Stimulation of Mesothelial Cell Proliferation by Exudate Macrophages Enhances Serosal Wound Healing in a Murine Model

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Examination of thermally induced serosal lesions in mice displayed collections of inflammatory cells, predominantly macrophages, on and surrounding the wound within 48 hours of injury. Furthermore, by 2 days a large number of uninjured mesothelial cells adjacent to the wound were synthesizing DNA. From these findings, it was hypothesized that macrophages play a major role in serosal repair by stimulating mesothelial cell proliferation. Again, using a murine model of mesothelial regeneration, depletion of circulating monocytes significantly delayed serosal healing whereas addition of peritoneal exudate cells to the wound site 36 hours before injury increased the healing rate. *In vivo* assessment of mesothelial cell proliferation using tritiated thymidine incorporation and autoradiography demonstrated that peritoneal exudate cells stimulated mesothelial cell proliferation ($12.44 \pm 1.63\%$ labeling index, compared with controls in which medium only was used $4.48 \pm 0.71\%$). The mesothelial proliferation was predominantly because of macrophage-secreted products with molecular weights of 36 to 53 kd or 67 to 100 kd. These data support the hypothesis that macrophages play an important role in serosal healing by stimulating mesothelial cell proliferation. (*Am J Pathol* 2002, 160:681–692)

The mesothelium is composed of an extensive monolayer of specialized cells that line the body's serous cavities and internal organs. Traditionally this layer was thought to be a simple tissue with the sole function of providing a slippery, nonadhesive and protective surface to facilitate intracoelomic movement. However, the mesothelium is now recognized as a dynamic cellular membrane with many important functions, including transport and movement of fluid and particulate matter across the serosal cavities,^{1,2} secretion of glycosaminoglycans, extracellu-

lar matrix proteins, proinflammatory cytokines, and growth factors^{3–7} and antigen presentation.^{8,9}

Injury to the mesothelium after surgery, infection, combined ambulatory peritoneal dialysis, and exposure to particulates such as asbestos, triggers a sequence of events that lead to regenerative and reparative processes. A breakdown in the regulation of these processes can result in the development of conditions such as adhesion formation, pleural fibrosis, and mesothelioma. To understand the pathophysiology of such conditions, we need to understand the mechanisms regulating normal mesothelial repair. Mesothelial healing differs from that of other epithelial-like surfaces because both small and large wounds heal in the same time period. Therefore, it is unlikely that healing occurs solely by centripetal migration of mesothelial cells from the edge of the wound into the wound center. Several hypotheses have been proposed for the origin of the regenerating mesothelium.¹⁰ Most evidence supports a bimodal mechanism involving proliferation and centripetal migration of cells at the edge of the lesion and implantation of free-floating mesothelial cells onto the wound surface.^{11,12}

Mesothelial cells surrounding a lesion and on the apposing serosal surface begin proliferating 24 to 48 hours after injury.^{11,13} It has been proposed that these cells are stimulated to proliferate by mediators released from inflammatory cells, predominantly macrophages, that accumulate on the wound surface within 48 hours.^{14–16} Macrophages are a major source of growth factors for many cell types and several of these factors have been shown to stimulate mesothelial cell proliferation *in vitro* and *in vivo*.^{12,16–18} Furthermore, it has been suggested that macrophages present on the serosal surface after injury may also provide a protective cover in the absence of an intact mesothelial membrane¹¹ and, through the secretion of neutral proteases such as plasminogen activator, elastase, and collagenase,^{19,20} remove tissue debris and fibrin, enabling mesothelial cells to resurface the injured site and reconstitute the coelomic space.

Cell depletion studies using whole body X-irradiation^{11,14} and silicon dioxide treatment,¹⁴ supported these

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proposals as a decrease in monocyte/macrophage numbers lead to a decrease in the mesothelial healing rate. However, these experiments did not selectively deplete monocytes and macrophages but affected other cell types as well. In this study, selective depletion of monocytes/macrophages using cytotoxic liposomes reduced the rate of mesothelial healing and addition of peritoneal exudate cells (PECs) before injury increased the rate of healing. Furthermore, we demonstrated that exudate macrophages stimulate mesothelial cell proliferation through the secretion of products with molecular weights of 36 to 53 kd and 67 to 100 kd.

Materials and Methods

Animals

Male BALB/c mice, 8 to 12 weeks old, and 12-week male guinea pigs were used in this investigation. Approval for all animal experimentation was obtained from the Animal Welfare Committee of the University of Western Australia.

Preparation of the Various Cell Populations

A series of different cell populations including peritoneal resident and exudate cells, thymocytes, spleen cells, and neutrophils were collected to determine their mitogenic effect on normal uninjured murine testicular mesothelium and their effect on healing rate.

Peritoneal Resident and Exudate Cells

Resident murine peritoneal cells were collected by lavage in 3 ml of RPMI 1640 medium (Gibco, Melbourne, Australia) containing 1000 U/ml heparin. PECs were obtained by intraperitoneal injection of 1 ml of thioglycollate broth (BDH, Poole, UK) 48 hours before lavage. On average, each mouse yielded $\sim 3 \times 10^6$ resident cells, or after thioglycollate stimulation, 2.5×10^7 exudate cells.

Macrophage Isolation from PECs

Macrophages were isolated from PECs by two methods: discontinuous density gradient or cell adhesion.

