STUDIES ON THE EFFECT OF SELECTIVE MEMBRANE FILTRATION ON THE DIFFERENTIATION OF TISSUE CULTURES

M. KUMEGAWA, T. NAKAMURA, and T. YAMADA. From the Department of Oral Anatomy and the Department of Internal Medicine, Josai Dental University, Saitama, Japan

INTRODUCTION

Rose's circumfusion system for multipurpose culture chambers favors the cultivation of fetal tissues and organs in a highly differentiated state for protracted periods (1–5). This result is achieved by rapidly recirculating a highly oxygenated and pH-controlled nutrient through each of 12 fluid-integrated chambers containing cultures established by the cellophane-sheet method (6). The culture microenvironment formed between a cover slip and a sheet of cellophane is continuous with the circulating nutrient through the small pores in the cellophane (24 Å) which only permit a transit between these two compartments of a dialysate of the nutrient. Rose (1) described 3 advantages of cellophane membranes: (*a*) they prevent the entrance of large molecular components of the nutrient into the culture microenvironment; (*b*) they prevent the washing out of tissue products of high molecular weight, and (*c*) they compress the tissues onto the cover glass.

Since it was uncertain whether the dialysate of the cellophane-filtered nutrient was wholly sufficient for the explanted tissues, experiments were planned to investigate the relation of the culture results to a variety of membrane pore sizes. Besides the morphological observations, four key enzymes for carbohydrate metabolism in liver explants were assayed and correlated with the levels of differentiation observed in vitro. The activity of glucokinase to hexokinase increases with the differentiation of liver in vivo. Therefore, these enzymes are useful markers in studying the differentiation of cultured liver. Glucose-6-phosphate dehydrogenase is one of the marker enzymes for anabolic activity in tissues, and pyruvate kinase activity parallels anaerobic glycolysis. Cellular activities, therefore, can be estimated further by analyzing the activities of these enzymes.

MATERIALS AND METHODS

Liver, rib, and salivary gland (submandibular and sublingual) cultures of 14–15-day old fetal mouse were established in multipurpose culture chambers, as reported earlier (1). The effect of five kinds of transparent membranes was studied (Table I). Three circumfusion systems, each containing 15 chambers, were used in the experiments; five chambers with different membranes were used for each of the three tissues. The circumfusion systems used 400 ml of medium 199 supplemented with calf serum (15%). Half of nutrients was exchanged once a week.

The morphological characteristics of differentiation of the living cells and tissues were observed by phase-contrast microscopy. After 2 wk, the cultures were terminated. The salivary glands were stained with periodic acid-Schiff method (PAS) (after diastase digestion) for mucopolysaccharide; the salivary glands and livers were prepared with thin-section procedures and were observed with an electron microscope; and the ribs were fixed on cover slips in phosphate-buffered glutaraldehyde, stained 2–4 h in toluidine blue (0.1% in 30% alcohol), dehydrated, cleaned, and whole-mounted for light microscopy. The differentiation of the rib cultures was investigated for chondroitin sulfate by its metachromasia.

The tissue cultures were removed from the chambers and the activities of hexokinase and glucokinase were assayed by the method of Dipietro and Weinhouse (7); glucose-6-phosphate dehydrogenase by the method of Glock and McLean (8); and pyruvate kinase by the method of Bücher and Pfleiderer (9). 1 U of enzyme was defined as that amount which caused a formation of 1 μmol of product/min. The content of protein was determined by the method of Lowry et al. (10), with the use of human serum albumin as the standard.

RESULTS

Table I illustrates the morphological results of the three tissues and organs cultured on cover glasses under five different membranes in chambers of the circumfusion systems. The results were the same for all three, i.e., the cells of the flattened explants and outgrowth retained their specific differentiated characteristics. Reconstructed cellulose SM 11533 was the most favorable for the maintenance of tissue and organ differentiation. With the exception of SM 11530 (pore size > 70,000 mol wt), the larger the pore size the more favorable were the conditions for culture differentiation. The tissues cultured with the SM 11530 membranes lost their structure and became ball-like masses after several days of cultivation. However, fibroblasts did not

emigrate from explants cultured with the SM 11530 membranes any more than from those cultivated with the other membranes.

