Macrophages Express a Plasma Membrane Receptor for Basement Membrane Laminin

THOMAS K. HUARD, PhD,
HERBERT L. MALINOFF, MD, and
MAX S. WICHA, MD

From the Division of Hematology and Oncology, Department of Internal Medicine, Simpson Memorial Research Institute, University of Michigan, Ann Arbor, Michigan

Laminin, a noncollagenous extracellular matrix glycoprotein, can serve as an attachment factor for epithelial and endothelial cells to basement membrane collagen. The authors have observed that inflammatory macrophages have the capacity to bind exogenous laminin in a time-, temperature- and dose-dependent manner. The laminin binds to these cells via a specific plasma membrane receptor which is detectable on several macrophage subpopulations as well as isolated macrophage membranes. The isolated receptor is a trypsin-sensitive protein with an apparent molecular weight of 70,000 which binds laminin with relatively high affinity, Kd $\cong 4 \times 10^{-9}$ M. The inflammatory macrophages attach preferentially to a substrate of laminin compared to Type I or Type IV collagen. Thus, the authors postulate that during tissue inflammation macrophages utilize the laminin receptor to bind to vascular basement membranes. (Am J Pathol 1986, 123:365–370)

MONONUCLEAR phagocytes play an important role in host defense against infectious agents and tumors.[1] Although it has been speculated that cell surface properties of these phagocytes direct their accumulation at sites of inflammation or tumor, little is known about the molecular mechanisms involved. We have recently found that mouse peritoneal macrophages express the glycoprotein laminin on their surface.[2] This glycoprotein with a molecular weight of approximately 800,000 is a component of all basement membranes,[3] and is believed to mediate the attachment of a variety of normal endothelial and epithelial cells to basement membrane collagen.[4,5] In addition, there is increasing evidence that some metastatic tumor cells utilize laminin as an attachment factor during the metastatic process.[6,7] We now report that in addition to displaying cell surface laminin, macrophages have the ability to bind exogenous laminin via a specific plasma membrane receptor. We have isolated a molecule from these cells (~70,000 mol wt) which retains the ability to bind exogenous laminin with high affinity (Kd $\cong 4 \times 10^{-9}$ M). The expression of this receptor may promote the interaction of these cells with vascular basement membranes, with microbes at sites of inflammation,[8] and with malignant tumor cells.[9]

Materials and Methods

Cell Isolations

The macrophages used in these studies were collected by peritoneal lavage from 3–5 week-old C57BL/6J mice inoculated 4 days previously with 1 ml sterile thioglycollate (Difco, Detroit, Mich) intraperitoneally as previously described.[2] The cells were sedimented at 300*g* for 5 minutes at 4 C, and contaminating erythrocytes were lysed by hypotonic shock with distilled water. The cells were reconstituted in Hanks' balanced salt solution, washed twice in Dulbecco's phosphate-buffered saline (M.A. Bioproducts, Gaithersburg, Md) supplemented with 1% bovine serum albumin (Sigma, Fraction V, St. Louis, Mo) and adjusted to 1×10^6 cells/ml in this buffer (PBS–1% BSA). In selected experiments we also utilized macrophages isolated from mammary adenocarcinomas. These cells were generously provided by Dr. Gloria Heppner, of the Michigan Cancer Foundation in Detroit. The J774.1 a macrophage-like cell line, were cultured in suspension in RPMI-1640 supplemented with 10% fetal bovine serum and antibiotics.[2]

Cell Binding of Laminin

Laminin was isolated from the EHS tumor by the method of Timpl et al,[10] and the purity was verified by SDS polyacrylamide gel electrophoresis on 5% gels.[11]

Supported by Grants IM-407 and BC-357 from the American Cancer Society and CA-25641 and HD 16721 from the National Institutes of Health, DHHS, and a grant from the Pardee Foundation.

Accepted for publication January 10, 1986.

Address reprint requests to Thomas K. Huard, PhD, University of Michigan, Simpson Memorial Research Institute, 102 Observatory, Ann Arbor, MI 48109.

