Synthesis and Transport of Cholesterol in Aortic Cells: Radioisotopic Studies in Cell Culture

The metabolism of cholesterol in aortic cells is of particular interest in studying the nature of atherosclerosis. However, the detailed cellular mechanisms involved in the synthesis or transport of cholesterol still remain to be clarified. We have devised an aortic cell culture system in which quantitative measurements of lipid metabolism can be made at the cellular levels using small quantities of radioisotopes[1-3]. The present experiments were conducted with cultured endothelial cells derived from chick aortic tissue to study (1) the incorporation of C^{14}-acetate into sterols in lipid fractions, and (2) the transport of C^{14}-cholesterol across the cell membrane. Furthermore, the site of transport of H^3-cholesterol in aortic cells of rhesus monkey was also examined by autoradiographic technique.

The procedures of chick aortic cell culture have been made as previously described[1-3]: aortic cells prepared from striped intimal layers were resuspended as 10^6 cells/ml in Eagle's minimum essential medium (MEM)[4] plus 10% calf serum and planted 5 ml or 15 ml per flask. In order to study the cholesterol synthesis, aortic cells grown for 1 week in monolayers were incubated in MEM with sodium C^{14}-1-acetate[5] for 6 h at 37 °C. The extraction and purification followed by the fractionation of cellular lipids were carried out by the same procedure as reported previously[3]: sterols were determined by the acetic acid-ferrous sulphate reagent[6], and digitonin precipitable radioactivity was counted as sterols by a liquid scintillation spectrometer. Table I shows that the incorporation rate of labelled acetate into sterol fraction in the aortic cells is maximum in both primary and secondary generations when the comparisons are made among the lipid fractions.

In the next experiment, the mode of the transport of cholesterol into aortic cells was investigated. After the aortic cells are exposed to C^{14}-4-cholesterol[7] in MEM (0.1 μC/ml) for the indicated time at 37 °C, the radioactivity was determined in the same way mentioned above. The preliminary experiment has revealed that the incorporation rate of C^{14}-cholesterol into aortic cells parallels the incubation time for at least 6 h. The regression equation of 5 day culture of primary cells determined 6 samples at every 2 h is: $Y = 1.52x - 0.571$, where x is incubation hours and Y is activities of intracellular labelled carbon, expressed as dpm/μg of total lipid weight. The results prompted us to make a succeeding study of the effect of human lipemic serum (HLS) on the cholesterol uptake by the aortic cells, since it should be important to know whether the incorporation of cholesterol across the cell membrane interferes with pre-existing lipids. The aortic cells were incubated with 10% HLS in MEM for 3 days to prepare lipid loaded aortic cells, which may be comparable to lipoid cells in natural atheromatous lesions. The addition of HLS did not show any toxic action of the cells of the intact chick aorta. The data obtained, as shown in Table II, indicate that the transport rates of labelled cholesterol into aortic cells loaded with lipids are significantly less than the untreated controls: 44–54% of the control values ($p < 0.001$) for 2, 4 and 6 h. Furthermore, the suppressing effect of pre-existing lipids was reversible. The replacement with MEM for the succeeding 2 days resulted in the reduction of the suppressing effect of lipidosis: the radioactivity of C^{14} is 84–91% of the control values. The findings seem to be contrary to the observation by LAZZARINI-ROBERTSON[8] in which the cells from intact vessels showed less incorporation of cholesterol than the atheromatous cells. This may be due to, in part, the different contents of lipids in aortic cells or different fashion in culture environment.

To elucidate the site of transport of cholesterol, an autoradiographic study was carried out with the thoracic aortae, which were removed from 2 sacrificed male monkeys (*Macaca mulatta*), weighing about 4.2 kg of body weight. The aortic segments were dissected horizontally and planted on cover slides in Leighton tubes[4]. After the incubation with Puck's nutrient medium[4] with 15% fetal calf serum for 72 h at 37 °C, they were exposed to H^3-7α-

Table I. Lipid synthesis in fractions in aortic cells

Generation of cells	No. of sample	Specific activity of synthesized lipids (dpm/μg)			
		Total lipids	Phospho-lipids	Neutral fat	Sterols
Primary	8	307 ± 33[a]	107 ± 30	425 ± 73	581 ± 88
Secondary	8	210 ± 75	67 ± 15	222 ± 32	319 ± 61

[a] Means ± standard errors. 1-week-old aortic cells in each generation were incubated with the media containing C^{14}-1-acetate (0.44 μc/ml) for 6 h.

