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Abstract

In vitro cell transformation of human embryo cells could be induced by the DNA purified from SV 40. The result shows clearly that cell transforms a part of viral DNA into the genome. In addition, for the purpose of clarifying th∼ biological differences between the normal and transformants the alteration of cell membraneous structures of transformants (hamster and mouse fibroblasts) were observed from mechanism of phagocytosis. The iron colloid particles are taken up by normal diploid fibroblasts but not by the human and hamster transformants. This fact suggests a differ∼nce in the molecular arrangement of the cell membranes between the normal and transformants. In the presence of histones, however, the transformants phagocytize the colloid particles very actively. The results show that cell membranes of transformants are altered in the molecular structure r∼sponsible for the surface charge. In addition, there is no remarkable quantative differences of sialic acids on the cell surfaces of non-malignant and malignant transformants so that phagocytic activity might be correlated to the alteration of molecular composition of cell membrane itself rather than of cell surfaces, i. e, sialic acids.

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MALIGNANT CELL TRANSFORMATION BY THE SV-40 DNA AND PHAGOCYTIC ACTIVITY RELATED TO ALTERATION OF CELL MEMBRANES

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The malignant transformed cell is characterized by alterations of the cell surface presumably leading to a loss of phagocytic activity, i. e, Ehrlich ascites tumor cells and transformant induced by SV 40 (1) (2). It is possible that these changes on the cell surface are responsible directly or indirectly for the loss of the regulatory restraint on DNA synthesis which characterizes the normal phagocytic cell. Thus, changes of cell surface might give some insight into the events leading to malignant cell transformation.

Antigenic changes of the cell surfaces, i. e, transplantation of antigens, has been reported by KLEIN and others (3, 4). It seems important, therefore, to determine whether any bilogical differences in components of cell membranes can be disclosed between normal and virus-transformed cells.

The differences between normal and transformed cells in electrostatic charge and acid mucopolysaccharides are also important. In the previous investigations (1, 2) it has been revealed that phagocytic activity relates to electrostatic charge on cell surface and electrostatic charges of the cells closely correlated to the specific carbohydrate sequences in an oligosaccharides chain (5) and hydrophobic lipid residues. Substances may adhere to the cell surface which may influence the electro-negative surface charge or may be solely taken by the cell. Thus, the changes in molecular structure of membranes of transformed cells may be explained by a modification of the mechanism of phagocytic activity in normal and transformed cells. Precise obserrations of human, hamster and also other mouse fibroblast cell lines transformed by SV 40 and SV 40 DNA, indicated loss or decrease of phagocytic activity. In addition, to allow

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more clear interpretation, electron microscopic observation was extended to compare and analyze acid mucopolysaccharides on the cell surface of normal, spontaneously transformed cells and viral transformed cells. As a result it was found that acid mucopolysaccharides of the cell membranes were digested with the proteolytic enzymes, trypsin, neuraminidase and chondroitinase ABC, and possible mechanisms by which these changes in the composition of cell membrane, particularly glycoproteins were observed by electron microscope.

MATERIALS AND METHODS

SV 40 strain, its propagation, purification and biological activities: SV 40-777 was supplied by Dr. SHIMOJO, National Institute of Health, Tokyo, Japan. For propagation of the virus, Vero cell line was obtained through the courtesy of Dr. Ito of Toshiba Chemical Industry (6). The cells were cultured with McCoy 5A (7) supplemented with 20 per cent calf serum (penicillin 100 units/ml and streptomycin 100 μg/ml).

For purification 20 ml of concentrated crude virus suspension obtained after 1-week propagation (TCID 50 10^8) was layered gently over 9 ml of saturated KBr solution and centrifuged at 25,000 rpm for 3 hours. The virion preparation thus obtained was further separated into two fractions by CsCl density gradient centrifugation in SW 39 rotor of a Spinco Model L at 35,000 rpm for 20 hours. Fractions were collected and 1 ml of phosphate buffered saline was added to each fraction.

