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Isolation, culture and characterization of human peritoneal mesothelial cells

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Isolation, culture and characterization of human peritoneal mesothelial cells. This study establishes a reproducible technique for the culture of human peritoneal mesothelial cells. Direct explants, as well as enzymatically degraded specimens, of human omentum have been used as the source of cells. Cells were grown on collagen and gelatin coated matrices and were maintained in supplemented Ham's F-12 medium containing 10% (vol/vol) Fetal calf serum. Morphologically and ultrastructurally, the cells formed a homogeneous population. They were polygonal when confluent and devoid of contaminating fibroblasts, endothelial cells and macrophages. Cultured mesothelial cells co-expressed cytokeratin and vimentin and synthesized laminin, fibronectin, mesosecrin, non-specific esterase and collagen Types I and III but not Type IV. Ultrastructural features included numerous surface microvilli, cytoplasmic vesicles and an abundant endoplasmic reticulum. The stimulation of mesothelial cells by the calcium ionophore A23187 demonstrated that the two major products of arachidonic acid metabolism were prostacyclin and prostaglandin E_2. The peritoneal mesothelial cell may be pivotal in the initiation of the inflammatory response during peritonitis and its establishment in culture will provide the basis for an in vitro model of peritoneal inflammation.

A monolayer of simple squamous epithelial cells of mesodermal origin forms the limiting serosal membranes that line the pleural, pericardial and peritoneal cavities. These mesothelial cells act as a permeability barrier regulating the passage of fluid and solutes between the systemic circulation and the body cavities.

Peritoneal infection is the major complication of continuous ambulatory peritoneal dialysis (CAPD), an increasingly common therapy for end-stage renal disease. In these circumstances the interaction of invading bacteria with the mesothelial lining may be pivotal in initiating the inflammatory response. The establishment of human mesothelial cells in culture would therefore provide the basis for an in vitro investigation of both phagocyte-mesothelial cell and bacterial-mesothelial cell interactions.

Although several detailed in vitro studies on cultured mesothelial cells have been reported, these have been limited to the isolation of cells from human serous effusions [1–7] or those obtained from animal serosal tissue [8–11]. Growth of cells from human ovarian carcinoma ascites fluid has been achieved using specific growth factors [12], feeder layers [4] and specific attachment substrates [6, 13]. A recent study has reported mesothelial-like cells from a collagenase digest of human omentum [10], but their characterization was incomplete.

Due to the conflicting evidence that exists with regard to the availability and homogeneity of cell types obtained from serous effusions [14] and in particular to avoid contamination by malignant cells [7, 15], we have utilized direct explants of human omentum as well as cells obtained by enzymatic disaggregation of this tissue. Both approaches have successfully and reproducibly yielded homogeneous human mesothelial cell cultures, the identity of which has been confirmed by immunohistochemical and ultrastructural criteria.

Methods

Omentum explant culture

Specimens of human omentum were obtained from consenting patients undergoing elective abdominal surgery. Blunt dissection removed excess fat and provided predominantly transparent samples of tissue. The omentum was washed in several changes of sterile phosphate buffered saline (PBS) (Oxoid, Basingstoke, UK) pH 7.3, and finely divided into approximately 1 mm^2 segments. These were washed in PBS three times to remove any contaminating red blood cells. Explants were seeded into collagen or gelatin coated 25 cm^2 tissue culture flasks (Falcon, Becton Dickinson, Oxford, UK), at a density of approximately 1/cm^2. Excess PBS was removed by aspiration and replaced with a minimum volume (0.5 to 1 ml) of Ham's F-12 medium (Flow Laboratories, Herts, UK), containing 20% (vol/vol) fetal calf serum (FCS) (Flow Laboratories), supplemented with penicillin 100 U ml^{-1} and streptomycin 100 mg ml^{-1} (Gibco, Uxbridge, UK), transferrin 0.5 $\mu g\ ml^{-1}$, insulin 0.5 $\mu g\ ml^{-1}$ (Sigma Chemicals, Poole, UK) and 2 mM glutamine (Flow Laboratories). In some experiments the above medium was further supplemented with one of the following growth factors (final concentration): hydrocortisone (0.4 $\mu g\ ml^{-1}$), sodium selenite (5 ng ml^{-1}), cholera toxin (10 ng ml^{-1}), epidermal growth factor (10 ng ml^{-1}).

Fresh medium (a minimum volume of 0.5 to 1.0 ml, sufficient to maintain viability) was added on alternate days until the

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explants adhered. At this stage, the volume of medium was increased to 5 ml with Ham's F-12, prepared as above, but containing 15% (vol/vol) FCS. Cells were cultured at 37°C in a humidified atmosphere of 95% air:5% CO_2.

Enzymatic disaggregation of omentum

Omental specimens were subjected to enzymatic disaggregation [modified from Verbrugh et al, 16]. Briefly, a piece of washed omentum (approximately 6 cm^2) was incubated with 15 ml of a solution containing 0.125% (wt/vol) trypsin, (Difco Laboratories, West Moseley, UK), 0.01% (wt/vol) EDTA (Sigma) and 0.1% (wt/vol) glucose (BDH Chemicals Ltd, Poole, UK), for 20 minutes at 37°C with continuous rotation. After incubation the suspension was centrifuged at 50 ×g for five minutes, at 4°C. The supernatant was discarded together with the digested omentum which remained on the surface throughout centrifugation. The cell pellet was washed once in culture medium containing 10% (vol/vol) FCS, resuspended in the same medium to a volume of 5 ml and seeded in 25 cm^2 matrix coated tissue culture flasks. Half the medium was exchanged 24 hours after seeding and thereafter fully replaced once every three days.

Passage of cell cultures

Confluent cells were washed with PBS and then incubated with 2 ml of PBS containing 0.125% (wt/vol) trypsin, 0.01% (wt/vol) EDTA and 0.1% (wt/vol) glucose for 4 to 5 seconds, at room temperature. Most of the buffer was removed, leaving a thin film of the liquid covering the cells, and cultures were then incubated for 10 to 15 minutes at 37°C until the cells detached as monitored by phase contrast microscopy. Free cells were suspended and washed once in supplemented, antibiotic free Ham's F-12 containing 10% (vol/vol) FCS, centrifuged at 50 × g for five minutes at 4°C, and seeded at a density of 1 to 2 × 10^4 cells/cm^2 in collagen or gelatin coated flasks. Cell number was estimated using an improved Neubauer chamber.

