collagenase that attacks type IV collagen, whereas nonmetastatic tumor cells do not (9). However, such activity is also found in normal cells that are able to traverse basement membranes (10). Local degradation of basement membranes is probably required for cells to cross these barriers.

Fibronectin and laminin are glycoproteins used by cells to bind to collagenous matrices (7, 8, 11). Metastatic tumor cells may be exposed to these proteins at various times during their transport in the body and during their colonization of target tissues. Fibronectin is present in serum and other body fluids (12) as well as in connective tissues, whereas laminin is found exclusively in basement membranes (13). Since both proteins influence the adhesion, growth, motility, and differentiation of cells (11), we examined the metastatic potential of mouse melanoma cells after culturing them in medium containing laminin or fibronectin. We found that fibronectin suppressed invasive activity, whereas laminin increased invasiveness. These changes were correlated with the ability of the cells to bind to and invade basement membranes in vitro and with their ability to bind laminin.

## Modulation of the Metastatic Activity of Melanoma Cells by Laminin and Fibronectin

**Abstract.** *Metastatic mouse melanoma cells have a high affinity for the basement membrane and the ability to degrade it; these properties may allow tumor cells to invade the membrane and disseminate. In this study it was found that the metastatic potential of mouse melanoma cells varied when the cells were exposed in culture to fibronectin or laminin. After removal of fibronectin or exposure to laminin, the cells had an increased affinity for basement membrane collagen, were more invasive of basement membranes in vitro, and produced more lung colonies in vivo. These changes are correlated with and may be due to an increase in the laminin-binding capacity of the tumor cell surface.*

Metastasis is one of the major causes of mortality in cancer. The propensity to metastasize is found only in a minority of the cells in a malignant tumor (1). To metastasize, tumor cells must enter and then leave the circulatory system, evade host defenses, and proliferate in a secondary site. Lines of tumor cells with increased metastatic potential have been

obtained in vivo by various techniques, including serial transfer of cells from metastatic lesions, cloning cells from metastatic lesions, and culturing the cells that penetrate the walls of isolated organs in vitro (2). The metastatic activity (3) and certain phenotypic characteristics, such as plasminogen activator (4), that are associated with invasiveness are

When melanoma cells of low metastatic activity (B16F1) were grown for 1 week in medium lacking fibronectin, the cells attached more rapidly and to a greater extent to type IV collagen substrates than cells cultured with fibronectin (Fig. 1). The increased attachment to type IV collagen substrates was maximal after the first passage and was not increased further, even after eight passages in fibronectin-free medium. Similar results were obtained with the more metastatic cell lines BL6 and B16F10.

Growth of the cells was also increased by removal of fibronectin from the culture medium. Although melanoma cells grow well in culture under standard culture conditions (50  $\mu\text{g}/\text{ml}$  of fibronectin) (1), the number of melanoma cells (BL6, B16F10, and B16F1) in 5-day cultures was increased 50 percent by utilizing media from which the fibronectin has been removed by affinity chromatography (14) and increased an additional 50 percent if laminin (50  $\mu\text{g}/\text{ml}$ ) was added at the onset of the culture period.

In some experiments cells were cultured on plastic petri dishes coated with fibronectin (50  $\mu\text{g}$  per dish). After being cultured for 1 week on such dishes in fibronectin-free medium or on tissue culture plastic in medium supplemented with fibronectin (50  $\mu\text{g}/\text{ml}$ ), B16F1 cells were examined for their ability to attach to type IV collagen. In both cases the rate of attachment and the total number of attaching cells was the same (43 percent attachment after 90 minutes). In other experiments we assessed the growth of B16F1 cells on fibronectin substrates formed by drying the protein on plastic petri dishes. After 1 week, growth of cells on fibronectin-coated dishes (50  $\mu\text{g}$  per dish) was identical to that of cells on tissue culture plastic in medium containing 50  $\mu\text{g}$  of fibronectin per milliliter. These results suggest that cells respond in a similar way to fibronectin in medium or as a substrate.

