CELL CULTURING OF SWINE AORTIC
SMOOTH MUSCLE CELLS

———————

A Thesis

Presented to

the Faculty of the School of Sciences and Mathematics

Morehead State University

———————

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

———————

by

Rita E. Bustos

2 May 1979

Accepted by the faculty of the School of Sciences and Mathematics, Morehead State University, in partial fulfillment of the requirements for the Master of Science degree.

_____
Director of Thesis

Master's Committee: _____, Chairman

_____

Howard S. Setser

_____
May 8, 1979
(date)

ABSTRACT

CELL CULTURING OF SWINE AORTIC
SMOOTH MUSCLE CELLS

Rita Bustos, M. A.
Morehead State University, 1979

Director of Thesis: _____

Presently there are many possible causes of athero-
sclerosis being studied, and smooth muscle cell cultures
can be a very important tool in the study of the disease.
Smooth muscle cells were obtained from swine abdominal
aorta and the cells were cultured under sterile procedure.

Two methods of culturing were included, the explant
method and the enzyme method; of the two, the explant
method was found more efficient under the conditions of
this study. Methods of culturing and sterile procedure
are emphasized.

Accepted by: _____, Chairman

_____

_____

ACKNOWLEDGEMENTS

I wish to express my gratitude to Dr. David Saxon for all the encouragement and guidance he provided during the course of this study. I also wish to thank Dr. David Magrane and Dr. Howard Setser for their advise and support all through my graduate work. I am grateful to Dr. Ted Pass for the loan of his equipment, and Mr. Allen Lake for his help with the photographs. I am also grateful to Dr. Betty Sisken, Dr. H. Swim, and Ann Brian of University of Kentucky for their advice.

My special thanks to Afaf Alshamma, Milford Jarrells, and Jim Salyer for their help during this work. I would like to thank my fiancé, Truman Duncan, for all his help with the experiment, photographs, and encouragement he offered while working on this thesis. Finally I wish to express gratitude to my parents for their constant support and love.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

The "response to injury" hypothesis of athero-
sclerosis began with the work of Virchon (Ross and
Glomset, 1976; Goldstein, et al., 1977). Presently, there
are many possible causes of the disease being studied, and
many factors investigated that may effect the development
of the process once it is initiated. The cause for the
initial damage to the endothelium of the artery may vary,
however once the endothelium is damaged, the wall of the
vessel becomes much more permeable to plasma constituents
which enter the intima and smooth muscle cells in the
media. As a result, these cells begin to proliferate at
a much faster rate than normal (Ross and Glomset, 1973 and
1976; Yeung, 1976).

Since smooth muscle cell proliferation is a main
component observed in the development of atherosclerosis,
smooth muscle cell culturing can be a very important tool
in the study of the disease. The cultures provide a very
effective way of isolating one of the components of the
lesion and studying it in detail; thereby eliminating, or
holding constant, some of the many variables involved.

The method of using smooth muscle cell cultures to
test for causative agents of the disease is a good start,

but not definitive proof of cause since whether or not
smooth muscle cells in vitro react to stimuli in the same
fashion as cells in vivo is devatable. Ross (1971) and
Brown, et al., 1976 noted that cultured and subcultured
cells retain the same characteristics of differentiated
smooth muscle cells. Chamley, et al., (1977) however,
found that cells from primary cultures remain differenti-
ated only for about nine days and the cells could no
longer be induced to contract after nine days. Also, none
of the subcultures could be induced to contract, there-
fore he concludes they were not differentiated smooth
muscle cells. Separate studies by Blose, et al., (1975)
and Fowler, et al., (1977) also support Chamley's results.
The studies of Chamley, et al., (1977) also show that de-
differentiated cells do not stain with antibodies against
myosin. These cells also tend to have much more of the
cellular organelles usually present during mitotic activi-
ty, such as ribosomes, rough endoplasmic reticulum, mito-
chondria and golgi complexes (Chamley-Campbell, et al.,
1979).

Several substances have been shown to increase
proliferation of smooth muscle cells by the method of cell
culturing, such as hyperlipemic serum (Mahley, et al.,
1977; Chen, et al., 1977; Coltoff-Schiller, et al., 1976),
insulin (Ledet, 1976), and platelet factor (Chamley-
Campbell, et al., 1979). The exact nature of the platelet

growth factor has not been determined, although Ross and Glomset, (1976) discovered that by adding it to smooth muscle cell cultures the cells showed a great increase in rate of growth. They state that the platelet factor is non-dialyzable, relatively heat stable, and probably basic (Ross, et al., 1977); adding that it is possibly derived from the pituitary gland, taken up by platelets and concentrated in granules or synthesized in the megakaryocyte during development (Antoniades and Scher, 1977). Holmsen and Day, (1970) suggest this factor includes serotonin and adenine nucleotides. The platelet factor does require plasma constituents to induce the completion of more than one mitotic cycle (Vogel, et al., 1978; Ross, et al., 1974).

