FOLIA ANGIOLOGICA

INTERNATIONAL JOURNAL OF CLINICAL ANGIOLOGY

Editores: Prof. Dr. Marcello Comel, Milano - Prof. Prof. h. c. Dr. Dr. h. c. Norbert Klüken, Essen

Herausgeber:
Prof. Dr. med. M. Comel — Prof. Prof. h. c. Dr. med. Dr. h. c. N. Klüken

Verleger:
VERLAG A · F · KOSKA
Schaperstraße 18 · D-1000 Berlin 15 · Tel. (030) 8 83 90 41

Anzeigenannahme: VERLAG A · F · KOSKA
Schaperstraße 18 · D-1000 Berlin 15 · Tel. (030) 8 83 90 41
Zimmermanngasse 1 · A-1095 Wien · Tel. (0043 222) 42 46 89

Erscheinungsweise: Monatlich

Studies on Cultured Coronary Endothelial Cells

S. NEES, B. WILLERSHAUSEN-ZÖNNCHEN, A. L. GERBES, E. GERLACH

Physiologisches Institut der Universität München

SUMMARY

Studies on Cultured Coronary Endothelial Cells

A method was developed for isolation and cultivation of endothelial cells from coronary vessels of guinea pig hearts. The cultured cells revealed typical morphological criteria of endothelial cells in vivo. Exceptionally high contents of adenine nucleotides (ATP, ADP, AMP) and of their degradatives (adenosine, inosine, hypoxanthine) were found in confluent endothelial cell cultures as determined by specially elaborated HPLC techniques. Adenine nucleotide levels were slightly influenced by the growth state of the cell cultures and remained unchanged when cells were incubated for 3 days in purine-free medium. The cultured coronary endothelial cells proved to be sensitive to lack of oxygen, as evidenced by the fast breakdown of ATP concomitant with an enhanced release of adenosine. Measurements of specific activities of enzymes involved in degradation and synthesis of adenine nucleotides lend additional support to the view that an active adenine nucleotide metabolism is a characteristic feature of coronary endothelial cells.

ZUSAMMENFASSUNG

Untersuchungen an Endothelzellen aus Koronargefäßen

Es wurde eine Methode zur Isolierung und Kultivierung von Endothelzellen aus Koronargefäßen von Meerschweinchen ausgearbeitet. Die in der Gewebekultur gezüchteten Zellen zeigten die gleichen morphologischen Eigenschaften wie Endothelzellen in situ. Durch quantitative HPLC-Analysen wurde nachgewiesen, daß Endothelzellen aus konfluenten Kulturen außergewöhnlich hohe Spiegel an ATP, ADP und AMP enthielten; auch die Konzentrationen von Adenosin, Inosin, Hypoxanthin und Adenin waren relativ groß. Der Adenin-Nucleotid-Gehalt änderte sich nur wenig in Abhängigkeit vom Wachstumszustand der Zellkulturen; auch nach einer Inkubation von 3 Tagen in Purin-freien Nährmedien blieben die Adenin-Nucleotid-Spiegel konstant. Es konnte gezeigt werden, daß Koronarendothelzellen sehr empfindlich auf Sauerstoffmangel mit einem raschen ATP-Abbau und einer gesteigerten Freisetzung von Adenosin in das Kulturmedium reagieren. Durch Aktivitätsmessungen verschiedener kataboler und anaboler Enzyme des Adenin-Nucleotid-Stoffwechsels ergaben sich weitere Hinweise, daß sich gezüchtete Koronarendothelzellen durch einen besonders aktiven Adenin-Nucleotid-Stoffwechsel auszeichnen.