The ability of the murine melanoma cells to penetrate the extracellular matrix of devitalized human amnion with its associated basement membrane was used as an *in vitro* test of cell invasiveness. Cells cultured in medium containing fibronectin were less invasive than cells grown for two passages in the absence of fibronectin (Fig. 2). Similarly, cells grown on a fibronectin substrate showed the same invasiveness as cells cultured in medium with fibronectin. B16F1 melanoma cells, the least invasive strain tested, showed the greatest increase, followed by B16F10 cells. The most invasive line, BL6, showed the least change in ability to penetrate the

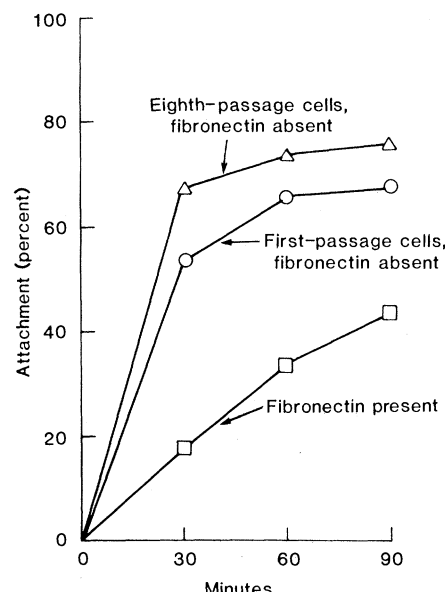


Fig. 1. Attachment of B16F1 melanoma cells to type IV collagen substrates after growth in standard culture medium (RPMI 1640 plus fetal bovine serum adjusted to 50  $\mu\text{g}$  of fibronectin per milliliter) or in standard culture medium with fibronectin-free serum (less than 0.1  $\mu\text{g}/\text{ml}$ , as determined by enzyme-linked immunosorbent assay). Both first- and eighth-passage B16F1 cells grown in the absence of fibronectin were used. After 1 week of growth the cells were trypsinized and  $10^5$  cells were added to 35-mm-diameter dishes coated with type IV collagen (10  $\mu\text{g}$  per dish). At the indicated times the plates were rinsed to remove nonadherent cells and the adherent cells were trypsinized and counted electronically. Each point is the mean of results for triplicate assays that differed from one another by less than 10 percent.

amnion, perhaps because the original selection of this line was based on its invasive potential.

Cells cultured under the same conditions were also assayed *in vivo* for production of lung colonies. The cultured BL6 cells were harvested, washed, and injected into the tail vein of syngeneic male mice (1, 2, 7). Twenty-five days later the mice were killed and their lungs were removed and examined for tumors. Cells grown without fibronectin produced more lung colonies than cells grown with mouse or calf fibronectin (Table 1). Cells grown in medium supplemented with mouse laminin also gave

rise to more lung lesions. In confirmation of previous observations (7), we found that coadministration of melanoma cells with antibody to laminin or the cell-binding fragment of laminin reduced the number of lung colonies. These results indicate that fibronectin suppresses the invasive potential of these cells whereas laminin increases it.

Previous studies have shown a correlation between the metastatic potential of tumor cells and the number of laminin receptors on the cells (15). The changes in adhesion and metastatic activity observed here suggested that changes in laminin receptors could be occurring. We measured the amount of laminin on the cell surface by indirect immunofluorescence and found that the B16F1 cells displayed more laminin on their surface when cultured without fibronectin. We