Preparation of matrix substrates

Twenty-five cm^2 flasks were treated for 15 minutes at room temperature with either 2.5 ml rat tail collagen (1 mg/ml) prepared according to the method of Bornstein [17] or 2.5 ml of 0.1% (wt/vol) gelatin (denatured collagen; Sigma), both solutions being made up in PBS, pH 7.3. Excess buffer was removed, the surface allowed to air dry, and the flasks rinsed three times with 2.5 ml PBS.

Transmission electron microscopy

All equipment and reagents used for transmission electron microscopy (TEM), were obtained from EMScope (Ashford, UK) unless otherwise stated. Primary and first passage confluent mesothelial cells grown on Thermonox coverslips precoated with collagen were fixed in 2.5% (vol/vol) glutaraldehyde in 0.2 M cacodylate buffer, pH 7.4, at 4°C. After one hour fresh cacodylate buffer was used to wash the cells. The coverslips were then cut into 1 to 2 mm strips and post-fixed in 2% (vol/vol) osmium tetroxide for two hours at room temperature. Following a further wash in cacodylate buffer cells were subjected to graded ethanol dehydration and propylene oxide (BDH) immersion. Cells were then placed in propylene oxide: araldite 1:1 (vol/vol) followed by fresh araldite at 35°C before being left to polymerise at 60°C for 48 hours. Ultrathin (500 nm) sections were cut perpendicular to the cell surface on a Cambridge/Huxley Ultramicrotome Mark II, stained with uranyl acetate and lead citrate and examined using a Phillips 300 TEM.

Scanning electron microscopy (SEM)

Fresh specimens of human omentum pinned flat, and confluent cultures of human mesothelial cells grown on collagen coated sterilized 24 mm^2 glass coverslips (BDH), were fixed in 2.5% (vol/vol) glutaraldehyde in 0.2 M cacodylate buffer, pH 7.4. Whole tissue and confluent cultured cells were washed in cacodylate buffer before being post-fixed in 1% (vol/vol) osmium tetroxide for approximately four hours. Both types of preparation were then extensively washed with distilled water and then subjected to graded ethanol dehydration; the latter included 3 × 30 minute immersions in 100% ethanol with the specimens being left in 100% ethanol until they were critically point dried in a CPD 750 (EMScope Ltd.). At this stage the dried specimens were mounted on 12.5 × 10 mm aluminium stubs with an electroconductive adhesive before being gold sputter-coated (cycle time 2.5 min, deposition current 15 mA, chamber vacuum 0.1 Torr; Sputter coater, EMScope) and examined using a Jeol JSM-840A scanning electron microscope.

Immunohistochemistry (IHC)

Monoclonal antibodies to human cytokeratin (diluted 1:20 vol/vol with PBS), vimentin (1:10 vol/vol PBS) and desmin, (1:20 vol/vol PBS; Dako Ltd., Bucks, UK) were used. In addition polyclonal antibodies to human fibronectin (1:10 vol/vol PBS; Serotec, Oxford, UK), human factor VIII (gift of Dr. D.C. West, Christie and Holt Radium Institute, Manchester, UK), human laminin (gift of Dr. J. Couchman, Birmingham Alabama, USA) and human collagens I, III, V (each diluted 1:40 vol/vol with PBS; Bionuclear Services Ltd., Reading, UK) and type IV collagen (1:25 vol/vol PBS; Bionuclear Services, London, UK) were used. The fluorescein conjugated second antibodies were: rabbit anti-mouse, rabbit anti-goat, and goat anti-rabbit from ICN Biomedicals Ltd., High Wycombe, UK and swine anti-sheep from Serotec. Human peritoneal fibroblasts cultured separately were included as positive controls for vimentin and fibronectin, and human kidney sections as positive controls for the remainder of the antibodies tested. Human anti-mesosecrin (gift of J.G. Rheinwald, Boston, Massachusetts, USA) was also tested on mesothelial cells prepared as for the other IHC tests except that cells were maintained in Ham's F-12 supplemented with only 0.1% (vol/vol) FCS for 24 hours prior to fixation. The appropriate non-immune serum and PBS in place of each primary antibody were used as controls for non-specific fluorescence.

Primary and first passage monolayers, cultured on gelatin coated petriperm (Heraeus, Brentwood, UK), were fixed in 1:1 (vol/vol) cold acetone:methanol and incubated with 40 to 50 μl of the suitably diluted primary antibody for 45 minutes at room temperature in a humidified chamber. The cells were washed three times with PBS containing 0.8% (wt/vol) bovine serum albumin (PBS-BSA), and then incubated with the appropriate fluorescein conjugated second antibody for 45 minutes in the dark in a humidified chamber. Cells were then extensively washed with PBS-BSA, mounted and examined using a Leitz fluorescence microscope.

The presence of non-specific esterase and Fc receptors was determined using the methods of Stuart, Habeshow and Davidson [18] and Bianco and Pyrowski [19], respectively. For the Fc receptor assay, sheep erythrocytes in Alsevers medium (Tissue Culture Services Ltd., Slough, UK) were sensitized using rabbit anti-sheep red blood cell stroma IgG (7s) (Sigma), diluted 1:128 with PBS. Adherent human peritoneal macrophages prepared from eight-hour dwell CAPD effluent according to the method of Alobaidi et al [20] were used as positive controls for both the non-specific esterase and Fc receptor assays.

Calcium ionophore stimulation

Primary or first passage mesothelial cells were seeded at a density of 2×10^4 cells/cm^2 in collagen coated 48-well tissue culture plates (Costar, Northumbria Biologicals, UK). At confluency, the cells were washed × 2 in Tyrodes-gelatin and 1 ml of 10 μM calcium ionophore A23187 (Calbiochem-Behring, Cambridge, UK) diluted in Tyrodes-gelatin was added per well. Tyrodes-gelatin contained the following reagents, all from BDH unless otherwise stated: 136.7 mM NaCl; 0.27 mM KCl; 5.0 mM $MgCl_2 \cdot 6H_2O$; 0.4 mM $NaH_2PO_4 \cdot 2H_2O$; 1.36 mM $CaCl_2 \cdot 2H_2O$; 5.0 mM D-glucose, 12.0 mM $NaHCO_3$ and 0.1% (wt/vol) Gelatin (Difco Labs); and was always prepared fresh on the day of the experiment [21]. Incubations were performed in 5%CO_2:95% air in a humidified atmosphere at 37°C. At the end of the incubation period, the supernatants were removed, centrifuged, and stored at −20°C until required.

