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INFORMATION ON MEMBRANES DERIVED FROM ELECTRICAL SURFACE CHARGE MEASUREMENTS

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For nearly a century it has been recognized that the surfaces of living cells bear a negative electrical charge. This observation forms the basis of several techniques, known collectively as "electrophoresis," in which an electric field is applied to a suspension of cells to cause their movement towards the positive electrode. The electrophoretic method has been widely used as a preparative procedure for achieving separations of heterogeneous mixtures of living cells with differing electric surface charge properties. A second application of electrophoresis, primarily during the past two decades, has been its use as an analytical tool for characterizing the chemical identity and the density of ionized groups at the cell surface. By using electrophoresis in conjunction with selective chemical modification techniques, such as the enzymatic cleavage of different molecular components of the plasma membrane, it has been possible in many instances to uniquely identify those biochemical moieties that lie at the outermost limits of the membrane. These superficial chemical constituents of the plasma membrane form an interface between the cell and its environment, and thereby serve as a chemical "signature" that determines many of the tissue-specific interactions and immunolo-

gical properties of cells. Several other chemical properties and associated biological functions of the cell surface coat have been revealed by electrophoretic measurements of surface charge. For example, it has been demonstrated in the author's laboratory that changes in the content of negatively charged carbohydrate components of the cell surface lead to the recognition and removal of old red blood cells from the circulation by macrophages in the spleen (J. Lab. Clin. Med. 89, 581-591 (1977)). Another application of electrophoresis in the author's laboratory has been to quantitate the binding of drugs such as chlorpromazine·HCl (thorazine) to the cell surface (Biochim. Biophys. Acta 511, 152-162 (1978)). In the present paper, consideration will be given to yet another application of electrophoresis that has been the subject of recent investigations, namely, the characterization of changes that occur in the surface properties of cultured fibroblastic cells when they are transformed by an oncogenic virus. Our focus in these studies has been both the characterization of changes in cell surface macromolecules following transformation, and the elucidation of the underlying cellular biochemical mechanisms that create these changes.

As a prologue to the discussion of changes in electrical surface properties of virally-transformed cells, it is worth noting some general characteristics of cultured cells after transformation with oncogenic chemical or viral agents. The defining characteristic of transformed cells grown in a culture system is their loss of sensitivity to cell contact phenomena, as indicated by the absence of density-dependent inhibition of cell division and cell movement. This behavior certainly implicates abnormal cell surface properties as an important factor in the phenotypic expression of the transformed state. In fact, many atypical surface properties of transformed cells have been documented, including reduced adhesiveness, fewer intercellular junctions, increased transport of nutrients across the plasma membrane, the appearance of new immunological markers (antigens)

at the cell surface, and major shifts in the macromolecular composition and "fluidity" of the membrane. These and other features of the transformed cell surface have been the subject of several thousand publications in the world's scientific literature from the late 1950's until the present time. Surprisingly few studies, however, have been devoted to the electrical surface properties of transformed cells. The type of electrical measurements reported in the remainder of this paper therefore occupy a somewhat unique place in the catalog of observations that have been made on the surface characteristics of transformed cells.

The system chosen for this study was cultured mouse fibroblasts transformed by Simian Virus 40 (SV 40), a small DNA virus that is a member of the papova family which also includes the polyoma and papilloma viruses. The total DNA content of the SV 40 virus is only 3.4×10^6 daltons, which corresponds to about 5000 nucleotide base pairs. This amount of DNA can encode roughly 170,000 daltons of protein, and in fact, it is known that only four proteins are produced by SV 40 virus particles. Three of these proteins are components of the virus coat, and the fourth is a protein called the "T antigen," which appears in the nucleus of infected cells and provides a convenient marker for transformation. In studies of cell surface chemistry, we have used three different lines of cultured mouse fibroblasts, and six lines of SV 40 transformed fibroblasts. The transformed cell lines exhibit a loss of contact inhibition of growth, and are able to achieve cell densities in culture that are approximately ten times above that of a confluent monolayer. In addition, the transformed cells contain intranuclear T-antigen markers (based on an assay procedure in which fluorescent antibody is bound to the T antigen), and the cells are capable of forming tumors when removed from cell culture and injected subcutaneously into recipient mice.