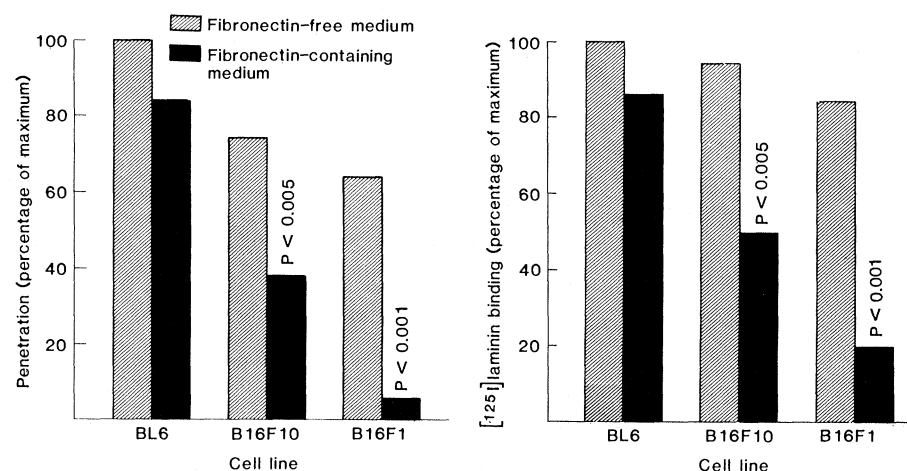


Fig. 2 (left). Invasion of human amnion (basement membrane and stromal tissue) *in vitro* by BL6, B16F10, and B16F1 cell grown for one passage in the presence or absence of serum fibronectin. The amnion was prepared as described by Liotta (20). Cells ( $5 \times 10^5$ ) were placed in the upper chamber in RPMI 1640 medium supplemented with 1 percent NuSera. After 5 days the Nuclepore filters were stained with hematoxylin and eosin and the cells on the filters were counted. Each point is the mean of results for triplicate assays that differed from one another by less than 10 percent. The maximum penetration of the BL6 cells cultured in the absence of fibronectin was assigned a value of 100 percent. Fig. 3 (right). Comparison of the binding of [ $^{125}\text{I}$ ]laminin to BL6, B16F10, and B16F1 cells grown with and without serum fibronectin. All binding experiments were conducted as previously described (15). Equilibrium binding was reached after 90 minutes for all cell lines tested. Maximum binding of [ $^{125}\text{I}$ ]laminin to BL6 cells cultured in the absence of fibronectin was assigned a value of 100 percent.

also measured the binding of [<sup>125</sup>I]laminin to melanoma cells that had been cultured with or without fibronectin. There was a greater binding of labeled laminin to cells grown without fibronectin (Fig. 3). The difference was greatest in the least metastatic lines (B16F1 and B16F10). As shown in Fig. 4, uptake of labeled laminin followed typical receptor-binding kinetics and was saturable. Receptor-binding fragments of laminin derived by proteolysis—the P1, C1, and α3 fragments (15)—blocked binding of labeled laminin (Fig. 4), as did an excess of unlabeled laminin. In contrast, no difference in the uptake of labeled laminin was observed in the presence of an excess of fibronectin, indicating that fibronectin does not compete directly for the laminin receptor (Fig. 4). These studies suggest that culture of these cells with fibronectin reduces their ability to bind laminin, but not by a direct reaction between fibronectin and the laminin receptor.

It appears that laminin and fibronectin act in an opposing fashion on processes likely to be critical to the metastatic activity of the melanoma cells. The ability of the cells to bind to type IV collagen and laminin and to invade the basement membrane and stroma are probably key events in the escape of cells from the tumor as well as in their arrest in capillary beds and their penetration into tissues. Such events could also be related to clearance of tumor cells from the circulation by the reticuloendothelial

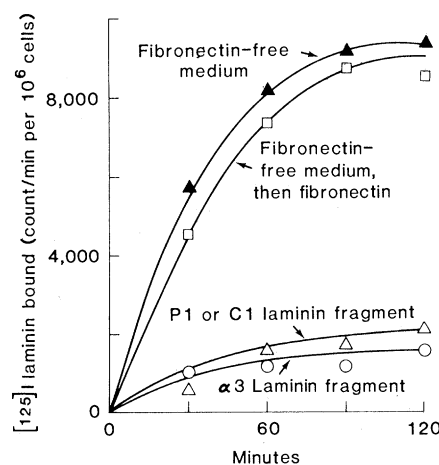


Fig. 4. Binding of [<sup>125</sup>I]laminin to B16F10 murine melanoma cells after culture in medium with or without fibronectin. The time course of binding with or without a 1000-fold excess of fibronectin or purified unlabeled protease-derived fragments of laminin (100 µg/ml) is shown.

system, since fibronectin has opsonic activity (16). Furthermore, these observations suggest that the B16F1 and B16F10 cells have a greater potential for metastasis than they express. Possibly fibronectin acts to suppress metastasis of tumor cells.