The purified laminin was radiolabeled with ^{125}I by the lactoperoxidase method to a specific activity of 4 μCi/μg as previously described.[12] Total laminin binding to cells was assessed by incubating 5 × 10^5 cells in 200 μl of PBS–1%BSA containing ^{125}I-laminin (5 × 10^5 cpm/100 μl). The tubes were incubated for the indicated time and temperatures, centrifuged for 8 minutes at 300*g*, washed three times with cold (4 C) PBS–1% BSA, and counted in a gamma counter. For determination of specific laminin binding, cells were incubated as above but with the addition of a 100-fold excess of nonradioactive laminin (10 μg/ml). The amount of laminin specifically bound was calculated as the difference between total counts per minute bound in the absence and the presence of the unlabeled laminin and represented 50–70% of the total laminin bound.

Isolation of the Laminin Receptor

To isolate the specific laminin binding molecule, 1.5 × 10^7 cells were treated with 2 ml of extraction buffer containing 0.5 M NaCl, 50 μg/ml N-ethylmaleamide (NEM), 50 μg/ml phenylmethysulfonylfluoride (PMSF), and 1% Nonidet P-40. The cells were sonicated and incubated with stirring overnight at 4 C. The cell extract was then sedimented at 1300*g* for 15 minutes at 4 C for removal of debris, and the supernatant was dialyzed against buffer containing 0.5 M NaCl, 0.1 M $NaHCO_3$, 50 μg/ml PMSF, 50 μg/ml NEM, pH 8.3. Five milliliters of the extract was fractionated at 4 C by affinity chromatography on a column of laminin (10 mg) coupled to Sepharose 4B (6 ml) as previously described.[13] The column was washed with 20 ml of buffer containing 0.5 M NaCl, 0.1 M $NaHCO_3$, pH 8.3. The bound material was eluted with 8 ml of buffer containing 0.1 M glycine-HCl, pH 2.4, and the eluate was neutralized to pH 7.0 with 1 M Tris. Samples containing 50 μg of protein were reduced by boiling with β-mercaptoethanol and analyzed by electrophoresis on 7.5% SDS-polyacrylamide gels.[11] Trypsin sensitivity of the eluted material was determined by pretreatment with 10 μg trypsin for 30 minutes at 37 C prior to gel electrophoresis.

Laminin Binding to the Isolated Receptor

To determine the parameters of laminin binding to the isolated receptor, 1.0 μg of eluted protein was spotted on each of replicate 6 mm disks of nitrocellulose (8 μ Millipore) and incubated for 60 minutes at 37 C as already described.[13] The disks were then placed in PBS–3% BSA for 60 minutes at 37 C to allow protein to bind to unoccupied sites on the nitrocellulose. The disks were rinsed three times in PBS–3% BSA to remove unbound protein. Indicated amounts of ^{125}I-laminin were then added to the disks with or without a 100-fold excess of unlabeled laminin in a final volume of 50 μl. The disks were incubated for 90 minutes at 37 C, washed with 1 ml PBS–3% BSA, and counted in a gamma counter. Specifically bound laminin was determined as the amount of total radioactivity bound in the absence of cold laminin minus the radioactivity bound in the presence of cold laminin. Specific binding represented approximately 80% of the total radioactivity bound. The affinity of laminin for the isolated receptor was determined by the method of Scatchard.[14]

Isolation of Macrophage Membranes

To verify that laminin binding to macrophages was a cell surface phenomenon, we measured the specific binding of laminin to isolated macrophage membrane vesicles. The cells were collected as usual and lysed by sonication at 4 C in PBS–1% BSA containing PMSF (50 μg/ml) and NEM (50 μg/ml). The cell lysate was sedimented in 0.3 M sucrose at 15,000*g* for 20 minutes at 4 C. The supernatant was collected and sedimented at 100,000*g* for 60 minutes at 4 C and the pellet reconstituted in PBS–1% BSA with PMSF and NEM to a membrane concentration equivalent to 1 × 10^7 cells/ml. Specific binding of laminin to the isolated membranes was measured as described above for intact cells.

