AD-A065 330 ARMY RESEARCH INST OF ENVIRONMENTAL MEDICINE NATICK MASS F/G 6/16
AN IN VITRO SYSTEM FOR STUDYING FREEZE THAW DAMAGE TO ENDOTHELI--ETC(U)
JAN 79 L R TRUSAL, C J BAKER , A GUZMAN

UNCLASSIFIED USARIEM-M-4/79 NL

| OF |
AD
A065330

END
DATE
FILMED
4-79
DDC

SECURITY CLASSIFICATION OF THIS PAGE *(When Data Entered)*

REPORT DOCUMENTATION PAGE	READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER: M 4/79	2. GOVT ACCESSION NO. / 3. RECIPIENT'S CATALOG NUMBER
4. TITLE *(and Subtitle)*: An In Vitro System for Studying Freeze Thaw Damage to Endothelial Cells in Monolayer Culture.	5. TYPE OF REPORT & PERIOD COVERED: Final rept.
	6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s): L. R. Trusal, C. J. Baker, A. Guzman	8. CONTRACT OR GRANT NUMBER(s)
9. PERFORMING ORGANIZATION NAME AND ADDRESS: Experimental Pathology Division, US Army Research Institute of Environmental Medicine, Natick, MA 01760	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS
11. CONTROLLING OFFICE NAME AND ADDRESS: 25p.	12. REPORT DATE: 2 Jan 1979
	13. NUMBER OF PAGES
14. MONITORING AGENCY NAME & ADDRESS *(if different from Controlling Office)*: USARIEM-M-4/79	15. SECURITY CLASS. *(of this report)*: Unclassified
	15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

LEVEL II

16. DISTRIBUTION STATEMENT *(of this Report)*

Unlimited

This document has been approved for public release and sale; its distribution is unlimited.

17. DISTRIBUTION STATEMENT *(of the abstract entered in Block 20, if different from Report)*

Unlimited

DDC REPRODUCED MAR 6 1979

18. SUPPLEMENTARY NOTES

19. KEY WORDS *(Continue on reverse side if necessary and identify by block number)*

endothelial cells
Leighton tubes
freeze-thaw
polymethylpentene coverslip

20. ABSTRACT *(Continue on reverse side if necessary and identify by block number)*

The study of freeze-thaw damage to endothelial cells necessitated the development of a suitable in vitro system. After investigating various substrates, Leighton tubes containing a plastic coverslip proved to be the most versatile for studying in vitro alteration to endothelial cells following thaw. The polymethylpentene coverslip is the key to its versatility. It may also be easily cut to allow portions of the same monolayer to be processed for examination by phase contrast, bright field, and both scanning and transmission electron microscopy. At the same time, the attached handle allows for easy processing.

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE

SECURITY CLASSIFICATION OF THIS PAGE *(When Data Entered)*

040 850 LB

without disturbing the monolayer. Endothelial cells also remain better attached to the plastic substrate than to glass coverslips following a freeze-thaw insult. The Leighton tube itself is ideal for temperature studies because it is water tight, allowing submersion in refrigerated water baths, and the media covering the cells may be assayed for cellular enzymes and ions released into the media following thaw. In this manner, biochemical markers may be correlated with the same cells used for all forms of microscopy.

Title: An In Vitro System for Studying Freeze-Thaw Damage to Endothelial Cells in Monolayer Culture

Authors: Lynn R. Trusal and Carol J. Baker
Experimental Pathology Division
US Army Research Institute of Environmental Medicine
Natick, MA 01760

Running Head: Versatility of Leighton Tubes

Correspondence Address: Dr. Lynn R. Trusal
Experimental Pathology Division
US Army Res. Inst. of Envir. Med.
Natick, MA 01760

Summary

The study of freeze-thaw damage to endothelial cells necessitated the development of a suitable in vitro system. After investigating various substrates, Leighton tubes containing a plastic coverslip proved to be the most versatile for studying in vitro alteration to endothelial cells following thaw. The polymethylpentene coverslip is the key to its versatility. It may also be easily cut to allow portions of the same monolayer to be processed for examination by phase contrast, bright field, and both scanning and transmission electron microscopy. At the same time, the attached handle allows for easy processing without disturbing the monolayer. Endothelial cells also remain better attached to the plastic substrate than to glass coverslips following a freeze-thaw insult. The Leighton tube itself is ideal for temperature studies because it is water tight, allowing submersion in refrigerated water baths, and the media covering the cells may be assayed for cellular enzymes and ions released into the media following thaw. In this manner, biochemical markers may be correlated with the same cells used for all forms of microscopy.