Quantitation of arachidonic acid (AA) metabolites

Prostaglandin E_2 (PGE_2), prostacyclin (PGI_2) (measured as 6-keto-$PGF_{1\alpha}$), thromboxane (TXB_2), leukotriene B_4 (LTB_4) and leukotriene C_4 (LTC_4) were measured by specific radioimmunoassays. Synthetically prepared prostaglandin standards were obtained from Sigma. Leukotriene standards were synthetically prepared (gift of Dr. B. Spur) and purified by reverse phase high pressure liquid chromatography (RP-HPLC) prior to use. Specific polyclonal antibodies (Bioclinical Services Ltd, Cardiff, UK) were used for the prostaglandin estimations. The antibodies to LTB_4 and LTC_4 were a gift of Dr. J. Rokach (Merck Frosst, Montreal, Canada) [22]. Tritiated eicosanoids were obtained from Amersham International (Aylesbury, UK), and were: [^{3}H] 6-keto-$PGF_{1\alpha}$, 157 Ci/mmol; and [^{3}H] PGE_2, 184 Ci/mmol; [^{3}H] TxB_2, 180 Ci/mmol; [^{3}H] LTB_4, 210 Ci/mmol and [^{3}H] LTC_4, 40.2 Ci/mmol.

The radioimmunoassays were performed in duplicate in total volumes of 300 μl Tris-Isogel buffer (0.1 M Tris-HCl, 0.14 M NaCl, 0.1% wt/vol gelatin) [21], and contained 100 μl samples of supernatant or synthetic standard, 100 μl of radiolabelled-eicosanoid and 100 μl of antibody in 3.5 ml polypropylene test tubes (Sarstedt, Leicester, UK). The reaction mixtures were incubates at 4°C overnight for all products other than LTB_4 when the assay was incubated at 37°C. Bound and free eicosanoid were separated by the addition of 250 μl of cold dextran T70 (Pharmacia LKB, Milton Keynes, UK) coated charcoal (Norit SX-1, BDH) both 1% wt/vol in Tris-Isogel buffer and centrifuged immediately at 250 × g for 10 minutes at 4°C. Supernatants were decanted and mixed with 3.5 ml of Optiphase MP scintillant (LKB Instruments, Poole, Dorset, UK) and the radioactivity measured in a Rakbeta liquid scintillation counter (LKB, Turku, Finland). Synthetic LTB_4 and LTC_4 were detected over the linear portion of the radioligand binding curve at concentrations ranging from 0.1 to 4 ng/ml while PGE_2, TxB_2 and 6-keto-$PGF_{1\alpha}$ were measured over the linear part of the curve at concentrations ranging from 0.8 to 2.5 ng/ml.

Characterization of AA metabolites

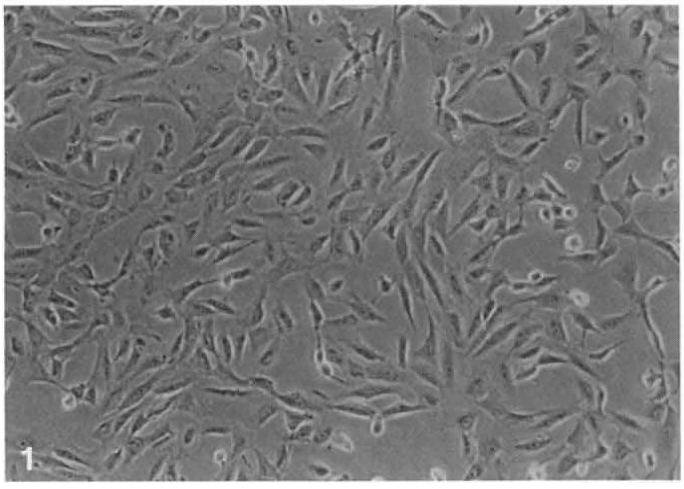
Approximately 1×10^7 first passage mesothelial cells were stimulated with 10 μM calcium ionophore for an appropriate time, the supernatants mixed with an equal volume of methanol at −20°C overnight and subsequently centrifuged at 11000 × g for one minute at room temperature to remove the precipitated proteins. The supernatant (2 ml) was injected directly onto a 5 μ C18 Nucleosil column (4.6 × 25.0 cm, Hichrom Ltd., Theale, Reading, UK) protected by a 2 cm guard column of the same material. Polar AA metabolites were eluted at a flow rate of 1 ml/min in methanol:water:acetic acid 65:35:0.01, respectively, at pH 5.6 for 30 minutes and monitored by UV absorption at 270 nm on a Gilson Holochrome dector (Anachem Ltd., Luton, UK).

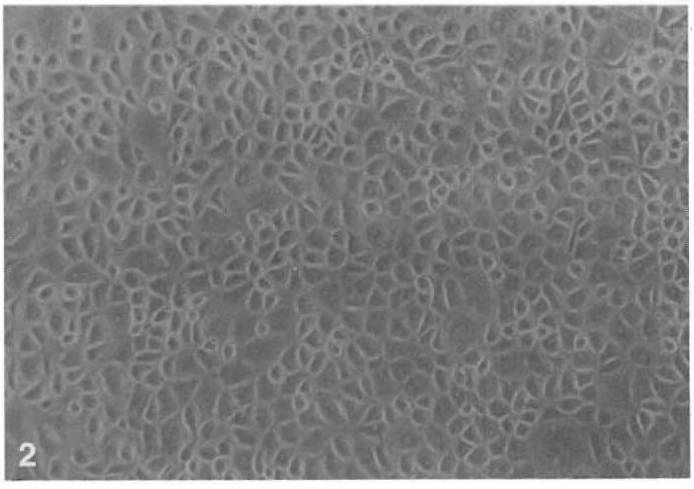
One milliliter fractions were collected and evaporated to dryness under negative pressure in a Univap vaccum centrifuge (Uniscience Ltd, Wandsworth, London, UK). The residue was dissolved in 1 ml of Tris-Isogel buffer and examined for immunoreactive 6-keto-$PGF_{1\alpha}$, PGE_2, TXB_2, LTB_4 and LTC_4. The retention times for synthetic standards were: ^{3}H 6-keto-$PGF_{1\alpha}$ seven minutes, PGE_2 11 minutes, TXB_2 nine minutes, LTB_4 26 minutes.

