Potential Role of Bone Marrow-Derived Cells in the Turnover of Mesothelium

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Abstract :

Background : The exact mechanism of mesothelial repair remains undetermined. Although accumulated evidences strongly support the concept that peritoneal free-floating cells and subserosal mesenchymal precursors were involved in new mesothelial regeneration, an alternative hypothesis which propose bone marrow cells as a source of new mesothelium can not be neglected. Given that highly plastic bone marrow cells can reconstitute tissue of mesoderm origin, together with the fact that peritoneal free-floating cells mainly came from bone marrow, and the nature of subserosal connective tissue precursors resemble that of bone marrow mesenchymal stem cell, it is possible that bone marrow cells may reconstitute mesothelium. The purpose of this study is to examine the validity of the hypothesis that bone marrow derived cells are an alternative source of mesothelial progenitors which contribute to mesothelial turnover.

Method: Twenty female wild mice received eGFP- and sex-mismatched bone marrow transplantation from male eGFP donor following lethdal dose irradiation. Five recipient mice were then euthanized at 2^{nd} wk, 4^{th} wk, 6^{th} wk and 6^{th} month after bone marrow transplantation respectively. Peritoneal tissue section obtained from abdominal organs were subject to immunohistochemical staining (IHC) for identification of eGFP protein and chromogenic in situ hybridization (CISH) for y-chromosome detection. Combined y-chromosome CISH and cytokeratin IHC technique was undertaken to confirm the expression of mesothelial phenotype by donor marrow cells.

Results : In recipients either 2^{nd} wk, 4^{th} wk, 6^{th} wk or 6^{th} month post-transplantation, only scanty eGFP containing cells could be identified within mesothelial layer of abdominal wall, intestine, mesentery or liver. The amount was very rare, accounting

for far less than 1% of total counted mesothelial cells in recipients either sacrificed at 2^{nd} wk, 4^{th} wk, 6^{th} wk or 6^{th} month post-transplantation. *There was no difference in the incidence of eGFP containing cells between recipients sacrificed at different timeing.* The incorporation of donor marrow cells into mesothelium was further confirmed by the detection of y-chromosome containing cells within mesothelial layer, but the detection rate is even fewer than that of eGFP containing cells. Combined y-chromosome CISH and cytkeratin IHC proved that the donor marrow cells which were incorporated into mesothelial layer also exhibited mesothelial marker cytokeratin, indicating that incorporated bone marrow cells have been differentiated into mesothelial phenotype.

Conclusion : Bone marrow derived cells can contribute to new mesothelium However, this mechanism does not plays a significant role in mesothelial turnover. Major source of cells involved in mesothelium regeneration come from other progenitors, rather than bone marrow derived cells.

Introduction:

The mesothelium consist of a single layer of flattened mesothelial cells which line the serosal cavities. It plays important roles in maintaining normal serosal integrity and function. Following injury, mesothelium itself can regenerate and repopulate the mesothelial layer of peritoneal cavity. Although the underlying mechanism regulating mesothelial repair has been substantially investigated, the exact source of new mesothelium remains controversial.

In early days, the mesothelium was believed to heal by centripetal migration from the damaged area as what occurs in the healing process of skin [1 *Cunningham 1926*]. But later experimental observation showed that entire wound surface became epithelialized simultaneously [2], rather than gradually from the border as in epithelization of skin wound. In addition, a large peritoneal wound healed at the same speed as a small one [3-7]. It is thus unlikely that new mesothelium arise solely by proliferation and centripetal migration of cells from the wound edge. Additional sources of progenitor cells should exist. Different hypothesis have been proposed for the origin of progenitor cells in regenerating mesothelium.

The concept that new mesothelium arises from submesothelial cells was first raisd by Robbin et al [*1919,*8] who suggested that new serosa might form by ingrowth from undamaged serosa as well as by transformation of fibroblast which migrated upward from the base of the wound. William [9, *1955*], studying the healing of parietal defect in rabbits, proposed that the new mesothelium arose mainly from subjacent connective tissue cells because the process of repair took place simultaneously and rapidly over the whole wound. Ellis et al [3,*1965*] noticed a two stage process for mesothelial healing: an initial wave of phagocytic cells responsible for clearing of

