ADENOSINE DIPHOSPHATE METABOLISM BY CULTURED HUMAN UMBILICAL ENDOTHELIAL CELLS

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1. Introduction

Recent work has emphasized the ability of endothelial cells to split adenosine diphosphate (ADP), leading to the formation of adenosine [1,2]. This property of endothelium could play a role in the reactions mediated by adenosine: local vasodilation and inhibition of platelet aggregation. Several investigations have shown that adenosine is produced when the metabolic activity of the tissues is increased [3] or when the oxygen supply is reduced [4–7]. Inhibition of platelet aggregation by adenosine has been demonstrated [8–10].

There is much evidence that functions of cultured cells may vary depending upon the experimental conditions [11]. These considerations led us to investigate the effect on ADP metabolism of changes in the physical state of the cell, in the cell density and in the age of the culture. The present work shows that there is a progressive functional dedifferentiation of primary cultures leading to the loss of ADP degrading activity.

2. Materials and methods

2.1. *Cell culture*

Endothelial cells from human umbilical veins were prepared by the method in [12], and plated at an initial density of 40 000 cells/cm^2 in 35 mm Petri dishes. Only primary cultures were used in this study.

2.2. *Incubation with [^{14}C]ADP*

Adherent cells were washed 4 times with prewarmed medium 199 without serum, and incubated immediately with ADP. Suspensions of cells were prepared by scraping the culture dish with a plastic spatula. Collagenase-dissociated cells were obtained by digestion with collagenase (0.1%, w/v) in EDTA (0.01%, w/v), glucose (0.1%, w/v) and bovine albumin (0.3%, w/v). The cells were centrifuged at 1000 × *g* for 10 min, washed in medium 199, centrifuged again and resuspended in 1 ml medium 199. The cell number was determined in replicate cultures.

The cells were incubated in 1 ml of prewarmed medium 199 containing 10 μM of [8-^{14}C]ADP (spec. act. 52 mCi/mmol, Radiochemical Centre, Amersham) in a CO_2 incubator. At the end of the incubation, 200 μl of an unlabelled mixture of adenine derivatives (ATP, ADP, AMP, adenosine, adenine, hypoxanthine, inosine), each compound at 10 mM final concentration, was added. The supernatant of adherent cells or whole cell suspension was filtered through a 0.2 μm Millipore membrane, and used directly for chromatography.

2.3. *Preparation of soluble cell extracts*

Soluble cell extracts were prepared only from adherent cells. The cells were washed 4 times with 0.15 M NaCl, and extracted for 30 min at 4°C with 0.5 ml 0.7 N $HClO_4$ supplemented with 100 μl of the mixture of unlabelled adenine derivatives. The cells were then scraped and centrifuged. The perchloric extract was neutralized with cold 0.1 M Tris–10% (w/v) KOH [13] and chromatographed.

2.4. *Repartition of the radioactivity in the different fractions*

Radioactivity counts were performed on aliquots

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of the extracellular supernatant (10 μl), NaCl washings (1 ml), neutralized perchloric extract (100 μl), cellular proteins solubilized in 1 N NaOH (500 μl) and $KClO_4$ precipitate eluted with 1 N NaOH (500 μl). Counting efficiency in Unisolve (Koch and Light) was determined by the addition of an internal standard (25×10^{-3} μCi in each of the fractions). The results were expressed as dpm.

2.5. *Chromatography of the adenine compounds*

Thin layer PEI cellulose (Schleicher and Schull) was used for the 2 chromatography systems described [14]. The spots were identified by reference to marginal markers in ultraviolet light and by autoradiography. The spot radioactivities were counted in 1 ml distilled water and 10 ml Unisolve scintillation liquid. For each compound the results were calculated as the percentage of the sum of all spot radioactivities. This percentage was converted into nmol for the supernatant, and into pmol for the cellular extract, by taking into account the radioactivity of these 2 fractions and the specific activity of [^{14}C]ADP.

3. Results

3.1. *Distribution of the radioactivity in the extracellular and cellular compartments of adherent cells*

The counting efficiency (E) showed only minor differences between the supernatant ($E = 0.98$), the washings ($E = 0.97$) and the soluble cellular perchloric extract ($E = 0.93$). For the fractions containing NaOH, there was a noticeable quenching: solubilized protein ($E = 0.39$), $KClO_4$ eluted fraction ($E = 0.59$). These values were used to determine the distribution of the radioactivity. After 5 min incubation with 10 μM [^{14}C]ADP, >98% of the radioactivity was in the supernatant (table 1). In the cell the radioactivity was mainly found in the perchloric extract and was negligible in the proteins and in the $KClO_4$ precipitate.