Key words: endothelial cells, Leighton tubes, freeze-thaw, polymethylpentene coverslip.

Introduction

Endothelial cells have been studied because of their importance to a variety of medical conditions including thrombosis (1,2,3), atherosclerosis (4,5), microangiopathy (6) and thromboangitis (6). Because of their strategic location at the blood-tissue interface they are also important in the pathophysiology of frostbite.

Frostbite often leads to tissue necrosis as a direct result of post-thaw hemostasis. This vascular thrombosis is believed to be initiated by freeze damage to endothelial cells which are normally nonthrombogenic when intact (1,2). Disrupted endothelial cells can expose subendothelium which can cause platelet aggregation. This initiates thrombosis leading to increased vascular permeability, edema, red blood cell sludging and eventual tissue necrosis.

In order to later study endothelial cell freeze-thaw damage as it relates to both temperature and duration of exposure, it was necessary to develop a suitable in vitro system for studying cellular alterations caused by freezing. Such an in vitro system allows more precise control of variables not possible in vivo.

Towards this aim, we describe the use of Leighton tubes containing a plastic coverslip for growth and processing of bovine endothelial cells following a freeze-thaw injury. Such a system permits cellular examination of the monolayer by phase contrast, bright field, scanning electron microscopy (SEM) and transmission electron microscopy (TEM). In addition, release of cellular enzymes as markers of cellular damage may be correlated with the same cells used for all forms of microscopy.

Materials & Methods

Cell Preparation

Aortas from newly slaughtered calves served as the source of endothelial

cells. Aortas were transported from the slaughter house to the laboratory on ice with both ends clamped with hemostats to maintain sterility as much as possible. Endothelial cells were obtained by 1 mg/ml collagenase perfusion (Type 1, Worthington Bioch. Corp., Freehold, NJ) essentially as previously described (7). Following incubation for 15 min at 37°C, the collagenase was collected and pooled with a rinse solution consisting of Earl's balanced salt solution (Ca^{++} and Mg^{++} free, pH 7.4) and the remaining loose endothelial cells from the luminal surface. After centrifugation at 750 rpm for 5 min, the cells were resuspended in media, counted and seeded as follows: in Leighton tubes (Costar, Cambridge, MA) containing a plastic coverslip (9x55 mm), on glass coverslips (11x22 mm), teflon coated glass coverslips (8) or collagen (.25%) coated glass coverslips (9) at a density of $5\text{-}6x10^5$ cells/ml. Growth media consisted of Medium 199 (Microbiological Associates, Walkersville, MO) containing 25 mM HEPES buffer and supplemented with 20% fetal calf serum, 2mM L-glutamine, 50µg/ml gentamicin and 2.5µg/ml fungizone. Preparations were placed in a 37°C incubator until coverslips reached confluency which was usually within 2-3 days. Only primary cultures were used to decrease the chance of mycoplasma contamination and improve comparisons to in vivo conditions. Testing for mycoplasma was conducted (Bio Assay Systems, Inc., Cambridge, MA) as previously described (10) with negative results.

Freeze-Thaw Procedure

For freeze-thaw studies, experimental Leighton tubes were submerged in an ethylene glycol refrigerated water bath (Neslab, Portsmouth, NH). Leighton tubes were water tight and coverslips did not require transfer to new containers for freezing. All types of glass substrates required transfer to 1 oz glass prescription bottles for freezing. The rate of freezing was approximately 0.5°C/min and was

monitored with a thermocouple permanently mounted in a sample Leighton tube containing the same amount of media (1 ml) that covered the cells. The media froze between -11 to -13°C. The frozen media was lowered to -15 or -20°C, and when the appropriate temperature was reached the cultures were thawed at a rate of 0.75°C/min. Control Leighton tubes were maintained at 37°C until processed with experimentals.