Viral DNA: The extraction and biological assay; For the extraction of viral DNA the purified virions were dialysed against saline citrate (0.15 M NaCl, 0.015 M sodium citrate) and incubated at 37°C for 2 hours in the presence of 0.1 mg/ml pronase P, by the method of YOSHIIKE (8, 9). From this material DNA was isolated by extracting with phenol three times. Phenol was removed by dialysis against 0.14 M NaCl, 0.01 M phosphate buffer pH 7.3. Biological assay of DNA was made by the procedure as described in the previous reports (10)(11). With the DNA samples obtained, sensitivity to DNase and heat resistance were tested. The DNA samples completely lost its infectivity by the treatment with DNase at 37°C, for 20 min, which caused no reduction in infectivity of complete virus.

Cells used for transformation: The cells from human embryo (6 weeks old), golden syrian hamster embryos, their kidneys, and mouse embryos (ddN mice) in primary culture were used for the observation of cell transformation. The embryo cells were grown in McCoy 5 A supplemented with 20 per cent calf serum respectively. Cells, grown on the glass, were infected with DNA and SV-40 complete virus particles. After 2 hours' adsorption at 37°C, the medium was discarded and then fresh medium added. Cells grown on coverslips of Leighton tubes were stained with Giemsa and hematoxylin-eosin for morphological observation. Immunofluorescent antibody reaction for tumor antigen was also examined by the indirect method.

Phagocytosis of colloidal iron: For this purpose the cells were given colloidal iron particles in the form of iron chondroitin sulphate (Cs-Fe) in culture media. Cs-Fe which had been supplied by Dainippon Seiyaku Co. Osaka, Japan, was a colloidal solution, pH 7.21 and 1 ml of solution contained 4 mg of iron. The size of the iron colloidal particles estimated by electron microscopy ranged from about 30 Å in the smallest unit to about 200 Å in the aggregated group particles. The colloid particles in Hanks' sulution was of negative charge as revealed by the paper electrophoresis in veronal-acetate buffer pH 7.4. Iron was combined so firmly with chondroitin sulphate that the two components were hardly separable by electrophoresis. The iron colloid solution was added to the culture media, in a 1 : 3 volume, in the Leighton tubes in which the cells had been grown on the coverslips, and 1, 2, 3, 12, 24, 48 and 72 hours after the addition of iron colloid, the cells were washed by Hanks' solution, dried, fixed with ethanol, stained by PERLS' method for iron and Kernechtrot, and observed under light microscope.

For the observation of the effect of polycation on the phagocytic activity, histones were used. These were extracted from calf thymus by the modified methods of JOHNS (12) and UI (13). Two fractions, arginine-rich and lysine-rich histones were used. Besides these, histone type II (Sigma Co.) was also used. These were added to the culture medium at the respective concentration to observe changes in phagocytic vacuoles.

Enzymic treatment for identification of acid mucopolysaccharides: To identify acid mucopolysaccharides on the cell surfaces, cells of non-malignant and malignant transformants were treated by protease and acid mucopolysaccharidase and then observed with electron microscope comparatively. One part of each cell was treated with neuraminidase 0.5 or 1.0 mg/ml (Sigma, from Cl. perfringes, purified, type V) or cholera filtrate 1.0 mg/ml (Sigma, from Vibrio cholera) in Hanks' solution at 37°C for 1 hour after pre-washed with Hanks' solution. Another part was pre-washed with Tris-Hanks'* solution and then treated with chondroitinase ABC 0.1 or 0.25 units/ml (Seikagaku Fine Biochemicals) in Tris-Hanks' solution at 37°C for 1 hour. Some samples of each cell were treated with neuraminidase and then chondroitinase ABC, or chondroitinase ABC and then neuraminidase. The cells treated in Hanks' or Tris-Hanks' solution containing no enzymes at 37°C for 1 hour served as the controls. After treating with the enzymes these cells were washed by the same media used in the pre-washing, fixed with 1.25 per cent glutaraldehyde in Milloning's (1961) phosphate buffer pH, 7.4 in cold for 30 minutes and rinsed with the buffer.