Discontinuous Density Gradient: Polystyrene 10-ml centrifuge tubes were coated with fetal calf serum (FCS) for 2 minutes immediately before use. PECs from 15 mice were filtered through nylon cloth, counted, and resuspended in 30 ml of a 45% Percoll (Pharmacia, Uppsala, Sweden)/phosphate buffered saline (PBS) solution. Two ml of 30, 45, 50, and 55% concentrations of Percoll were layered into the centrifuge tube, the highest concentration at the bottom and RPMI 1640 medium at the top. The cells were centrifuged at $280 \times g$ for 20 minutes at 4°C and various fractions of the gradient collected. The top three cell fractions, which contained the greatest proportion of macrophages, were combined, centrifuged at $300 \times g$ for 10 minutes, washed twice in PBS and once in medium, counted, and reappplied to a second Percoll

gradient. Fractions 2 and 3 were combined, washed as previously described, and counted. Fractions 6 to 9 in the first Percoll gradient contained predominantly lymphocytes and granulocytes and had a very low proportion of macrophages. These fractions were washed as described for fractions 1 to 3 and seeded into plastic culture flasks for 30 minutes to remove macrophages by adhesion. Cells were counted and resuspended in medium at 1×10^7 cells/ml.

Adhesion Method for Retrieval of Macrophages: Culture flasks (75 cm^2) were coated with 3 ml of FCS and left at 4°C overnight. The flasks were washed with warm sterile PBS and incubated with PBS at 37°C for 10 to 15 minutes. The PBS was removed and the cell suspension, consisting of 6 ml of RPMI 1640 medium containing 10% FCS and 5.7×10^7 PECs, were added to each flask. Cells were incubated for 1 hour at 37°C and the nonadherent cells removed. Nonadherent cells were washed, resuspended in medium, and placed in plastic culture flasks for 30 minutes to deplete remaining macrophages. This procedure was repeated three times. Adherent cells were washed three times with PBS and overlaid with a solution of prewarmed PBS containing 0.02% ethylenediaminetetraacetic acid (Sigma, St. Louis, MO, USA). After a 20-minute incubation, adherent cells were dislodged by vigorous pipetting. Both cell populations were washed twice with PBS and twice with medium, counted, and resuspended at 1×10^7 cells/ml.

Fixed PECs

PECs from four mice were resuspended and fixed overnight in 2.5% glutaraldehyde in 0.1 mol/L of phosphate buffer, washed in five changes of PBS and two changes of RPMI 1640 medium, each for 30 minutes, and resuspended in medium at 1×10^7 cells/ml.

Thymocytes and Spleen Cells

The thymuses and spleens from four mice were finely minced and passed through a fine sieve. Cells were centrifuged at $300 \times g$ for 5 minutes and washed twice in cold RPMI 1640 medium. Spleen cells were cultured for 30 minutes on plastic flasks to deplete macrophages. Activated spleen cells were prepared by culturing cells (5×10^6 /ml) for 48 hours in medium containing $1 \mu\text{g}/\text{ml}$ of phytohemagglutinin (BDH, Wellcome, UK) and 100 U/ml interleukin-2 (Cetus Corp, Emeryville, USA) with 5% FCS, 2 mmol/L L-glutamine, 0.1 mmol/L nonessential amino acids, 1 mmol/L sodium pyruvate (Gibco), 100 U/ml penicillin/streptomycin, and 50 $\mu\text{mol}/\text{L}$ 2-mercaptoethanol (BDH). Cells were washed twice, counted, and resuspended in medium at 1×10^7 cells/ml.

Neutrophils

Neutrophils were prepared from bone marrow cultures.²¹ Briefly, bone marrow cells were obtained by flushing out femurs of mice with medium using a 23-

gauge needle and seeded in a 75-cm² culture flask at a concentration of 2.5×10^6 cells/ml in 20 ml of medium containing 15% FCS, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 23.8 mmol/L NaHCO₃, 10 mmol/L HEPES, 7.5×10^{-5} mol/L monothioglycerol, 10^{-6} mol/L hydrocortisone 21-hemisuccinate (Sigma), and 100 U/ml penicillin/streptomycin. At weekly intervals, half of the supernatant was removed and replaced with an equal volume of fresh medium. After 3 weeks all of the supernatant was removed from the adherent cell cultures and replaced with 20 ml of medium containing freshly isolated bone marrow cells. Nonadherent cells in the spent medium were harvested weekly and replaced with fresh culture medium. These nonadherent cells were fractionated on discontinuous Percoll gradients: 81, 65, and 55% in PBS. Cells banding between the 65% and 81% Percoll solutions were harvested, washed, and resuspended in RPMI 1640 medium at 1×10^7 cells/ml.

A sample of each prepared cell population was cyto-centrifuged onto a glass slide, stained for nonspecific esterase,²² and a differential count performed. Cell viability was assessed by dye exclusion using a 0.25% solution of trypan blue in PBS and preparations used only if cell viability was >95%. All cells were counted and resuspended in medium at between 2×10^6 and 1×10^7 cells/ml.

Preparation of Macrophage-Conditioned Medium (CM) and Lysates

Peritoneal exudate macrophages were obtained from 150 mice and isolated by adhesion on culture flasks (1×10^8 cells/flask). The cells were incubated in 10 ml of RPMI 1640 containing 125 μ mol/L phorbol myristate acetate (PMA) (Sigma) for 12, 18, 24, and 48 hours. The CM was removed, centrifuged at $1000 \times g$ for 10 minutes, the supernatant filtered through a 0.22- μ m Millipore filter, dialyzed to remove the PMA, and concentrated approximately sixfold using Centriprep-10 concentrators (10-kd molecular weight cutoff; Amicon, Beverly, MA, USA). Adherent cells in each flask were washed twice with PBS and once with medium and overlaid with 1 ml of medium. The cells were freeze-thawed three times at 20°C, the lysates centrifuged, filtered, and dialyzed to remove the PMA. Control medium containing PMA was also dialyzed and concentrated.