Chamley's method of cell culturing uses collagenase and elastase, versus the explant method used by Ross and others. It is possible that the use of these enzymes alters the physiological state of the cells, for this reason both methods were used in this study. It should be emphasized that it is likely that cells in culture do not react exactly the same as cells in vivo, therefore one may not always directly relate the results; however finding a significant increase in the rate of growth in cultures is certainly a good indication that this may occur in vivo and therefore greater investigation of that

factor is warranted.

Swine were selected as a model system for several reasons: a) Their cardiovascular system is basically the same as in humans. b) The distribution of coronary arteries of swine is like that of humans. c) Changes produced by growth and the aging process that lead to atherosclerosis are also very similar in human and swine aortas (Ratcliffe and Lunginbuhl, 1971; Jarmolych, et al., 1968). d) Many species transport their cholesterol primarily as HDL, these species are resistant to atherosclerosis; however a few species such as humans, most swine, and monkeys carry much of their cholesterol in the bloodstream as LDL and are susceptible to the disease process (Nicolosi, et al., 1977).

Male animals were used because several studies indicate that the male sex hormones may affect the development of atherosclerosis (Khan, et al., 1977). In a study by Fisher-Dzoga, et al., (1974) the addition of estrogen to rabbit aortic smooth muscle cells in culture inhibited the proliferative effects of hyperlipemic serum.

The aortic media was used to provide the smooth muscle cells necessary to start the cultures because it is entirely made up of smooth muscle cells (Ross and Glomset, 1976).

This study involved the isolation of swine smooth muscle cells by the enzyme dispersal and explant methods. The investigation also included an analysis of the problems of culturing these smooth muscle cells, particularly with respect to pH changes, and contamination by bacteria and fungi.

CHAPTER II

MATERIALS AND METHODS

Removal of Abdominal Aorta

Smooth muscle cell cultures were prepared from the abdominal aorta of healthy, 8 to 10 week old, Yorkshire swine that had been fed a normal diet of Purina Pig Chow. They were chloroformed and the aortic segment surgically removed under a laminar flow laboratory hood. All instruments used were sterilized at $122^{\circ}C$ for 30 minutes. The surgical procedure involved cleaning the skin area with 70% ethanol, and making the initial incision with a scalpel without penetrating into the peritoneal cavity. The peritoneum was then cleansed; entrance into the abdominal cavity was then performed with sterile instruments.

The abdominal aorta was clamped on both sides with hemostats, and scissors were used to remove a piece of abdominal aorta 5 to 7.5 cm long. The aortic section was placed on a clean culture dish containing culture medium. The fat was removed, the portion of aorta rinsed three times in culture medium and then divided into two segments of equal size. One of these segments was maintained in culture medium for 2 to 3 hours while the explants were prepared; subsequently this segment was used to pre-

6

pare cultures as described by Chamley (1977).

Cell Culturing

The following procedures are essentially those of
Ross (1971), Brown, et al., (1976), Mahley, et al., (1977),
Fowler, et al., (1977), and Chamley, et al., (1977). Care
was taken to clean all work areas, and sterilize all in-
struments and glassware to avoid culture contamination.
Glassware was cleaned with a solution of concentrated
nitric acid (20 mL) and sulfuric acid (1 L), then it was
well rinsed and soaked in a solution of sodium bicarbonate
to neutralize the surface (Swim, personal communication).
The medium was sterilized by filtering it through a
disposable millipore filter (Millipore Corp.), 25 mm in
diameter and with a pore size of 0.22 μm, into a sterile
culture medium bottle. Sterile disposable Falcon culture
dishes (60 mm x 15 mm) and 30 mL sterile Falcon flasks
were used. Pipettes (with cotton plugs), centrifuge tubes,
stoppered bottles for storage of culture medium, forceps
and razor blades were also sterilized.

Culture medium was prepared within 24 hours prior
to use. The components and proportions are listed in
Table 1, and they were modified from Ross (1971), Fowler,
et al., (1977), Brown, et al., (1976) and Sisken (personal
communication). This medium was used to rinse the tissue

Table I. Medium Components for Swine Aorta Smooth Muscle Culture.