Before the fixed cells were stained with the colloidal iron solution for acidic carbohydrates, they were washed with distilled water and then 30 per cent acetic acid. The staining method was principally the same as that of MOWRY (1963). After staining they were washed with 30 per cent acetic acid, distilled water and the phosphate buffer. Then they were post-fixed with 1 per cent OsO_4 in cold for 1 hour, dehydrated with ethanol series and embedded in epoxy resin through propylene oxids. Thin sections were cut with a Porter-Blum MT-1 microtome,

* Tris-Hanks' solution used does not contain Ca, Mg and phosphate, and is buffered at pH 7.4 with 0.002 M Tris-HCl.

stained with an alkaline lead solution and observed under a Hitachi HU 11 A electron microscope.

RESULTS

The human embryo cells in primary culture were exposed to the viral DNA. Three months after the addition of DNA, some colonies were found to show some morphologic changes with malignant transformation. The transformed cells showed the appearance of epitheloid cells, some of which were of multinuclear giant cells (Photo. 2) and lost their contact inhibition. Cell transformations were also observed on cell lines from hamster and mouse embryo cells, both of which were homogenous fibroblasts. Immunologic tests revealed that the transformed cells had specific tumor antigen.

Prior to the observation on phagocytic activity related to properties of cell membranes of these cells toxicity of histones was checked, because the histones have inhibitory activities of DNA synthesis in cells. The toxicity tests of histones were solely made on the transformed hamster cells. Experiments revealed that an excess of histone in the culture medium suppressed the growth or cell division of the transformed cells: *i. e.*, the arginine-rich histone in the concentration of 2 μg to 100 μg/ml gave no inhibitory effect on the growth of the cells, but at the concentration of 250 μg/ml the histone suppressed the growth of the transformants. At the concentration of 450 μg/ml the division or growth of the transformants was completely stopped (2).

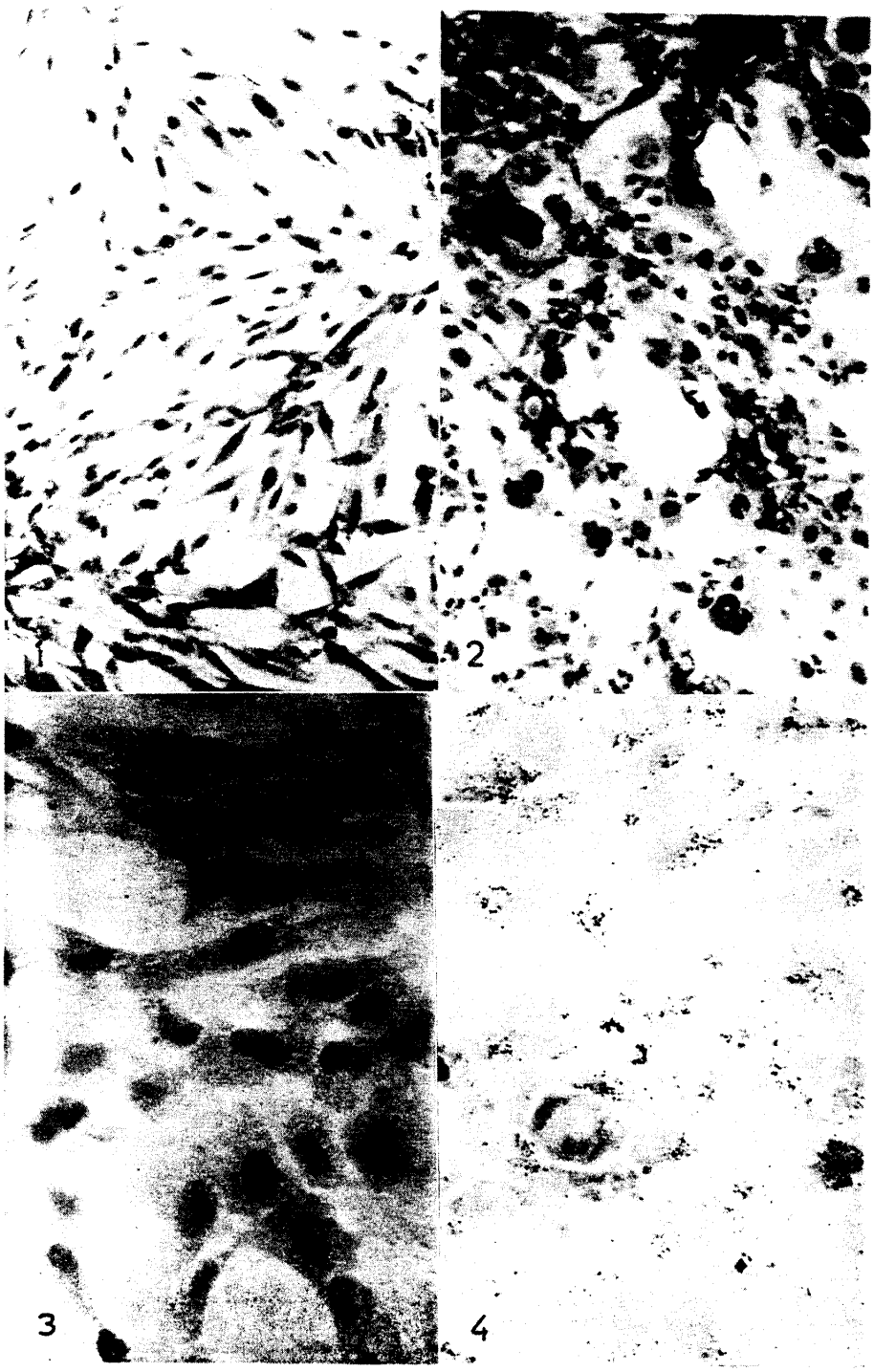
Referring to the data just mentioned, the effect of the histones on the phagocytosis of the cells to iron colloid was observed at the concentration of 100—250 μg/ml. By 24-hour cultivation with the Cs-Fe non-malignant fibroblasts of human, hamster and mouse, phagocytized the colloidal iron particles moderately, but the transformed fibroblasts of human and hamster showed no phagocytic activity as revealed by PERLS'