3.2. *Distribution of the ADP metabolites*

In the supernatant 2 main metabolites were found: AMP, which decreased after 5 min incubation; adenosine, which was predominant at 30 min (fig.1).

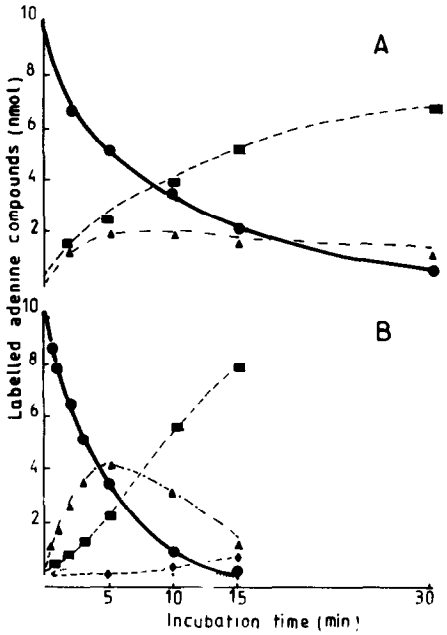

Fig.1. Distribution of the labelled adenine compounds in the supernatant of cultured cells incubated with 10 μM [^{14}C]ADP: (A) adherent cells; (B) collagenase-dissociated cells. In both cases the cell number was $300-360 \times 10^3$. (•) ADP, (▲) AMP, (■) adenosine, (*) ATP, (♦) inosine.

Table 1
Distribution of radioactivity in endothelial cell culture incubated with 10 μM [^{14}C]ADP for 5 min

Supernatant	Washing				Cellular extract	Protein	$KClO_4$
	1	2	3	4			
95	3.5	0.3	0.08	0.01	0.45	0.007	0.006

Adherent cells (400 000) were treated as in section 2. Results are expressed as percentage of the total radioactivity

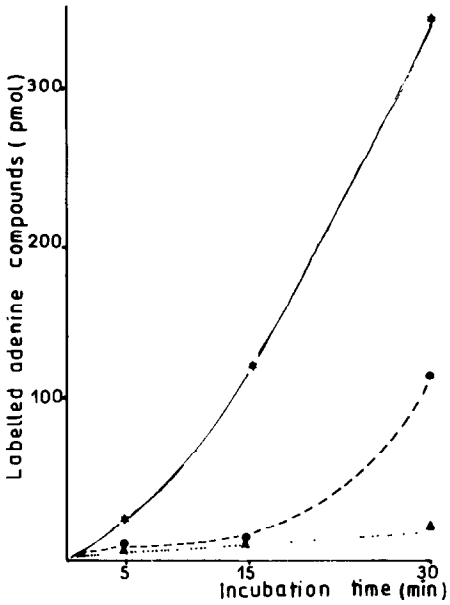

Fig.2. Distribution of the labelled adenine compounds in the soluble cell extract after incubation of 800 × 10^3 adherent cells with 10 μM [^{14}C]ADP, (*) ATP, (•) ADP, (▲) AMP.

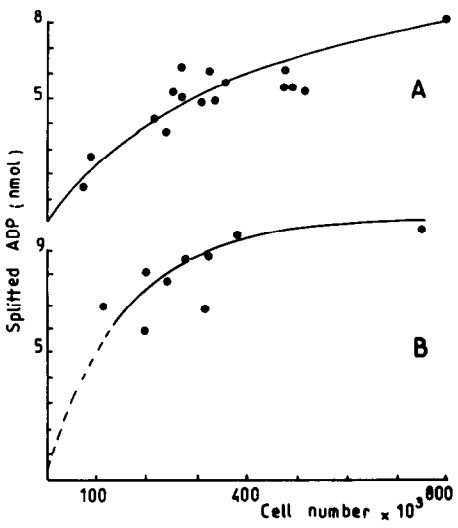

Fig.3. Effect of cell number on ADP degradation: (A) adherent or (B) collagenase-dissociated cells were incubated with [^{14}C]ADP for 5 min. In all cases the culture time was 5–7 days.

ADP degradation by collagenase-dissociated cells was increased compared to adherent cells. The half-life of ADP in presence of 300 000 cells was 324 ± 120 s and 480 ± 60 s, respectively. Some minor metabolites such as adenosine triphosphate and inosine were detected when the cells were dissociated.

Radioactivity was rapidly incorporated into nucleotides, mainly into ATP (fig.2).

