Binding and Spreading of Hepatocytes on Synthetic Galactose Culture Surfaces Occur as Distinct and Separable Threshold Responses

Janet A. Oka and Paul H. Weigel

Division of Biochemistry, Department of Human Biological Chemistry and Genetics, University of Texas Medical Branch, Galveston, Texas 77550

Abstract. Isolated rat hepatocytes bind to synthetic flat polyacrylamide matrices containing covalently attached galactose residues in a sugar-specific and concentration-dependent manner. Cell binding is mediated by the asialoglycoprotein receptor and occurs as a threshold response at or above a critical concentration of sugar in the matrix. Hepatocytes in the presence or absence of serum were able to spread on these synthetic galactose surfaces and were morphologically similar to cells on tissue culture plastic. Cell spreading also occurred as a threshold response but at a much higher critical concentration of sugar than for the cell-binding response. Above the critical concentration for spreading, the area occupied by a cell increased as the sugar concentration increased. By manipulating the galactose content of the matrix, cell spreading and cell binding can be differentiated as independent and separable threshold responses to the extracellular substratum.

CELLULAR interactions with the extracellular matrix and with neighboring cell surfaces are important during morphogenesis, development, tumorigenesis, metastasis, wound repair, and the maintenance of tissue differentiation (8, 13, 16, 20, 33, 36). Many investigators have attempted to understand how the extracellular matrix influences cell behavior and how cells in turn change the structure and function of the matrix. Most studies of this difficult problem have used complex tissue or cell culture-derived extracellular matrices whose composition and structure are not well defined. Alternatively, proteins or other molecules have been adsorbed to glass or plastic culture surfaces (6, 9-12, 18, 19, 25, 31, 34). These surfaces have the disadvantage that the adsorbed molecules can be dissociated or displaced from the substratum during an experiment. Glass and plastic surfaces have also been chemically derivatized to allow various test molecules to be attached covalently (2, 7, 24, 32, 37). Derivatized surfaces have the disadvantage that they are often chemically undefined. Additionally, the glass or plastic substratum itself is usually adhesive for cells and it is difficult to control the amount of the immobilized test molecule.

We have developed a well-defined system in which a pure compound is covalently attached to a flat inert non-ionic polyacrylamide matrix (28, 29, 42). Using this approach we previously showed that rat hepatocytes specifically adhere to galactose surfaces (27, 38, 39, 41). These cells do not bind to polyacrylamide alone or to matrices containing other sugars such as glucose, mannose, or N-acetylglucosamine. Cell binding requires calcium ions and is mediated by asialoglycoprotein (or galactosyl) receptors, which form a large cluster at the cell substratum interface (41). This receptor specifically binds molecules with terminal galactose or N-acetylgalactosamine residues (4). Other cell types can bind to different carbohydrate surfaces. For example, chicken hepatocytes bind specifically to N-acetylglucosamine surfaces (35, 39), whereas rat Kupffer cells (P. H. Weigel, unpublished observations) and rabbit alveolar macrophages (22) specifically recognize mannose surfaces. The above studies demonstrated that, for each cell type, adhesion occurred only as a threshold response at or above a critical concentration of sugar in the substratum. Below the critical concentration, cells did not bind; above that concentration, binding occurred and became maximal with only a small (e.g., 15%) increase in sugar content. In the present study we wanted to determine whether hepatocytes were also able to spread on galactose surfaces and if this ability could be demonstrated to be a separate threshold response distinct from the cell-binding threshold response.

Materials and Methods

Chemicals

N,N-Methylenebisacrylamide (electrophoresis grade) was from Eastman Kodak Co., Rochester, NY. Acrylamide, from Polysciences, Inc., Warrington, PA, was recrystallized twice from 95% ethanol. Collagenase (type I) and dexamethasone were from Sigma Chemical Co., St. Louis, MO. Williams' Medium E, insulin, penicillin, and streptomycin were from Gibco, Grand Island, NY. Gentamicin sulfate (Garamycin) was from Schering Corp., Kenilworth, NJ. N-Acrylamidomethyl 2-aminocarbonyl(ethy) 1-deoxy-l-thio-D-galactopyranoside (which has the following structure:...
Galactose-S-CH₂-CO-NH-CH₂-NH-CO-CH=CH₂) was synthesized by the procedure of Lee et al. (23). Formaldehyde (20%) was from Polysciences, Inc. Fetal calf serum was from HyClone Laboratories, Sterile Systems, Inc., Logan, UT. L-glutamine was from Flow Laboratories, Inc., McLean, VA. Hepes was from Research Organics, Inc., Cleveland, OH.