Macrophage Attachment to Laminin-Coated Plastic

Substrata of laminin-coated plastic were prepared by allowing 0.5 ml of buffer (PBS–1% BSA) containing laminin (20 μg/ml) to dry onto the surface of wells of a 24-well microtiter tissue culture plate (Costar) at ambient temperature. Dried wells were then stored at 4 C until used. Just prior to use, the wells were rinsed gently with 1 ml of sterile H_2O for removal of unbound material. (With laminin labeled with ^{35}S-Met at least 90% of the protein was retained under these conditions.) Control plates were treated in a similar manner with buffer alone. For these experiments, the macrophages were radiolabeled with $Na_2{}^{51}CrO_4$ as previously described for malignant tumor cells.[25] To assay for attachment to the laminin-coated substrata, 5 × 10^5 cells (1 × 10^6/ml in PBS–1% BSA) were incubated in each of triplicate wells for varying time periods at ambient temperature (25 C). At the indicated times, the nonadherent cells were removed and the wells washed with cold (4 C) PBS–1% BSA three times with gentle agitation. The remaining adherent cells were lysed in 2% SDS, 1 M NaOH, neutralized with an equal volume of 1 M HCl, and brought to 10 ml with Unogel scintillation

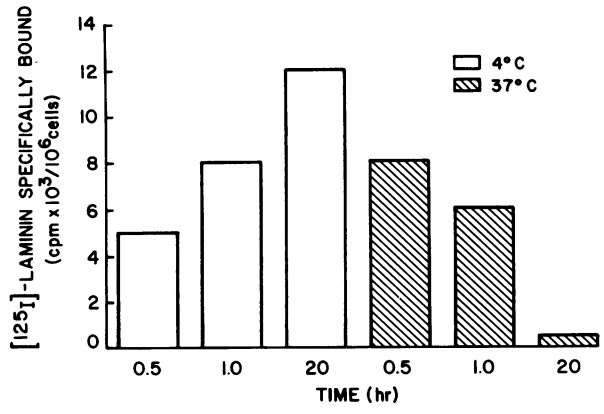

Figure 1—Specific binding of laminin to macrophages. ^{125}I-laminin specifically bound is the total ^{125}I-laminin bound minus ^{125}I-laminin bound in presence of a 100-fold excess of cold laminin. □, Laminin binding at 4 C; ▨, at 37 C. Values represent the mean of triplicate samples from three similar experiments (SEM ≤ 10%).

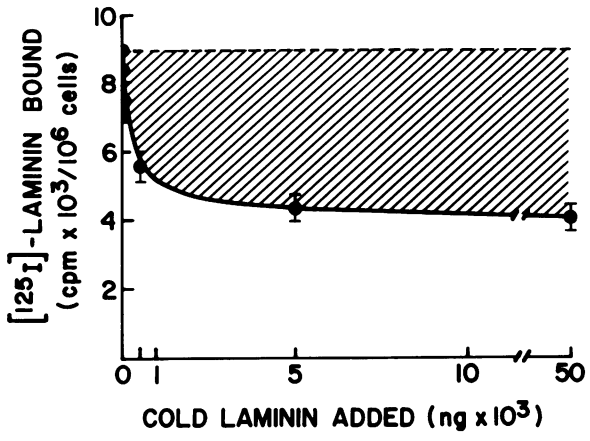

Figure 2—Laminin binding to macrophges. ^{125}I-Laminin binding in the absence (– –) or presence (●) of varying amounts of cold laminin. The *shaded area* represents the amount of ^{125}I-laminin specifically bound at each concentration of cold laminin. Values represent the mean (± SEM) of triplicate samples from one representative experiment.

fluid (Becton-Dickinson, Orangeburg, NY) for counting. The percent of cells remaining attached were calculated from the formula:

$$\%\ \text{cells attached} = \frac{\text{cpm attached}}{\text{total cpm added}} \times 100$$

In each experiment Type IV collagen and Type I collagen were used for comparison of macrophage attachment to other extracellular matrix components. Type IV collagen was purified from the EHS tumor,[15] and Type I collagen from rat tail tendons[16] and 25 μg was dried onto tissue culture plates as described for the laminin.