Molecular Weight Fractionation of CM

CM was fractionated using Centriprep concentrators and gel filtration. A 5-ml sample of CM was diluted to 15 ml with medium and then concentrated to 3 ml in a Centriprep 100 (100-kd molecular weight cutoff). This was repeated twice to give a solution containing predominantly molecules >100 kd. The effluent was collected and the procedure repeated using a Centriprep 30 (30-kd molecular weight cutoff) to yield a solution containing molecules predominantly 30 to 100 kd. This was repeated using a Centriprep 10 to give a solution with molecules 10 to 30 kd. The CM fraction 30 to 100 kd was

further fractionated on a Sephadex G75 column (Pharmacia, Piscataway, NJ, USA) equilibrated with PBS (pH 7.4).²³ Proteins were eluted from the column with PBS and the optical density of each eluted fraction measured at 280 nm. Various fractions of the eluted CM were pooled, dialyzed, freeze-dried, and then reconstituted in RPMI 1640.

Mesothelial Healing Model

To examine the role of different exudate cell populations in mesothelial repair, a murine model of mesothelial healing was used as previously described.²⁴ Briefly, male mice were anesthetized, their scrotum and tunica vaginalis incised, and their testes exposed. A 60°C heated metal probe, with a 2-mm diameter tip, was applied to the surface of the testis for 3 seconds. The testis was reinserted into the testicular sac and the scrotum sutured. The wound was washed with 70% ethanol and the animal placed in a recovery cage. Animals were killed 1 to 8 days after injury and the extent of healing assessed by scanning electron microscopy (SEM).

Endogenous Peroxidase Staining to Assess Origin of Wound Macrophages

Five male 12-week guinea pigs were anesthetized and their right testes injured as previously described for the mouse: two sites were injured on each testis. Guinea pigs were used in this study because of strong endogenous peroxidase staining of their monocyte/macrophage populations. Groups of two and three animals were killed by cervical dislocation 24 and 48 hours after injury, respectively, their injured testes fixed in 1% glutaraldehyde in 0.165 mol/L sodium cacodylate-hydrochloric acid buffer (pH 7.4) for 10 minutes, and preincubated in a solution of 0.1% 3,3'-diaminobenzidine tetrahydrochloride (Sigma) in 0.165 mol/L cacodylate buffer at room temperature for 45 minutes. Two testes from each group were incubated in freshly prepared preincubation solution (pH 6.5) containing 0.01% H₂O₂ for 30 minutes. The tissues were washed in three changes of cacodylate buffer for 10 minutes each, postfixed in 1% osmium tetroxide for 30 minutes at 4°C, washed in three changes of buffer, and processed for transmission electron microscopy. The control testis was prepared by omitting H₂O₂ in the incubation solution. The proportion of different cell types present on the healing testicular surface was determined and >200 macrophages were examined for peroxidase staining patterns at each time point.

Effect of Various Cell Populations on the Rate of Mesothelial Healing

Measurement of Healing Rate by SEM

The effect of the addition or depletion of various cell populations on the rate of testicular mesothelial healing was investigated by SEM. The normal healing rate was determined by injuring the right testis of four groups of 10

mice as previously described and killing them 5, 6, 7, and 8 days after injury. The injured testis of each mouse was removed, fixed overnight in 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer, and prepared for SEM. The extent of healing was determined by the proportion of the lesion that was covered by a confluent layer of mesothelial cells (demonstrated by expression of surface microvilli). For this study, healing rate was expressed as the number of completely healed testes over the total number of testes examined.

Depletion of Inflammatory Cell Populations and Assessment of Mesothelial Healing by SEM

Monocyte Depletion by Liposomes: Multilamellar liposomes, cytotoxic to monocytes and macrophages, were prepared as described by Huitinga and colleagues.²⁵ Briefly, 70.9 mg of phosphatidylcholine and 10.8 mg of cholesterol (both from Sigma) were dissolved in 8 ml of chloroform and added to 3.6 mg of *p*-aminophenyl- α -D-mannopyranoside (Sigma), dissolved in 2 ml of methanol, and dried using a rotary evaporator. The lipid was dissolved in chloroform and dried again before adding it to 10 ml of a 0.189 g/ml solution of the cytotoxic agent, dichloromethylene diphosphonate. The preparation was kept at room temperature for 2 hours, sonicated for 3 minutes at 20°C, and kept at room temperature for a further 2 hours. The liposomes were centrifuged at $100,000 \times g$ for 30 minutes and finally resuspended in 4 ml of PBS; Cl₂MDP PCMAN.

To ascertain the monocyte-depleting effect of the treatment, six mice were injected with 200 μ l of Cl₂MDP PCMAN liposomes, with three mice receiving a second injection of 100 μ l of liposomes 24 hours after the first injection. Blood was collected by cardiac puncture from the three mice that received a single dose of liposomes 24 and 48 hours after injection and the other three mice 24 hours after the second injection. A white blood cell differential count was performed on all blood samples.

To determine the effect of depleting circulating monocytes on the rate of mesothelial healing, 10 mice were treated by an intravenous injection of 200 μ l of Cl₂MDP PCMAN liposomes, followed by injections of 100 μ l of Cl₂MDP PCMAN liposomes at 24-hour intervals for 2 days. The right testis of each mouse was injured 24 hours after the first injection. The mice were sacrificed 8 days after injury and the lesion examined by SEM. The left testis of each animal was removed and examined as control.

T Cell Deficiency: The testicular mesothelium of 10 BALB/c nude mice was injured as previously described and the degree of healing assessed by SEM 8 days after injury.

Addition of Inflammatory Exudate Cells to Testicular Lesions and Assessment of Healing by SEM

Addition of Cells at Time of Injury: The right testes of two groups of 10 mice were injured as previously described

and 50 μ l of a 5×10^8 PEC preparation injected into the testicular sac before closure. PECs were also injected at the same concentration into the uninjured left testicular sac as a control. Animals were allowed to recover for 5 and 6 days before being killed, and both injured and uninjured testes examined by SEM.