**Cell Culture**

Hepatocytes were prepared by a collagenase perfusion procedure (40) using male Sprague-Dawley rats (Harlan Sprague-Dawley Inc., Houston, TX). Final cell suspensions were >90% viable and single cells. Cells were seeded at 0.5-1.0 × 10⁶ cells/cm² on tissue culture plastic or on synthetic galactose surfaces in Williams' Medium E containing 10 mM Hepes, pH 7.4, 50 mM/ml insulin, 2 mM l-glutamine, 5 × 10⁻⁷ M dexamethasone, 50 μg/ml gentamycin, 100 U/ml penicillin, and 100 μg/ml streptomycin with or without fetal calf serum. After 45 min at 37°C, the dishes were washed twice to remove nonbound cells, fresh complete medium containing 5% fetal calf serum, unless stated otherwise, was added, and the incubation was continued. The extent of cell binding was quantitated by a lactate dehydrogenase assay (35). Cells were cultured on the galactose surfaces by both the System I and System II procedures recently described (29). Flat 20% (wt/vol) polyacrylamide matrices containing various amounts of covalently incorporated galactose residues were prepared by the co-polymerization of acrylamide, bisacrylamide, and varying amounts of the galactosyl-acrylamide monomer described above (see references 39 and 42 for details). These matrices are nonionic and porous to molecules smaller than ~3.3 kD but are not penetrated by larger molecules (29). Cell processes such as microvilli therefore cannot penetrate into the matrix.

**Measurement of Cell Area**

The area occupied on the substratum by individual cells was measured from photographs of cells taken with a Lietz Diavert microscope using a Wild automatic camera. The negatives were projected onto a piece of paper and the enlarged outline of individual cells was traced. The area occupied by each cell was then determined using a planimeter (Numonics Corp. Landsdale, PA). Sample size ranged from 33-89 cells for each time point or galactose surface tested. The nonspread control to which cell areas were compared were cells fixed in suspension (2% formaldehyde, room temperature, 30 min). To assess the effect of gravity on cell deformation, cells were also allowed to settle onto polyacrylamide surfaces containing no galactose and their area was determined. This sagging or deformation of cells passively resting on a substratum causes about a 17% increase in the projected average area of these cells. This does not represent true spreading and was corrected for in the data presented here. Typically, at least 75% of the cells measured were single cells not in contact with other cells, the remainder were mostly doublets. The average area occupied by all cells sampled did not differ from the average area of the single cells by more than ~2-3%.

The relationship between cell spreading and the galactose concentration of the matrix was determined using both linear and curvilinear regression analysis programs (SciMed Analysis, Evanston, IL). Assessments of the appropriate regression model for each data set were based on minimizing the residual sum of squares for each function; the "best fit" curves were based on a χ² goodness of fit statistic.

**Results**

A marked difference in the degree of cell spreading was observed when freshly isolated rat hepatocytes were plated on synthetic culture surfaces containing different concentrations of galactose (Fig. 1). As the amount of galactose in the matrix was increased, the number of cells exhibiting a morphology similar to cells on tissue culture plastic (Fig. 1 A) increased and the extent to which individual cells spread onto the substratum also appeared to increase. Hepatocytes which were maximally spread on galactose surfaces looked essentially the same as cells on plastic (compare Fig. 1, A and E) except that the former cells had an abundance of large lamellipodia anchored to the substratum (Fig. 1, E and F).