RESUME

Recherches sur des cellules endothéliales des vaisseaux coronaires du coeur

Une méthode d'isolation et d'élevage des cellules endothéliales d'origine coronaire du cobaye a été développée. Toutes les cellules, élevées en culture de tissus, montraient les propriétés morphologiques typiques des cellules en situ. L'analyse quantitative HPLC des cultures confluentes prouvait un contenu extraordinairement élevé des nucléotides d'adénine et de leurs produits de dégradation, qui ne dépendait que peu de l'état de croissance et du contenu de purines du milieu alimentaire. Une sensibilité remarquable des cellules endothéliales coronaires au manque d'oxygène, une dégradation simultanée rapide du triphosphate d'adénosine et une libération augmentée d'adénosine pouvaient être montrées. Le mesurage des activités enzymatiques de plusieures enzymes cataboliques et anaboliques du métabolisme des nucléotides d'adénine supporte notre opinion, que les cellules endothéliales d'origine coronaire, cultivées en culture de tissus, soient caractérisées par un métabolisme très actif des nucléotides d'adénine.

Introduction

Although it is generally accepted that endothelial cells are involved in several biological processes (1–6) our knowledge about their intermediary metobolism is rather limited. The possibility to culture endothelial cells provides the opportunity to set up in vitro systems for studying certain aspects of their metabolic and functional properties. However, as yet experience with cultured endothelial cells was only obtained with cells isolated from human umbilical vein (7) as well as from larger vessels of various animals (8–9). To our knowledge suitable methods for isolation and cultivation of coronary endothelial cells have not been elaborated so far.

Our interest in the coronary endothelial cell type originated from recent findings of Schrader and Gerlach (10), who showed that in guinea pig hearts at least two different adenine nucleotide compartments exist. Both these compartments can contribute to the production of vasoactive adenosine, which is considered to play an important role in the metabolic regulation of coro-

nary blood flow. Since the coronary endothelium might constitute one of the adenine nucleotide compartments it seemed promising to develop adequate methods for the isolation and cultivation of coronary endothelial cells and to study some pertinent features of their adenine nucleotide metabolism.

Materials and Methods

Culture medium M 199, fetal calf serum (mycoplasma tested and virus screened), streptomycine-penicilline-solution and L-glutamine were purchased from Seromed, München. Crude collagenase (Cl. histolyticum) type II and trypsin were obtained from Worthington Biochemical Corporation, Freehold, N.J., USA. Reagents for fixation and embedding tissue samples prior to electron microscopy (glutaraldehyde; osmiumtetroxide; epon 812; 2,4,6-tri(dimethylaminomethyl)phenol; dodecenyl succinic acid anhydride; methylnadic anhydride) were obtained from Serva, Heidelberg. Nucleotides, nucleosides and bases for calibration were purchased from Boehringer Mannheim, all other chemicals of highest available purity from Merck, Darmstadt. Substituted silica column packings (Nucleosil-NH_2 and Nucleosil-C_{18}) for HPLC were obtained from Macherey und Nagel, Düren.

Preparation of cells and cell culture: Guinea pig hearts were cannulated through the aorta and the coronary system washed free of blood was filled with an isotonic buffer solution containing collagenase and trypsin (0.1 % each). After an exposure of 20 min perfusion was started again and all endothelial cells which had been detached were collected from the perfusate by centrifugation. Subsequently, the cells were washed with culture medium (M 199, containing 60 mg penicilline and 120 mg streptomycine per liter, supplemented with 20 % fetal calf serum [FCS] and 2 mM glutamine and seeded in culture dishes. Cultivation was performed at 40° C in a humidified air atmosphere containing 3 % CO_2. Confluency of the cell cultures was reached after 2 to 4 weeks.

Transmission electron microscopy: Cultured cells were fixed for 1 h in 2.5 % glutaraldehyde, 0.09 M cacodylate buffer (pH 7.4), 5 % sucrose, postfixed in 1 % OsO_4 for 1 h, stained with a saturated solution of tungsten phosphoric acid and uranylacetate in ethanol-water 75 : 25 (v/v), dehydrated with ethanol and embedded in epon 812.

Scanning electron microscopy: Cells were fixed as decribed before. After dehydration in ethanol they were critical-point dried and shadowed with gold.

Analysis of nucleotides, nucleosides and bases: Monolayers of endothelial cells in culture dishes were extracted with 0.4 N perchloric acid. Separation of the different purine compounds in the neutralized cell extracts was carried out by application of HPLC (Varian 8500) using weak anion exchange columns for the separation of nucleotides and reverse phase columns for nucleosides and bases. The concentrations of the individual metabolites in the eluates were continuously recorded using a sensitive UV-detection device (Varian UV-Vis detector „Variscan").