Table II. Suppressing effect of human lipemic serum on C^{14}-cholesterol uptake into chick aortic cells

Experiment		C^{14}-cholesterol present (h)			
		1	2	4	6
1[a]	% of radioactivity (control = 100%)	84	91	88	84
	p value[c]	< 0.01	> 0.05	< 0.01	< 0.05
2[b]	% of radioactivity (control = 100%)	–	54	44	48
	p value[c]	–	< 0.001	< 0.001	< 0.001

Aortic cells were incubated with 10% human lipemic serum (HLS: total lipids, 1050 mg%; total cholesterol, 334 mg%) in minimum essential medium for 3 days, and then replaced with regular media for 2 days in experiment 1. In experiment 2, however, the replacement was omitted. C^{14}-cholesterol (0.1 μC/ml in media) was added for the indicated hours. [a] Total No. of tests equals 4 using 6 controls and 6 HLS treated. [b] Total No. of tests equals 3 using 4 controls and 4 HLS treated. [c] Significance value based on Student t-test.

[1] K. MURATA, J. J. QUILLIGAN JR. and L. M. MORRISON, Experientia *21*, 637 (1965).

[2] K. MURATA, J. J. QUILLIGAN JR. and L. M. MORRISON, Nature *213*, 1030 (1967).

[3] K. MURATA, J. J. QUILLIGAN JR. and L. M. MORRISON, Biochim. biophys. Acta *144*, 473 (1967).

[4] D. J. MERCHANT, R. M. KAHN and W. H. MURPHY, in *Handbook of Cell and Organ Culture* (Burgess Publish. Co., Minn. 1960).

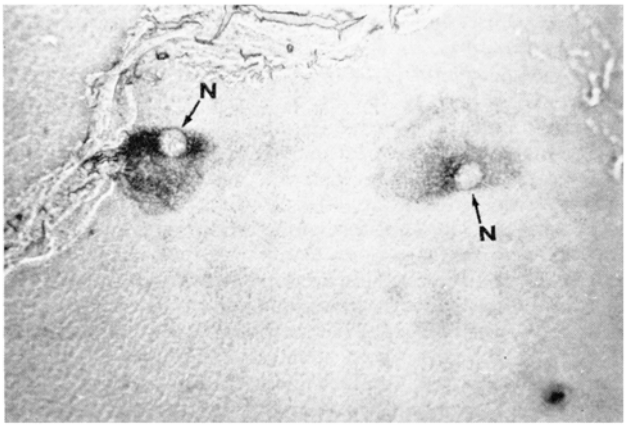
[5] Specific activity, 3.0 mC/mM; purchased from Volk Radiochemicals Calif.

[6] R. L. SERCY, L. M. BERG and R. C. JUNG, J. Lipid Res. *1*, 349 (1960).

[7] Specific activities of C^{14}-4- and H^3-7α-cholesterol are 30 mC and 4 C/mM, respectively: both purchased from New England Nuclear Cor., Mass.

[8] A. LAZZARINI-ROBERTSON, in *Lipid Metabolism in Tissue Culture Cells* (Wistar Institute Press, Philadelphia 1967), p. 115.

cholesterol[6] (6 μC/ml media) for 12 h. The cultured cells in slides were rinsed well and fixed with 10% formalin solution. Further autoradiographic procedure was made by the method reported previously[9]. The Figure illustrates that the high density of tritiated cholesterol is incorporated into cytoplasm of aortic cells. However, H^3-grains in nuclei are negligible. The first migrated intact cells from aortic segments were mostly round or oval and seem to be referred to as endothelial cells[10]. The same observation was made in fibroblastic aortic cells cultured more slowly than endothelial cells. Thus, evidence is presented that the cytoplasm is the site of the entry of H^3-cholesterol in monkey aortic cells.



Incorporation of tritiated cholesterol into cytoplasm of monkey aortic cells. Note the absence of H^3-grain in the nucleus (N). No staining. × 960.

Species differences appear to be of importance in understanding the metabolism of cholesterol in aortic cells[11]. However, the data obtained here clearly indicate that the synthesis and transport of cholesterol in aortic cells are very high in dynamic cellular levels in cultured environments. Analyses of such high metabolic activities in cells appear promising in studying cellular mechanisms which may be masked in vivo environments.

Zusammenfassung. Synthese und Transport von Cholesterin in Hühner-Aortazellen wurden untersucht: 1. grösste Inkorporationsrate von C^{14}-Acetat in der Sterin-Fraktion, 2. verminderte Aufnahme von C^{14}-Cholesterin in Aortazellen nach ihrer Inkubation mit lipämischem Menschenserum. Der Eintritt von H^3-Cholesterin in das Zytoplasma der Aortazellen von Affen wurde autoradiographisch festgestellt.