Processing for Light Microscopy

Following thaw, portions of the plastic coverslip used for light microscopy were rinsed briefly in phosphate buffered saline (PBS) (Ca^{++} and Mg^{++} free, pH 7.4) at room temperature to remove excess media and fixed in Carnoy's (1 part glacial acetic acid:3 parts absolute ETOH:6 parts chloroform) prior to Toluidine Blue O staining. The pieces of coverslip used for examination by phase contrast were mounted unfixed with the cell side down on glass coverslips (25x 60mm) for photography.

Endothelial cells were identified by phase contrast morphology (i.e. polygonal cells in close apposition)(11), transmission electron microscopy (i.e. numerous pinocytotic vesicles, 60-70 Å filaments, microtubules) (11) and the presence of Factor VIII antigen determined by indirect immunofluorescent staining (11). The rabbit antiserum to bovine Factor VIII was kindly supplied by Dr. Edward P. Kirby of Temple University.

SEM & TEM Processing

Those portions of the plastic coverslip used for SEM and TEM were processed as recently described (12). Briefly, coverslips were rinsed, fixed in 2.5% buffered glutaraldehyde, post fixed (1% buffered osmium tetroxide) and dehydrated in ethanol. Pieces of coverslips for SEM examination were critically point dried,

mounted on stubs, and sputter coated with gold-palladium for viewing. Those pieces of coverslips for TEM were embedded in Epon-Araldite from propylene oxide. After polymerization for 2 days (60°C), ultrathin sections were cut and stained with both uranyl acetate and lead citrate for viewing.

Enzyme Determinations

Immediately after thaw, media (1ml) was removed from the Leighton tube by syringe, filtered through a Millipore filter (Type GS) to remove cellular debris and refrigerated. Lactic dehydrogenase (LDH) determinations were performed as soon as all samples were collected. Measurements were made using the Worthington/Gilford LDH (P→L) kit (Worthington Bioch. Corp., Freehold, NJ) according to package insert SM 527020 and the Gilford Automated System, Model 3402 (Gilford Instru. Lab., Inc., Oberlin, OH). The six printed values were averaged for a reportable LDH activity in I.U./l at 30°C.

Statistical analyses were not performed since the data represented only one experiment. Inclosure of this information is for the purpose of illustrating the feasibility of enzyme determinations using this in vitro system without drawing conclusions to its statistical significance.

Results

Choice of Substrate

Since the endothelial cell cultures were subjected to freeze-thaw conditions, the ability of the substrate to permit normal monolayer growth and retain the cells following thaw was very important. All substrates except the teflon coated coverslips yielded good monolayer growth, with polygonal endothelial cells growing in close apposition. Teflon was found unsuitable because cells would not grow on the teflon droplets thereby interrupting the monolayer.

The integrity of the monolayer after freeze-thaw was also an important consideration. All glass substrates proved unsuitable in this regard since 90 to 95% of the cells detached after thaw, with only a few cells remaining around the periphery of the coverslip. Plastic coverslips were able to retain 75 to 95% of the monolayer following thaw. Occasionally the monolayer had a tendency to peel off in certain areas, but gentle handling minimized this occurrance. Figure 1 illustrates the subjective evaluation of the various substrates with regard to growth characteristics, integrity of the monolayer following thaw, differential staining and ease of processing for examination by light microscopy and scanning and transmission electron microscopy.

Light Microscopy

Bovine endothelial cells growing on plastic polymethylpentene substrates may be stained with a variety of histological stains or examined by phase contrast. Figure 2 illustrates control cultures maintained at 37°C, stained with Toluidine Blue O, while Figure 3 illustrates a photomicrograph of cells frozen to -20°C, thawed, and similarly stained. The small, round pyknotic cells remaining over the control monolayer (Fig. 2) are dead cells not rinsed away after seeding and attachment. In Figure 3, some cells appear normal in appearance (closed arrows) while others demonstrate pyknotic nuclei (open arrows).