Results

Cell culture

Explants. The initiation of a homogeneous mesothelial cell population was dependent on the adherence of tissue explants to the culture vessel surface. A minimum volume of 0.5 to 1 ml of medium containing 20% (vol/vol) FCS was found to be sufficient to maintain viability and at the same time facilitate explant adherence. Cellular outgrowths from adhered explants of human omentum were observed five days after initiation of the culture. Growth was well established by 10 days and confluency was usually obtained by 18 ± 1 day (N = 11). Morphologically the cells appeared bipolar, multipolar or even elongated during the initial growth phase (Fig. 1) but immediately prior to, and at confluency, they adopted a polygonal appearance characteristic of epithelial cells. Cell cultures appeared homogeneous and growth occurred as a monolayer (Fig. 2). The ability to enhance the outgrowth of cells from the tissue and to reduce the number of days required for monolayer formation was investigated by supplementation of the medium with the following compounds: hydrocortisone (0.4 μg ml^{-1}), sodium selenite (5 ng ml^{-1}), cholera toxin (10 ng ml^{-1}) and epidermal growth factor (10 ng ml^{-1}). Of these, only hydrocortisone had a beneficial effect, significantly reducing the number of days to reach confluency from 18 ± 1 day (N = 11) to 13 ± 2 days (N = 12; $P < 0.001$) days. It had, however, no effect on the life span of cell cultures. Epidermal growth factor also increased the rate of cell turnover but its routine addition to the culture medium was precluded by its ability to induce a





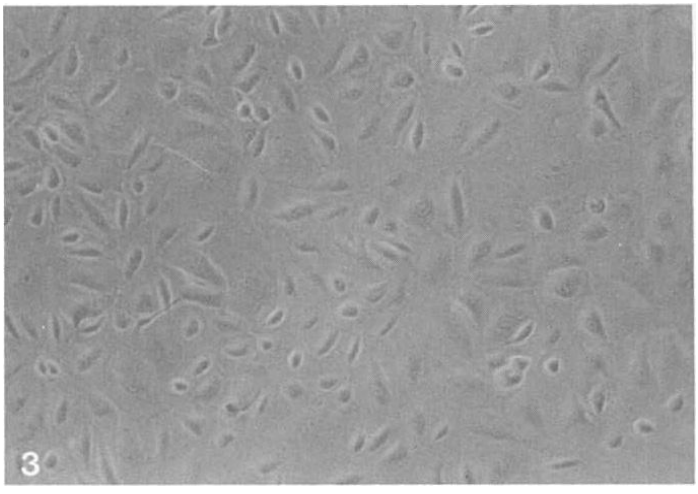


Fig. 1. *Sub-confluent human mesothelial cells.* The multipolar and elongated morphology occurs during the initial growth phase in culture. Magnification ×125.

Fig. 2. *Confluent human mesothelial cell outgrowth from an explant of omentum.* The cells have adopted the polygonal appearance characteristic at confluency. Magnification ×125.

Fig. 3. *Fourth passage mesothelial cell culture demonstrating the loss in uniformity of the monolayer accompanied by the presence of senescent cells.* Magnification ×125.

reversible change in mesothelial cell morphology from a polygonal to an elongated fibroblast like appearance.

Enzymatic disaggregation. Confluent mesothelial cells were also obtained by limited treatment of the omentum with trypsin-EDTA. This approach yielded on average three times the number of confluent cultures per unit of tissue compared to the explant method. Enzymatic digestion is clearly more efficient than the explant method since it allows the direct plating of disaggregated cells. The plating of 1×10^4 cells/cm^2 resulted in confluency in the presence of hydrocortisone in 8 ± 1 day ($N = 12$). The low concentration of trypsin used in this study (0.125% wt/vol) together with the time of exposure of the omentum to the enzyme resulted in an optimal yield of homogeneous mesothelial cells. Increasing the concentration of trypsin and/or the time of digestion beyond 25 to 30 minutes caused a significant fibroblast contamination.

Propagation of cells. The confluent cells from both preparation methods were successfully passaged when seeded on either collagen or gelatin coated matrices. Cells were subcultured at a range of seeding densities from 3×10^3 to 5×10^4 cells/cm^2. Provided the seeding cell density was not less than 1 to 2×10^4 cells/cm^2 the polygonal morphology observed at confluence was maintained up to the sixth passage. Under these conditions the cell density at confluency was $5.3 \pm 0.8 \times 10^5$/cm^2 ($N = 12$). The time to reach confluency for primary, secondary and tertiary passaged cells did not vary and was approximately eight days ($N = 3$, for each passage).

Cells seeded onto plastic did not proliferate beyond the third passage whereas those seeded onto collagen or gelatin were successfully cultured to the sixth passage. However, cells cultured on collagen or gelatin beyond the third passage did exhibit a gradual increase in cell size with the appearance of large, non-proliferating or senescent cells (Fig. 3). Subsequently there was a reduction in proliferative capacity of fourth and fifth passage cultures. Sixth passage cells lost viability when subcultured, demonstrated by a failure to reattach to the culture vessel surface. No cells with the morphological appearance of fibroblasts or macrophages were observed in the polygonal cell cultures.

Cells derived from both explants and enzyme digestion were characterized by TEM, SEM and IHC. For these studies, primary or first passage confluent cells were used.

Ultrastructure

TEM showed the presence of numerous surface microvilli, abundant cytoplasmic vesicles and a well developed endoplasmic reticulum (Fig. 4). In addition large numbers of mitochondria were present. Weibel-Palade bodies were not observed. SEM verified the abundant microvilli on the surface of the mesothelial cells of the omentum (Fig. 5A) and the retention of

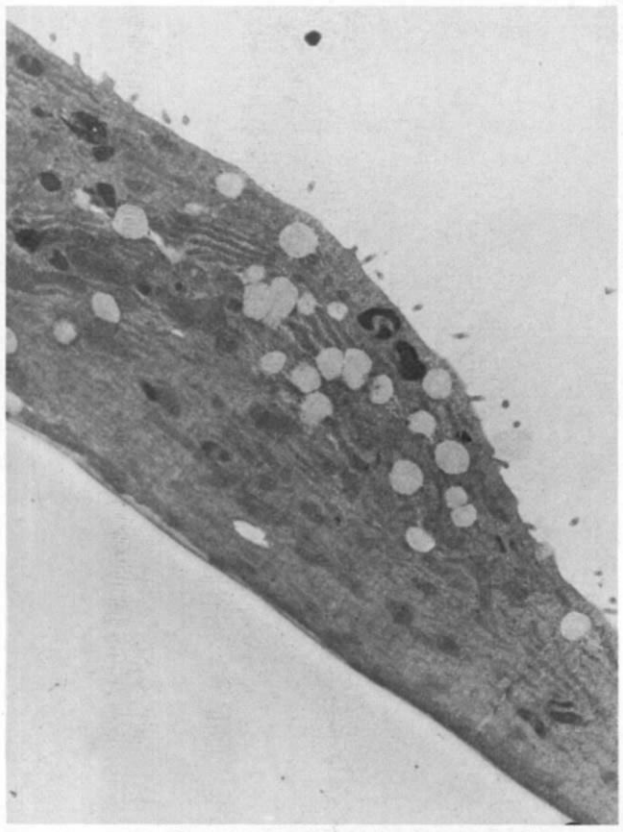


Fig. 4. *Transmission electron micrograph of human mesothelial cells stained with uranyl acetate and lead citrate.* Numerous surface microvilli, abundant cytoplasmic vesicles, a well developed endoplasmic reticulum and mitochondria are evident. Magnification ×6,800.

these structures on the cultured cells (Fig. 5B). Although the density of cell-cell bridging and microvilli were reduced in the cultured cells (Fig. 5B) these features were, however, retained and demonstrate that the cultured cells maintain their mesothelial phenotype. At no time were cells resembling fibroblasts or macrophages observed.