Activities of the four key enzymes for carbohydrates metabolism in mouse liver cultured with the five different membranes are shown in Table II. The glucokinase, which is one of the marker enzymes in mammalian adult liver, was the highest in specific activity in those liver explants cultured under SM 11533. However, activity was not detected in livers cultivated under SM 11530. The values of the ratios of glucokinase to hexokinase in livers cultured under the various membranes were in good agreement with the morphological observations. Mouse livers cultured under cellulose acetate and SM 11533 contained high glucose-6-phosphate dehydrogenase and pyruvate kinase activity, whereas those livers cultured under the remaining three membranes showed low glucose-6-phosphate dehydrogenase and pyruvate kinase activity.

Thus, among the five membranes used for cultivating these fetal tissues and organs in circumfusion systems, membranes of SM 11533 and cellulose acetate were the most favorable for both functional and morphological differentiation.

TABLE I

Morphological Results of Fetal Mouse Salivary Gland, Liver, and Rib Explants Cultured under Various Membranes

Membranes	Thickness	Water filtration rate (μl/cm²/760 mHg/min)	Filtration components*	Salivary gland‡	Liver§	Rib‖
	μm		*mol wt*			
Cellophane[a]	30	1.10	≤ 12,400 (2%)	+ (++)	+ (+)	+
Cellulose acetate[b]	100	25.2	≤ 40,000 (2%)	++ (+++)	+++ (++)	+++
Reconstructed cellulose[c]						
SM 11536	90	2.50 ~ 10.0	< 50,000	++ (++)	++ (++)	++
SM 11533	100	10.0 ~ 30.0	< 70,000	++++ (++++)	++++ (++++)	++++
SM 11530	100	30.0 ~ 100	> 70,000	− (−)	− (−)	−

[a] Dialysis tubing cellophane 36/32, Visking Company, Chicago, Ill.

[b] HF 35, KP 00, Eastman Chemical, Chicago, Ill.

[c] Sartorius-Membranfilter GmbH, D-34, Gottingen, W. Germany.

* Components are not filtrated from nutrient supplemented with 15% calf serum, but from serum-free nutrient.

‡ Salivary gland: phase-contrast microscopy; maintaining of glandular structure was graded as ++++, +++, ++, +, and −. PAS reaction; quantity of mucous secretion was graded as (++++), (+++), (++), (+) and (−).

§ Liver: Phase-contrast microscopy; numbers of hepatocytes were graded as ++++, +++, ++, +, and −. Electron microscopy; maintaining of bile canaliculi and rough-surfaced endoplasmic reticulum, and quantity of glycogen and microbodies were graded as (++++), (+++), (++), (+) and (−).

‖ Rib: toluidine blue staining; degree of metachromasia was graded as ++++, +++, ++, + and −.

TABLE II

Enzyme Levels of Fetal Mouse Liver Cultured under Various Membranes

Membranes	Specific activities of enzymes (mU/mg protein)				
	Glucokinase	Hexokinase	Glucokinase/ Hexokinase	Glucose-6-phosphate dehydrogenase	Pyruvate kinase
Cellophane	1.40	23.0	0.06	13.7	596
Cellulose acetate	9.50	30.6	0.31	43.8	826
Reconstructed cellulose					
SM 11536	6.30	21.5	0.29	15.8	625
SM 11533	16.3	19.0	0.86	32.5	843
SM 11530	0.00	10.3	0.00	13.0	350

DISCUSSION

If the tissue mass of a culture system exceeds a certain size, the cells die from a lack of oxygen and nutrient in the central area. The perfusion or stirring of the medium has been tried by many to improve their supply. However, Trowell (11) found that a rapid movement of nutrient was harmful because of the leaching out of important constituents from the cells being perfused. In the system we used, the membranes selectively prevented the perfusing nutrient from leaching out tissue products of various sizes, and the rapid circulation of the oxygen-saturated nutrient supplied a favorable environment for the cultures. It has been said that growth and differentiation are antagonistic. The great inhibition of growth found in fetal cultures under dialysing membranes is considered to be due to the prevention of the entrance of the large molecular components of growth promoting factors in the serum (12) of the nutrient into the microenvironment of the culture. Great suppression of glucose-6-phosphate dehydrogenase suggests also the growth inhibition of tissues under the membranes (5).