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[9] K. MURATA, Experientia *23*, 732 (1967).
[10] T. KASAI and O. J. POLLAK, Z. Zellforsch. mikrosk. Anat. *62*, 743 (1964).
[11] D. L. AZARNOFF, Proc. Soc. exp. Biol. Med. *98*, 680 (1958).

Histone bei dem Phycomyceten *Allomyces arbuscula* (Butl.)

Das Vorkommen von Histonen in höheren Organismen ist gesichert, dagegen ist über ihre Existenz und Funktion bei Mikroorganismen noch wenig bekannt. Seit den Untersuchungen von ALLFREY[1] und BONNER[2] setzt sich immer mehr die Auffassung durch, dass die basischen Proteine der Zellkerne, vor allem Histone, eine wichtige Rolle als Regulatoren der Genfunktion spielen. Da auch bei dem Phycomyceten *Allomyces arbuscula* eine Reihe interessanter gengesteuerter Differenzierungsprozesse beschrieben wurden[3–6], erschien es lohnend, das Vorkommen von Histonen bei Gametophyt und Sporophyt dieses Pilzes zu analysieren.

Es war nicht möglich, Kerne von Zytoplasma und Hyphenwandresten zu trennen; wir verwendeten darum als Ausgangsmaterial lyophilisiertes Mycel, das homogenisiert und mit Äther und Aceton entfettet wurde. Die Extraktion der basischen Proteine erfolgte mit 0,5 *N* HCl bei 0 °C.

100 mg Mycel ergaben eine Ausbeute von ca. 200 μg basischen Proteinen (Bestimmung nach LOWRY). Mit Hilfe der Polyacrylamid-Elektrophorese nach ORNSTEIN[7] und DAVIS[8] in der Modifikation von SHEPHERD und GURLEY[9] wurden Proben von 80 μg aufgetrennt. Die Figur zeigt das Ergebnis: Die Trägergele enthalten mindestens 16 Bänder. Die Bändermuster von Sporophyt und Gamethophyt sind qualitativ identisch, und zwar sowohl für undifferenziertes als auch differenziertes (Hyphen mit Sporangien bzw. Gametangien) Mycel.

Quantitative Unterschiede sind ebenfalls nicht mit Sicherheit zu konstatieren.

Zur Untersuchung der Histone gingen wir von 1000 mg lyophilisiertem Mycel aus und folgten der für pflanzliches Material ausgearbeiteten Methode von IWAI[10]. Dabei wurde das Mycel homogenisiert und zunächst mit 1,5 *M* NaCl zweimal bei 0 °C extrahiert. Nach Präzipitierung der im Überstehenden befindlichen Desoxynukleoproteine durch Verdünnung auf 0,5 *M* NaCl und Zufügen von 2 Vol. Äthanol (24–48 h) erfolgte aus dem sehr geringen Präzipitat die Extraktion des Histonanteils mit 0,5 *N* HCl bei 0 °C. Diesem Extrakt wurden 2 Vol. Äthanol (pH 10) zugefügt. Nach 48 h bei 0 °C war kein oder nur äusserst geringes Präzipitat zu bemerken. Die Eiweissbestimmung nach LOWRY im Zentrifugat ergab keine messbare Proteinmenge. Nach Polyacrylamid-Elektrophorese[9] traten keine Bänder auf. Aus diesem Ergebnis

[1] V. G. ALLFREY, V. C. LITTAU und A. E. MIRSKY, Proc. natn. Acad. Sci. *49*, 414 (1963).
[2] J. BONNER und R. C. HUANG, J. molec. Biol. *6*, 169 (1963).
[3] S. K. DELONG, Am. J. Bot. *52*, 999 (1965).
[4] P. FÄHNRICH, Z. Vererblehre *92*, 8 (1961).
[5] C. STUMM, Z. Vererblehre *89*, 521 (1958).
[6] G. TURIAN, Devl. Biol. *6*, 61 (1963).
[7] L. ORNSTEIN, Ann. N.Y. Acad. Sci. *121*, 321 (1964).
[8] B. J. DAVIS, Ann. N.Y. Acad. Sci. *121*, 404 (1964).
[9] G. R. SHEPHERD und L. R. GURLEY, Analyt. Biochem. *14*, 356 (1966).
[10] K. IWAI, in *The Nucleohistones* (Ed. J. BONNER und P. Ts'o, Holden-Day, San Francisco 1964).