Type of Constituents	mL of constituent in 100 mL of medium
[1]Dulbecco's Modified Eagle Medium	88.5 cc
Fetal Bovine Serum	10.0 cc
MEM Nonessential Amino Acid Solution	1.0 cc
Penicillin-Streptomycin Solution (5000 units of each per mL)	0.7 cc
[2]Dilute HCl	added until pH of the medium reaches 7.1 or 7.2.
[3]Fungizone-Amphotericin B (250 mcg/mL)	25 μg

[1]The constituents of Dulbecco's Modified Eagle Medium are given in Table II (Appendix).

[2]The dilute solution was prepared with 0.5 mL of concentrated HCl to 20 mL of triple distilled water and then sterilized.

[3]Added to only half of the cultures.

removed from the swine and to culture the cells. Fungizone was added to half of the cultures and compared with cultures without it. Fungizone is used by Fowler, et al., (1977) but not used by most other workers in the field. Prepared culture medium was stored at 4 to 6°C.

Explant Method of Culturing Smooth Muscle Cells

The section of aorta was cut longitudinally, then the intima, adventitia, and part of the outer media were removed by stripping it with forceps and a scalpel. The remaining layer of the aortic media was washed three times in culture medium and then divided with a sterile razor blade into pieces about 0.5 to 1.0 mm square (Ross, 1971; Brown, et al., 1976). Several small grids were made on the bottom of each culture dish with a scalpel; a piece of tissue was then placed on each grid (Swim, personal communication). To avoid dislocation of the explants when transfering the dishes to the incubator, one or two drops of culture medium were carefully added to each section to keep it moist. The culture dishes were then transfered to an incubator, filled with 1.5 mL of culture medium and then perfused with a 95% O_2 and 5% CO_2 gas mixture for approximately 30 minutes. They were maintained in a moist air Psycrotherm environmental incubator (see pages 11 and 12) with a 95% O_2 and 5% CO_2 gas mixture until

confluence, which took approximately 35 to 40 days. The culture medium was changed every other day.

Chamley's Method of Culturing Smooth Muscle Cells (Enzyme Dispersal)

In this method, described by Chamley, et al., (1977) the piece of aorta was stripped of its adipose tissue, everted, sealed at both ends with a sterile clip and immersed in a collagenase (1 mg/mL) solution for 30 minutes. Then the segment was removed and rinsed in trypsin (1.25 mg/mL) twice, leaving it immersed for 3 minutes each time, at 37°C to remove the endothelium. The vessel was then placed in a 4 mL solution of collagenase (1 mg/mL) and elastase (0.5 mg/mL), in a 1:1 ratio, for two hours with occasional gentle agitation. The vessel was removed, the solution placed in sterile test tubes and centrifuged at 1500 rpm (5422 g's) for 6 minutes. The supernatant was discarded, the pellet redissolved in 3 mL of culture medium (Chamley, et al., 1977). The culture was placed in a flask, loosely capped and left in the moist air incubator with a 95% O_2 and 5% CO_2 gas mixture for two hours. Then the cap was tightened.

The culture medium was changed every other day and perfused with the 95% O_2 and 5% CO_2 gas mixture each time. The cultures were grown to confluency.

Incubator

The dessicator-incubator which was designed and used in the Psycrotherm incubator is illustrated in figure 1. It illustrates the inlet pipette for the gas mixture introduction into the dessicator and an exhaust pipette for the gas mixture. Both were only opened for about 30 minutes after opening the dessicator to let the gas mixture perfuse the cultures.

Figure 1. Single incubator contained in a Psycrotherm incubator
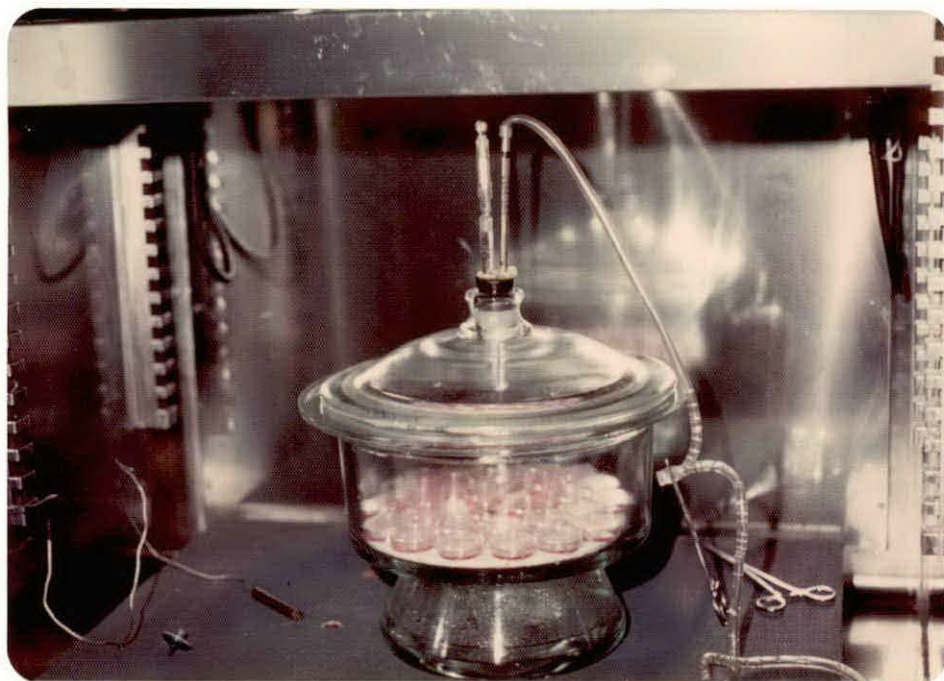
The next illustration is basically the same design as figure 1 but provides for an additional incubator to be used. The extra incubator provided a type of isolation chamber for cultures that were suspected of being contaminated. The chambers were necessary for two purposes; one, to retain the gas mixture and the other to retain the air misture and thus prevent evaporation of the culture medium.