3.3. *Effect of the cell number*

For this investigation, the cells were seeded at different densities and studied after 5–7 days in culture. For a 5 min incubation the amount of degraded ADP increased non-linearly with the number of cells (fig.3A,B). The uptake of radioactivity by adherent cells was proportional to the number of cells at the rate of 13 pmol .10^6 $cells^{-1}$ .min^{-1} (fig.4).

3.4. *Effect of culture time*

It was observed that as culture time increased there was a decrease in the ADP splitting activity in spite of the increasing cell number. Moreover, the formation of ATP by dissociated cells was enhanced (table 2).

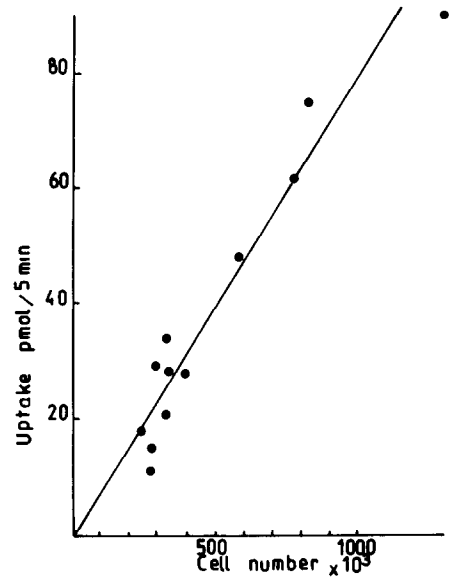

Fig.4. Effect of cell number on the uptake of radioactivity by adherent cells incubated with [^{14}C]ADP for 5 min. Culture time was 5–8 days. Linear regression was: $Y = [(0.08)10^{-3}]$ $X + 0.43$ ($P < 0.01$).

Table 2
Effect of the culture time on ADP metabolism

Days	Cell no. × 10^3	ADP	AMP	Adenosine	ATP
Adherent cells					
5	266	3.8	2.1	3.7	n.d.
11	632	4.8	1.9	3.0	n.d.
18[a]	710	7.1	1.6	0.8	0.2
Scraped cells					
5	266	3.1	3.8	2.5	0.2
11	632	6.6	1.8	0.7	0.6
18	710	7.1	1.4	0.5	0.6
Collagenase-dissociated cells					
5	266	1.3	4.4	3.9	n.d.
11	632	1.1	2.6	5.9	0.06
18	710	3.0	3.3	3.3	0.2

[a] Spontaneous detachment of the monolayer during incubation

These results were obtained at the times indicated after plating the cells of the same umbilical vein in replicate Petri dishes and after studying them in different physical states. The cells were incubated with 10 μM [^{14}C]ADP for 5 min. The distribution of the labelled adenine compounds was analyzed in the cell supernatant. The results are expressed in nmoles. n.d., non detected

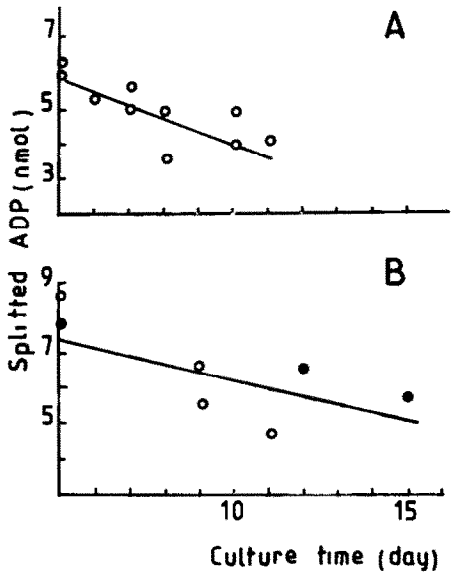

Fig.5. Effect of culture time on ADP degradation. Cells were incubated with 10 μM [^{14}C]ADP for 5 min: (A) adherent cells 300 000 ± 50 000; (B) collagenase-dissociated cells 250 000 ± 50 000. Linear regressions were $Y = -(0.32)\,X + 7.8$ ($P < 0.02$) for (A) and $Y = -(0.24)\,X + 8.6$ ($P > 0.10$) for (B). Dark circles correspond to the results of the same culture dissociated at different times.

In order to discriminate between the effects of cell density and of culture age, a comparison was made between different cultures of similar mean density at different times after seeding. In adherent cells, the ability to degrade ADP decreased linearly for 5–11 days (fig.5A). The collagenase-dissociated cells showed the same tendency but with a greater variability (fig.5B).