All glass coverslips presented no problems for viewing by either phase contrast or bright field. Plastic coverslips presented no problems for viewing by either phase contrast or bright field when mounted on large glass coverslips using PBS and inverted for viewing. Typical permanent mounting on glass slides hindered examination at higher magnifications unless the coverslips were mounted on slides cell side up and, in turn, covered with a glass coverslip. This may then be sealed

with clear fingernail polish allowing the cells to be viewed through only the thickness of the glass coverslip.

SEM

Scanning electron microscopy was possible with either glass or plastic substrates, but plastic ones were more versatile because they could easily be cut into appropriate sizes for mounting on aluminum stubs and retained the monolayer better following thaw.

Figure 4 is a scanning electron micrograph of bovine endothelial cells maintained at 37° (control). Nuclei can be seen to rise slightly from the centers of the cells. Figure 5 represents two endothelial cells following a freeze-thaw insult at -20°C. The large cell is fairly representative of the extensive cytoplasmic disruption caused by ice crystal formation within the cell. Other cells viewed (not illustrated) demonstrated varying degrees of damage, from craters and holes in the plasma membrane to little or no external damage detectable when viewed by SEM.

TEM

The important test for the substrates was their ability to be processed for transmission electron microscopy. Glass coverslips proved very poor in this regard because of the difficulty in separating the Epon-Araldite from the glass following polymerization. Teflon coated coverslips separated well but were unsuitable in cell growth characteristics. Glass and glass coated with collagen tended to shatter, splinter or cleave into layers upon separation using liquid nitrogen immersion. Plastic coverslips embedded for either parallel or perpendicular sectioning always allowed quick and easy separation of the coverslip from the embedding resin (12).

Figure 6 is a transmission electron micrograph of a bovine endothelial cell maintained at 37°C. Cellular integrity is intact including nuclear membranes, and

mitochondria. Numerous pinocytotic vesicles (V), characteristic of endothelial cells, can be seen near the cell periphery.

An endothelial cell frozen to -15°C is illustrated in Figure 7. Freeze-thaw alterations include separation and distortion of the nuclear membrane (black arrows), and changes in the internal mitochondrial membrane (white arrow). Our observations demonstrated that other cells exhibited more extensive internal ultrastructural damage consistent with the external changes evident with SEM (Fig. 5).

Enzyme Data

LDH was assayed for its presence in the media following thaw in experimental cultures and after incubation at 37°C for controls. Figure 8 illustrates that LDH levels increased greatly following a freeze-thaw cycle. Release of LDH did not appreciably occur until after freezing at -12°C even though the cells were supercooled. The small number of cells did not present quantitation problems and as few as 5×10^5 cells released as much as 100-300 I.U./l @ 30°C following a freeze-thaw state.

Discussion

Any in vitro cell culture system designed to study cellular injury must be versatile enough to allow processing and subsequent examination by the major forms of microscopy. It is also desirable to use other parameters, such as the release of enzymes, to evaluate cellular damage. We have found the Costar Leighton tube containing a polymethylpentene plastic coverslip to be such a system; suitable for studying freeze-thaw damage to bovine endothelial cells by four types of microscopy (phase contrast, bright field, SEM, TEM) and release of cellular enzymes.

The Leighton tube is particularly suited for temperature studies which require submersion of the cell culture in a refrigerated water bath. It is self-contained, water tight and does not require transfer of the coverslip to another container for freezing. It was our experience that individual glass coverslips (11x22mm) proved difficult to manage in this regard and required transferring between the culture dish and a glass prescription bottle for freezing. The coverslips were often broken and the monolayer damaged because of excessive handling. The presence of a handle on the Leighton tube coverslip permitted easy handling and decreased the chance of disturbing the monolayer.

Temperature equalization between the water bath and the media temperature did not prove to be a problem. By means of a thermocouple implanted in an identical Leighton tube containing the same amount of media, it was possible to demonstrate that the temperature of the media was only $1^{o}C$ higher than the external water bath temperature. Therefore, there was not a long latent period for temperature equalization between the water bath and the media covering the cells. The assumption is made that there is little or no appreciable difference between the freezing temperature of the media and the cells.

We also found that endothelial cells remained attached better to plastic than to glass following a freeze-thaw insult. Prior use of glass coverslips showed that most of the cells detached following thaw. Plastic coverslips were able to retain most of the monolayer following thaw.