Addition of Cells before Injury: PECs were injected in 0.5 ml of medium (1×10^7 cells/ml) into each testicular sac of 20 mice and 36 hours later, the right testes injured. Groups of 10 animals were sacrificed 5 and 6 days after injury and the healing rate assessed by SEM.

In Vivo Assay for Mesothelial Cell DNA Synthesis

This method was performed as previously described.¹² Both testes of five mice were used in each experimental group. Briefly, animals were anesthetized and a small incision (~4 mm in length) made in the scrotal skin, exposing the tunica vaginalis. Various concentrations of PECs (0 to 2×10^7 cells/ml), cultured immune and inflammatory cells, and macrophage CM and lysates were injected in 0.5-ml aliquots into each testicular sac, exposing all areas of mesothelium on the testis. The incision was closed using a suture, the wound washed with 70% ethanol, and the animal placed in a recovery cage. Animals were injected intravenously with tritiated thymidine (³H-TdR) (1 μ Ci/g body weight, specific activity 6.7 Ci/mmol; Amersham, Sydney, Australia) 30 minutes before sacrifice. Animals were killed by cervical dislocation 6 to 36 hours after injection. The testes were removed and mesothelial imprints obtained.¹² Approximately 85% of the mesothelium was routinely obtained from each testis. The slides were coated with L4 photographic liquid emulsion (Ilford Imaging, Knutsford, UK) and exposed at 4°C for 4 weeks protected from light. Slides were subsequently developed with D-19 developer (Ilford Imaging, Mt Waverley, Australia) and stained with hematoxylin.

Preliminary studies determined that the optimum cell concentration and exposure time required to yield maximal mesothelial cell proliferation was 1×10^7 cells/ml after 36 hours of exposure. Therefore, all further experiments were performed under these conditions.

Assessing Autoradiographs of Imprints for DNA Synthesis

Imprints were subjected to cell counting if 100 or more cells were in direct contact. The labeling index represented the number of labeled nuclei over the total number of cells counted and expressed as a percentage. All imprints throughout this study were counted double blind. Statistical analysis was performed after arcsine transformation of all data, an adjustment required for comparison of proportional values.²⁶ The probability of differences between mean values was determined using Student's *t*-test on transformed data, and considered significant if $P < 0.05$. All experiments performed in this study were repeated at least once.

Figure 1. Scanning electron micrograph of serosal lesion edge 24 hours after injury, showing aggregates of small spherical cells (**arrowheads**) and larger fan-shaped (**small arrows**) and plump elongated (**large arrows**) cells. Original magnification, $\times 370$.

Results

Ultrastructure of Healing Serosal Lesion

Examination of the edge of injured testicular serosa by SEM 24 hours after injury demonstrated aggregates of small spherical cells among larger fan-shaped and plump elongated cells (Figure 1). Transmission electron microscopy showed that the small spherical cells were inflammatory cells and the larger fan-shaped and plump elongated cells were mesothelial cells.¹¹

Origin of Testicular Wound Macrophages

The proportion of different cell types present on the injured serosal surface of guinea pig testes was determined morphologically at 24 and 48 hours after injury. The proportion of resident and exudate wound macrophages was determined by their peroxidase staining patterns.²⁷ At 24 hours macrophages were the predominant cell type at the edge and center of the wound (61%) followed by neutrophils (32%), with occasional lymphocytes, eosinophils, and mast cells. By 48 hours the number and proportion of macrophages (71%) and lymphocytes (6%) increased with a fall in neutrophils (19%). These cell proportions were comparable to figures obtained for injured mouse testes although at 24 hours there was a slightly greater proportion of neutrophils on the testicular surface in the mouse (41% neutrophils, 53% macrophages).

Table 1. Proportion of Resident and Exudate Macrophages Present on the Surface of Injured Guinea Pig Testes at the Early Stages of Mesothelial Healing

Hour lesion	Resident (%) (NE, RER)	Exudate (%)			Cell number
		(G)	(NE, RER, G)	(-ve)	
24	38.9	(17.7	7.9	35.5) = 61.1	203
48	50.3	(11.2	7.3	31.2) = 49.7	205

Cells are categorized on the basis of their endogenous peroxidase-staining pattern: resident macrophages stain positive for nuclear envelope (NE) and rough endoplasmic reticulum (RER); exudate macrophages are negative for NE and RER and positive for granules and phagocytic vacuoles (G); and cells with peroxidatic activity in the NE, RER, and G are exudate-resident cells that were classified as exudate. Cells that do not have any peroxidatic activity (-ve) were also classified as exudate. All values are expressed as a percentage of the total number of cells examined.

Table 2. The Effect of Reducing or Adding Various Cell Types on Mesothelial Healing

Experiment	Number of completely healed testes/total number of testes examined, day			
	5	6	7	8
Normal	0/10	6/10	10/10	10/10
T cell depleted (BALB/c nude)	ND	ND	ND	10/10
Macrophage depletion	ND	ND	ND	3/10
Addition of PECs at $t = 0$	0/10	6/10	ND	ND
Addition of PECs at $t = -36$ hour	5/10	7/10	ND	ND

ND, not done.

Peroxidase staining was present in the nuclear envelope and rough endoplasmic reticulum of resident macrophages, in granules of monocytes and granules and phagocytic vacuoles of exudate macrophages, and in granules, rough endoplasmic reticulum and the nuclear envelope of exudate-resident macrophages (ie, cells that demonstrate a transition between the two activation states). Exudate-resident macrophages and cells with no peroxidase staining were classified as exudate cells. It was demonstrated from examination of more than 200 macrophages at each time point after injury, that at 24 hours there was a greater proportion of exudate (61%) compared to resident (39%) macrophages, but an equal proportion of resident and exudate macrophages by 48 hours (Table 1). No peroxidase staining was observed in any cells of the testis when H_2O_2 was omitted from the incubation solution.