Determination of enzyme activities: Specific activities of enzymes involved in nucleotide metabolism were measured in a 200 x g membrane preparation as well as in a soluble 200 000 g supernatant fraction of endothelial cells. Enzyme assays were performed using standard procedures. Substrates and products of the enzyme catalyzed reaction were separated by HPLC.

Results and Discussion

Growth behaviour and cytology: As was revealed by scanning electron microscopy the endothelial cells were selectively detached from their basement membranes during the exposure of the coronaries to trypsin-collagenase solution. The coronary perfusate subsequently collected contained small clumps of rounded cells. After seeding in complex culture medium the cells attached to the substratum within 24 h. The clusters of cells increased in size and gradually coalesced to form monolayers after 2 to 4 weeks. Primary cultures consisted of about 98 % endothelial cells, contaminated with about 2 % fibroblasts and smooth muscle cells.

Selected confluent cultures of guinea pig coronary endothelial cells could be serially subcultured and maintained for at least 4 months. Primary and subcultured cells appeared similar with respect to shape and typical tight packing in a strict monolayer. The generation time of coronary endothelial cells in culture during the logarithmic phase of growth (G_1 phase) turned out to be 18 h, and was thus very similar to that of aortic endothelial cells in vivo after artificial wounding of the intima (11). Addition of thrombin in physiological concentrations to the culture medium (1 μg/ml) resulted in a marked reduction of the generation time to 16 h, and accelerated DNA-synthesis in resting cells (G_0 phase) by more than 30 %.

Phase contrast and interference microscopic pictures of endothelial cultures (Figure 1a and 1b) demonstrate that the individual cells were of uniform appearance: elongated (20–30 μm wide, 50–80 μm long), with single ovoid nuclei containing 2 to 3 nucleoli and surrounded by a perinuclear granular region and a broad peripheral cytoplasm with distinct borders. The granules consisted mainly of mitochondria, as could be shown by use of specific staining techniques (Janus green); they did not contain neutral lipids (no staining with oil red O).

Ultrastructural analysis of the cytoplasm of cultured and in vivo coronary endothelial cells by use of electron microscopy revealed a high degree of conformity. As can be seen from the transmission electron micrograph in Figure 1c the cytoplasm of cultured cells contained large numbers of vesicles, clusters of long and unbranched mitochondria, smooth and rough endo-