Another advantage to the use of the large (9x55mm) plastic coverslips is their ability to be easily cut for processing for the four types of microscopy. Following thaw, the coverslip can be briefly rinsed to remove excess media. The first portion of the coverslip may then be cut off and mounted cell side down on a large glass

coverslip for phase contrast examination. Another portion or portions may be excised and fixed for staining by different histological stains such as Toluidine Blue O, Hemotoxylin and Eosin, and Acridine Orange. The remaining piece of coverslip with attached handle may then be fixed with glutaraldehyde and post fixed in osmium tetroxide in preparation for SEM and TEM examination. Following dehydration, the piece of coverslip for SEM examination may be excised and critical point dried; while the last portion of the coverslip is embedded in resin prior to TEM examination, in either cross section or "en face". This procedure has been previously discussed in detail (12). Thus, portions of the same monolayer may be processed separately and examined by four forms of microscopy.

In addition to using the in vitro system for examining freeze-thaw alterations in endothelial cells on both the light and electron microscopy level, we were interested in evaluating the versatility of the Leighton tube as a means of studying cellular damage via release of cellular enzymes and ions. Thus, we assayed the media for the presence of glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), creatine phosphokinase (CPK), lactic dehydrogenase (LDH) and Sodium (Na^+) and Potassium (K^+). Results of approximately 5 experiments illustrated that GOT, GPT and CPK could not be detected in significant amounts above control levels using between 5-6 x 10^5 cells and spectrophotometric methods of detection. Likewise, Na^+ and K^+ concentrations were also unchanged as measured by flame photometry. Thus, only LDH was released in appreciable quantities to be detected following the freeze-thaw state. This proved to be very reproducible with one experiment illustrated in Figure 8. Although the Leighton tube capacity for monolayer growth (area 5 cm^2) is relatively small, the small amount of media (0.75-1.0 ml) necessary to cover the

cells probably facilitates detection of enzymes released into the media. Although bovine endothelial cells are not believed to contain high amounts of GOT, GPT or CPK, other cultures, such as hepatocytes, might be expected to release appreciable quantities of GOT and GPT when subjected to stresses such as heat, cold or cytotoxic chemicals.

In order to later improve in vivo comparisons and decrease the chance of overgrowth by smooth muscle and bipolar cells, we chose to use only primary cultures of endothelial cells. The use of such primaries sometimes presents the problem of obtaining enough cells for processing by several procedures. Many of the techniques using plastic substrates utilize large flasks for cell culturing. This method, although satisfactory, requires a greater number of cells and a longer growth period to achieve a monolayer. With Leighton tube cultures, we found that as few as 5×10^5 cells yielded a confluent monolayer in two days. The coverslip is large enough to use for SEM, TEM and light microscopic examination without needing millions of cells.

It was not the intent of this investigation to go into extensive detail concerning specific ultrastructural changes caused by freezing and thawing of endothelial cells. This will be the subject of a later paper. More specifically, it was our intent to demonstrate the feasibility of using the Costar Leighton tube with plastic coverslip as a suitable system to study, using various methods, freeze-thaw damage to endothelial cells. This system will now be used to study the effects of various temperatures and durations on the micro and macrostructure of this cell type.

In conclusion, the Leighton tube with a plastic coverslip was found to be very versatile for studying the effects of various temperatures on bovine endothelial

cells. Its advantages are summarized as follows: the Leighton tube is self-contained and water tight allowing submersion for temperature studies, release of cellular enzymes may be correlated with the same cells used for microscopy examination, the large plastic coverslip allows all forms of microscopy to be done on the same monolayer, the plastic coverslip is easy to cut into various sizes for versatility in processing, the coverslip handle allows easy processing without disturbing the monolayer, the cell monolayer remains better attached to the plastic coverslip following a freeze-thaw injury, and plastic coverslips permit easy resin embedding and separation for electron microscopy.

Acknowledgement

The author gratefully acknowledges the contributions of the following individuals: Dr. Wilbert Bowers for his advice and council, Albert Guzman for preparation of electron microscopy samples and Sandra Lamont for manuscript preparation.

Disclaimer Statement

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision, unless so designated by other official documentation.