Figure 2. Double incubator design contained in the Psycrotherm incubator.

RESULTS AND DISCUSSION

Cell growth and proliferation of swine smooth
muscle cells in primary culture was obtained. The first
time growth was obtained, the cultures were contaminated
by bacteria very early and the proliferation was minimal
(growth was confirmed by Ann Brian - Dr. Swim's laboratory
at University of Kentucky). The second time, the cultures
did proliferate for four weeks without contamination.
This was confirmed by observation with an inverted phase
microscope.

Photographs in figures 3, 4, 5, and 6 illustrate
the growth of smooth muscle cells in primary culture after
four weeks. Smooth muscle cells must be stained with
labelled smooth muscle myosin to conclusively prove that
the cells are in a dedifferentiated state (Groschel-
Stewart, et al., 1975; Blose, et al., 1975). This study
did not include myosin staining of the cells in culture.
However, since the cells in culture were proliferating it
can be assumed that these cells are in the dedifferentia-
ted state, because this is a prerequisite for the cells
to be in a mitotic state.

The rate of proliferation was not as rapid as the
rate reported in previous studies by Ross and Glomset,
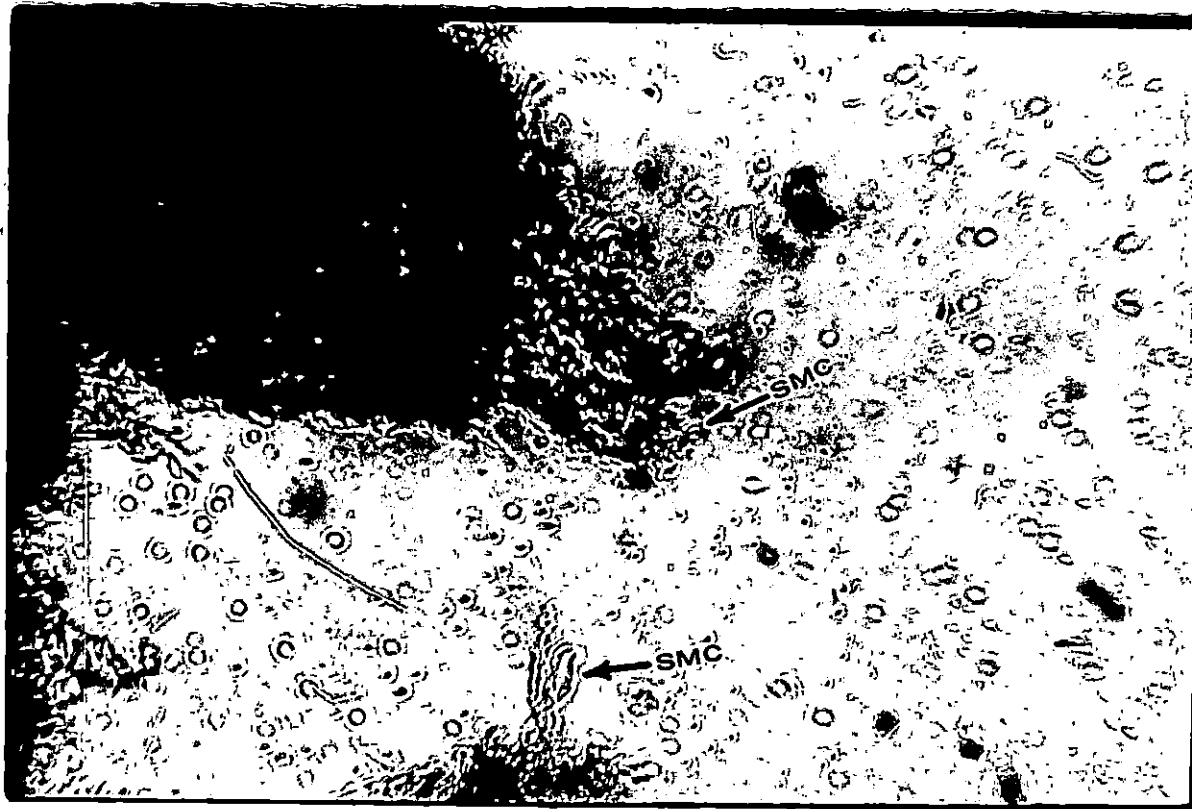(1973 and 1976) and Chamley, et al., (1977). The constit-