Results

To determine whether laminin could interact with the plasma membrane of macrophages, we measured the capacity of ^{125}I-laminin to bind to these cells. Specific binding was calculated as the proportion of the total laminin bound that could be displaced from the cell surface by a 100-fold excess of unlabeled laminin. As

Table 1—Binding of ^{125}I-Laminin to Macrophage Subpopulations

Cell population	Cold laminin* added	Total cpm† bound	Cpm specifically‡ bound	%
Inflammatory macrophage§	–	3420 ± 310		
	+	1390 ± 89	2030	59%
J774.1‖	–	4532 ± 14		
	+	2802 ± 265	1730	38%
Tumor macrophages¶	–	3311 ± 344		
#168	+	1391 ± 179	1920	58%
		4552 ± 405		
#66	+	1982 ± 216	2570	56%
	–	4313 ± 344		
#4526	+	1771 ± 126	2542	59%
Macrophage membranes**	–	6800 ± 340		
	+	4045 ± 203	2755	41%

* Binding of ^{125}I-laminin to macrophage subpopulations for 60 minutes at 4 C assayed with (+) and without (–) excess purified cold laminin (100-fold excess; 10 μg/ml).

† Mean counts per minute ^{125}I-laminin bound/0.5 × 10^6 cells or cell equivalents.

‡ Total counts per minute bound (–) minus counts per minute bound in presence (+) of excess cold laminin.

§ Peritoneal macrophages isolated 4 days after thioglycollate injection.

‖ J774.1 is a macrophagelike cell line obtained from ATCC and cultured in suspension in RPMI-1640 with 10% FCS and antibiotics.

¶ Macrophages isolated by weak adherence to Teflon from murine mammary carcinomas: #66 and #4526 were isolated from highly metastatic tumors and #168 from a low-metastatic tumor.

** Macrophage membranes isolated by ultracentrifugation of lysates of sonicated peritoneal macrophages.

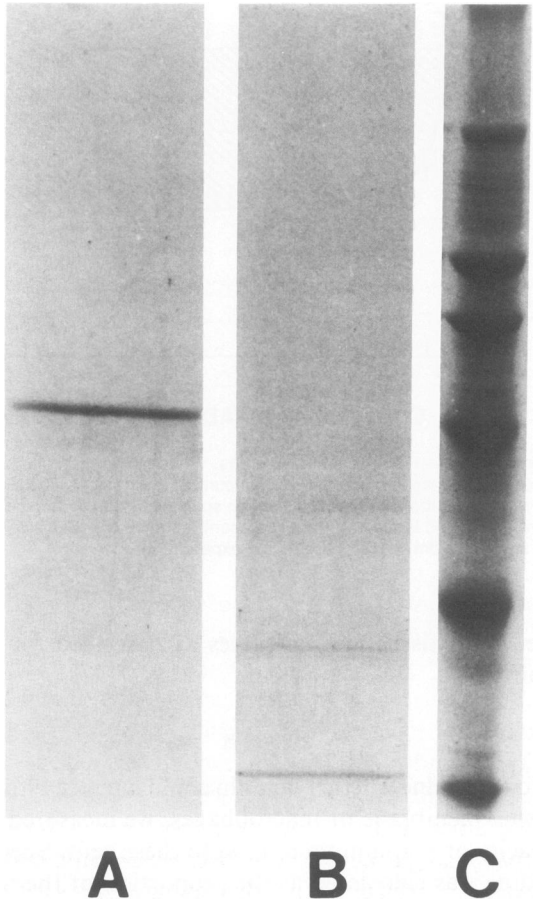

Figure 3—Characterization of laminin binding protein from macrophages. Electrophoresis of reduced laminin binding protein on 7.5% polyacrylamide gels (**A**), trypsin digested laminin binding protein (**B**), and molecular weight standards (**C**), including myosin 200K, β-galactosidase 116K, phosphorylase B 96K, bovine serum albumin 66K, ovalbumin 45K, and carbonic anhydrase 29K.

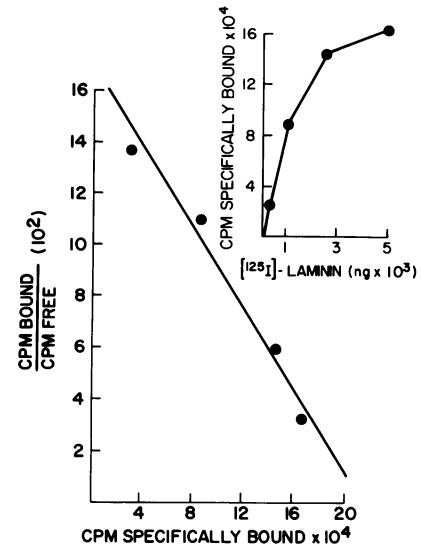

Figure 4—Laminin binding to isolated macrophage receptor. Scatchard analysis of ^{125}I-laminin specifically bound to macrophage-derived laminin receptor protein immobilized on nitrocellulose; Kd ≅ 4 × 10^{-9} M. **Inset**—Dose-response curve of the specific binding of ^{125}I-laminin to the isolated receptor protein.