traumatic debris, followed by a second wave of fibrlblast infiltration in peritoneal wound. They believed that submesothelial fibroblast were responsible for healing of the defect and could differentiation into new mesothelium. In a similar observation, Raftery et al [4,*1973*] confirmed the two stage process of mesothelial healing and proposed that new mesothelium arised from subperitoneal connective tissue cells, which may be either primitive mesenchymal cells or fibroblasts. Subsequently, Bolen et al [10] demonstrated a cytokeratin staining in subserosal cells of inflamed healing serosa, which was normaly absent in subserosal layer. They thus hypothesized that submesothelial spindle cells are multipotent and can differentiate into mesothelial cells upon activation. Now, it is generally agreed that sub-serosal mesenchymal precursors might provide cells for new mesothelium. Recent advance in the knowledge of regenerative medicine suggest that mesenchymal stem cells (MSC) are present in bone marrow as well as in peripheral tissue of adult animal. They are responsible for the maintenance of multiple tissue or organs of mesoderm origin. The subserosal progenitors which are thought to be the source of new mesothelium are probably tissue resident MSC. This postulation can be supported by the observation of Lucas et al [11] who showed that intraperitoneal injection of MSC isolated from skeletal muscle of neonatal rats could reduce peritoneal adhesion.

Another well accepted hypothesis proposed that new mesothelium come from free-floating cells within exudates of the peritoneal cavity [7,12-18]. Cameron and colleagues [12,*1957*] described that island of cells appeared on the surface of wound within 24 hr of injury, and spread out from the island to form new sheet of mesothelium. They proposed mesothelial healing involved attachment of free-floating mesothelial cells which were detached from adjacent or opposing peritoneum. Johnson et al [13,*1962*] covered peritoneal defect with sheet of polythene and showed there was considerable delay in healing of wound. In contrast,

there was rapid seeding of cells on free surface of wound and polythene. These cells soon formed a continuous mesothelial layer. They agreed to the concept of Cameron that free-floating cells came from mesothelium and contributed to new mesothelium. However, they also proposed that circulating monocyte and tissue macrophage are capable of transformation into mesothelial cells under certain circumstance. Eskeland [14, 1964] placed a double comprtment Millipore capsule within peritoneal cavity of rat, they found the filter in comprtment which contained peritoneal exudates became covered with typical fibroblast and cells of the same appearance as proliferating mesothelium. They further demonstrated in EM study [7, 1966] that surface of peritoneal wound were rapidly covered by macrophage and these cells gradually attained characteristic of mesothelial cells. Thus, they concluded that peritoneal macrophage transformed to fibroblast, and finally differentiated into mesothelial cells. Ryan et al [15,*1973*] reported that free-floating peritoneal cells settled on the denuded surface, spread out, attached to one another, and developed feature typical of mature mesothelial cells. They favored the concept that mesothelial regeneration result from differentiation of multipotent mononuclear cells, but not detached mesothelial cells, which were free-floating in peritoneal cavity.

It is now generally agreed that peritoneal free floating cells are an important source of new mesothelium. However, the origin of these free floating progenitors is controversial. Many authors believed that peritoneal free-floating progenitors came from desquamated mesothelial cells of the serosal lining, and this concept has gained support from recent studies which demonstrated incorporation of exogenous mesothelial cells into peritoneal layer following intraperitoneal transplantation of mesothelial cells [Foley-Comer 19, Nagy 20, Bertman 21]. However, the idea that peritoneal free floating precursors came from bone marrow cells, such as monocyte, lymphocyte, macrophage or mononuclear precursors had been advocated by some

investigators [Johnson 13, Ryan 15, Eskeland 7] *based on LM & EM findings and the fact that most free-floating cells populations in peritoneal fluid of normal animal came from bone marrow. [28,29,30]*. Wagners [22] , who studies the histology pattern of serially transplanted mesothelioma, believed that the free floating mononuclear population implicated in repair of mesothelium were primitive stem cells originated from bone marrow, and he proposed that such primitive stem cells might be the source of dimorphic mesothelioma.