The technique that we have employed for measuring the electrical surface charge properties of these various cell lines is the microscope method of elec-

trophoresis, which was originally devised by Northrup and Kunitz and is commonly referred to as "microelectrophoresis." In this method, cells detached from culture dishes are placed as a single-cell suspension in a thin glass chamber (less than 1 mm in depth), which is in direct liquid contact with a pair of electrodes that provide an electric field of known strength (usually about 5 V/cm). The cells are viewed through a high-power microscope fitted with an eyepiece containing a calibrated reticule, and the rate of cell migration in response to application of the electric field is determined by stopwatch measurements of the time required to traverse a given distance (typically 15 to 30 μm). The rate of migration per unit field strength is known as the "electrophoretic mobility," and is frequently on the order of -1 (μm/sec)/(V/cm) for living cells in a physiological salt solution (the minus sign indicates that the cell bears a net negative charge, and thus migrates toward the anode). From theoretical considerations, the electrophoretic mobility can be shown to have a nearly linear relationship to the density of electrically charged groups located within a narrow 10 Å zone at the outermost reaches of the cell surface. Several reviews have been published that describe in detail the theoretical considerations underlying the calculation of cellular surface charge density from the measured electrophoretic mobility (D.A. Hayden, pp. 94-158 in Recent Progress in Surface Science, Vol. 1 (K.G.A. Pankhurst and A.C. Riddiford, editors), Academic Press, N.Y., 1964; J.Th.G. Overbeek and P.H. Wiersema, pp. 1-52 in Electrophoresis, Vol. 2 (M. Bier, editor), Academic Press, N.Y., 1967; T. Tenforde, pp. 43-105 in Adv. in Biol. and Med. Phys., Vol. 13 (J.H. Lawrence and J.W. Gofman, editors), Academic Press, N.Y., 1970).

Microelectrophoretic measurements have indicated that there is a close similarity in the surface charge properties of different lines of cultured mouse fibroblasts. The measured electrophoretic mobilities range from -1.01 to -1.03 (μm/sec)/(V/cm) for the 3T3 fibroblast cell lines derived from both Swiss and Balb

mice by G. Todaro, as well as for an embryonic fibroblast line derived in the author's laboratory from a Balb mouse. From these mobilities, the calculated surface charge density is close to 0.02 coulomb/m^2, which, when translated into more obvious units, corresponds to one negatively charged group per 785 $Å^2$ of cell surface. If these charges are uniformly distributed, the center-to-center spacing is 28 Å.

The surface charge densities of six different SV 40 transformed cell lines was found to be reduced relative to their non-transformed counterparts. Also, the transformed cell lines were observed to be quite heterogeneous in their surface charge properties as demonstrated by mobilities ranging from -0.76 to -0.94 (μm/sec)/(V/cm). The lowest mobility, -0.76, corresponds to one charged group per 1090 $Å^2$. The transformed cell line with this low charge density was originally derived by G. Todaro from a Balb mouse fibroblast culture, and denoted as the SV-T2 cell line. For simplicity, the data presented in the remainder of this paper will be restricted to SV-T2 cells and to their non-transformed 3T3/Balb counterparts.

Having established that SV 40 transformation leads to a reduced density of negatively charged groups at the fibroblast surface, the next step in our investigation was to identify the biochemical groups responsible for this membrane alteration. The first set of experiments involved the measurement of cellular surface charge as a function of the pH of the suspending medium, thus giving the electrophoretic equivalent of an acid-base titration curve. From a comparison of the surface charge versus pH characteristics for 3T3 and SV-T2 cells, we determined that the transformed cell surface had a substantially lower density of strongly acidic groups ($2 \lesssim pK_a \lesssim 4$). The next step in the process of identifying these chemical moieties was to use enzymatic procedures to selectively remove various constituents of the membranes of 3T3 and SV-T2 cells, and then to investigate their resulting surface charge characteristics. Through this approach, we discovered that the surface charge densities of 3T3 and SV-T2 cells were reduced to a nearly

identical level following treatment with the enzyme trypsin, which cleaves acidic glycoproteins (protein-carbohydrate complexes) from the cell surface. More specifically, the surface charge density of 3T3 cells was reduced by 42% following trypsin treatment, whereas that of SV-T2 cells was reduced by only 30%. The remaining difference in the surface charge densities of trypsin-modified 3T3 and SV-T2 cells was 0.13×10^{-2} coulomb/m^2, as compared with a 0.55×10^{-2} coulomb/m^2 difference for the native (unmodified) cells. From these numbers, it can be estimated that 75% of the acidic chemical groups responsible for the difference in surface charge properties of 3T3 and SV-T2 cells are associated with trypsin-susceptible glycoproteins at the cell surface.