Figure 1. Chart illustrating a subjective evaluation of four different substrates used for growth and processing of bovine endothelial cells.

Figure 2. Light micrograph of a control (37°C) culture of bovine endothelial cells stained with Toluidine Blue O. Round pyknotic cells are dead cells not removed with rinses following seeding and attachment. x 205.

Figure 3. Light micrograph of bovine endothelial cells frozen to –20°C and then thawed. Some cells appear like controls (white closed arrows) while others demonstrate pyknotic nuclei (white open arrows). Toluidine Blue O, x 830.

Figure 4. Scanning electron micrograph of control (37°C) bovine endothelial cells growing on the plastic substrate. Nuclei can be seen to bulge from the center of the cells. x 2965.

Figure 5. Scanning electron micrograph of freeze-thawed (–20°C) bovine endothelial cells. The large cell exhibits extensive cytoplasmic destruction as the result of ice crystal formation. x 5450.

Figure 6. Transmission electron micrograph of a control (37°C) bovine endothelial cell. Nuclear membrane and mitochondria appear normal. Pinocytotic vesicles (V) and 60-70 Å filaments (F) are evident. x 19,000.

Figure 7. Transmission electron micrograph of a freeze-thawed (–15°C) bovine endothelial cell. The nuclear membrane has separated and moved away from the peripheral chromatin (black arrows) and some mitochondria display distorted internal membrane structures (white arrow). x 11,825.

Figure 8. Graph illustrating amounts of LDH released from a monolayer culture of bovine endothelial cells at various temperatures (°C). Freeze occurred at –12°C. Solid line represents LDH detected in media from tubes containing cells while the dashed line represents corresponding controls containing only media.

References

1. Ashford, T. P. and D. G. Freiman. 1967. The role of the endothelium in the initial phases of thrombosis. Am. J. Pathol. 50:257-273.

2. Bunting, S., S. Moncada and J. R. Vane. 1977. Antithrombotic properties of vascular endothelium. Lancet 2:1075-1076.

3. Nordoy, A., B. Svensson and J. C. Hoak. 1978. The inhibitory effect of human endothelial cell monolayers on platelet reactions and its inhibition by aspirin. Thromb. Res. 12:597-608.

4. Clopath, P. 1978. Arteriosclerotic and atherosclerotic lesions induced in swine by single and repeated endothelial cell injury. Artery 4:275-288.

5. Lewis, J. C. and B. A. Kottle. 1977. Endothelial damage and thrombocyte adhesion in pigeon atherosclerosis. Science 196:1007-1009.

6. Bell, E. T. 1960. Diabetes Mellitus. Charles C. Thomas, Springfield, IL

7. Macarak, E. J., B. U. Howard and N. A. Kefalides. 1977. Properties of calf endothelial cells in culture. Lab. Invest. 36:62-67.

8. Chang, J. P. 1974. A new technique for separation of coverglass substrate from epoxy-embedded specimens for electron microscopy. J. Ultra Struct. Res. 37:370-375.

9. Heyner, S. 1963. In situ embedding of cultured cells or tissue, grown on glass, in epoxy resins for electron microscopy. Stain Technol. 38:335-336.

10. Russell, W. C., C. Newman and D. H. Williamson. 1975. A simple cytochemical technique for demonstration of DNA in cells infected with mycoplasmas and viruses. Nature 253:461-462.