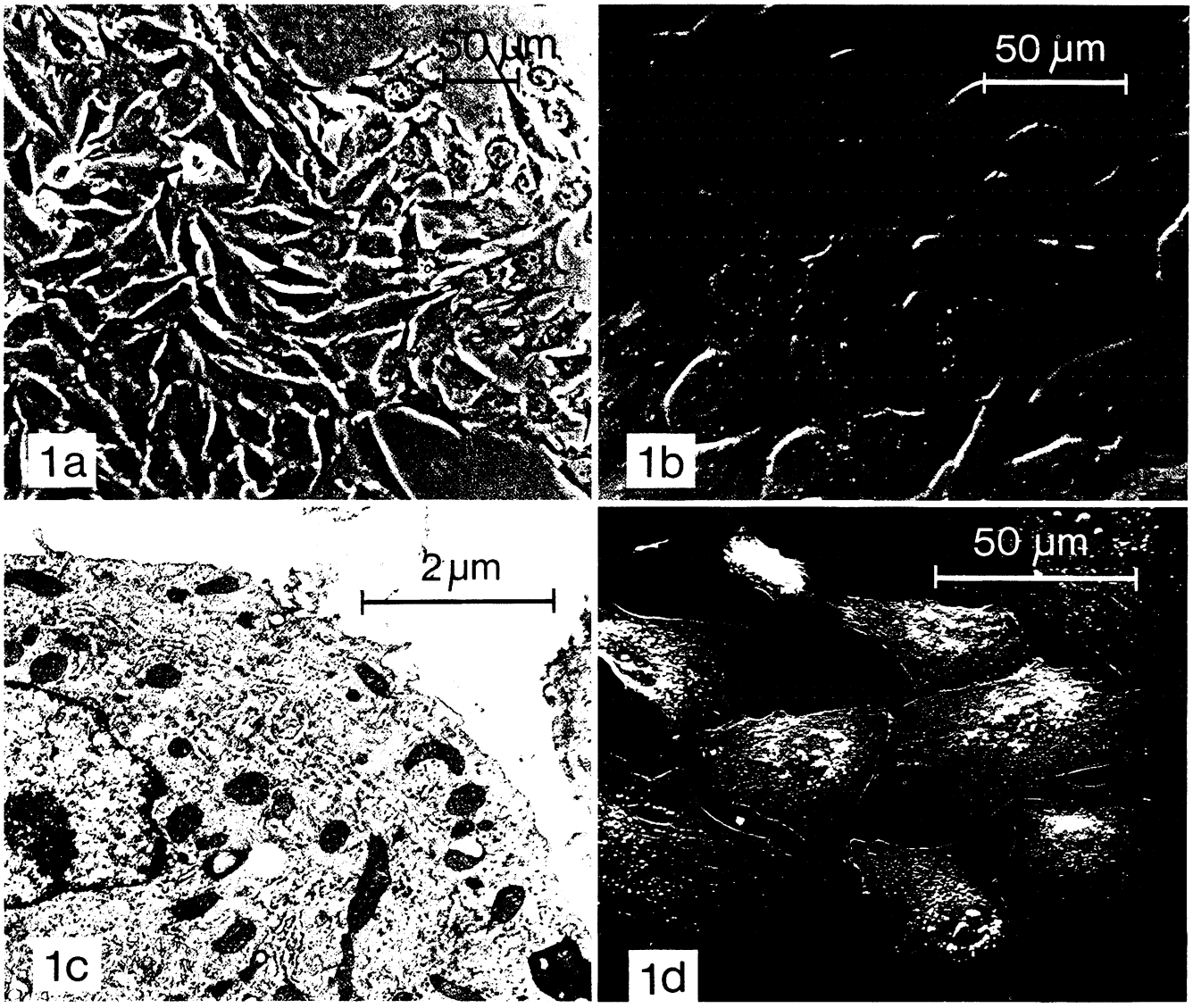

Figure 1:
Cultured coronary endothelial cells: a) Phase contrast micrograph; b) interference micrograph according to NGMARSKI; c) transmission electron micrograph; d) scanning electron micrograph.

plasmic reticulum, and abundant numbers of fine and coarse filaments. Weibel-Palade bodies. typical markers of endothelial cells from greater blood vessels, were seldomly found; therefore, the cells in culture originated mainly from small vessels.

In scanning electron micrographs (Figure 1d) the cultured endothelial cells appeared as homogenous flat cells with prominent nuclei and with numerous blebs and pits in or near the surface, especially in the perinclear region. Many bundles of thick filaments could be detected which connect different parts of the cytoplasm with the cell mebrane and which might be involved in the regulation of size and shape of the cells.

All these morphological findings indicate that the cultured coronary endothelial cells do not differ from the respective cells in vivo. This view could be further substantiated by studies of the gelelectrophoretic cytosolic protein pattern of freshly isolated coronary endothelium and of cultured cells, which proved to be very similar.

Some features of adenine nucleotide metabolism in coronary endothelial cells: In Table 1 mean values from three individual series of analyses concerning contents

Table 1:
Content of adenine nucleotides and their dephosphorylated degradatives in confluent coronary endothelial cells and in myocardial tissue of guinea pigs. Mean values from three analyses, in each of which 30 mg endothelial cells were extracted.

	Endothelial cells nmoles/g	Myocardium nmoles/g
ATP	11 960	4 280
ADP	2 760	1 050
AMP	630	160
Σ ATP, ADP, AMP	15 350	5 490
Adenosine	87	2
Inosine	100	1.2
Adenine	60	0.5
Hypoxanthine	50	0.9

of adenine nucleotides and their dephosphorylated degradatives in non-growing confluent endothelial cell cultures (G_0 phase) are listed. For reasons of comparison respective data determined in normoxic myocardial tissue are also given. It is obvious that endothelial cells contain extraordinarily high amounts of ATP, ADP and AMP. The sum of the adenine nucleotides (Σ ATP, ADP, AMP) reaches with more than 15 μmoles/g a value which is about three times higher than the total adenine nucleotide content of cardiac tissue. It shall be emphasized that to our knowledge comparably high values of adenine nucleotides have not been reported so far for any other mammalian tissue.