13

Figure 3. A photograph indicating proliferating smooth muscle cells (SMC) growing from an explant (arrow) and a few isolated smooth muscle cells at the bottom of the picture (100 x).
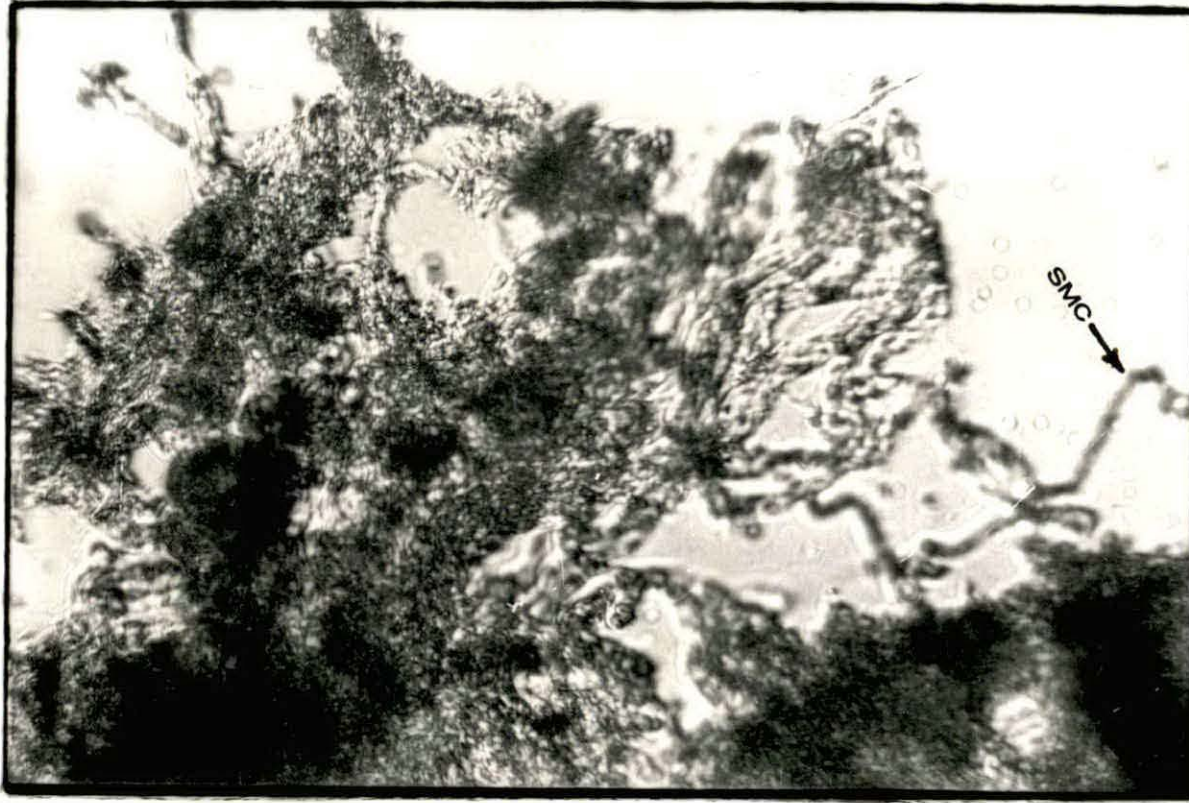
Figure 4. Photograph of a pellet of dedifferentiated smooth muscle cells (SMC) after dislodging the cells with trypsin. Note the arrow indicating single cells (300 x).
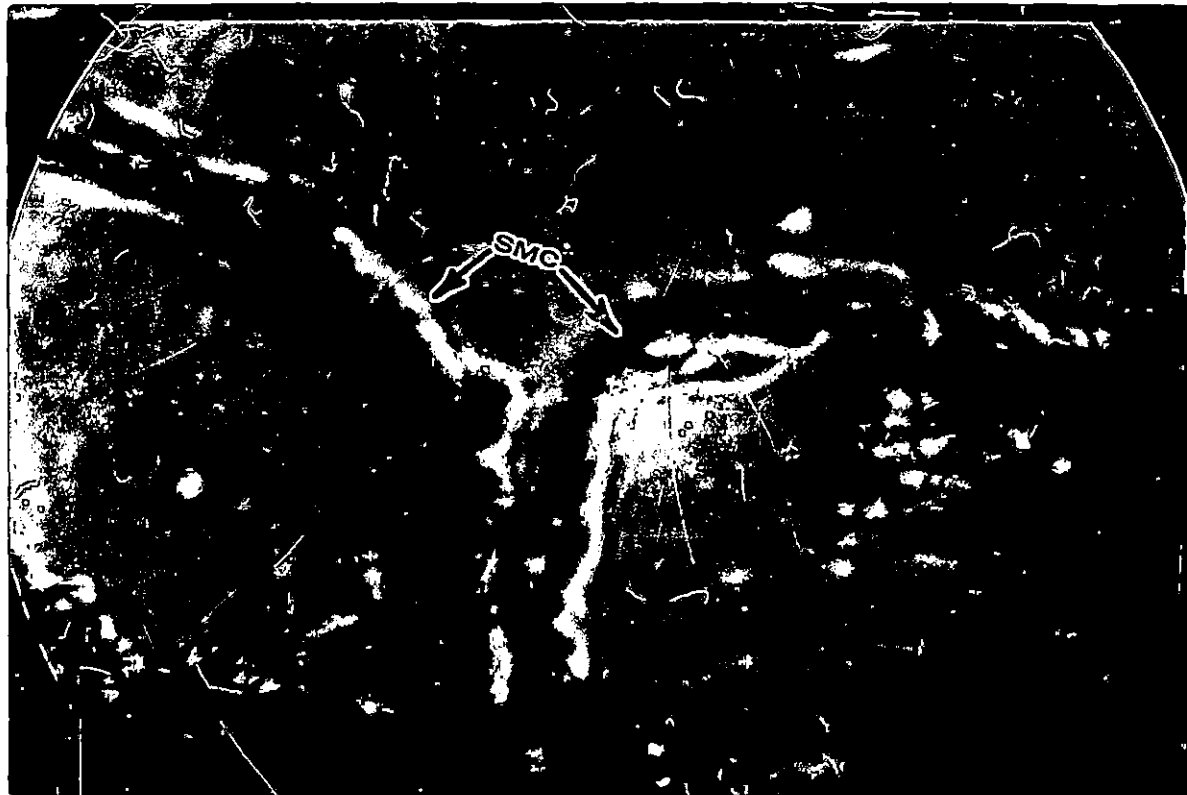
at