Embryologically, mesothelium and bone marrow cells both arise from mesoderm [23]. In adult animal, bone marrow cells still possess wide range of plasticity. They can differentiate into various types of adult cells both in vivo and in vitro. The highly plastic bone marrow cells have a role in maintenance and repair of nonhematopoietic tissue [24-28]. Given the facts that (1) bone marrow cells and mesothelium both derived from mesoderm (2) bone marrow cells are pluripotent and they can reconstitute tissue of mesoderomal origin (3) bone marrow contain MSC which resemble subserosal mesenchymal precursor (4) the major cells population in peritoneal fluid, such as monocyte, lymphocyte and macrophage, came from bone marrow [29-31], it is possibly that bone marrow derived cells may provide source for new mesothelium

In this work, we would like to examine the validity of this hypothesis by undergoing GFP- and sex-mismatched bone marrow transplantation in female wild type mice. Then, we use eGFP and y-chromosome as trackers to detect the presence of donor bone marrow cells in mesothelial layer of recipients. Furthermore, we examine whether donor marrow cells express mesothelial phenotype by combined cytokeratin immunostaining (IHC) and y-chromosome chromogenic in situ hybridization (CISH).

Material and Methods:

Design

Twenty female, wild type mice were given lethal dose irradiation and bone marrow transplantation from male, GFP donor. Then, five recipients were euthanized at 2nd, 4th, 6th week and 6th month after bone marrow transplantation separately. Peritoneal organ/tissue of recipients were obtained to detect markers of donor marrow cells (eGFP and y-chromosome). Combined y-chromosome in situ hybridization with immunohistochemistry was employed to confirm the expression of mesothelial phenotype in donor marrow cells.

Animal

All animal procedures were carried out in accordance with protocol approved by the institutional committees for animal research of Chang Gung Memorial Hospital *and ethical guideline of American Physiological Society (??).* Wild FVB mice were purchased from National Laboratory Animal Center, and transgenic eGFP mice (FBV/N) were obtained from Level Biotechnology Inc, Taipei, Taiwan [32]

Bone Marrow Transplantation

Male transgenic mice that ubiquitously express eGFP were used as donor. To harvest bone marrow, animals were euthanized by cervical dislocation under general anesthesia, then, the femur and tibia were removed. Using a syringe and 23 g needle, the bone marrow cells were flushed into a sterile 4 ml tube. After RBC lysis and sieving through 50 μm mesh, cells were washed, counted and then ready for use.

Eight-week-old female wild type FVB mice were used as recipients. Each

recipient animal received whole-body gamma ray irradiation with a dose of 9 Gray to ablate their bone marrow. Then, unfractionated bone marrow cells (5 X 10^6 nucleated cells in 50 μl PBS) obtained from male transgenic eGFP mice were transfused through tail vein within 24 hr of irradiation.

Treatment of Tissue Section

Peritoneal organs which contain mesothelium, such as liver, intestine, mesentery, pancreas and anterior abdominal wall, were harvested at the time of sacrifice. Harvested organs were initially placed in 4% paraformaldehyde/10% sucrose in PBS at 4℃ to hold eGFP protein within cytoplasm. This fixation procedure took 1 hour for every 2 mm of tissue penetration needed. Then, organs/tissues were fixed in 10% neutral buffered formalin overnight and embedded in paraffin wax. Sections of 4μm thickness were obtained for both immunohistochemical staining and chromogenic in situ hybridization.

Immunohistochemical Staining

Paraffin-embedded sections were dewaxed and incubated with 3% hydrogen peroxide in methanol and taken through graded alcohols to PBS. Antigen retrivel was achieved by pressure cooker using 10 mM citrate buffer, PH 6.0, at 100℃.
Non-specific immunoglobulin binding sites were blocked with goat serum.

For demonstration of eGFP protein in mouse tissue, slides were incubated with rabbit anti-GFP antibody (Chemicon, CA) at 4℃overnight at a dilution of 1:400. Sections were then washed in PBS and incubated with *biotinylated secondary body conjugated with* streptavidin–horseradish peroxidase(HRP) system (DAKO, CA) for 60 min at room temperature. Following washes in PBS, enzymatic detection with diaminobenzidine (DAB) substrate (DAKO, CA) was applied for 10 min at room

temperature.

Chromogenic In situ hybridization

Sections were incubated in 1 M sodium thiocyanate for 10 min at 80ºC, washed in PBS, and then digested in 0.4% pepsin in 0.1 M HCl for 10 min at 37ºC. The reaction was quenched in 0.2% glycine in double concentration PBS and the sections were then rinsed in PBS, post-fixed in 4% paraformaldehyde in PBS, dehydrated through graded alcohols, and air dried.