Photo. 1. Non-malignant human cell lines (fibroblast). Giemsa stain, 4th passage. 10×20

Photo. 2. *In vitro* human transformed cell lines induced by DNA purified from SV 40. 15th. passage. Giemsa stain. 10×20

Photo. 3. Human SV 40 DNA transformed cells cultivated with colloidal iron in culture media for 24 hours. No iron particles are found in any transformants. Perls' reaction and Kernechtrot stain. 15th. passage. 10×40

Photo. 4. Human SV 40 DNA transformed cells (15th. passage) cultivated with colloidal iron and histone (100 μg/ml) in culture media for 24 hours. Transformants show remarkable phagocytic activities containing a numerous colloidal particles in their cytoplasm. Perls' reaction and Kernechtrot stain. 10×20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reaction (Photo. 3). But mouse (ddN strain) viral transformants and spontaneous transformants of fibroblasts phagocytized colloidal iron particles considerably as compared with human and hamster transformed fibroblasts.

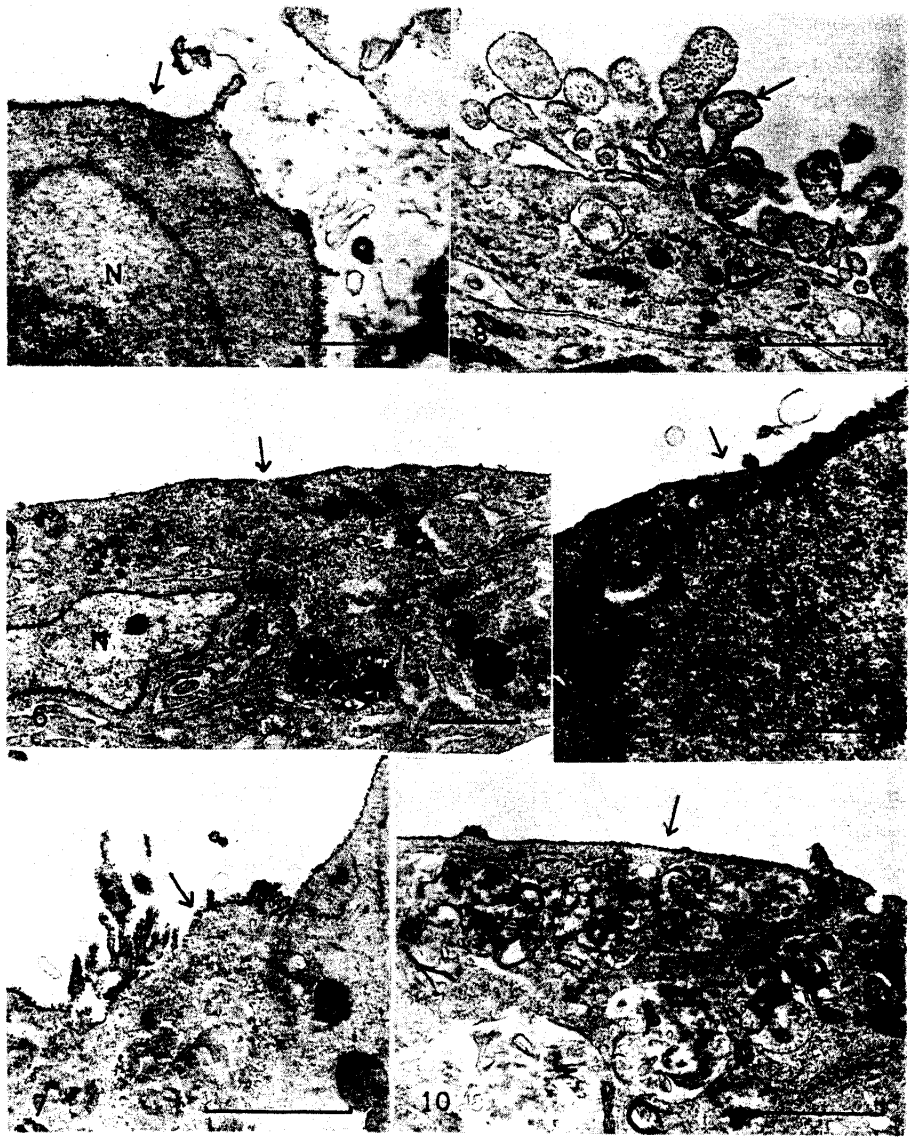
While in the presence of histones as polycation, the human and hamster transformants cultivated with Cs-Fe for 24 hours showed a marked phagocytic activity containing numerous colloidal particles in their cytoplasms (Photo. 4). Electron microscopic observation of these transformants cultured with the iron colloid and histones revealed that the transformed cells ingested a number of iron colloid particles of about 200 Å in diameter by the 24 and 48-hour cultivation. In transformants of human and hamster cells a massive accumulation of the colloid particles was seen in their phagocytic vacuoles (1).