Our previous studies on the interaction of rat hepatocytes with galactose culture surfaces were performed in the absence of serum. Therefore, we verified that the sugar specificity of this interaction was not altered by 5% fetal calf serum, which was usually present during long term culture (Table I). Hepatocytes still bound only to surfaces containing galactose and not to other carbohydrates such as mannose, glucose, N-acetylglucosamine, or maltose, or to polyacrylamide alone. Other experiments (not shown) demonstrated that the cell spreading threshold response (presented below) occurred and binding was maximal within 2-3 h over the galactose concentration range tested. Furthermore, the spreading response also occurred even in the absence of serum. For example, after 24 h in complete medium without serum, hepatocytes on surfaces containing 58 mM galactose had increased their average area by 44%. In the presence of 2.5 and 5% fetal calf serum these cells had spread and increased their area by 54 and 80%, respectively. Although the response was greater than that in the absence of serum, cell spreading was clearly not dependent on the presence of serum. The quantitative difference in spreading is probably due to the known beneficial effects of serum on cell maintenance and the viability of hepatocytes in culture (15, 26). Additionally, the ability of hepatocytes to spread on the galactose surfaces was independent of whether or not cells were also contacting other cells. Isolated single cells were able to spread at least as much as cells which were involved in cell-cell contacts on the substratum.

The cell binding threshold response occurred at ~3 mM galactose (see Fig. 2 B). Therefore, although hepatocytes were able to bind to all of the galactose surfaces pictured in Fig. 1, the degree of cell spreading was clearly different. This difference was quantitated by measuring the area occupied by cells on the surface and then plotting the distribution of cell areas (Fig. 3) and the mean area of a cell population as a function of the galactose concentration (Fig. 2 A). Regression analysis showed that the data were better described by an exponential rather than a linear function, supporting the conclusion that the increase in the mean area of the cell population occurs as a threshold response. The results from this analysis verified the visual impression that cell spreading occurred only above a critical amount of sugar in the matrix.

The distribution of individual cell areas (Fig. 3) and the

---

**Table I. Effect of Serum on the Specificity of Hepatocyte Binding to Glycoside Surfaces**

| Sugar            | Cell binding response (percent of maximum) |
|------------------|-------------------------------------------|
|                  | Minus 5% FCS | Plus 5% FCS |
| Galactose        | 100          | 100         |
| N-Acetylglucosamine | 6           | 5            |
| Mannose          | 5            | 3            |
| Glucose          | 7            | 3            |
| Maltose          | 7            | 2            |
| None             | 4            | 6            |

Cells in Williams' medium E with or without 5% (vol/vol) fetal calf serum were allowed to bind to the flat polyacrylamide surfaces containing the indicated glycosides for 60 min at 37°C in the System II culture system (29). The culture surfaces were washed three times with 0.2 ml PBS, removed from the culture system, and put in 1 ml of 0.1 M potassium phosphate pH 7.0 containing 0.5% Triton X-100. The number of cells bound to the surfaces was then determined by a spectrophotometric assay to quantitate lactate dehydrogenase activity (35). The sugar content of the matrices used were all >88 mM.
Figure 1. Photomicrographs of cells cultured on galactose surfaces. Hepatocytes were cultured for 12 h and then photographed. The galactose concentrations of the culture surfaces were (A) tissue culture plastic; (B) 38 mM; (C) 58 mM; (D) 78 mM; (E) 115 mM; (F) 170 mM. The distribution of cell areas from this experiment is shown in Fig. 3. Bar, 10 μm.
mean area of cells spread on the substratum did not increase until the galactose concentration in the matrix exceeded ~30 mM (Fig. 2 A). The mean cell area then increased to a maximum of ~45% above 70 mM galactose. Below 30 mM galactose, the distributions of cell area (not shown) and the distribution of cell areas in a nonspread control population (Fig. 3 A) and a population spread maximally on tissue culture plastic (Fig. 3 F), we chose an area of 112.5 \mu m^2. This value was chosen because <3% of the nonspread control population was larger than 112.5 \mu m^2 (Fig. 3 A) whereas >96% of the population spread on plastic was larger (Fig. 3 F). This value is in the upper range of the nonspread distribution and the lower range of the fully spread distribution. Since the two distributions overlap only slightly (<7%), a cell can be classified with good reliability as spread or nonspread if its area is, respectively, greater or less than 112.5 \mu m^2. The percentage of the cell population with areas greater than this cutoff was plotted versus galactose concentration (Fig. 2 B). Regression analysis again indicated that the cell spreading data are better fit by an exponential rather than a linear function, supporting the conclusion that the response occurs as a threshold phenomenon at a critical concentration of galactose. The threshold for cell spreading occurred at ~30 mM galactose in the matrix. Below that concentration down to the binding threshold at 3 mM galactose, essentially no cells were spread, although they were bound to the substratum. There was a distinct separation between the two cellular responses; each occurred at a very different sugar concentration.