Then, FITC-conjugated mouse Y chromosome probe (Star-FISH, Cambio, Cambridge, UK) was added to the center of each tissue sections and covered with a coverslip of suitable size. The edges were sealed with a thin layer of rubber cement to prevent the evaporation of probe solution during incubation. Hybridization was performed by heating slide to 60°C for 10 min, and incubating at 37°C for 16-18 hrs. After removal of the rubber cement and coverslip, the slides were washed in 50% formamide/2x SSC at 37°C, then washed with 2x SSC, incubated with 4x SSC/0.05% Tween/5% milk powder for 10 min at 37°C . Slides were washed with PBS and were submerged in 3% H_2O_2 to quench endogenous peroxidase activity. After washing for 2 min three times with PBS, endogenous biotin blocking is performed with casein blocking solution. The slides were incubated with 1:3000 peroxidase-conjgated rabbit polyclonal anti-fluorescein (Abcam, Cambridge, UK) for 60 min at room temperature. Signal detection is performed using DAB (DAKO) as the chromagen. The slides are counterstained with hematoxylin, cleared in xylene, and mounted with Permount (Fisher Scientific).

Combined Immunohistochemistry and Chromogenic In Situ Hybridization

Analysis

Sections were sequentially stained for mesothelial marker(cytokeratin staining by immunohistochemical method) to identify cells of mesothelial phenotype, followed by Y chromosome in situ hybridization to identify bone marrow derived cells.

Step 1 : Cytokeratin immunohistochemistry

Four-micrometre sections were dewaxed, their endogenous peroxidases blocked and then were taken through graded alcohols to PBS. Slide were incubated with rabbit *anti-pancytokeratin* primary antibody (DAKO, CA) overnight at 4ºC, then followed by biotinylated secondary antibody and streptavidin-alkaline phosphatase (Vector Laboratory). Color was developed with Vector Red Substrate (Vector Laboratory) .

Step 2 ISH for Y chromosome – indirect detection

Cytokeratin-stained sections were processed as described above in the section of "chromogenic in situ hybridization". Then, slides were rinsed in PBS and followed by a light haematoxylin counterstain.

Flowcytometry

The presence of eGFP expressing cells in blood or bone marrow was examined by flowcytometric study to confirm the successful engraftment and repopulation of donor marrow cells..

In brief, blood and bone marrow obtained from either wild type mice, eGFP donor or transplant recipients were treated with RBC lysing solution, washed with PBS, then analyzed with FAC scan at a cell concentration of 1×10^6 cell/ml.

Using forward and side scatter parameters, we eliminated dead cells and debris

from the analysis (mainly in blood sample) to obtain a histogram. eGFP was excited by an argon laser and fluorescence was detected using a 530/30 nm bandpass filter in the FL1 channel.

Results:

Efficacy of bone marrow transplantation

Flowcytometric analysis revealed that almost all mononuclear cells within bone marrow of recipient were expressing eGFP (Figure 1). In blood of recipient, most mononuclear cells (after gating to exclude dead cells and debris of RBC) were also expressing eGFP. This indicated that bone marrow of recipient was well reconstituted with donor eGFP containing cells. Thus, bone marrow transplantation was successful with well engraftment and repopulation of donor bone marrow cells.

Identification of eGFP containing cells on mesothelium of peritoneal organ

In eGFP positive control, *??? %* of mesothelial cells were positively stained with anti-GFP antibody.

In section of anterior abdominal wall of recipients, only very few anti-GFP positive cells can be detected within mesothelial layer in whole slide (Figure 2A), accounting for less than 1% of total counted mesothelial cells

In liver section, some eGFP containing cells can be identified in endothelium of vascular system (not shown), hepatocyte (not shown), but only one or two eGFP containing cells can be found in mesothelium covering liver surface in whole slide(Figure 2B).

In intestine section, lots of eGFP containing cells can be found to distribute diffusely within stroma of villi, and a few GFP containing cells in epithelial layer of villi. In contrast, only scanty eGFP containing cell can be identified within serosa layer of intestine and mesentery (Figure 2C, 2D), accounting for far less than 1% of mesothelial cells covering intestine.

The incidence of eGFP positive cells in mesothelial layer were all much less than 1% in recipient sacrificed either at 2 wk, 4 wk, 6 wk or 6 months after bone marrow transplantation.