shown in Figure 1, laminin specifically bound to the macrophages in a time- and temperature-dependent manner. At 4 C binding increased with time and reached a plateau by 18 hours. In contrast, at 37 C, specific binding was high within 30 minutes, with a rapid decline in binding after that period. As shown in Figure 2, specific binding to the macrophages increased with the addition of increasing amounts of exogenous laminin. We observed a similar pattern of specific laminin binding to a macrophage-like cell line, J774.1, as well as to macrophages isolated from several different metastatic tumors (Table 1). In addition, we detected specific laminin binding to isolated macrophage plasma membrane vesicles (Table 1), which suggests that the laminin binding component is a cell surface receptor.

In order to isolate a moiety from the macrophages responsible for laminin binding, the cells were extracted in buffer containing 1% NP-40 detergent. The extract was subjected to affinity chromatography on a column of laminin coupled to Sepharose 4B. Bound material was eluted with glycine-HCl, pH 2.4, and the eluate was analyzed by SDS-gel electrophoresis (Figure 3). When reduced, the sample produced a single band, ~70,000 mol wt when compared with known standards. This laminin binding protein was completely degraded by trypsin treatment into low-molecular-weight fragments.

The binding of exogenous laminin to the isolated laminin receptor protein was studied by immobilizing the receptor on nitrocellulose filter disks. After un-

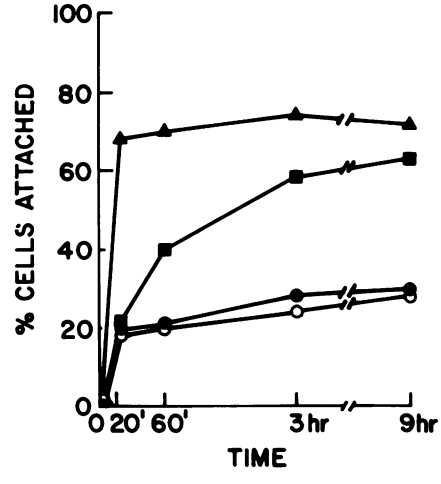

Figure 5—Macrophage attachment to extracellular matrix components. Attachment of 5 × 10^5 ^{51}Cr-labeled macrophages to plastic tissue culture wells pretreated with buffer (▲), 20 μg/ml laminin (■), 50 μg/ml Type IV collagen (●) or 50 μg/ml Type I collagen (○). % Cells attached = cpm attached ÷ total cpm added × 100 for each time interval of incubation.

bound sites on the nitrocellulose disks were blocked with albumin, the disks were incubated with ^{125}I-laminin. Again, specific laminin binding was determined in the presence of a 100-fold excess of unlabeled laminin. As shown in Figure 4 (inset), ^{125}I-laminin bound specifically to the isolated receptor in a concentration-dependent manner. There was no inhibition by albumin or whole serum (which contains fibronectin) for binding to the laminin receptor (data not shown). Scatchard analysis of laminin binding to the isolated receptor (Figure 4) was consistent with a single class of high-affinity receptors for laminin with a Kd $\cong 4 \times 10^{-9}$ M.

To determine whether the presence of the cell surface laminin receptor could be utilized by the cells to attach to basement membranes, we evaluated whether the macrophages could attach to substrata of plastic tissue culture wells coated with several extracellular matrix components. As shown in Figure 5, at all time intervals the macrophages exhibited preferential attachment to laminin substrata, compared with the other extracellular matrix components, including Type IV collagen and Type I collagen.