Figure 6.   Photograph of sections of two dedifferentiated smooth muscle cells
            (SMC at 700 x).

uents used in the culture medium were the same as those
used by the studies just mentioned. The slower rate of
growth may have involved an inability to maintain a con-
stant pH of 7.4. The pH of the medium immediately before
its addition to the cultures was between 7.0 and 7.1.
Exposure of the culture medium to atmosphere would change
the pH to 7.4 - 7.5 before it was completely perfused with
the 95% O_2 and 5% CO_2 mixture that maintained a fairly
constant pH. At times the pH would change to 7.7 to 7.8
before it would come to equilibrium. This change of pH
did not result in necrosis of the smooth muscle cells;
however, it is likely that the change in pH would affect
them enough to delay mitotic events. Although the pH did
change the cells remained alive. Trypan blue, a vital
stain, was used to verify that the cells remained alive
throughout the entire period of incubation. This stain
is not internalized by living cells. Methylene blue,
another vital stain, which is internalized by living cells,
was also used, and indicated that the cells were alive.

The grids on the bottom of the culture dishes, were
effective in the attachment of explants. Yet this did
present some minor difficulty in trying to microscopical-
ly distinguish between the grid scratches and cells
growing out of the explant. This difficulty was alleviated
by gently swirling the culture medium under an inverted

microscope and producing movement of the attached cells.
After a few days this was not necessary since the cells
could be seen growing out of the grids, and were easily
distinguished.

The size of the cells seen was from 120 μm to
about 190 μm in length, this is in agreement with the
100 μm to 200 μm in length reported in the literature
(Chamley-Campbell, et al., 1979).