Morphologically, most eGFP containing cells within mesothelial layer showed flat, squamous shape resembling that of mesothelial cell.

Confirm the presence of donor marrow cells within mesothelium by y-chromosome marker identification

To confirm the findings of anti-GFP immunostaining, we use CISH to identify y-chromosome marker containing cells within mesothelial layer of peritoneal organ.

In male positive control, only ?? % of meosthelial cells were positive for y-chromosome markers. As sections were 4 μ thick, only part of nucleic y-chromosome are present in a 4 μ section, the detection rate of y-chromosome is lower than that of eGFP protein. The much lower occurrence of y-chromosome positive cells in serosa layer may be due to the fact that it is not easy to preserve mesothelil layer after pepsin treatment during the process of CISH.

However, y-chromosome marker positive cells can hardly be detected, usually only one or two y-chromosome positive cells can be found within mesohtelium in whole slide section (Figure 3A, 3B). This amount is even fewer than that of eGFP containing cells.

Combined y-chromosome CISH and cytokeratin histochemisty

To confirm that donor marrow cells which were incorporated into mesothelium have differentiated into mesothelial phenotype, we use technique combining y-chromosome CISH and cytokeratin IHC. The results showed that some y-chromosome positive cells within mesothelium were also positive for cytokeratin

immnohistochemical staining (Figure 3C, 3D). However, only several cells which coexpress y-chromosome and cytokeratin can be identified within mesothelium after reviewing several slides section.

Discussion:

In this experiment, we for the first time demonstrated that bone marrow derived cells can be integrated into mesothelial layer of peritoneum, changed to flat, elongated shape, and expressed cytokeratin antigen. Previous investigators had hypothesized that bone marrow cells may contribute to new mesothelium. However, there has been no direct evidence to support this postulation. The findings in current experiment provide direct evidence to support the hypothesis that bone marrow derived cells may differentiate into mesothelial phenotype and participate in mesothelial turnover, although at a very low level.

As unfractionated whole bone marrow cells were transplantated in this experiment, we can not tell which component of bone marrow cells contribute to this differentiation process. *Circulating bone marrow cells include MSC, hematopoietic stem cell (HSC), endothelial progenitor cell (EPC) as well as mature white blood cells.* However, we believe bone marrow MSC is the most likely source because of the following reasons. First, mesothelium and mesenchyme both originate from mesoderm in embryo[23]. Some evidence further suggested that mesothelium arised from primitive mesenchyme [34]. Second, pluri-potent MSC exist in bone marrow as well as in peripheral tissue of adult animal. They can reconstitute damaged tissue of mesenchymal origin. Third, the sub-serosal mesenchymal precursors involved in mesothelial regeneration may be actually a tissue resident mesenchymal stem cell. As different source of MSC share similar characteristic, bone marrow MSC should be able to provide cells for generating new mesothelium if subserosal MSC could contribute to new mesothelium. The possibility of MSC to participate in mesothelial repair has been examined by Lucas et al [11] who showed that intraperitoneal

injection of MSC isolated from skeletal muscle of neonatal rats could reduce adhesion caused by peritoneal injury. They, based on this observation, postulated that the MSC had the capacity to differentiate into mesothelial cells and repaired the injured mesothelium. Hematopoietic stem cell (HSC) is another possible source. Although HSC is mainly responsible for the maintenance of hematopoietic cells lineages, it has also been demonstrated that HSC may contribute to the repair of multiple non-hemotopoietic tissue in adult animal [24,27]. Thus, the possibility of HSC to be a source of new mesothelial cell can not be ruled out. Another possibility is a transformation process which turned mature monocyte lymphocyte, or macrophage into mesothelial phenotype. Nevertheless, no laboratory evidence to date can support the presence of such transformation process. To delineate which component of bone marrow cells contribute to new mesothelium, future experiment of bone marrow transplantation by using different trackers to label different component of donor marrow cells may help to answer this question.

Most data regarding the contribution of bone marrow cells to organ repair came from studies with specific organ injury. But the role of bone marrow stem cells in daily cell turnover is another issue which deserves attention. In current study, our recipients which were sacrificed at sixth month post-transplantation can be considered as a model to observe the normal turnover of mesothelium. Since only rare eGFP containing cells can be identified within peritoneum of recipients, the role of bone marrow cells in the normal turnover of mesotehlium should be very minor.