As shown in Table I, acid mucopolysaccharides localized on the cell membranes were observed by colloidal iron stain post-treatments of protenases and mucopolysaccharidases. According to results of enzymic treatment non-malignant, SV 40 transformed hamster fibroblasts (Photo. 5) as well as transformed mouse fibroblast (Photos. 9, 10) and Ehrlich actites tumor cells possessed presumably acid mucopolysaccharides without digestion by trypsin. But these colloid iron stainable substances on the cell surface of hamster fibroblasts could be digested with neuraminidase (Photo. 6) or neuraminidase supplemented with chondroitinase ABC in contrast to mouse fibroblasts (Photos. 9. 10). On the other hand, by treatment of chondroitinase ABC the colloid iron stainable substances were not digested by these enzymes (Photos. 7. 8). So substances on the cell surfaces of hamster fibroblasts were presumably sialic acids but cell

TABLE 1. COLLOIDAL IRON STAIN FOR ACID MUCOPOLYSACCHARIDES

	Vero	Hamster		Mouse		
		Control cell	SV 40 transformant	3T3	SV 40 transformant**	Ehrlich ascites tumor cell
Washed with Hanks' solution	+	+	+	+	+	+
Washed with Ca, Mg-free, Tris-buffered Hanks' solution		+	+		+	+
Treated with trypsin (Merck)		+	+		+	+
Treated with neuraminidase (purified, from Cl. perfringes,)		−	−		+	−
(crude, Vibrio cholera filtrate,)		−	−		+	±
Treated with chondroitinase ABC (Seikagaku Kogyo)		+	+		+	+
Treated with neuraminidase, then, chondroitinase ABC		−	−		+	−

** ddN mouse strain.



Electron microscopic findings of cell surfaces.