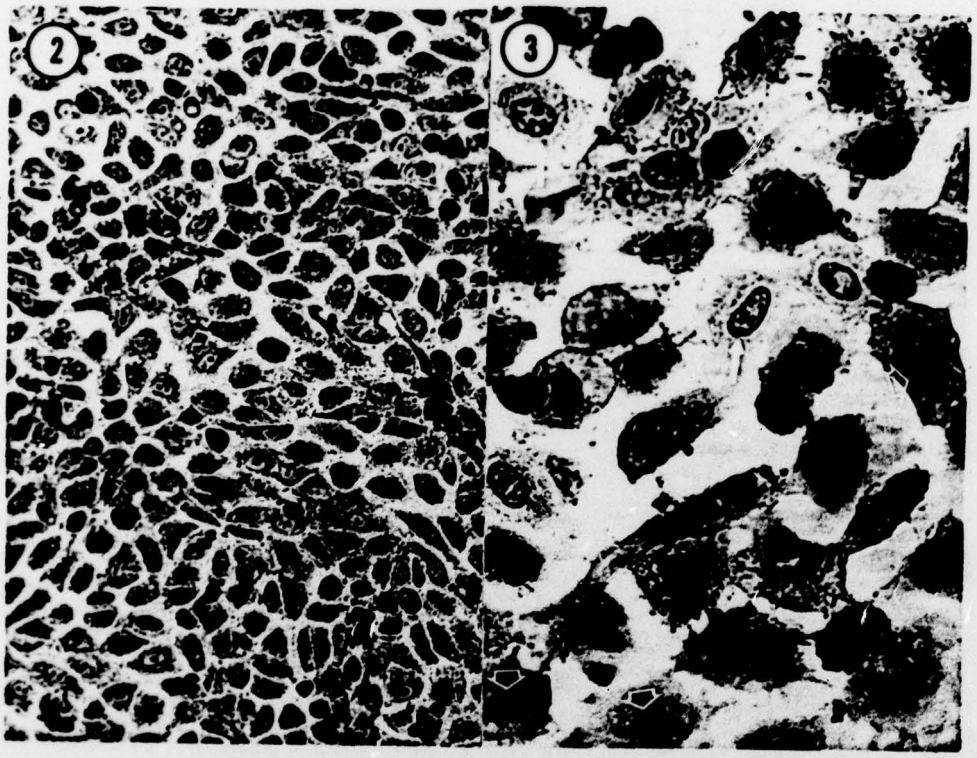
11. Gospodarowicz, D., K. D. Brown, C. R. Birdwell and B. R. Zetter. 1978. Control of Proliferation of human vascular endothelial cells: Characterization of the response to human umbilical vein endothelial cells to fibroblast growth factor, epidermal growth factor and thrombin. J. Cell Biol. 77:774-788.

12. Trusal, L. T., C. J. Baker and A. J. Guzman. Processing of endothelial cell monolayers grown on polymethylpentene substrates for transmission and scanning electron microscopy. (Submitted to Stain Technol.)

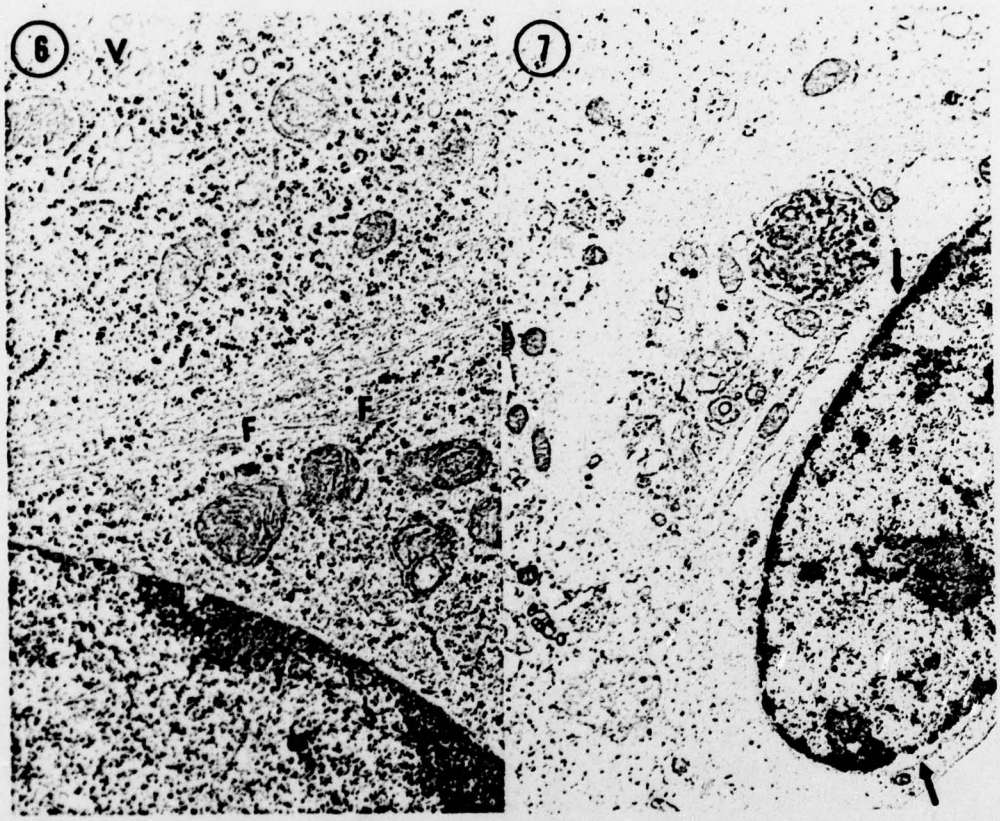
(1)