The mechanism by which these attachment proteins modulate the metastatic activity of cells is not known. However, it seems that the increase in availability of laminin receptors enhances the cells' ability to bind basement membranes. Binding of the cells to laminin may initi-

ate a phenotype in the cell distinct from that induced by fibronectin. This could include production, activation, or release of enzymes (for example, type IV collagenase) that participate in the degradation of basement membranes.

Transformation of cells is associated with a reduced expression of fibronectin and laminin on their surfaces (17). In general, this makes the cells less adherent to substrates. It has been observed that B16F1 melanoma cells grown in vitro in a spherical configuration exhibit a marked increase in metastatic capability in vivo (18). The highly metastatic cells in a tumor have a phenotype different from that of the other tumor cells (1), characterized by a larger number of laminin receptors and a greater capacity to bind to and degrade basement membranes (7–9, 15). Since fibronectin and laminin influence cell shape (19) and react with different receptors, it is not surprising that they can influence metastatic activity as well as have antagonistic effects on cells. This might be an example of a yin-yang mechanism whereby occupancy of one receptor suppresses expression of the other. Such a mechanism might be important not only in the metastatic process but also in normal cells—including myoblasts, hepatocytes, and endothelial cells—that can utilize more than one attachment factor (11, 12, 19).

VICTOR P. TERRANOVA

Laboratory of Developmental  
Biology and Anomalies, National  
Institute of Dental Research,  
Bethesda, Maryland 20205

JEANNETTE E. WILLIAMS

LANCE A. LIOTTA

Laboratory of Pathology,  
National Cancer Institute,  
Bethesda, Maryland 20205

GEORGE R. MARTIN

Laboratory of Developmental  
Biology and Anomalies, National  
Institute of Dental Research

Table 1. Metastasis of BL6 cells in vivo after culture for 1 week in the presence of extracellular matrix components. The standard growth medium consisted of RPMI 1640 supplemented with fetal bovine serum, which gave a fibronectin concentration of 50 µg/ml, plus glutamine, penicillin (10 U/ml), and streptomycin (100 µg/ml). Removal of fibronectin from the serum was accomplished by passing whole serum over a gelatin affinity column (14). Murine fibronectin was obtained from the serum of syngeneic C57/BL mice by gelatin affinity chromatography followed by heparin Sepharose chromatography. Purified laminin was added at a concentration of 50 µg/ml. In some experiments antibodies to laminin or fibronectin (1 µg/ml) (7) were added to certain dishes on the last day. P1 or C1 laminin fragments were added at a concentration of 0.25 µg/ml. All cells were grown in culture until confluent for one passage. A suspension of cells ( $2 \times 10^5$  in a volume of 0.1 ml), determined to be viable by the trypan blue dye exclusion test, was then injected into the tail veins of syngeneic, pathogen-free male mice 6 weeks of age. Twenty-five days later the mice were killed and their lungs were removed and immersed in Bouin's fixative. Tumors were counted with a dissecting lens ( $\times 10$ ). Twenty-four animals were used per assay group. Values are mean numbers of tumors  $\pm$  standard deviations. The metastatic potential of cells grown with fibronectin differed significantly from that of cells grown without fibronectin ( $P < 0.001$ ). Addition of laminin significantly increased the number of lung colonies ( $P < 0.001$ ). N.D., not determined.

Addition	Concentration	Pulmonary metastases	
		Fibronectin in growth medium	No fibronectin in growth medium
None		38 $\pm$ 12	137 $\pm$ 20
Murine fibronectin	50 µg/ml	N.D.	52 $\pm$ 14
Antibody to fibronectin	1 µg/ml	36 $\pm$ 10	N.D.
Laminin	50 µg/ml	134 $\pm$ 16	183 $\pm$ 34
P1 or C1 laminin fragment	1 µg/ml	6 $\pm$ 4	4 $\pm$ 3
Antibody to laminin	1 µg/ml	5 $\pm$ 4	5 $\pm$ 3