The main problem in culturing the cells was prevent-
ing bacterial and fungal contamination. Penicillin,
Streptomycin and Fungizone were only efficient when very
sterile procedure was followed and the culture medium
was filtered before adding it to the cell cultures. The
cultures did become detectively contaminated with bacteria
and fungi within approximately ten hours. This probably
occured because the millipore filter did not function
properly. If the temperature of the autoclave used to
sterilize the millipore filter is only 3 to 4°C higher
than the required temperature, the filter may shrink.
The temperature of the autoclave used to sterilize the
filters was about 6°C higher than recommended; this may
have allowed the passage of microscopic organisms into
the culture medium. In one of the experiments Fungizone
was added to half of the cultures. However, no fungal
contamination appeared in any of those cultures at that

time. Fungizone was also tested in bacterial medium and found ineffective in controlling fungal growth at the concentration that it could be used in the smooth muscle cell cultures. Culture medium removed from the cultures was plated on bacterial and fungal plates to check for contamination. At the start of the experiment, the culture medium that remained after cutting the blood vessel into small pieces for the explant was also placed on bacterial plates to test for contamination.

Once the cultures were contaminated, it did not prove feasible to remove the bacterial or fungal contamination since even if the culture medium was changed every $\frac{1}{2}$ hour for 6 hours, the contamination remained. The entire body surface of each swine was washed in disinfectant soap before operating to decrease contamination of the cultures. Also sterilized water, rather than unsterilized, was used in the incubator to moisturize the air.

In conclusion, of the two methods analyzed, the explant method was found more efficient than the enzyme method. Techniques to prevent bacterial and fungal contamination, and to provide a good surface for cell attachment have been investigated. It is hoped that the development of these techniques will ease the difficulty of growing smooth muscle cells in culture. Smooth muscle cells are a very important component of the disease

process of atherosclerosis. Their growth in culture
provides an important advantage of studying the disease
in an environment where variables can be controlled.

LITERATURE CITED

LITERATURE CITED

Antoniades, H. N. and C. D. Scher. 1977. Radioimmunoassay
    of a human serum growth factor for Balb/c3T3 cells:
    derivation from platelets. Proc. Natl. Acad. Sci.
    USA 74:1973-1977.

Blose, S. H., E. Lazarides, and S. K. Chacko. 1975. Loss
    of differentiated phenotype in the progeny of aortic
    smooth muscle cells. J. Cell Biol. 67:34a.

Brown, B. G., R. Mahley, and G. Assman. 1976. Swine aortic
    smooth muscle in tissue culture: some effects of
    purified swine lipoproteins on cell growth and morpho-
    logy. Circ. Res. 39:415-424.

Chamley, J. H., G. R. Campbell, J. D. McConnell, and
    U. Groschel-Stewart. 1977. Comparison of vascular
    smooth muscle cells from adult human, monkey and
    rabbit in primary culture and in subculture. Cell
    Tissue Res. 177:503-522.

Chamley-Campbell, J., G. R. Campbell, and R. Ross. 1979.
    The smooth muscle cell in culture. Physiol. Rev.
    59:1-61.

Chen, R. M., G. S. Getz, K. Fischer-Dzoga, and R. W.
    Wissler. 1977. The role of hyperlipidemic serum on
    the proliferation and necrosis of aortic medial cells
    in vitro. Exp. Mol. Pathol. 16:259-374.

Coltoff-Schiller, B., S. Goldfischer, A. M. Adamany, and
    H. Wolinsky. 1976. Endocytosis by vascular smooth
    muscle cells in vivo and in vitro: roles of vesicles
    and lysosomes. Am. J. Pathol. 83:54-60.

Fisher-Dzoga, K., D. Vessenlinovich, and R. W. Wissler.
    1974. The effect of estrogen on the rabbit aortic
    medial tissue culture cells. Am. J. Pathol. 74:52a.

Fowler, S., H. Shio, and H. Wolinsky. 1977. Subcellular
    fractionation and morphology of calf aortic smooth
    muscle: studies on whole aorta, aortic explants,
    and subcultures grown under different conditions.
    J. Cell Biol. 75:166-184.

Goldstein, J. L.,and M. S. Brown. 1977. Atherosclerosis:
    the low-density lipoprotein receptor hypothesis.
    Metabolism. 26:1257-1275.

Gröschel-Stewart, U., J. H. Chamley, J. D. McConnell, and
   G. Burnstock. 1975. Comparison of the reaction of
   cultured smooth and cardiac muscle cells and fibro-
   blasts to specific antibodies to myosin. Biochem.
   43:215-224.

Gröschel-Stewart, U., J. H. Chamley, G. R. Campbell, and
   G. Burnstock. 1975. Changes in myosin distribution
   in dedifferentiating and redifferentiating smooth
   muscle cells in tissue culture. Cell Tiss. Res.
   165:13-22.