4. Discussion

The present work confirms the role played by endothelium in the clearance of circulating ADP. It is, however, difficult to compare ADP disappearance in vivo (1–2 min) as reported [15] with its half-life in a culture of a small area of endothelial cells studied in stagnant conditions. In some experiments, dissociated cells were agitated and the half-life of 10 μM ADP added to 300 000 cells was considerably decreased (2–3 min) which appeared more in agreement with in vivo data.

The adenosine newly formed from ADP was taken up slowly by the cells in comparison to the rate of ADP splitting: ADP hydrolysis was ~80-fold faster than uptake of radioactivity. This difference led to the accumulation of adenosine in the surrounding medium, since adenosine deamination appeared to be very slow. The adenosine uptake by cultured pig and bovine endothelial cells [16] was 5-fold greater than our values. Such discrepancies may be related to vessel origin or to species differences, as has been proposed for serotonin degradation [17].

Endothelial cell culture is a useful model for studying isolated organized endothelial tissue. However, dissociated cells may also be of interest for some investigations. The data obtained suggested that alterations might occur after dissociation. The increase in ADP splitting activity may be related to the greater surface area exposed or to cell injury during the manipulation. Such an increase in enzymatic

activity has been reported for the angiotensin I converting enzyme [18].

The dissociation may also expose the cellular surfaces in contact with the culture dish and with other cells. It is possible that the formation of ATP by dissociated cells may occur at the contact surfaces, since spontaneously detached monolayers exhibited the same property. This hypothesis would require further investigation.

The cell density effect was investigated since some functional properties may vary with the degree of cell confluence, as has been observed for LDL binding to endothelial cells [19]. Our results showed that, providing the culture age was similar, ADP splitting activity increased with the cell density, the nonlinear response being due to the insufficiency of substrate for the enzymatic reaction.

The effect of culture age appeared independent of cell density. The continuous decrease in ADP splitting activity suggested a functional dedifferentiation during culture. This functional loss did not seem to be associated with morphological modification of the cells, which keep their polygonal shape.

Acknowledgements

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References

[1] Dosne, A. M., Legrand, C., Bauvois, B., Bodevin, E. and Caen, J. P. (1978) Biochem. Biophys. Res. Commun. 85, 183–189.
[2] Glasgow, J. G., Shade, R. S. and Pitlick, F. A. (1978) Thromb. Res. 13, 255–266.
[3] Dobson, J. G., Rubio, R. and Berne, R. M. (1971) Circ. Res. 29, 375–384.
[4] Mentzer, R. M., Rubio, R. and Berne, R. M. (1975) Am. J. Physiol. 229, 1625–1631.
[5] Berne, R. M. and Rubio, R. (1974) Adv. Cardiol. 12, 303–317.
[6] Henderson, R. J. and Griffin, C. A. (1978) Fed. Proc. FASEB 37, 1349.
[7] Heistad, D. D. (1978) J. Clin. Invest. 62, 133–140.
[8] Born, G. V. R. and Cross, M. J. (1963) J. Physiol. 168, 178–195.
[9] Born, G. V. R., Honour, A. J. and Mitchell, J. R. A. (1964) Nature 202, 761–765.
[10] Rosenberg, M. C. and Holmsen, H. (1968) Biochim. Biophys. Acta 155, 342–352.
[11] Harris, M. (1964) Cell culture and somatic variations, p. 547, Holt, Rinehart and Winston, New York.
[12] Jaffe, E. A., Nachman, R. L., Becker, C. G. and Minick, C. R. (1973) J. Clin Invest. 52, 2745–2756.
[13] Rosing, J. and Slater, E. C. (1972) Biochim. Biophys. Acta 267, 275–290.
[14] Chivot, J. J. E., Leibrandt, M. J., Rouvroy, H. L., Simeon, J. and Caen, J. P. (1974) Clin. Chim. Acta 53, 69–77.
[15] Brashear, R. E. and Ross, J. C. (1969) J. Lab. Clin. Med. 73, 54–59.
[16] Pearson, J. D., Carleton, J. S., Hutchings, A. and Gordon, J. L. (1978) Biochem. J. 170, 265–271.
[17] Small, R., Macarak, E. and Fisher, A. B. (1977) J. Cell Physiol. 90, 225–232.
[18] Johnson, A. R. and Erdos, E. G. (1977) J. Clin. Invest. 59, 684–695.
[19] Vlodavsky, I., Fielding, P. E., Fielding, C. J. (1978) Proc. Natl. Acad. Sci. USA 75, 356–360.