Photo. 5. Hamster SV-40 transformed cell. Iron stainable substances (↓) on the cell surface of the hamster transformant. The substances are not digested with trypsin.

Photo. 6. Hamster SV-40 transformed cell. The colloid iron stainable substances on the cell surface are digested with neuraminidase (↓). (1.0mg/ml). N: Nucleus.

Photos. 7 and 8. Hamster SV-40 transformed cells. The colloid iron stainable substances on the cell surfaces are not digested with chondroitinase ABC (0.25 units/ml) (↓).

Photo. 9. Mouse SV-40 transformed cell. The colloid iron stainable substances on the cell surface are not digested with neuraminidase (1.0mg/ml) (↓). N; Nucleus.

Photo. 10. Mouse (ddN strain) SV-40 transformed cell. The colloid iron stainable substances on the cell surface are not digested with chondroitinase ABC (0.25units/ml) (↓). P: Phagosome.

surfaces of mouse fibroblasts were not composed of sialic acids. There is no difference in the quantity of sialic acids between normal and transformed hamster fibroblasts by electron microscopic observation.

DISCUSSION

In the previous investigation it was reported that fibrosarcoma, hemangioma, and intestinal carcinoma could be induced by partially purified DNA from SV 40 (10, 11). The present investigation demonstrated that human cells were also transformed into malignant cells *in vitro* by viral DNA purified from SV 40. These facts indicate that the oncogenicity of virus is the viral DNA itself and presumably the viral DNA is incorporated into the genome of the host cell, resulting in alteration of the cell characteristics.

Just as demonstrated, the cells transformed by viral DNA and virus itself acquire the characteristic properties of antigenicity in cell membranes and nuclei. On the cell surfaces they have the surface antigen, and specific tumor antigen in the nucleus: these features are thought to be specific to malignant cells. Recently, it is also believed that the surface change of the cell is altered by the malignant transformation, which should be closely correlated to the change in molecular architecture. ABERCROMBIE (14) and AMBROSE (15) elucidated that by malignant transformation the negative surface charge increases compared to non-malignant cells from which the tumor cell was derived. Recently, our investigations and co-workers' (16)(17) have revealed that the phagocytic activity of the cell is related to the charge of the cell surface, demonstrating that the adsorption of a particle to the cell surface which is largely dependent on the charge of the cell surface and the phagocytizing particles, is the first and essential step of phagocytosis. The engulfing of the cytoplasm at the site of the adsorption of the particles should be formed by the local lysis of molecular architecture understood as cooperative phenomena in the terms of high molecular physics. For instance, Ehrlich ascites tumor cells, which do not phagocytize the negatively charged metal colloid particles, show a striking phagocytic activity of the colloidal particles in the presence of histones and other polycation (1) (16) (17). On the other hand, macrophages present in the Ehrlich tumor ascites phagocytize the metal colloid particles very actively. But it has been noted that each of iron colloid particles adheres to the cell surface with a certain distance but not covering the whole surface area. Macrophage should have positively charged specific groups at a certain distance on the cell suface to adsorb negatively

charged colloid particles, though the charge of the cell may be negative as a whole. Thus the differences in phagocytic activity between macrophages and tumor cells in ascites directly indicate the difference of molecular structural arrangement in the surfaces of these two different cells.

On the basis of these findings of the surface charge related to structures and phagocytic mechanism a similar phenomenon was observed in normal diploid fibroblast and their transformants induced by viral DNA and virus itself, particularly in human and hamster cell lines. By 20- or 24- hour incubation with the iron colloid particles the non-malignant fibroblast phagocytized the iron particles actively but the transformant did not. In the presence of arginine-rich histones, 100 or 200 μg/ml, which gave no suppressing effect on cell growth, but the transformant phagocytized the colloid particles very actively (Photo. 4).