Holmsen, H. and H. J. Day. 1970. The selectivity of the
   thrombin-induced platelet release reaction: subcel-
   lular localization of released and retained constit-
   uents. J. Lab. Clin. Med. 75:840-857.

Jarmolych, J., A. S. Daoud, J. Landau, K. E. Fritz, and
   E. McElvene. 1968. Aortic media explants cell
   proliferation and production of mucopolysaccharides,
   collagen and elastic tissue. Exp. Mol. Pathol.
   9:171-188.

Khan, M. A., F. L. Earl, T. M. Farber, E. Miller, M.
   Husain, E. Nelson, S. Gertz, M. Forbes, M. Rennels,
   and F. Heald. 1977. Elevation of serum cholesterol
   and increased fatty streaking in egg yolk: lard fed
   castrated miniature pigs. Exp. Mol. Pathol. 26:
   63-74.

Ledet, T. 1976. Growth of rabbit aortic smooth muscle
   cells in serum from patients with juvenile diabetes.
   Acta Path. Microbiol. Scand. 84:508-516.

Mahley, R. W., T. L. Innerarity, K. H. Weisgraber, and
   D. L. Fry. 1977. Canine hyperlipoproteinemia and
   atherosclerosis. Am. J. Pathol. 87:205-226.

Nicolosi, R. J., J. L. Hojnacki, N. Llansa, and K. C.
   Hayes. 1977. Diet and lipoprotein influence on
   primate atherosclerosis. Proc. Soc. Exp. Biol. Med.
   156:1-7.

Ratcliffe, H. L. and H. Lunginbuhl. 1971. The domestic
   pig: a model for experimental atherosclerosis.
   Atheroscler. 13:133-136.

Ross, R. 1971. The smooth muscle cell. II. Growth of smooth muscle in culture and formation of elastic fibers. J. Cell Biol. 50:172-186.

Ross, R. and J. A. Glomset. 1973. Atherosclerosis and the arterial smooth muscle cell. Science. 180: 1332-1339.

Ross, R., J. Glomset, B. Kariya, and L. Harker. 1974. A platelet-dependent serum factor that stimulates the proliferation of arterial smooth muscle cells in vitro. Proc. Nat. Acad. Sci. USA. 71:1207-1210.

Ross, R. and J. A. Glomset. 1976. The pathogenesis of atherosclerosis (second of two parts). N. Engl. J. Med. 295:369-377.

Ross, R., A. Vogel, J. Glomset, B. Kariya, E. Raines, and M. J. Rivest. 1977. Platelet factor - the principal stimulant in serum for DNA synthesis of cells in culture. J. Cell Biol. 75:86a.

Sisken, B.: Personal Communication. Department of Anatomy. Albert B. Chandler Medical Center, University of Kentucky. September, 1978.

Swim, H. E.: Personal Communication. Department of Medicine. Albert B. Chandler Medical Center, University of Kentucky, March, 1978 and December, 1978.

Vogel, A., E. Raines, B. Kariya, M. J. Rivest, and R. Ross. 1978. Coordinate control of 3T3 cell proliferation by platelet-derived growth factor and plasma components. Proc. Natl. Acad. Sci. USA 75:2810-2814.

Yeung, D. L. 1976. Relationships between cigarette smoking, oral contraceptives, and plasma vitamins A, E, C, and plasma triglycerides and cholesterol. Am. J. Clin. Nutr. 29:1216-1221.

APPENDIX

Table II. Constituents of Dulbecco's Modified Eagle Medium.

INORGANIC SALTS	mg/L
CaCl$_2$ (anhydrous)	200.00
Fe(NO$_3$)$_3$. 9H$_2$O	0.10
KCl	400.00
MgSO$_4$. 7H$_2$O	200.00
NaCl	6400.00
NaHCO$_3$	3700.00
NaH$_2$PO$_4$. H$_2$O	125.00

OTHER COMPONENTS

Glucose	4500.00
Phenol red	15.00
L-Arginine . HCl	84.00
L-Cystine	48.00
L-Glutamine	584.00
Glycine	30.00
L-Histidine HCl . H$_2$O	42.00
L-Isoleucine	105.00
L-Leucine	105.00
L-Lysine HCl	146.00
L-Methionine	30.00
L-Phenylalanine	66.00
L-Serine	42.00
L-Threonine	95.00
L-Tryptophane	16.00
L-Tyrosine	72.00
L-Valine	94.00

VITAMINS

D - Ca pantothenate	4.00
Choline chloride	4.00
Folic acid	4.00
i - Inositol	7.20
Nicothinamide	4.00
Pyridoxal HCl	4.00
Riboflavin	0.40
Thiamine HCl	4.00

25