From previous biochemical studies on the composition of membrane glycoproteins, we strongly suspected that a large fraction of the charge-determining groups removed from the surfaces of 3T3 and SV-T2 cells by trypsin treatment were sialic acid residues. These molecules are 9-carbon sugars that contain a very strongly acidic carboxyl group ($pK_a \approx 2.6$), and constitute a major fraction of the carbohydrate component of cell surface glycoproteins. In order to evaluate the role of sialic acid molecules as determinants of the surface charge properties of 3T3 and SV-T2 cells, we measured the electrophoretic mobilities of both cell lines following treatment with the enzyme neuraminidase, which selectively cleaves sialic acid residues from cell surface glycoproteins. The results of this experiment conformed closely to our expectation, insofar as neuraminidase reduced the surface charge densities of 3T3 and SV-T2 cells by nearly the same amount as trypsin. The total charge reduction was 10 - 25% less than with trypsin, but nevertheless, the observed effects of neuraminidase clearly indicated that a difference in the quantity of sialic acid residues at the surfaces of 3T3 and SV-72 cells is primarily responsible for the difference in their electrical charge properties.

The next aspect of this study--and in many ways the most challenging aspect--was our attempt to determine the underlying cellular biochemical mechanism that leads to an alteration in fibroblast surface charge properties following SV 40 transformation. After chasing down several blind alleys, our attention turned to the potential role of cyclic 3' : 5'-adenosine monophosphate (cAMP). This small molecule, which has been termed a "second messenger," serves to regulate a wide variety of key phosphorylation reactions in the synthesis and metabolism of cellular proteins, lipids and nucleic acids (see N.D. Goldberg, pp. 185-201 in Cell Membranes (G. Weissmann and R. Claiborne, editors), H.P. Publ. Co., N.Y., 1975). It is termed a second messenger because its production within the cell is triggered by the binding of hormones such as epinephrine to the cell surface. Work performed in other laboratories had shown that the cAMP levels in SV 40 transformed cells are substantially reduced relative to non-transformed fibroblasts; furthermore, several investigators had reported that elevation of the intracellular cAMP level produced a phenotypic reversion in the surface properties of transformed cells towards those observed for normal fibroblasts. For example, evidence was obtained that cAMP elevation leads to an increased amount of acidic glycoprotein and a decreased number of tumor-specific antigens at the surface of transformed cells. It was also reported that transformed cells with elevated cAMP were more adhesive and grew in culture as flatter cells that were morphologically similar to non-transformed fibroblasts.

Starting from these observations, we began a series of experiments in which the intracellular cAMP content of SV-T2 cells was chemically manipulated by several well-established methods. In the first experiment, SV-T2 and 3T3 cells were grown for three days in a medium containing both cAMP (in its dibutyryl form) and theophylline (1,3-dimethylxanthine), which is a potent inhibitor of the cyclic nucleotide phosphodiesterase enzymes that metabolize cAMP within the cell. When microelectrophoresis was performed on these cells, it was observed that the negative surface charge density of 3T3 cells was unaffected by the presence of cAMP

and theophylline in the growth medium, but that the charge density at the surface of SV-T2 cells had risen to a level identical to that of the 3T3 cells. The same experimental observation was made for SV-T2 cells grown in the presence of theophylline alone, i.e., without added cAMP. Finally, an elevation of SV-T2 surface charge density to the same level as that of 3T3 cells was also observed when the growth medium contained either glucagon or L-epinephrine (adrenalin), both of which serve as hormonal stimulants of intracellular cAMP production.

In summary, the general picture emerging from these studies is that SV 40 transformation leads to a reduced content of acidic glycoproteins at the surface of mouse fibroblasts, and that this biochemical alteration may be a consequence of a depression in the intracellular level of cAMP. An important extension of these experiments will be to thoroughly evaluate the full spectrum of abnormal cell surface properties that result from altered cyclic nucleotide levels following transformation.