Effect of Various Cell Types on the Rate of Mesothelial Healing

Control Animals

The healing rate of testicular injuries was assessed after the addition or depletion of various PEC populations in the murine model (Table 2). Examination by SEM of control testicular lesions revealed a continuous surface layer of mesothelial cells on all lesions by day 7, and therefore were considered completely healed. Only 6 of 10 testes had healed by day 6 and no lesions were totally healed by day 5. Therefore, a reduction in the healing rate was considered significant if complete healing had not been achieved by 8 days after injury in test animals. Conversely, the healing rate was considered to have

Table 3. White Blood Cell Profile for Normal and Liposome-Injected Mice

Hours after initial injection	Normal, %						Liposome injected, %					
	WCC, $\times 10^9/l$	N	L	M	E	B	WCC, $\times 10^9/l$	N	L	M	E	B
24	3.4	62	28	9	1	0	4.1	67	33	0	0	0
48	5.2	60	35	5	0	0	8.1	69	19	11	1	0
48*	3.3	54	40	6	0	0	4.4	63	38	0	0	0

Three hundred cells were counted for each MGG-stained whole blood preparation. WCC, white cell count; N, neutrophils; L, lymphocytes; M, monocytes; E, eosinophils; B, basophils.

*Animals received second liposome injection 24 hours after the first injection.

significantly increased if complete healing had occurred by day 5.

Monocyte-Depleted Animals

Examination of blood from mice receiving single or double doses of Cl_2MDP PCMAN liposomes revealed that 24 hours after injection, despite an increase in the total white cell count compared to normal, because of an increase in neutrophils and lymphocytes, monocytes had been eliminated from the blood. Conversely, at 48 hours, monocytes were observed in the blood at a greater concentration than normal. However, the increase in monocyte number observed at 48 hours in animals receiving only a single liposome injection was prevented by a subsequent liposome injection 24 hours after the first (Table 3).

Animals treated with Cl_2MDP PCMAN liposomes to reduce circulating monocytes demonstrated a failure to completely heal in 7 of 10 mice examined at day 8 (Table 2). Examination of uninjured testes from animals treated with Cl_2MDP PCMAN liposomes demonstrated normal mesothelial cell morphology.

T-Cell-Depleted Animals

Examination of testicular wounds in T-cell-deficient BALB/c nude mice at 8 days after injury showed that all testes had completely healed (Table 2).

PEC-Injected Animals

Examination of 6-day lesions in animals injected with PECs at the time of injury demonstrated complete healing in 6 of 10 mice examined. Complete healing was not observed in any mouse by day 5 after injury. The mesothelium of animals exposed to PECs 36 hours before injury demonstrated complete healing in 5 of 10 testes examined by day 5 and 7 of 10 by day 6 (Table 2).

Mitogenic Effect of PECs and Control Cells on Uninjured Mesothelium

Different concentrations of PECs and exposure times were assessed by 3H -TdR incorporation and autoradiography to optimize conditions for measuring stimulation of mesothelial cell proliferation *in vivo*. Mesothelium on the testes of mice that received no injections into the testic-

ular sac displayed a very low labeling index [mean percentage 3H -TdR labeled cells $\pm$ SE of mean (SEM): $0.25 \pm 0.05\%$]. The optimal conditions for measuring stimulation of mesothelial cell proliferation was demonstrated to be at 1×10^7 cells/ml measured at 36 hours after injection ($12.44 \pm 1.63\%$, compared with $4.48 \pm 0.71\%$ in controls receiving only medium) and these conditions were used for all subsequent experiments.

The effect of fixed PECs and viable quiescent thymocytes, injected at equivalent concentrations, on mesothelial cell proliferation was assessed to confirm that stimulation of DNA synthesis in mesothelium exposed to PECs was not because of mechanical artifact. The labeling index of mesothelium exposed to fixed PECs or viable thymocytes was not significantly greater than for controls receiving only medium (Figure 2). Testicular mesothelium was also exposed to equivalent concentrations of resident peritoneal cells as PECs to determine whether peritoneal cells required activation before they were mitogenic for mesothelial cells. No significant difference in the labeling index between resident cells and controls receiving only medium was demonstrated (Figure 3).

Macrophages, isolated from a population of PECs by discontinuous density gradient and adhesion methods (93.8% and 90.2% macrophages, respectively; Table 4) and macrophage-depleted cell subpopulations obtained

Figure 2. The mitogenic effect of fixed PECs and quiescent thymocytes (Thym.) on mesothelial cells. Error bars, SEM. *, $P < 0.0005$. **Inset:** An autoradiograph of a mesothelial imprint after a 36-hour incubation with PECs. Unlabeled mesothelial cell nuclei are lightly stained with hematoxylin (arrows) whereas the nuclei of labeled cells are overlaid with black silver grains (arrowheads). Original magnification, $\times 25$.

Figure 3. The mitogenic effect of different functional states of peritoneal cells on mesothelial cells. The mitogenic effect of peritoneal resident cells (PRCs) and PECs are compared to medium-only controls. Error bars, SEM. *, $P < 0.0005$.

from the same PEC population (Table 4), were prepared at equal concentrations (1×10^7 cell/ml) and injected into the testicular sacs of mice to compare the mitogenic effect of different cell types in PECs on mesothelium. The labeling indices obtained after injection of macrophages isolated by both enrichment techniques were significantly higher than the labeling index for control medium (adhesion method: $22.87 \pm 1.44\%$, compared with $4.23 \pm 1.02\%$ in controls receiving only medium and density gradient method: $13.06 \pm 2.10\%$, compared with $5.15 \pm 0.93\%$ in controls receiving only medium; $P < 0.005$, Figure 4). The labeling index for macrophages isolated by the adhesion method was greater than for total PECs. However, there was no significant difference in the labeling index between macrophages isolated using the discontinuous density gradient method and total PEC medium (adhesion method: $22.87 \pm 1.44\%$ for macrophages, compared with $13.61 \pm 1.42\%$ for total PECs; $P < 0.0005$ and density gradient method: $13.06 \pm 2.10\%$ for macrophages, compared with $13.60 \pm 1.14\%$ for total PECs; Figure 4).