Discussion

The mononuclear phagocytes, the monocytes and macrophages, play an integral role in host defense processes.[1] Their capacity to infiltrate sites of inflammation and/or tumor are well documented. Our observation of the expression of cell-surface laminin on stimulated macrophages suggests a putative role for this basement membrane glycoprotein in macrophage function.[2] This has already been documented for another extracellular matrix component, fibronectin.[17,18] We have more recently determined that exogenous laminin can influence macrophage uptake and ingestion of microorganisms[8] and can significantly promote the attachment of malignant tumor cells to macrophages.[9] Bohnsack et al have observed an influence of laminin on the phagocytosis of erythrocytes as well.[19] Our current observations indicate that macrophages can interact with laminin by a specific cell-membrane receptor. Macrophages were shown to bind exogenous laminin in a dose-, time-, and temperature-dependent manner. Macrophage binding of laminin was optimal at 4 C. At 37 C we observed diminished laminin binding with time, and analysis of supernatants revealed that most (>80%) of the laminin was shed from the cell surface (not shown). This is similar to other macrophage receptor–ligand interactions such as the Fc-receptor for IgG[20] or specific carbohydrate receptors[21] which undergo rapid ligand-induced catabolism at 37 C. We are currently investigating whether this represents degraded laminin of laminin shed during ligand-induced endocytosis. We also observed laminin binding to a macrophage-like cell line (J774.1) and macrophages isolated from malignant tumors. In addition, we have observed similar binding of exogenous laminin to human peripheral blood monocytes and polymorphonuclear leukocytes (not shown).

In our preliminary characterization of the receptor molecule, we have verified that the receptor was a plasma membrane protein, because it was detectable in membrane vesicles prepared from intact macrophages. The isolated protein was sensitive to trypsin digestion and had an approximate molecular weight of 70,000. Trypsinization of the macrophages destroyed their capacity to bind exogenous laminin as well (not shown). By Scatchard analysis of laminin binding to the isolated receptor, we estimated an affinity for laminin of 4×10^{-9} M. These properties are similar to the laminin receptors we and others have isolated from several types of malignant tumor cells.[13,22,23] Although we were able to isolate approximately 100 μg of receptor protein from 1.5×10^7 macrophages, we had no indication of the efficiency of our isolation technique in order to accurately determine a precise concentration of receptor protein per cell. From the specific activity of the ^{125}I-laminin bound (4 μCi/μg), however, we estimate at saturation a range of $3–4 \times 10^3$ receptor molecules/cell. This estimate is based on each molecule of laminin binding to single receptor site. Because laminin may be polyvalent for cell binding,[26] this estimate may vary substantially. In addition, this estimate does not take into account the fact that these cells already express endogenous cell-surface laminin, and, thus, many of the plasma membrane laminin receptors may already be occupied.

The role of this receptor in the biologic function of macrophages is unknown. We have observed that the macrophages can attach to laminin substrata and thus may utilize this receptor during extravasation to sites of inflammation and/or tumor. Our observations that these cells acquire the capacity to bind to a substratum of laminin *in vitro* are in accordance with the results of Hart and Giavazzi, who observed that both monocytes and macrophages acquire the capacity to bind to laminin following *in vitro* culture.[24] The increase in macrophage adherence to laminin substrata with time may indicate a time-dependent up-regulation of cell-surface laminin receptors.

We and others have now shown that exogenous basement membrane laminin can influence several activities of macrophages, including interactions with microbial pathogens, uptake of opsonized erythrocytes, cell attachment, and conjugation to malignant tumor cells.[8,9,19] Our current observations suggest that the expression of a cell-surface receptor specific for laminin

may be a key element in the capacity of laminin to modulate these activities.