#### References

1. I. J. Fidler, *Nature (London)* **242**, 148 (1973); and M. L. Kripke, *Science* **197**, 893 (1977); M. G. Brattain, J. Strobel-Stevens, J. Fine, M. Webb, A. M. Sarraf, *Cancer Res.* **40**, 2142 (1980); K. G. Danielson, L. Anderson, H. Hosich, *ibid.*, p. 1812; M. A. Cifone and I. J. Fidler, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 6949 (1981); G. Poste, J. Doll, I. J. Fidler, *ibid.*, p. 6226; K. M. Miner, T. Kawaguchi, G. W. Uba, G. L. Nicolson, *Cancer Res.* **42**, 4631 (1982).
2. G. L. Nicolson, K. Brunson, I. J. Fidler, *Cancer Res.* **38**, 4105 (1978); V. A. Pollach and I. J. Fidler, *J. Natl. Cancer Inst.* **69**, 127 (1982); G. Poste *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 6574 (1982).
3. J. E. Talmadge, J. R. Starkey, W. C. Davis, A. L. Cohen, *J. Supramol. Struct.* **12**, 227 (1979); A. Neri and G. L. Nicolson, *Int. J. Cancer* **28**, 731 (1981).
4. L. Ossowski and E. Reich, *Cell* **33**, 323 (1983).
5. R. Vracko, *Am. J. Pathol.* **77**, 314 (1974); J. E. Talmadge and I. J. Fidler, *J. Natl. Cancer Inst.* **69**, 975 (1982); *Nature (London)* **297**, 593 (1982);