**Discussion**

Other investigators have studied hepatocyte adhesion and spreading on surfaces containing various proteins (6, 19, 34). Rat hepatocytes were able to spread on substrata containing fibronectin and native and denatured collagen but did not spread onto surfaces coated with asialoceruloplasmin (34), although this glycoprotein is recognized by the asialoglycoprotein receptor (4). The apparent difference between this result and the present study can be readily explained by the difference in the amount of immobilized galactose on the substratum. Higher concentrations of galactose can be obtained by the procedures used here (42) than by the adsorption of a large glycoprotein. The amount of galactose on these latter surfaces was probably below the critical concentration needed for cell spreading.

Threshold phenomena are extremely prevalent in chemical, biochemical, cellular, and organ processes, and they provide an important way to regulate and control biological processes. Threshold binding responses occur when cells or liposomes interact with a wide range of surfaces containing, for example, carbohydrates (5, 22, 27, 39), lectins (1, 21, 30), antibodies to cell surface proteins (17, 21), or extracellular matrix proteins (18). By their very nature, threshold phenomena lend themselves to exquisitely sensitive control since very small changes in the concentration of the molecule with which a cell interacts can mean the difference between an all or none response. A very small change in the concentration of the ligand can be viewed as an on/off switch. For example, only a 10–15% change in the sugar concentration of the matrix is necessary in order for hepatocytes to go from a nonbinding to a binding state or vice versa (39).

Several studies have shown that cell spreading on protein coated surfaces can also occur as a threshold response (2, 3, 18). However, it was not demonstrated to what extent the threshold responses for cell binding and cell spreading were independent. The system used here (29, 42) allows excellent control over the amount of galactose immobilized in the extracellular culture substratum. This has enabled us to demonstrate, to our knowledge for the first time, that the cellular
responses of binding and spreading on a carbohydrate surface can be clearly separated and controlled by varying the amount of immobilized carbohydrate.

The reason why these two cellular processes occur at different critical concentrations of galactose is unknown. One possible explanation is that the force (tension) generated between the substratum and the cell surface must attain a minimum value per unit surface area of contact in order for spreading to occur. This force would depend on the total energy of all receptor–galactose interactions within that area and may be significantly higher than that needed for simple cell binding. The required energy value might be obtained either by increasing the number of receptor–substratum interactions or by increasing the association constant of a constant number of interactions. Hardy et al. (14) recently proposed a model to explain how surface galactosyl receptors might be able to recognize different numbers and arrangements of galactose residues present in different oligosaccharide structures. They suggest that receptors form a matrix of binding sites that can bind a variety of structures with different affinities. Such behavior of the galactosyl receptor could explain how two different cellular responses involving different physical forces and energy requirements could occur as distinct threshold responses at different ligand densities in the substratum.

The synthetic cell culture system used here has potential advantages which may be exploited to study cell spreading and the interaction of cells with extracellular surfaces. For example, by varying the ligand concentration of the substratum, one might slow down or stop the overall process (e.g., a “freeze-frame” effect) and thereby allow transient intermediate stages in the spreading process to be identified and studied in more detail. The prevalence of large lamellipodia on cells spread on galactose surfaces (Fig. 1) might be explained by this effect.

We thank Dr. Robert LeBoeuf for very helpful discussion, Carolyn Chester for help with the tissue culture, and Betty Jackson for help in preparing the manuscript.

This work was supported by National Institutes of Health Grant GM 30218.

Received for publication 15 November 1985, and in revised form 7 May 1986.

References

1. Alecio, M. R., and R. R. Rando. 1982. Threshold effects on the lectin-mediated aggregation of liposomes: influence of the diameter of the liposomes. J. Membr. Biol. 67:137–141.