Another interesting feature of endothelial cells concerns the high levels of adenine nucleotide degradatives. As can be seen the contents of adenosine, inosine, adenine and hypoxanthine are about 1 to 2 orders of magnitude higher than the respective values for these compounds in the myocardium.

Additional experiments revealed that growth state of the cell cultures did not profoundly influence the total content of adenine nucleotides. As could further be shown, incubation of confluent cell cultures in purine free media for three days did not result in any detectable reduction of the adenine nucleotide content. On the other hand, endothelial cells proved to be sensitive to lack of oxygen. Brief periods of anoxic incubation (1.5 and 3 min, respectively) caused a pronounced decrease of ATP (18% and 27%, respectively) and an increase in ADP and AMP levels. Simultaneously, remarkable amounts of adenosine were formed and released from the cells into the incubation medium (107 nmoles/g and 218 nmoles/g, respectively). Obviously, energy production from anaerobic glycolytic processes was not sufficient to prevent the breakdown of nucleotides.

In Table 2 activity values of enzymes are listed which are directly or indirectly involved in degradation and synthesis of adenine nucleotides. Again for reasons of comparison respective figures are included for myocardial tissue. The following findings deserve particular attention: While 5'-nucleotidase activity in endothelial cells exceeded by far that of myocardial tissue, the activity of adenosine deaminase proved to be much higher in the myocardium. This pattern of enzyme activities may reasonably explain that adenosine is present in endothelial cells in rather high amounts compared with the small quantities of this nucleoside in the myocardium. Surthermore it is evident from the data in Table 2 that the activities of glucose-6-phosphate-dehydrogenase (Glu-6-PDH) and ribose phosphate pyrophosphonkinase (PRPP-synthease) are much higher in endothelial cells than in cardiac tissue. Lince these enzymes are known to be involved in the generation and utilization of ribose -5-phosphate, an essential intermediate in nucleotide synthesis ,it can be concluded that coronary endothelial cells possess a pronounced potency for nucleotide synthesis. As a matter of fact, we could demonstrate in preliminary experiments using 1-^{14}C-glycine and ^{14}C-labeled purine bases that nucleotide synthesis in endothelial cells proceeds via salvage and de novo pathways.

From all our findings it is evident that coronary endothelial cells are characterized by a very active adenine nucleotide metabolism. It is not yet possible, however, to decide whether the coronary endothelium in vivo can be actually regarded as important source for the release of adenosine from the heart.

Table 2:
Enzyme activities in cultured coronary endothelial cells and in cardiac tissue from guinea pigs.

	Specific activity (nmoles/min·mg) Endothelial cells	Cardiac tissue
5'-Nucleotidase (E.C. 3.1.3.5)	95	13.6
Alkaline phosphatase (E.C. 3.1.3.1)	14.6	24
AMP deaminase (E.C. 3.5.4.6)	1.1	2.2
Adenosine deaminase (E.C. 3.5.4.4)	3.4	28
Glu-6-P-dehydrogenase (E.C. 1.1.1.49)	12.7	4.2
PRPP synthetase (E.C. 2.7.6.6)	6.58	1.9
APR-transferase (E.C. 2.4.2.7)	0.7	0.2
GPR-transferase (E.C. 2.4.2.8)	0.3	0.1

Acknowledgements

The skilful technical assistance of Mrs. Marie-Luise Arnhofer and of Mr. Hans Sima is gratefully acknowledged. We are very much indebted to Prof. Dr. J. Staubesand, Department of Anatomy, University of Freiburg i. B., for the preparation of the transmission electron micrograph.

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S. NEES
Physiologisches Institut der Universität München
Pettenkofer Straße 12
D-8000 München 2