SM 11533 was found to be the most favorable membrane for tissue and organ differentiation, both morphologically and functionally. The pore size of cellophane is 24 Å and its filtration of water is only 1.10×10^{-3} ml/cm²/min. The filtration rate of the components of the nutrient supplemented with 15% calf serum are considered to be much less than that shown in Table I because of a masking by the serum protein on the membranes. The pore size of cellophane is, therefore, too small to filter efficiently the nutrient requirement for these cultures. Accordingly, an enlargement of the pore size should provide the culture with a more favorable microenvironment by increasing the filtration rate of the nutrient. Membrane SM 11533 was found to be a more effective molecular screen which not only permitted a greater fluid exchange between the compartments, but also inhibited a leaching out of important tissue products and the entrance into the microenvironment of large molecular components of the serum in the circulating nutrient.

The dedifferentiation of tissues and organs cultured under the RC 11530 membranes apparently was not affected by the entrance of larger molecular components (SM 11530), as their growth was not notably increased. It is more likely, therefore, that tissue and organ dedifferentiation was a response to the washing out of tissue products induced by the effective larger pore size of this membrane and the rapid flow of the circulating nutrient. This washing out prevented a "conditioning" of the medium in the culture microenvironment.

Lastly, the compression of tissues and organs onto the cover glasses is also an essential feature of the membrane technique for the consequent differentiation of the cultures. However, with membrane SM 11530, the cultures were not compressed, and this may be considered another reason for their dedifferentiation.

SUMMARY

Fetal mouse salivary gland, rib, and liver explants were cultured in circumfusion systems, using five transparent membranes with different porosities. Reconstructed cellulose SM 11533, which prevents a filtration of components of molecular weight $> 7 \times 10^4$, was the most favorable for the differentiation of these three tissues and organs. The explants could not be cultured well by covering membranes which permitted a passage of particles of a greater molecular weight, but differentiated cultures were sustained by covering membranes which permitted a passage of smaller-sized particles, i.e., $< 7 \times 10^4$.

The authors wish to express their thanks to Professor George G. Rose, The University of Texas Dental Branch, Houston, Texas, for reviewing and revising the manuscript. The skillful assistance of Miss S. Hosoda is gratefully acknowledged.

Received for publication 29 January 1974, and in revised form 11 April 1974.

REFERENCES

1. ROSE, G. G. 1967. *J. Cell Biol.* **32**:89.
2. ROSE, G. G., M. KUMEGAWA, and M. CATTONI. 1968. *J. Cell Biol.* **39**:430.
3. ROSE, G. G., M. KUMEGAWA, H. NIKAI, M. CATTONI, and F. HU. 1969. *Cancer Res.* **29**:2010.
4. ROSE, G. G., M. KUMEGAWA, H. NIKAI, M. BRACHO, and M. CATTONI. 1970. *Microvasc. Res.* **2**:24.
5. NAKAMURA, T., and M. KUMEGAWA. 1973. *Biochem. Biophys. Res. Commun.* **51**:474.

6. ROSE, G. G., C. M. POMERAT, T. O. SHINDLER, and J. B. TRUNNELL. 1958. *J. Biophys. Biochem. Cytol.* **4**:761.
7. DIPIETRO, D. L., and S. WEINHOUSE. 1960. *J. Biol. Chem.* **235**:2542.
8. GLOCK, G. E., and P. MCLEAN. 1953. *Biochem. J.* **55**:400.
9. BÜCHER, T. and G. PFLEIDERER. 1955. *Methods in Enzymol.* **1**:435.
10. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. *J. Biol. Chem.* **193**:265.
11. TROWELL, O. A. 1961. *La Culture Organotypique.* **101**:237.
12. TODARO, G. J., Y. MATSUYA, S. BLOOM, A. ROBBINS, and H. GREEN. 1967. Growth regulating substances for animal cells in culture. V. Defendi and M. Stoker, editors. *Wistar Inst. Symp. Monogr.* **7**:87.