2. Aplin, J. D., and R. C. Hughes. 1981. Protein-derivatised glass coverslips for the study of cell-to-substratum adhesion. Anal. Biochem. 113:144–148.
threshold effects and receptor modulation. J. Cell Sci. 50:89-103.
4. Ashwell, G., and J. Harford. 1982. Carbohydrate-specific receptors of the liver. Annu. Rev. Biochem. 51:53-84.
5. Blackburn, C. C., and R. L. Schnaar. 1983. Carbohydrate-specific cell adhesion is mediated by immobilized glycolipids. J. Biol. Chem. 258: 1180-1188.
6. Bianget, P. R. 1983. Role of a surface glycoprotein in the control of protein-mediated attachment of adult hepatocytes. Exp. Cell Res. 147:479-481.
7. Curtis, A. S. G., J. V. Forrester, C. McInnes, and F. Lawrie. 1983. Adhesion of cells to polyurethane surfaces. J. Cell Biol. 97:1500-1506.
8. Grinnell, F. 1978. Cellular adhesiveness and extracellular substrate. Int. Rev. Cytol. 53:65-144.
9. Grinnell, F., and D. G. Hays. 1978. Induction of cell spreading by substrate-absorbed ligands directed against the cell surface. Exp. Cell Res. 1180-1188.
10. Grinnell, F. 1984. Fibroblast adhesion to fibrinogen and fibrin substrata: requirement for cold-insoluble globulin (plasma fibrinectin). Cell. 19:517-525.
11. Grinnell, F. 1984. Fibroblast spreading and phagocytosis: similar cell responses to different-sized substrata. J. Cell Physiol. 119;58-64.
12. Harper, P. A., P. Brown, and R. L. Juliano. 1983. Fibronectin-independent adhesion of fibroblasts to extracellular matrix material: partial characterization of the matrix components. J. Cell Sci. 63:287-301.
13. Hay, E. D. 1981. Cell Biology of the Extracellular Matrix. Plenum Publishing Corp., New York. 1-409.
14. Hardy, M. R., R. R. Townsend, S. M. Parkhurst, and Y. C. Lee. 1985. Different modes of ligand binding to the hepatic galactose/N-acetylgalactosaminoglycans: the surface of rabbit hepatocytes. Biochemistry. 24:22-28.
15. Hasegawa, K., and M. Koga. 1981. A high concentration of pyruvate is essential for survival and DNA synthesis in primary cultures of adult rat hepatocytes in a serum-free medium. Biomed. Res. 2:217-221.
16. Hawkes, S., and J. L. Wang. 1982. Extracellular Matrix. Academic Press, Inc., New York. 3-413.
17. Huang, L. 1985. Incorporation of acylated antibody into planar lipid multilayers: characterization and cell binding. Biochemistry. 24:29-34.
18. Hughes, R. C., S. D. J. Pena, J. Clark, and R. R. Dourmashkin. 1979. Differentiation of polyacrylamide gels containing copolymerized omega-acrylamido-glycosaminoglycan substrates by adherent fibroblasts. J. Cell Biol. 121:307-314.
19. Johannson, S., and M. Håk. 1984. Substrate adhesion of rat hepatocytes: on the mechanism of attachment to fibronectin. J. Cell Biol. 98:810-817.
20. Kemp, R. B., and J. R. Hinchliffe. 1984. Matrices and Cell Differentiation. Alan R. Liss Inc., New York. 1-622.
21. Krueger, K. E., and C. G. Heilerqvist. 1981. Studies on intercellular adhesion. Induction of adhesion by multivalent ligands. J. Biol. Chem. 256: 8553-8560.
22. Lar gent, B. L., K. M. Walton, C. A. Hoppe, Y. C. Lee, and R. L. Schnaar. 1984. Carbohydrate-specific adhesion of alveolar macrophages to mannose derivatized surfaces. J. Biol. Chem. 259:1764-1769.
23. Lee, R. T., S. Cascio, and Y. C. Lee. 1979. A simple method for the preparation of polycrylamidylate gels containing thioglycolic acid ligands. Anal. Biochem. 95:260-269.
24. Maroudas, N. G. 1975. Adhesion and spreading of cells on charged surfaces. J. Theor. Biol. 49:417-424.