Immunohistochemistry

IHC staining of confluent cells demonstrated that all cells reacted positively for cytokeratin and vimentin (Fig. 6A,B) but did not stain for desmin. Furthermore, cytokeratin negative cells were not observed. The components of the mesothelial cell extracellular matrix were shown to consist of laminin (Fig. 6C), collagen Types I and III and fibronectin. Mesothelial cells synthesized mesosecrin and non-specific esterase but they did not synthesize factor VIII, collagen Type IV or Type V, nor did they express surface Fc receptor activity.

Quantitation and characterization of AA metabolites

Confluent monolayers of cultured mesothelial cells generated small quantities of immunoreactive PGE_2 and 6-keto-$PGF_{1\alpha}$ in the absence of exogenous stimuli. Over a 60-minute incubation period the cells produced 2.40 ± 1.03 ng PGE_2 and 2.15 ± 0.62 ng 6 keto-$PGF_{1\alpha}$ per 5×10^5 cells (mean ± SEM, $N = 7$). The addition of the calcium ionophore A23187 resulted in an increase in the generation of both cyclooxygenase metabolites to 8.29 ± 3.65 ng of PGE_2 and 4.75 ± 1.66 ng of 6-keto-$PGF_{1\alpha}$ per 5×10^5 cells over the same time period (Fig. 7). While the increase in PGE_2 synthesis was significant ($P < 0.025$, $N = 7$) the changes in 6-keto-$PGF_{1\alpha}$, although doubled, failed to reach significance (Wilcoxon matched-pairs rank test).

The pooled supernatants from 20 wells of confluent cells containing 86.22 ng of immunoreactive 6-keto-$PGF_{1\alpha}$ and 94.24 ng of immunoreactive PGE_2 were resolved by RP-HPLC. The immunoreactive peaks had retention times of seven minutes and 10 minutes for 6-keto-$PGF_{1\alpha}$ and PGE_2, respectively. The material eluting with the retention time of 6-keto-$PGF_{1\alpha}$ contained 64.1 ng of immunoreactive product giving a recovery of 74.3%. The material eluting with the retention time of PGE_2 contained 76.76 ng of immunoreactive product giving a recovery of 81.45%. TXB_2, LTB_4, and LTC_4 were not detected in the supernatant of control or ionophore stimulated cells.

Discussion

The present study describes a reproducible technique for the culture of human mesothelial cells. Cells of epithelial morphology possessing structural features characteristic of mesothelial cells were consistently obtained in defined culture conditions from both explants and trypsin-EDTA treatment of human omentum. Morphologically, histochemically and ultrastructurally, the cultured cells formed a homogeneous population and were devoid of contaminating fibroblasts (Table 1). Cells with the characteristics of endothelial cells and macrophages were also absent.

A feature of mesothelial cells in culture that has been recognized for some time is their adoption of a heterogeneous morphology [11, 23]. In the present study the cells appeared multipolar or even elongated when isolated and became polygonal only when confluent. Despite this, mesothelial cells remained morphologically distinct from spindle-like connective tissue fibroblasts. Fibroblast contamination of explant derived cell cultures was minimized, first by dislodging adhered explants from which fibroblastic outgrowths were evident, and second, by using passaging conditions that preferentially removed cells of epithelial morphology. This was done by allowing trypsinization to proceed only to the stage at which polygonal cells had detached while at the same time leaving the fibroblast-like cells adhered. The use of relatively large (approximately 6 cm^2) samples of omentum for limited enzymatic disaggregation (compared with 1 mm^2 used for explants) minimized contact between the enzyme and interstitial omental fibroblasts, thereby restricting fibroblast contamination of the cultures. The higher cell yield and, therefore, seeding density of the enzymatically-derived primary mesothelial cell cultures reduced the time required to attain confluency compared with that required by the explant-derived cells.

The use of collagen or gelatin-coated substrates rather than plastic alone has been reported to be important for maintaining epithelial cell growth and differentiation in culture [24]. In our studies mesothelial cells retained their morphology either on gelatin or collagen coated surface for up to six successive passages when seeded at a density of 1 to 2×10^4 cells/cm^2, whereas cells seeded onto plastic lost proliferative capacity after three subcultures ($N = 4$). The importance of coated substrates in human mesothelial cell growth and morphology is not fully understood, although a recent study has shown that

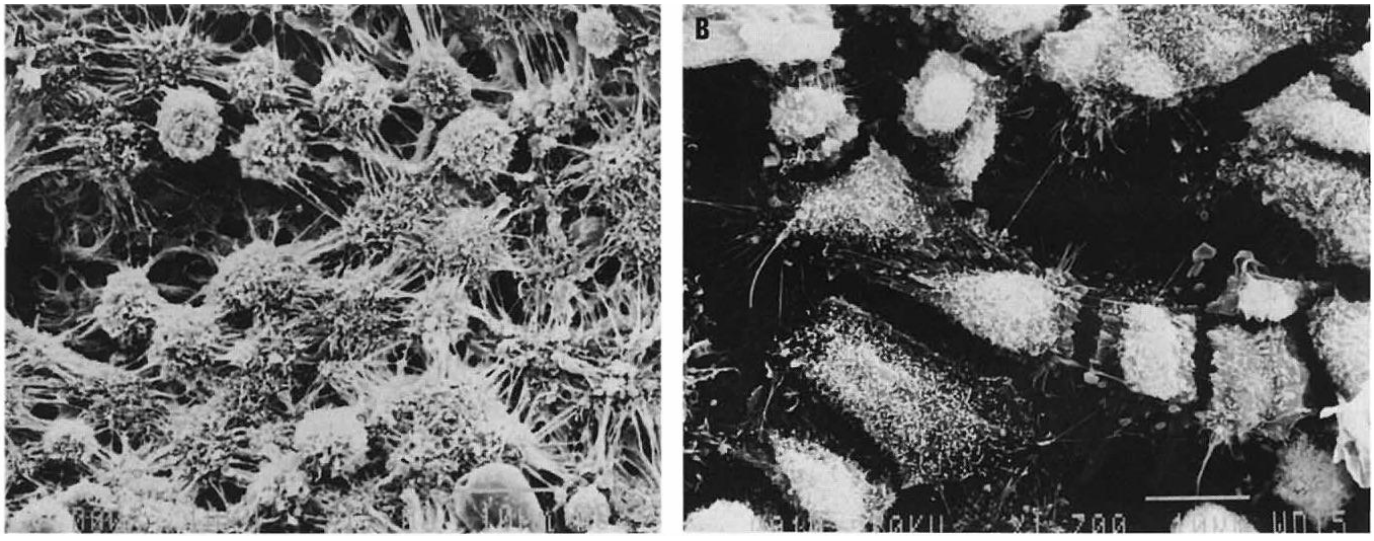


Fig. 5. A. *Scanning electron micrograph of human omentum with abundant surface microvilli (bar = 10 μm).* Magnification ×1700. **B.** Scanning electron micrograph of a cultured human mesothelial cell monolayer (Bar = 10 μm). Magnification ×1700.