The facts indicate definite changes to have occurred in the molecular structure of the cell surface or membrane by the transformation. Such a phenomenon should be induced by the decrease of the positively charged group or by the increase of negatively charged group, which may keep the negatively charged particles away or both. Thus evidence has been presented to support the observation that phagocytic activity gives important informations for the changes of the cell membranes of their molecular architecture in connection with malignant transformation. On the basis of the difference in phagocytic activities between non-malignant and malignant fibroblasts, acid mucopolysaccharides on the cell surfaces, which may keep negative charge, were discussed by enzymic digestion and electron microscopical observation. As shown, acid mucopolysaccharides are localized on the cell surfaces of non-malignant and transformed fibroblasts of hamster, mouse and also Ehrlich ascites tumor cells. No definite quantitative differences of mucopolysaccharides of these cells were observed by electron microscopy either. To identify these substances on the cell surfaces the cholera filtrate (crude neuraminidase), purified neuraminidase (Type. V), chondroitinase ABC, and neuraminidase plus chondroitinase ABC were used.

As a result iron stainable substances were degradated by neuraminidase only in hamster cells. This result indicates clearly that acid mucopolysaccharides on the surface of hamster cells contain at least sialic acids. In contrast to such hamster cell surfaces, the iron stainable substances seem to be associated with negatively charged substances other than sialic acids in mouse cells, which are not degradated by treatment with neuraminidase.

The first possibility of loss of phagocytic activity in transformants is

an increase in negatively charged substances on the membranes or cell surfaces, which might be sialic acids and correlated to phagocytosis. But the results suggest that the composition of acid mucopolysaccharides of hamster differs from that of mouse transformants which are decreased in phagocytic activity in spite of the fact that the acid mucopolysaccharides of the mouse cells are not degradated by neuraminidase treatment. In addition, Ehrlich ascites tumor cells phagocytize no Cs-Fe even after treatment with neuraminidase. These results suggest that the loss or decrease of phagocytic activities of transformants is not directly correlated to the increase of sialic acids on the cell surfaces. Therefore, other negatively charged substance, *i. e*, phosphate or glycolipid composed of cell membrane itself, also might be correlated to the decrease in phagocytic activity. Concerning sulfate it may be chondroitin sulfate but not likely to be related to the composition of negatively charged group on the cell surfaces by electron microscopic observation after the treatment with chondroitinase ABC (Photos. 7. 8. 10). Such differences in characteristics of cell surfaces in the fibroblasts of both mouse and hamster cells also suggest that sialic acids on the mouse cell surfaces might not be effected by neuraminidase because of a kind of barrier to this enzyme.

The second possibility of loss of phagocytic activity in malignant transformants might be explained that the cell surface of non-malignant cells has specific properties of affinity to Cs-Fe as compared to defective affinity of malignant transformants. In spite of differences of sensitivity to neuraminidase in mouse and hamster, loss or decrease of phagocytic activity might support this assumption. HAKOMORI (5) (18) and WU (19) *et al.* made reports of the comparative studies on the carbohydrate-containing membranes of normal and viral transformed fibroblasts of mouse cell lines (3T3 cell lines). Their results suggest that the composition of glycoprotein and glycolipids in the membrane of SV-40 transformed cell is relatively low in N-acetylneuraminic acid and galactosamine and high in glucosamine as compared with the composition of normal mouse fibroblasts. Our electron microscopic observations also show that there is no remarkable quantitative differences of sialic acids on the cell surfaces of non-malignant and malignant transformants. Now, the change of phagocytic activity will be explained by alteration of molecular composition of cell membrane itself rather than of cell surfaces, *i. e*, sialic acids.

CONCLUSION

In vitro cell transformation of human embryo cells could be induced

by the DNA purified from SV 40. The result shows clearly that cell transforms a part of viral DNA into the genome. In addition, for the purpose of clarifying the biological differences between the normal and transformants the alteration of cell membraneous structures of transformants (hamster and mouse fibroblasts) were observed from mechanism of phagocytosis. The iron colloid particles are taken up by normal diploid fibroblasts but not by the human and hamster transformants. This fact suggests a difference in the molecular arrangement of the cell membranes between the normal and transformants. In the presence of histones, however, the transformants phagocytize the colloid particles very actively. The results show that cell membranes of transformants are altered in the molecular structure responsible for the surface charge. In addition, there is no remarkable quantative differences of sialic acids on the cell surfaces of non-malignant and malignant transformants so that phagocytic activity might be correlated to the alteration of molecular composition of cell membrane itself rather than of cell surfaces, *i. e*, sialic acids.

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