Our recipients which were sacrificed 2 weeks after bone marrow transplantation may represent a model of mesothelial repair following acute irradiation injury. As serosal healing is usually complete within 7–10 days of injury, mesothelial repair should have completed within 2 weeks post-irradiation injury. The findings that *even fewer (????) anti-GFP containing cells within mesothelium in recipients*

sacrificed at 2nd week after transplantation than that of recipients sacrificed at 6th month indicates bone marrow derived cells also exerted little effect in the mesothelial repair following acute radiation injury.

Although the number of mesothelial cells which come from donor bone marrow cells is rare in either normal turnover condition or post-irradiation injury, the result of current study does not exclude the possibility of bone marrow derived cells to contribute a more active role in mesothelial repair following other types of peritoneal injury. Future studies using different model of peritoneal injuries are needed to determine the exact role of bone marrow cells in mesothelial repair.

The mechanism which turned the bone marrow derived cells into mesothelial phenotype has not been examied in current experiment. However, in our unpublished observation, we have found that many round GFP + cells could attach to wound surface, then flattened, and finally formed a continuous layer (Fig 4), a process which has been described by previous investigators[Eskeland 7, Cameron 12, Johnson 13, Ryan 15, Watters 18]. Some authors believed that the round cells are monocyte or macrophage and these cells finally differentiated into mesothelial cells [7,13,15], whereas others thought the round cells on wound surface came from reactive mesothelial cells which detached themselves from adjacent or opposing mesothelium[12,13]. By using GFP- and sex-mismatched transplantation model, we can clearly demonstrate that most round cells which attached on wound surface were GFP (+) donor marrow cells, rather than exofoliative mesothelial cells (Fig 4). Despite these GFP+ round cells could attach on wound surface, flatten and form continuous layer resembling that of mesothelium, ultimate double staining of y-chromosome and cytokeratin revealed that only very few new mesothelial cells express both y-chromosome and cytokeratin. This finding suggests that although

donor bone marrow cells activelyparticipated in the process of mesothelial repair, they only play a transient role. The bone marrow derived cells which cover the outer surface of mesothelial wound were subsequently replaced by new mesothelium which originated from other source of progenitors. Only a very small proportion of donor bone marrow cells were still left within mesothelial layer and differentiated into mesothelial phenotype. These findings are consistent with the hypothesis of Watters [18] who proposed that "subsequent to the attachment of the round cells from the peritoneal fluid, ultimate covering cells may come from certain cells from underlying connective tissue, which differentiate into mesothelium, or from surrounding detached mesothelial cells, and the round cells are lost. The round cells have served only as a temporary covering." Our finding is also in agreement with the postulation of Johnson [13] who proposed that new mesothelium in the main came from detached mesothelial cells, and only in special circumstance came from monocyte.

Ito et al [35] has used similar bone marrow transplantation model to examine the possibility of bone marrow cells to contribute to mesothelial turnover in rat. They reported that GFP + cells can be identified in submesothelial area, but no evidence which showed submesothelial GFP+ cells differentiated into new mesothelial population. The difference between our results and those of Ito et al may be due to the difference in the efforts we have paid to identify the presence of GFP + cells within mesothelium. As the amount of eGFP or y-chromosome positive cells are very rare in mesothelium of recipients, it took a lot of time and work to detect donor marrow cells within mesothelial layer. Usually, only one or two GFP+ or y-chromosome + cells can be found after reviewing several slides. Furthermore, mesothelium is easily sloughed off during the process of peritoneal specimen collection. Using frozen preservation, which was utilized by Ito et al, usually resulted in loos of mesothelial layer in our experience.

The overall results of this experiment indicate that bone marrow derived cells can contribute to normal turnover of mesothelium at very low level. However, the exact role of bone marrow cells in mesothelial remodeling await further studies using other model of peritoneal injury to delineate. If bone marrow cells can be induced to differentiate into mesothelial cell in ex vivo cell culture condition (a work we are undergoing now), it may provide us hope for future application of bone marrow derived cells as a source of cell therapy to rescue injured peritoneum.