EVALUATION OF CELL SUBSTRATES

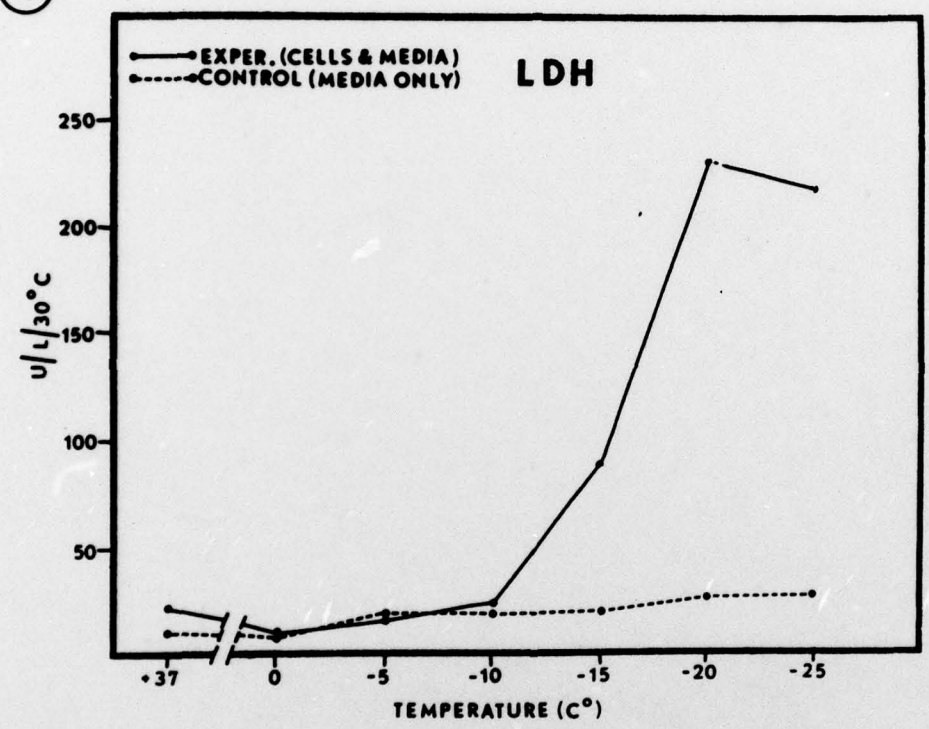
SUBSTRATE	CELL MONOLAYER			MICROSCOPY		
	GROWTH	INTEGRITY	STAINING	LIGHT	SEM	TEM
GLASS COVERSLIPS	EXCELLENT	POOR	EXCELLENT	EXCELLENT	EXCELLENT	POOR
GLASS COVERSLIPS (TEFLON COATED)	POOR	POOR	EXCELLENT	EXCELLENT	—	GOOD
GLASS COVERSLIPS (COLLAGEN COATED)	EXCELLENT	POOR	EXCELLENT	EXCELLENT	—	POOR
PLASTIC COVERLSIPS (COSTAR LEIGHTON TUBE)	EXCELLENT	GOOD	EXCELLENT	GOOD	EXCELLENT	EXCELLENT


2
3

4
5
10μ


6
V
F
F
7

(8)


EXPER. (CELLS & MEDIA)
CONTROL (MEDIA ONLY)
LDH
250
200
150
100
50
U/L/30°C
+37
0
-5
-10
-15
-20
-25
TEMPERATURE (C°)