The PEC population remaining after removal of the majority of macrophages (94.8% of macrophages were removed by adhesion and 100% by density gradient;

Figure 4. The mitogenic effect on mesothelial cells of macrophage-enriched (open bar) and macrophage-depleted (cross-hatched bar) subpopulations of total PECs (hatched bar) separated by adhesion and density gradient methods, when compared to controls receiving medium only (filled bar). Error bars, SEM. *, $P < 0.05$; #, $P < 0.005$; **, $P < 0.0005$. A significant increase in the labeling index of mesothelial cells was also demonstrated after incubation with a macrophage-enriched cell population (adhesion method) compared to total PECs ($P < 0.0005$).

Table 4) also induced a significant increase in the mesothelial labeling index ($P < 0.0005$), with values approximating those of the total PEC population (adhesion method: $15.03 \pm 1.68\%$, compared with $13.61 \pm 1.42\%$ for total PECs and 4.23 ± 1.02 in controls receiving only medium, and density gradient method: $9.97 \pm 1.96\%$, compared with $13.60 \pm 1.14\%$ for total PECs and $5.15 \pm 0.93\%$ in controls receiving only medium; Figure 4).

The majority of cells present in the macrophage-depleted PEC population were lymphocytes and neutrophils. To assess the potential of these cell types to stimulate DNA synthesis in mesothelial cells, resting and activated splenic lymphocytes and cultured neutrophils were assessed for mitogenic activity for mesothelium. Nonactivated splenic cells and neutrophils stimulated mesothelial cell proliferation more than medium only controls but the labeling indices were smaller than for the total PEC population (Figure 5). Activated spleen cells did not significantly induce DNA synthesis in mesothe-

Table 4. The Proportion of Cell Types Present in Various Cell Populations as Determined by Differential Counts on Nonspecific Esterase-Stained Cytocentrifuge Preparations

Cell population*	Cell type, %				Cells counted
	M	L	N	O	
Resident	41.4	53.8	3.8	1.0	584
PECs	76.3	6.4	15.7	1.6	654
Total PECs	66.6	10.2	22.2	1.0	612
Macrophage enriched	93.8	1.8	4.2	0.2	685
Macrophage depleted	0	34.8	52.4	12.8	622
Total PECs	70.8	4.8	22.0	2.4	576
Macrophage enriched	90.2	1.8	5.2	2.8	601
Macrophage depleted	5.2	20.8	52.5	21.5	907
Spleen	10.0	87.4	2.6	0	580
Spleen after adherence	2.1	96.7	1.2	0	521
BM-derived neutrophils	0	0	100	0	500

*PEC, peritoneal exudate cells; BM, bone marrow; M, macrophages; L, lymphocytes; N, neutrophils; O, other granulocytes and mast cells.

Figure 5. The mitogenic effect of various inflammatory and immune cell populations on mesothelial cells. The labeling index of spleen cells (primary and activated in culture) and cultured polymorphonuclear leukocytes (PMNs) are compared to controls receiving medium only (*, $P < 0.005$; **, $P < 0.0005$). *, $P < 0.005$ represents significance for the difference in the labeling index between PECs and spleen cells and PECs and neutrophils. Error bars, SEM.

lium more than the values obtained for the medium-only controls.

Mitogenic Effect of Activated Macrophage-CM and Lysates on Uninjured Mesothelium

Concentrated macrophage CM and lysates generated from cells incubated for 12 to 48 hours in medium containing PMA demonstrated a significant mitogenic effect for mesothelial cells *in vivo*. Maximal proliferation for lysates occurred when cells were incubated with PMA for 18 hours compared to 48 hours for CM (lysates: $9.20 \pm 1.02\%$ compared with $5.08 \pm 1.26\%$ in controls receiving only medium and CM: $7.23 \pm 1.16\%$ compared with $2.44 \pm 0.65\%$ in controls receiving only medium; $P < 0.025$; Figure 6). Fractionation of CM from PMA-activated macrophages demonstrated maximal mitogenic activity in the 30- to 100-kd fraction ($11.62 \pm 1.08\%$ compared with $3.13 \pm 0.72\%$ in controls receiving only medium; Figure 7). Subsequent column fractionation of the 30- to 100-kd fraction to obtain a narrower molecular weight range of the mitogenic molecule(s) demonstrated an increase in the mesothelial labeling index for several fractions with the greatest mitogenic activity in fractions 36 to 53 kd and 67 to 100 kd ($9.34 \pm 1.26\%$ and $7.42 \pm 1.26\%$, respectively, compared with $1.63 \pm 0.55\%$ in controls receiving only medium, $P < 0.0005$; Figure 8).