- I. J. Fidler, D. M. Gersten, I. R. Hart, *Adv. Cancer Res.* **28**, 149 (1978).
6. G. L. Nicolson, T. Irimua, R. Gonzalez, E. Ruoslahti, *Exp. Cell Res.* **135**, 461 (1981).
7. V. P. Terranova, L. A. Liotta, R. G. Russo, G. R. Martin, *Cancer Res.* **42**, 2265 (1982); I. Vlodavsky and D. Gospodarowicz, *Nature (London)* **289**, 304 (1981); J. C. Murray, L. A. Liotta, V. P. Terranova, in *Invasion and Metastasis* L. A. Liotta and I. R. Hart, Eds. (Nijhoff, The Hague, 1982), p. 309.
8. J. P. McCoy, R. V. Lloyd, M. S. Wicha, J. Varani, *J. Cell. Sci.* **65**, 139 (1984).
9. L. A. Liotta, J. Kleinerman, P. Catanzaro, D. Rynbrandt, *J. Natl. Cancer Inst.* **58**, 1427 (1977); L. A. Liotta, K. Tryggvason, S. Garbisa, P. Gehron-Robey, S. Abe, *Biochemistry* **20**, 100 (1981); L. A. Liotta, S. Abe, P. G. Robey, G. R. Martin, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 2268 (1979).
10. L. A. Liotta, U. L. Langer, S. Garbisa, *Biochem. Biophys. Res. Commun.* **98**, 184 (1981).
11. H. K. Kleinman, R. J. Klebe, G. R. Martin, *J. Cell Biol.* **88**, 473 (1981); S. L. Palm and L. T. Furcht, *ibid.* **96**, 1218 (1983); A. Baron Van-Overcooren, H. K. Kleinman, H. E. J. Seppä, B. Rentier, M. Dubois-Dalcq, *ibid.* **93**, 179 (1982); J. B. McCarthy, S. L. Palm, L. T. Furcht, *ibid.* **97**, 772 (1983); J. Varani, W. Orr, P. A. Ward, *J. Cell Sci.* **34**, 133 (1978).
12. D. F. Mosher, *Prog. Hemostasis Thromb.* **5**, 111 (1980); K. M. Yamada, *Annu. Rev. Biochem.* **52**, 761 (1983).
13. R. Timpl *et al.*, *J. Biol. Chem.* **254**, 9933 (1979); J. M. Foidart *et al.*, *Lab. Invest.* **42**, 336 (1980).
14. E. Engvall and E. Ruoslahti, *Int. J. Cancer* **20**, 1 (1977).
15. V. P. Terranova, C. N. Rao, T. Kalebic, I. M. Margulies, L. A. Liotta, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 444 (1983); H. L. Malinoff and M. S. Wicha, *J. Cell Biol.* **96**, 1475 (1983).
16. J. Molnar, F. B. Gelder, M. Z. Lai, G. E. Siegrivvy, R. B. Credo, *Biochemistry* **18**, 3305 (1979); F. A. Blumenstock *et al.*, *J. Biol. Chem.* **253**, 4287 (1978).
17. R. O. Hynes, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 3170 (1973); P. Ekblom, K. Alitalo, A. Vaheri, R. Timpl, L. Saxén, *ibid.* **77**, 485 (1980); E. G. Hayman, E. Engvall, E. Ruoslahti, *J. Cell Biol.* **88**, 352 (1981); G. L. Nicolson, *Biochim. Biophys. Acta* **458**, 1 (1976); E. G. Hayman, A. Oldberg, G. R. Martin, E. Ruoslahti, *J. Cell Biol.* **94**, 28 (1982); K. Alitalo, J. Keski-Oja, K. Hedman, A. Vaheri, *Virology* **119**, 347 (1982).
18. A. Raz and A. Ben-Ze'ev, *Science* **221**, 1307 (1983).
19. K. M. Yamada, *Cell Biology of the Extracellular Matrix*, E. D. Hay, Ed. (Plenum, New York, 1982); S. Johansson, J. Kjellen, M. Höök, R. Timpl, *J. Cell Biol.* **90**, 260 (1981); M. L. McGarvey, A. Baron-Van-Overcooren, M. Dubois-Dalcq, H. K. Kleinman, *Dev. Biol.*, in press; S. P. Sugrue and E. D. Hay, *J. Cell Biol.* **91**, 45 (1981); D. Gospodarowicz, G. Greenburg, C. R. Birdwell, *Cancer Res.* **38**, 4155 (1978); D. Gospodarowicz, G. Greenburg, J. M. Foidart, N. Savion, *J. Cell. Physiol.* **107**, 171 (1981); D. M. Scott, J. C. Murray, J. Barnes, *Exp. Cell Res.* **144**, 472 (1983); J. R. Couchman, M. Höök, D. A. Rees, R. Timpl, *J. Cell Biol.* **96**, 177 (1983); R. Carlsson, E. Engvall, A. Freeman, E. Ruoslahti, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 2403 (1981); S. Sell and E. Ruoslahti, *J. Natl. Cancer Inst.* **69**, 1105 (1982); E. J. Macarath and P. S. Howard, *J. Cell. Physiol.* **116**, 76 (1983); L. I. Gold and E. Pearlstein, *Biochem. J.* **186**, 551 (1980); M. Aumailley, H. Nowack, R. Timpl, in *Structural Carbohydrates of the Liver*, H. Popper, W. Reutter, E. Köttgen, F. Guder, Eds. (MTF Press, Boston, 1983), p. 373; R. Timpl, S. Johansson, V. Van Delden, I. Oberbauer, M. Höök, *J. Biol. Chem.* **255**, 8922 (1983).
20. L. A. Liotta, C. Lee, D. J. Morakis, *Cancer Lett.* **11**, 141 (1980).

11 January 1984; accepted 15 August 1984

## Inhibitors of Lysosomal Enzymes: Accumulation of Lipofuscin-Like Dense Bodies in the Brain

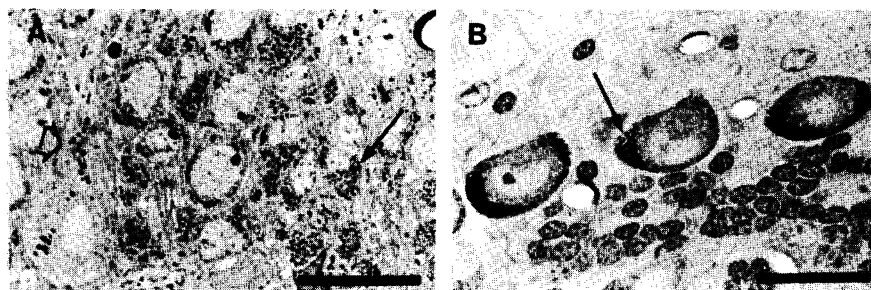
**Abstract.** *Injections of leupeptin (a thiol proteinase inhibitor) or chloroquine (a general lysosomal enzyme inhibitor) into the brains of young rats induced the formation of lysosome-associated granular aggregates (dense bodies) which closely resembled the ceroid-lipofuscin that accumulates in certain disease states and during aging. The dense material increased in a dose- and time-dependent fashion and was differentially distributed across brain regions and cell types. These observations provide clues to the origins of ceroid-lipofuscin and suggest means for studying the consequences of its accumulation.*