25. McKeegan, W. L., and R. G. Ham. 1976. Stimulation of clonal growth of normal fibroblasts with substrate coated with basic polymers. J. Cell Biol. 71:727-734.
26. Nakamura, T., O. Asami, K. Tanaka, and A. Ichihara. 1984. Increased survival of rat hepatocytes in serum-free medium by inhibition of a trypsin-like protease associated with their plasma membranes. Exp. Cell Res. 155:81-91.
27. Oka, J. A., D. A. Ray, K. Hacker, and P. H. Weigel. 1984. The interaction of rat hepatocytes with synthetic galactoside culture surfaces. J. Cell Biol. 99 (4, Pt. 2):161a. (Abstr.)
28. Raja, R. H., and P. H. Weigel. 1983. Covalent immobilization of hyaluronic acid oligosaccharides, proteins and amino-molecules on synthetic culture surfaces. J. Cell Biol. 97 (5, Pt. 2):314a. (Abstr.)
29. Raja, R. H., M. Grissom, M. Herzig, and P. H. Weigel. 1986. Synthesis of cell culture surfaces containing covalently immobilized proteins, oligosaccharides and amino molecules on a non-ionic inert matrix. J. Biol. Chem. 261:8505-8513.
30. Rando, R. R., G. A. Orr, and F. W. Bangarter. 1979. Threshold effects on the concanavalin A-mediated agglutination of modified erythrocytes. J. Biol. Chem. 254:8318-8323.
31. Rauvala, H., W. G. Carter, and S.-I. Hakomori. 1981. Studies on cell adhesion and recognition. I. Extent and specificity of cell adhesion triggered by carbohdrate-reactive proteins (glycosidases and lectins) and by fibronectin. J. Cell Biol. 88:127-137.
32. Rauvala, H., and S.-I. Hakomori. 1981. Studies on cell adhesion and recognition. III. The occurrence of alpha-mannosidase at the fibroblast cell surface, and its possible role in cell recognition. J. Cell Biol. 88:149-159.
33. Rollins, B. J., M. K. Cathcart, and L. A. Culp. 1982. Fibronectin-proteoglycan binding as the molecular basis for fibroblast adhesion to extracellular matrices. In The Glycoconjugates, Vol. 3. Academic Press, Inc., New York. 289-329.
34. Rubin, K., S. Johannson, M. Håk, and B. Obrink. 1981. Substrate adhesion of rat hepatocytes. On the role of fibronectin in cell spreading. Exp. Cell Res. 135:127-135.
35. Schnaar, R. L., P. H. Weigel, M. K. Kuhlenschmidt, Y. C. Lee, and S. Roseman. 1978. Specific adhesion of chicken hepatocytes to polycrylamidylate gels containing N-acetylgalcosamine. J. Biol. Chem. 253:7945-7951.
36. Treidel, R. L. 1987. The Role of Extracellular Matrix in Development. Alan R. Liss Inc., New York. 1-622.
37. Turley, E. A., and S. Roth. 1979. Spontaneous glycosylation of glycosaminoglycan substrates by adherent fibroblasts. Cell. 17:109-115.
38. Weigel, P. H., E. Schmell, Y. C. Lee, and S. Roseman. 1978. Specific adhesion of rat hepatocytes to immobilized beta-galactosides linked to polycrylamidylate gels. J. Biol. Chem. 253:330-333.
39. Weigel, P. H., R. R. Schnaar, M. K. Kuhlenschmidt, E. Schnell, R. T. Lee, Y. C. Lee, S. Roseman. 1979. Adhesion of hepatocytes to immobilized sugars: a threshold phenomenon. J. Biol. Chem. 254:10830-10838.
40. Weigel, P. H. 1980. Characterization of the asialoerythropoietin receptor on isolated rat hepatocytes. J. Biol. Chem. 255:6111-6120.
41. Weigel, P. H. 1980. Rat hepatocytes bind to synthetic galactoside surfaces via a patch of asialoerythropoietin receptors. J. Cell Biol. 87:855-861.
42. Weigel, P. H., R. L. Schnaar, S. Roseman, and Y. C. Lee. 1982. Preparation of polycrylamidylate gels containing copolymerized omega-acrylamido-alkyl glycosides. Methods Enzymol. 83:294-299.