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Legends :

Figure 1. Flowcytometric analysis of eGFP presenting cells in blood and bone marrow [magnification : all 1000 X]
(a) Blood of wild type mouse
(b) Bone marrow of wild type mouse
(c) Blood of eGFP donor mouse (gating to exclude dead cells and debris)
(d) Bone marrow of eGFP donor mouse
(e) Blood of recipient mouse (gating to exclude dead cells and debris)
(f) Bone marrow of recipient mouse

Figure 2. eGFP protein containing cells were detected in the peritoneal membrane of recipient [magnification : all 1000 X]
(A) anti-GFP positive cell (arrow) on parietal peritoneum of abdominal wall
(B) anti-GFP positive cell (arrow) on visceral peritoneum covering liver surface
(C) anti-GFP positive cell (arrow) on serosa layer of intestine
(D) anti-GFP positive cell (arrow) on peritoneum covering mesentery

Figure 3. y-chromosome detection in the peritoneal mesothelium of sex-mismatched transplantation recipients [magnification : all 1000 X]
(A) y-chromosome positive mesothelial cell (arrow) was incorporated into mesothelium covering surface of pancreas
(B) y-chromosome positive mesothelial cells (arrow) was found on serosa of intestine
(C) combined y-chromosome in situ hybridization (brown color) with cytokeratin immunohistochemistry (red color) revealed that y-chromosome positive cells (arrows) within mesothelial layer covering liver surface also expressed cytokeratin marker;
(D) y-chromosome positive cells (arrows) are also cytokeratin positive within the serosa of intestine (weakly staining of Vector Red following the in situ hybridization procedure)

Figure 4. anti-GFP immunostaining of liver of recipient mice which experience intraperitoneal chlorhexidine injection [magnification : ???? X]

(A) many GFP containing spherical cells (hollow arrow) attached on wound surface

(B) a few attached spherical cells flattened (arrow)

(C) more GFP containg round cells turned into flat shape(arrows)

(D) most GFP containing cells were flattened, resembling mesothelial morphology

(E) GFP containing cells form a continuous layer on the surface

Figure 1 :