During aging there is an accumulation in neurons and other cell types of a heterogeneous group of pigment granules referred to as lipofuscin (1). A similar material, usually termed "ceroid," develops in cells during a number of pathological conditions ranging from anoxia to vitamin deficiency (2), and is characteristic of a group of genetically inherited diseases called the "neuronal ceroid lipofuscinoses" (3). The origins and causes of ceroid or lipofuscin accumulation, as well as the consequences for the functioning of the cell, are poorly understood. The granules are rich in peroxidized lipids and polymerized phospholipids (4), and a combination of histochemical and ultrastructural evidence indicates that they are related to lysosomes (5). This evidence has led to the hypothesis that peroxidation of membrane lipids induced by free radicals

blocks the normal degradation of organelles and vesicles by lysosomes, resulting in the accumulation of residual bodies that are classified as ceroid-lipofuscin

(6). Alternatively, it has been proposed that ceroid-lipofuscin accumulation results from a defect in intracellular catabolism itself, followed by intralysosomal peroxidation and polymerization of the undigested material (7). We now report that inhibitors of lysosomal enzymes cause a rapid and massive increase in ceroid-lipofuscin in the brain cells of young rats.

Leupeptin and chloroquine, two well-established inhibitors of lysosomal proteinases (8, 9), were injected into the brains of Sprague-Dawley rats (40 to 60 days of age) with a constant infusion technique. The rats were implanted with osmotic minipumps (Alzet) that were attached to cannulae leading into the lateral ventricle of each rat (10). The pumps contained leupeptin (acetyl-leupeptin, Boehringer Mannheim, 8, 20, or 40 mg/ml; 2, 3, and 12 rats, respectively), chloroquine (Sigma, 40 mg/ml; 4 rats) or saline (9 mg/ml; 4 rats) and operated at a steady rate of 0.5  $\mu$ l/hour. After 2 weeks most of the animals were killed; three rats implanted with leupeptin-filled pumps (40 mg/ml) were allowed to survive for 8 to 10 weeks. In addition, two rats each received daily intraventricular injections of leupeptin (0.5 mg/5  $\mu$ l), chloroquine (0.5 mg/5  $\mu$ l), or saline (0.045 mg/5  $\mu$ l) for four consecutive days, and two rats received single intraventricular injections of leupeptin (0.5 mg/5  $\mu$ l) and were killed 8 hours later. Most animals were killed by perfusing with a mixed aldehyde fixative (11), and samples from various brain regions were dissected and processed for electron microscopy. Remaining portions of these brains were either sectioned on a freezing microtome (at 20  $\mu$ m thick) or were embedded and sectioned in paraffin (at 6  $\mu$ m thick) or glycol methacrylate (at 1 to 2  $\mu$ m thick). The following stains were then employed (12): periodic acid Schiff (PAS), Nile



**Fig. 1.** Bright-field photomicrographs from the brains of two rats that received a continuous infusion of leupeptin (0.5 mg/day) into the lateral ventricle via a cannula attached to a mini-osmotic pump. (A) A 1- $\mu$ m-thick section (in epon) through the dentate gyrus showing dense granular inclusions in granule cells (solid arrow) and in a pyramidal-like interneuron (open arrow); toluidine blue stain. (B) A 1- $\mu$ m section (in glycol methacrylate) through cerebellum showing densely staining PAS-positive inclusions in Purkinje cells (arrow). Scale bars, 25  $\mu$ m.