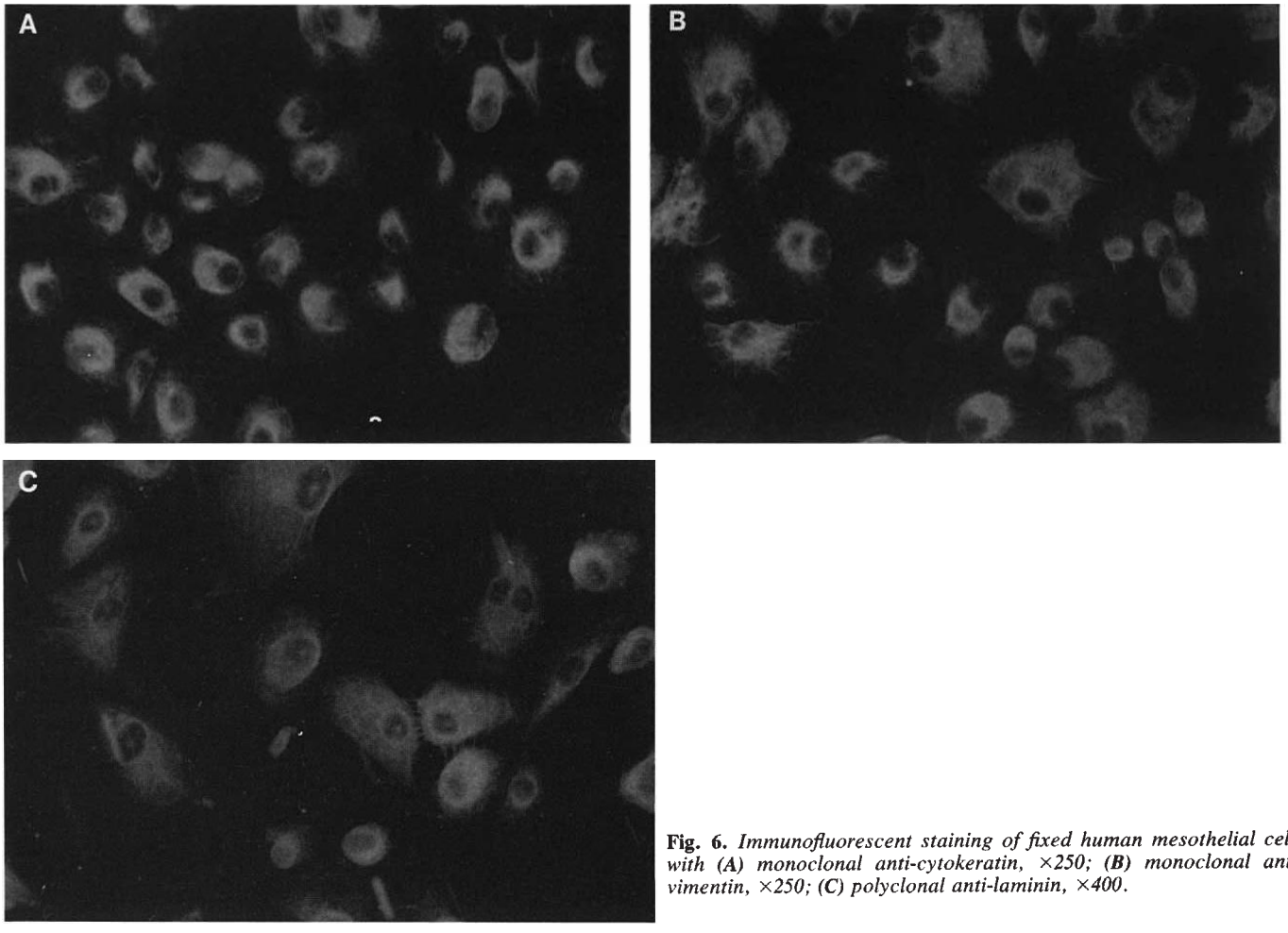


Fig. 6. *Immunofluorescent staining of fixed human mesothelial cells with (A) monoclonal anti-cytokeratin, ×250; (B) monoclonal anti-vimentin, ×250; (C) polyclonal anti-laminin, ×400.*

bovine corneal endothelial cell matrix is beneficial to human mesothelial cells obtained from ovarian carcinoma ascite fluid [6].

Ultrastructural examination of omentum-derived polygonal cells demonstrated the presence of numerous microvilli and cytoplasmic vesicles. Both these features are characteristic of mesothelial cells in vivo [25] and have previously been described in other ultrastructural studies [26–31]. The absence of

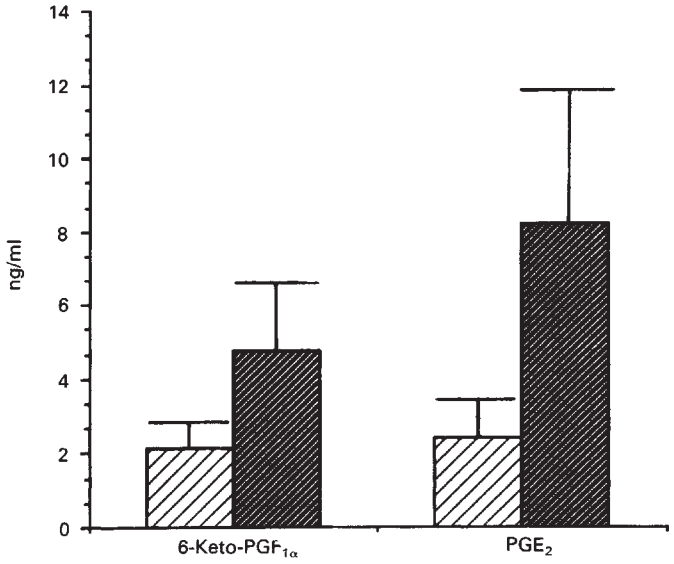


Fig. 7. *Effect of the calcium ionophore A23187 (10 μM, ▨) on the capacity of 5 × 10^5 human mesothelial cells to generate immunoreactive PGI_2 (measured as 6-keto-$PGF_{1\alpha}$) and PGE_2 after one hour incubation.* Symbol (▨) is Tyrodes. The mean ± SEM are given for each dose from seven separate experiments.