Discussion

It has previously been demonstrated, and again confirmed in this study, that under normal physiological conditions the mesothelium is a slowly renewing tissue with mitotic activity ranging from 0.16 to 0.25% in mice²⁸ and 0.5 to 3.0% in rats.^{14,29} However, after injury, the mitotic

Figure 6. The mitogenic activity of PMA-stimulated macrophage lysates (open bars) and concentrated-CM (solid bars) on normal mesothelium. Peritoneal exudate macrophages were incubated for various times in medium containing 125 $\mu\text{mol/L}$ of PMA. *, $P < 0.025$; **, $P < 0.001$.

activity of these cells increases with 30 to 60% of cells surrounding the injury synthesizing DNA by 48 hours.^{11,13}

It has been proposed that inflammatory cells, particularly macrophages, provide the signal to initiate mesothelial cell proliferation through the secretion of mitogenic factors.¹⁴⁻¹⁶ Inflammatory cells, predominantly macrophages, are clearly seen on the wound surface within 48 hours of injury. We demonstrated that the majority of these cells are exudate macrophages, particularly in the first 24 hours. In previous studies, whole body X-irradiation and silicon dioxide treatment demonstrated a reduction in the mesothelial healing rate after a decrease in circulating monocyte numbers.^{11,14} However, these treatments are not specific for monocytes/macrophages^{30,31}

Figure 7. Effects of different molecular weight fractions of PMA-stimulated macrophage-CM, separated by Centriprep centrifugation, on DNA synthesis in mesothelial cells, compared with controls receiving medium only. *, $P < 0.005$; **, $P < 0.0005$.

Figure 8. The effect of different molecular weight subfractions, separated on a Sephadex G75 column, of the 30- to 100-kd fraction of PMA-stimulated macrophage-CM, on DNA synthesis in mesothelial cells compared with controls receiving medium only. *, $P < 0.05$; **, $P < 0.01$; #, $P < 0.0005$.

and therefore the depressed mesothelial healing rate observed cannot conclusively be attributed to monocytes/macrophages alone. In the present study, cytotoxic liposomes were used *in vivo* that specifically depleted the monocyte/macrophage population.^{25,32,33} The liposomes contain dichloromethylene diphosphonate, a substance that is cytotoxic to cells only on ingestion. The mannose residues that are incorporated into the liposomes bind to specific receptors on monocytes and macrophages and induce phagocytosis, thereby selectively depleting these cell populations. In mice, it was demonstrated that peripheral blood monocytes were depleted by 24 hours after liposome injection, but subsequent liposome injections at 24-hour intervals were required to maintain low monocyte numbers. Examination of the healing rate of injured mesothelium after Cl₂MDP PCMAN liposome injections, demonstrated a failure of the mesothelium to heal in 7 of 10 animals examined. It is possible that death of the macrophages may have affected other cells that subsequently influenced the healing rate. However, it is more likely that depletion of the monocyte/macrophage population directly was the main cause of a reduced rate of mesothelial healing.

If macrophages initiate healing by stimulating mesothelial cell proliferation, we proposed that addition of macrophages to a serosal lesion before or at the time of injury, should result in an increase in the mesothelial healing rate. Injection of PECs into the testicular sacs at the time of injury did not induce a measurable increase in the healing rate, however, animals injected with PECs 36 hours before injury demonstrated a significant increase in the rate of mesothelial healing as 5 of 10 mice had completely healed by day 5 compared to 0 of 10 for untreated animals. In untreated animals, mesothelial cells surrounding a lesion are undergoing maximal division 2 days after injury.¹³ Addition of inflammatory cells to the testicular sac 36 hours before injury reduced this lag

phase to almost zero, thereby increasing the rate of the healing process. The healing rate was not increased in all animals examined, and this may have been because of differences in the concentration of inflammatory cells coming into direct contact with the testis, because cells are washed into the peritoneal cavity on injection.

To examine the effect of inflammatory cells on mesothelial cell proliferation, PECs, isolated cell populations from PECs, and cultured immune and inflammatory cells were injected into the testicular sacs of uninjured mice and proliferation assessed using tritiated thymidine incorporation and autoradiography. In the presence of PECs, the mitotic activity of normal uninjured testicular mesothelium was increased from 0.25% to values exceeding 12%. This increase was a specific response to these cells, and not a mechanical artifact, as fixed PECs and viable quiescent thymocytes did not stimulate DNA synthesis in mesothelial cells more than values for medium-only controls. In addition, resident peritoneal cells did not induce a significant increase in DNA synthesis in mesothelial cells more than controls suggesting that the activational state of peritoneal cells is important for mitogenic activity. Furthermore, injection of purified exudate macrophages into the testicular sacs of mice resulted in a marked stimulation of DNA synthesis in mesothelial cells. The labeling index of mesothelium exposed to macrophages isolated by the adhesion method was significantly higher than for the same concentration of cells in the total inflammatory population, whereas values from macrophages obtained using Percoll gradients approximated the value for the total PECs population. The higher values obtained for the adhesion method may have been because of exposure of the macrophages to FCS, either by increasing activation of these cells or, if serum proteins were bound to the macrophage surface, by providing a source of growth factors to the mesothelial cells.^{34,35} Alternatively, the increased mitogenic activity may have been because of adherence-activation.³⁶ In all cell populations examined for mitogenic activity, very few contaminating mesothelial cells were present and therefore addition of mesothelial cells is unlikely to be the cause of increased cell proliferation.

Populations of PECs depleted of macrophages also stimulated mesothelial cells to synthesize DNA, suggesting that cells other than macrophages can also stimulate mesothelial cell proliferation. Neutrophils and lymphocytes represented the greatest proportion of cells in the macrophage-depleted PECs population. When tested separately both cell types induced DNA synthesis in mesothelial cells, although the labeling indices were significantly lower than for PECs. Neutrophils and lymphocytes release many cytokines, some of which stimulate mesothelial cell proliferation¹⁸ and macrophage chemotaxis.³⁷ The mitogenic effect of neutrophils and lymphocytes observed in this study may be because of release of these cytokines. Alternatively, enhanced mesothelial cell proliferation may be because of macrophage recruitment. Although the lymphocyte population stimulated a degree of DNA synthesis in mesothelial cells, we could not demonstrate any support for a significant role by T cells. Injection of activated T lymphocytes, selected by

culturing spleen cells in medium containing interleukin-2 and phytohemagglutinin, did not significantly induce mesothelial cell proliferation. Furthermore, mesothelial healing was not impaired in immunoincompetent animals, suggesting that T cells are unlikely to play a significant role in mesothelial healing. Adjustment of macrophage-depleted PECs to concentrations equivalent to total PECs also demonstrated mitogenic activity for mesothelium. However, the macrophage-depleted fraction represented no more than 25% of the total PECs. Therefore, it is unlikely that the cells in this fraction were responsible for the majority of the mitogenic activity seen for total PECs.