References

1. Nelson D: Immunobiology of the Macrophage. New York, Academic Press, 1976
2. Wicha MS, Huard TK: Macrophages express cell surface laminin. Exp Cell Res 1983, 143:475–479
3. Foidart JM, Bere EW, Yaar M, Rennard SI, Gullino M, Martin GR, Katz SI: Distribution and immunoelectron microscopic localization of laminin, a noncollagenous basement membrane glycoprotein. Lab Invest 1980, 42:336–342
4. Terranova VP, Rohrbach DH, Martin GR: Role of laminin in the attachment of PAM 212 (epithelial) cells to basement collagen. Cell 1980, 22:719–726
5. Farquhar MG: The glomerular basement membrane, Cell Biology of the Extracellular Matrix. Edited by ED Hay. New York, Plenum Press, 1982, pp 353–357
6. Terranova VP, Liotta LA, Russo RG, Martin GR: Role of laminin in the attachment and metastasis of murine tumor cells. Cancer Res 1982, 42:2265–2269
7. Malinoff H, McCoy JP, Varani J, Wicha MS: Metastatic potential of murine fibrosarcoma cells is influenced by cell surface laminin. Int J Cancer, 1984, 33:651–655
8. Boekeloo SW, Huard TK: Laminin and immune intervention in periodontal disease (abstract). J Dental Res 1985, 64:197
9. Huard TK, Baney JL, Wood JFB, Wicha MS: A potential role for the extracellular matrix glycoprotein laminin in macrophage-tumor cell interactions. Int J Cancer 1985, 36:511–517
10. Timpl R, Rohde H, Robey PG, Rennard SI, Foidart JM, Martin GR: Laminin—a glycoprotein from basement membranes. J Biol Chem 1979, 254:9933–9937
11. Laemmli UK: Cleavage of structural protein during the assembly of the head of bacteriophage T-4. Nature 1970, 227:680–685
12. Thorell JI, Johansson BG: Enzymatic iodination of polypeptides with ^{125}I to high specific activity. Biochem Biophys Acta 1971, 251:363–369
13. Malinoff H, Wicha MS: Isolation of a cell surface receptor protein for laminin from murine fibrosarcoma cells. J Cell Biol 1983, 96:1475–1479
14. Scatchard G: The attraction of proteins for small molecules and ions. Ann NY Acad Sci 1949, 51:660–672
15. Timpl R, Martin GR, Bruckner P, Wick G, Wiedemann H: Nature of the collagenous protein in a tumor basement membrane. Eur J Biochem 1978, 84:43–52
16. Michalopoulos G, Pitot HC: Primary culture of parenchymal liver cells on collagen membranes. Exp Cell Res 1975, 94:70–78
17. Bevilacqua MP, Amrani D, Mosesson MW, Bianco C: Receptors for cold-insoluble globulin (plasma fibronectin) on human monocytes. J Exp Med 1981, 153:42–60
18. Czop JK, Kadish JL, Austin KF: Purification and characterization of a protein with fibronectin determinants and phagocytosis-enhancing activity. J Immunol 1982, 129:163–167
19. Bohnsack JF, Kleinman HK, Takahashi T, O'Saka JJ, Brown EJ: Connective tissue proteins and phagocytic cell function. J Exp Med 1985, 161:912–923
20. Silverstein SC, Steinman RM, Cohn ZA: Endocytosis. Ann Rev Biochem 1977, 46:669–722
21. Mellman IS, Plutner H, Steinman RM, Unkeless JC, Cohn ZA: Internalization and degradation of macrophage Fc Receptors during receptor-mediated phagocytosis. J Cell Biol 1983, 96:887–895
22. Rao CN, Barsky SH, Terranova VP, Liotta LA: Isolation of a tumor cell laminin receptor. Biochem Biophys Res Commun 1983, 111:804–808
23. Terranova VP, Rao CN, Kalebic T, Margulies IM, Liotta LA: Laminin receptor on human breast carcinoma cells. Proc Natl Acad Sci 1983, 80:444–448
24. Giavazzi R, Hart I: Mononuclear cell adherence in the presence of laminin. Exp Cell Res 1983, 146:391–399
25. Huard TK, Garagiola DM, LoBuglio AF: In vivo activation of monocyte and alveolar macrophage ADCC: A rabbit model, mediation of Cellular Immunity in Cancer by Immune Modifiers. Edited by MA Chirigos, M Mitchell, MJ Mastrangelo, M Krim. New York, Raven Press, 1981, pp 237–244
26. Timpl R, Johansson S, van Delden V, Oberbaumer I, Hook M: Characterization of a protease-resistant fragment of laminin mediating attachment and spreading of rat hepatocytes. J Biol Chem 1983, 258:8922–8927

Acknowledgments

We would like to thank Ms. Patti Alvarez, Ms. Lorena Abrego, and Mr. George Lowrie for their technical assistance and Ms. Sue Grech for her help in preparation of the manuscript. We would also like to thank Dr. Gloria Heppner and Mr. Keith Mahoney for providing us with tumor macrophages for these studies.