Weibel-Palade bodies, organelles characteristically associated with endothelial cells [32], further supports the identity of these cells. In addition, a well developed endoplasmic reticulum and abundant mitochondria were observed, indicative of a cell involved in active protein synthesis and supporting the view that the mesothelial cell is "far from a passive cell" [31].

The immunohistochemical demonstration of cytokeratin and vimentin, two classes of intermediate filaments which are constituents of epithelial and mesodermal cells, respectively [33], confirms their embryological origin and agrees with observations on intact mesothelium [33]. These findings are complemented by the absence of the endothelial cell marker factor VIII and the myogenic cell marker desmin.

Human mesothelial cells were positive for fibronectin which confirms the finding in the mouse [34], and suggests that these cells may play a role in the adhesion and migration of cells across the peritoneal lining in vivo. Furthermore, the presence of fibronectin, together with the basement membrane glycoprotein laminin, collagens I and III and the 46 kD glycoprotein, mesosecrin (thought to be a constituent of the extracellular matrix [35]) suggests that human peritoneal mesothelial cells in vitro, like their rat pleural counterparts, synthesize an organized extracellular matrix [36]. While the function of mesosecrin remains unknown [35], the other connective tissue components may be important in conferring selective permeability to the mesothelium in situ as well as contributing to the structural integrity of the peritoneum itself. The identification of nonspecific esterase as a component of human mesothelial cells is in agreement with several other authors [37–41], although there is some controversy as to whether it is an authentic feature of intact mesothelium [14]. Finally, the lack of Fc receptors on human mesothelial cells demonstrated here concurs with the finding by Geiling and co-workers [42] in rat mesothelium.

Human mesothelial cells in culture metabolize arachidonic acid to PGI_2 and PGE_2. In contrast, TXB_2, LTB_4 and LTC_4 were not detected. This finding is similar to those from studies of isolated rabbit peritoneal tissue and cultured rabbit mesothelial cells [43,44]. PGI_2, PGE_2 and TXB_2 are the three major cyclooxygenase products that have been detected in human peritoneal fluid either before or during episodes of peritonitis [45]. Our results therefore suggest that the mesothelial cell may be an important source of PGI_2 and PGE_2, but not TXB_2 in the peritoneal cavity. The capacity of mesothelial cells to synthesize these two major vasodilatory prostaglandins may facilitate peritoneal hyperemia early in the inflammatory response. In addition, both PGI_2 and PGE_2 may have a cytoprotective effect [46] on the peritoneum during inflammation by acting to retain the integrity of the mesothelial monolayer. Furthermore, PGE_2 may exert a negative feedback effect on the release of Interleukin 1 by peritoneal macrophages [47], thus regulating the inflammatory response in the peritoneal cavity.

The present study will facilitate the in vitro examination of the interaction of the mesothelium with phagocytic cells as well as with bacteria. Thus, the establishment of human mesothelial cells in culture provides the basis of a model for investigating the role of the mesothelium in host defense mechanisms during peritonitis. In addition the current study provides a useful technique to study in vitro the physiology of human mesothelium and its role in the transport of water and solutes.

Table 1. Comparison of confluent mesothelial and omentum-derived fibroblasts

	Mesothelial cell	Fibroblasts
Morphology	polygonal	elongated, stellate
Cytokeratin	positive	negative
Microvilli	present	absent
NSE	positive	negative
Mesosecrin	strongly positive	weakly positive
TxB_2	not synthesized	synthesized