It is well established that macrophages play a major part in wound healing.³⁸ One role is the secretion of factors that are mitogenic for a variety of cell types, including mesothelial cells.^{12,17,38} Therefore, it is highly probable that macrophages play an important role in mesothelial healing through secretion of growth factors. Testicular wound lavages injected into the testicular sacs of uninjured rats induced a significant increase in DNA synthesis in mesothelial cells.¹⁴ In the same model, a reduction in the number of macrophages and other white blood cells by whole body irradiation significantly decreased the mitogenic effect of the wound exudate cell population. From these findings, Fotev and colleagues¹⁴ proposed that macrophages in the wound exudate were primarily responsible for stimulating mesothelial cell proliferation. Furthermore, it has been reported that postsurgical macrophages secrete mitogenic factors for peritoneal serosal tissue repair cells that were claimed to be of mesothelial cell origin.¹⁶ However, it is not clear if these were mesothelial cells or fibroblasts because in other studies the authors referred to these cells as fibroblasts.^{39,40}

To show that macrophage-induced mesothelial cell proliferation is because of a secreted factor(s), CM and lysates from isolated activated PEC macrophages were assessed *in vivo*. Wound macrophages are normally activated and stimulated to produce growth factors by mediators released by dead and injured resident cells, inflammatory cells, and blood products. To generate high concentrations of these growth factors *in vitro*, PEC macrophages were first activated using PMA before collecting CM and lysates. A significant increase in mesothelial cell proliferation was demonstrated for lysates and CM obtained from macrophages cultured with PMA for 18 and 48 hours, respectively. This suggests that macrophages produce a mitogenic factor(s), with the intracellular concentration reaching a maximum at 18 hours, which is then secreted by the cell, attaining a concentration measurable by *in vivo* assay at 48 hours. The lack of mitogenic activity observed in the 24- and 48-hour PMA-stimulated macrophage lysates suggest that after secretion, either significant amounts of the factor(s) are no longer produced or it is secreted rapidly into the culture medium. Although more than 95% of the macrophages were viable after 48 hours in culture, the possibility that their ability to produce large quantities of protein had decreased must also be considered.

Initial molecular weight fractionation of the CM demonstrated that the majority of the mitogenic activity was within a molecular weight range of ~30 to 100 kd. This

fraction was further fractionated on a Sephadex G75 column to give a series of samples containing proteins in a narrower molecular weight range. Mitogenic activity was demonstrated in several fractions, however, the most significant activity was in the 36- to 56-kd and 67- to 100-kd fractions. It is possible that this latter fraction may contain growth factors complexed with other proteins.

Many growth factors are known to be mitogenic for mesenchymal cells. However, mesothelial cells are unique because they also proliferate in response to growth factors thought to be specific for epithelium such as hepatocyte growth factor^{7,41} and keratinocyte growth factor.⁴¹ Macrophages secrete many growth factors, some, such as tumor necrosis factor- α , platelet-derived growth factor, and fibroblast growth factor, have been shown to be mitogenic for mesothelium *in vitro*^{12,16-18} and *in vivo*.¹² Further studies are now required to determine which factors in the 36- to 53-kd and 67- to 100-kd fractions of macrophage CM are responsible for mesothelial cell proliferation *in vivo*.

Mesothelial cells can also be stimulated to secrete a variety of growth factors and chemokines, including platelet-derived growth factor, transforming growth factor- β , hepatocyte growth factor, keratinocyte growth factor, interleukin-6, endothelin-1, and monocyte chemoattractant protein-1 that can act as autocrine factors to promote mesothelial cell proliferation and migration.^{7,41-45} Therefore, it is possible that mediators secreted by macrophages stimulate an autocrine loop in mesothelial cells rather than directly stimulating cell proliferation.

Early morphology and histochemistry studies demonstrated that the mammalian mesothelium is essentially similar irrespective of species or anatomical site.^{10,46} However, other studies have shown that mesothelial cells isolated from different patients or different species do not all behave the same way.^{18,47,48} Therefore, although it is likely that the findings described in this study represent mesothelial healing on all serosal surfaces, further studies need to be performed before this can be confirmed.

In summary, the data obtained in this study clearly demonstrate that in addition to any fibrinolytic role in the healing process, inflammatory cells, in particular macrophages, stimulate testicular mesothelial cells to proliferate. Furthermore, the importance of macrophages in mesothelial healing was shown by significantly delaying mesothelial healing after depletion of circulating monocytes, whereas addition of macrophages to the wound site 36 hours before injury increased the healing rate. Macrophages are a major source of growth factors and it is likely that mesothelial proliferation is induced by one or more of these factors. Inflammatory exudate cells, in particular macrophages, induced mesothelial cell replication *in vivo*, which was attributed to 36- to 53-kd and 67- to 100-kd fractions of PMA-stimulated macrophage CM. Previous studies have demonstrated that many factors, including mediators produced by macrophages such as tumor necrosis factor- α , platelet-derived growth factor, and fibroblast growth factor, are mitogenic for mesothelial cells *in vivo*. The challenge is to determine which factor(s) play a crucial role in the mesothelial healing process.

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