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References

1. GOTZOS V: Culture in vitro de cellules de liquide peritoneal humain. *Acta Anat* 98:281–294, 1977

2. Mouriquand J, Mouriquand C, Petitpas E, Mermet M-A, Augusseau S, Paramello B: Short and long term behaviour of pleural effusion cultures (Report of 200 cases). *Pathol Biol (Paris)* 25:15–21, 1977
3. Whitaker D, Papadimitriou JM, Walters NM-I: The mesothelium: Techniques for investigation, origin, nature and behaviour. *J Pathol* 132:263–271, 1980
4. Wu Y-J, Parker LM, Binder NE, Beckett MA, Sinard JH, Griffiths GT, Rheinwald JG: The mesothelial keratins: A new family of cytoskeletal proteins identified in cultured mesothelial cells and non-keratinizing epithelia. *Cell* 31:693–703, 1982
5. Jap PHK, Mungyer GM, Ramaekers FCS, Poels LG, Van de Molengraft FJJ, Vooijs GP: Identification of human mesothelial cells in culture by electron microscopy and antibodies. *Cell Biol Int Rep* 8:274, 1984
6. Niedbala NJ, Crickard K, Bernacki RJ: Adhesion, growth and morphology of human mesothelial cells on extracellular matrix. *J Cell Sci* 85:133–147, 1986
7. Castor CW, Naylor B: Characteristics of normal and malignant mesothelial cells studied in vitro. *Lab Invest* 20:437–443, 1969
8. Watters WB, Buck RC: Scanning electron microscopy of mesothelial regeneration in the rat. *Lab Invest* 26:604–609, 1972
9. Coene M-C, Solheid C, Claeys M, Herman AG: Prostaglandin production by cultured mesothelial cells. *Arch Int Pharmacodyn* 249:316–318, 1981
10. Nicholson LJ, Clarke JMF, Pittilo RM, Machin SJ, Woolf N: The mesothelial cell as a non-thrombogenic surface. *Thomb Haemost* 52:102–104, 1984
11. Thiollet J, Jaurand MC, Kaplan H, Bignon J, Holande E: Culture procedure of mesothelial cells from rat parietal pleura. *Biomedicine* 29:69–73, 1978
12. Connell ND, Rheinwald JG: Regulation of cytoskeleton in mesothelial cells: Reversible loss of keratin and increase in vimentin during rapid growth in culture. *Cell* 34:245–253, 1983
13. Lechner JF, Tokiwa T, LaVeck M, Benedict WF, Banks-Schlegel S, Yeager H, Banerjee A, Harris GG: Asbestos associated chromosomal changes in human mesothelial cells. *Proc Natl Acad Sci USA* 82:3884–3888, 1985
14. Whitaker D, Papadimitriou JM, Walters MN-I: The mesothelium and its reactions: A review. *CRC Rev Toxicol* 10:81–144, 1982.
15. Singh G, Dekker A, Ladoulis CT: Tissue culture of cells in serous effusions: Evaluation as an adjunct to cytology. *Acta Cytologica* 22:487–489, 1978
16. Verbrugh HA, van Bronswijk H, Heezius EC, van der Meulen J, Oe PL, Donker AJM, Verhoef J: Cytotoxic effects of commercial CAPD fluids and of bacterial exoproducts on human peritoneal mesothelial cells in vitro. *Peritoneal Dial Bul (Suppl)* 7:S81, 1987
17. Bornstein MB: Reconstituted rat tail collagen used as a substrate for tissue cultures on coverslips, in Maximow slides and roller tubes. *Lab Invest* 7:134–142, 1958
18. Stuart AE, Habeshow JA, Davidson AE: *Handbook of Experimental Immunology*. Edited by DM Weir, Oxford, Blackwell Scientific Publications, 2:31.1–31.31, 1978
19. Bianco C, Pytowski B: *Methods for Studying Mononuclear Phagocytes*. Edited by DD Adams, PJ Edelson, HS Koren, 1981, pp. 273–282
20. Alobaidi HM, Coles GA, Davies M, LLoyd D: Host defense in continuous ambulatory peritoneal dialysis: The effect of the dialysate on phagocyte function. *Nephrol Dial Transplant* 1:16–21, 1986
21. Williams JD, Czop JK, Austen KF: Release of leukotrienes by human monocytes on stimulation of their phagocytic receptor for particulate activators. *J Immunol* 132:3034–3040, 1984
22. Rokach J, Hayes EC, Girard Y, Lombardo DL, Maycock AL, Rosenthal AS, Young RN, Zambani R, Zweerink HJ: The development of sensitive and specific radioimmunoassays for leukotrienes. *Prostaglandin Leuk Med* 13:21–25, 1984
23. Lewis WH: Mesenchyme and mesothelium. *J Exp Med* 38:257–262, 1923
24. Hay ED: Extracellular matrix. *J Cell Biol* 91:204–223, 1981
25. Odor DL: Observations of the rat mesothelium with the electron and phase microscopes. *Am J Anat* 95:433–465, 1954
26. Andrews PM, Porter KR: The ultrastructural morphology and possible functional significance of mesothelial microvilli. *Anat Rec* 177:409–426, 1973
27. Baradi AF, Rao SN: A scanning electron microscope study of mouse peritoneal mesothelium. *Tissue Cell* 8:159–162, 1976
28. Casley-Smith JR: The dimensions and number of small vesicles in cells, endothelial, mesothelial and the significance of these for endothelial permeability. *J Microscopy* 90:251–269, 1969
29. Cotran RS, Karnovsky MJ: Ultrastructural studies on the permeability of the mesothelium to horseradish peroxidase. *J Cell Biol* 37:123–137, 1968
30. Felix MD: Observations on the surface cells of the mouse omentum as studied with the phase contrast and electron microscopes. *J Nat Can Inst* 27:713–744, 1961
31. Dobbie JW, Zaki M, Wilson L: Ultrastructural studies on the peritoneum with special reference to Chronic Ambulatory Peritoneal Dialysis. *Scot Med J* 26:213–223, 1981
32. Weibel ER, Palade GE: New cytoplasmic components in arterial endothelia. *J Cell Biol* 23:101–112, 1964
33. LaRocca PJ, Rheinwald JG: Coexpression of simple epithelial keratins and vimentin by human mesothelial and mesothelioma in vivo and in culture. *Cancer Res* 44:2991–2999, 1984
34. Leak LV, Sun DC, Just EE: Localization and characterization of fibronectin in the peritoneal cavity of the mouse. *Anat Rec* 211: 106–114, 1985
35. Rheinwald JG, Jorgensen JL, Hahn WC, Terpstra AJ, O'Connell TM, Plummer KK: Mesosecrin: A secreted glycoprotein produced in abundance by human mesothelial, endothelial and kidney epithelial cells in culture. *J Cell Biol* 104:263–275, 1987
36. Rennard SI, Jaurand M-C, Bignon J, Kawanami O, Ferrans VJ, Davidson J, Crystal RG: Role of pleural mesothelial cells in the production of the submesothelial connective tissue matrix of lung. *Am Rev Resp Dis* 130:267–274, 1984
37. Whitaker D, Papadimitriou JM, Walters MN-I: The mesothelium: A cytochemical study of "activated" mesothelial cells. *J Pathol* 136:169–179, 1982.
38. Efrati P, Nir E: Morphological and cytochemical investigation of human mesothelial cells from pleural and peritoneal effusions: A light electron microscopy study. *Isr J Med Sci* 12:662–673, 1976
39. Whitaker D, Papadimitriou JM, Walters MN-I: The mesothelium: A histochemical study of resting mesothelial cells. *J Pathol* 132:273–284, 1980
40. Ramsey SJ, Tweddale DN, Bryant IR, Braunstein H: Cytologic features of pericardial mesothelium. *Acta Cytol (Baltimore)* 14:283–290, 1970
41. Clausen PP, Hojgaard K, Thommesen N: The diagnostic value of cytochemical staining for non-specific esterase in the search for cancer cells in effusions. *Acta Microbiol Scand* 87(A):347–352, 1979
42. Geiling D, Geiling HG, Halbhuber KJ, Frober R, Geyer G: Rat endo- and mesothelium lack Fc receptors. *Exp Pathol* 17: 171–175, 1979
43. Herman AG, Claeys M, Moncada S, Vane JR: Biosynthesis of Prostacyclin (PGI_2) and 12L-Hydroxy-5,8,10,14,-Eicosatetraenoic acid (HETE) by pericardium, pleura, peritoneum, and aorta of the rabbit. *Prostaglandins* 18:439–452, 1979
44. Coene M-C, Solheid C, Claeys M, Herman AG: Prostaglandin production by cultured mesothelial cells. *Arch Int Pharmacodyn* 249:316–318, 1981
45. Steinhauer HB, Gunter B, Schollmeyer P: Stimulation of peritoneal synthesis of vasoactive prostaglandins during peritonitis in patients on continuous ambulatory peritoneal dialysis. *Eur J Clin Invest* 15:1–5, 1985
46. Miller, TA: Protective effects of prostaglandins against gastric mucosal damage: Current knowledge and proposed mechanisms. *Am J Physiol (Gastrointest Liver Physiol)* 245:G601–G623, 1983
47. Kunkel SL, Chensue SW, Phan, SH: Prostaglandins as endogenous mediators of Interleukin 1 production. *J Immunol* 136: 186–192, 1986