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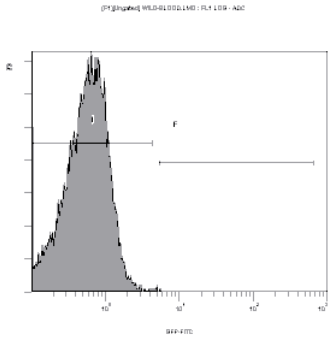
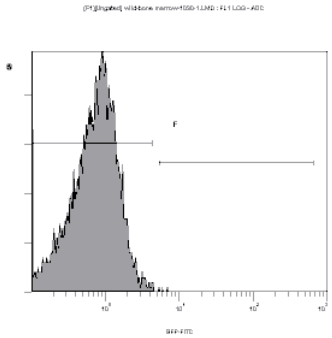
<tr><td>  </td><td>  </td></tr>
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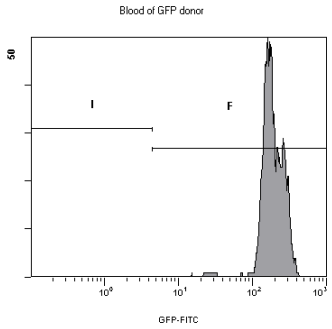
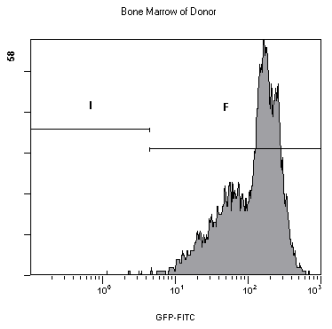
<tr><td> 
</td><td> 
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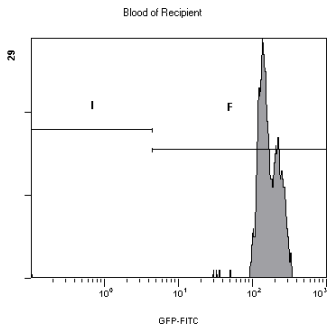
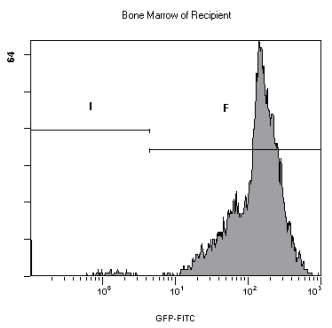
<tr><td>(e)</td><td>(f)</td></tr>
<tr><td> 
</td><td> 
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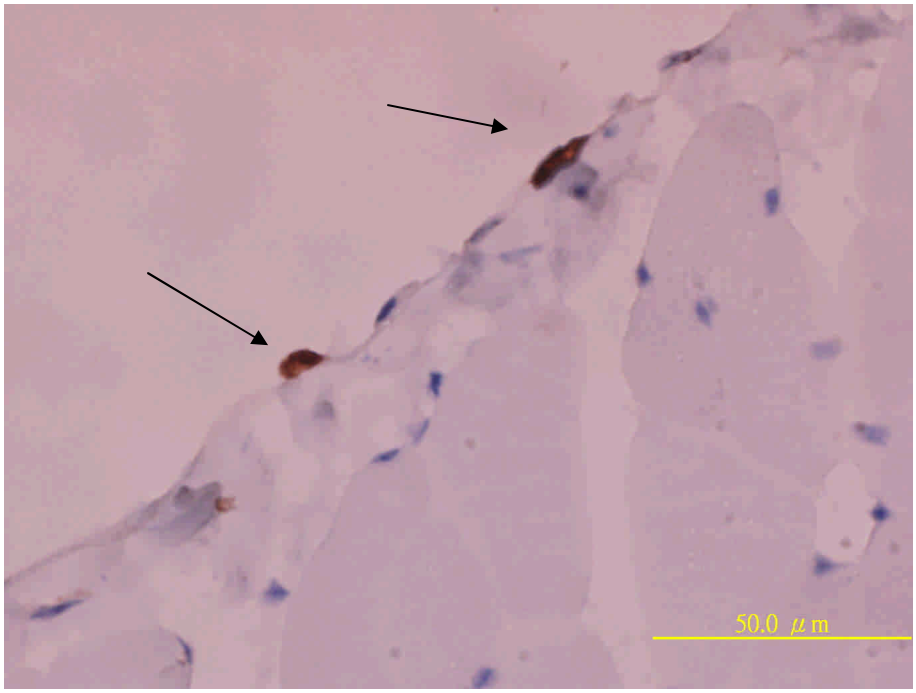
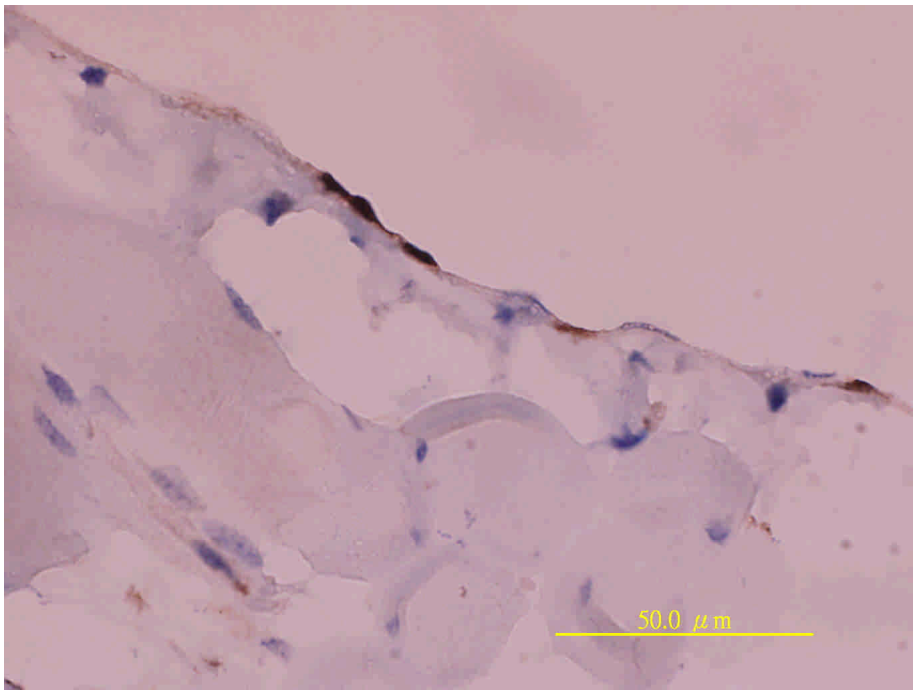
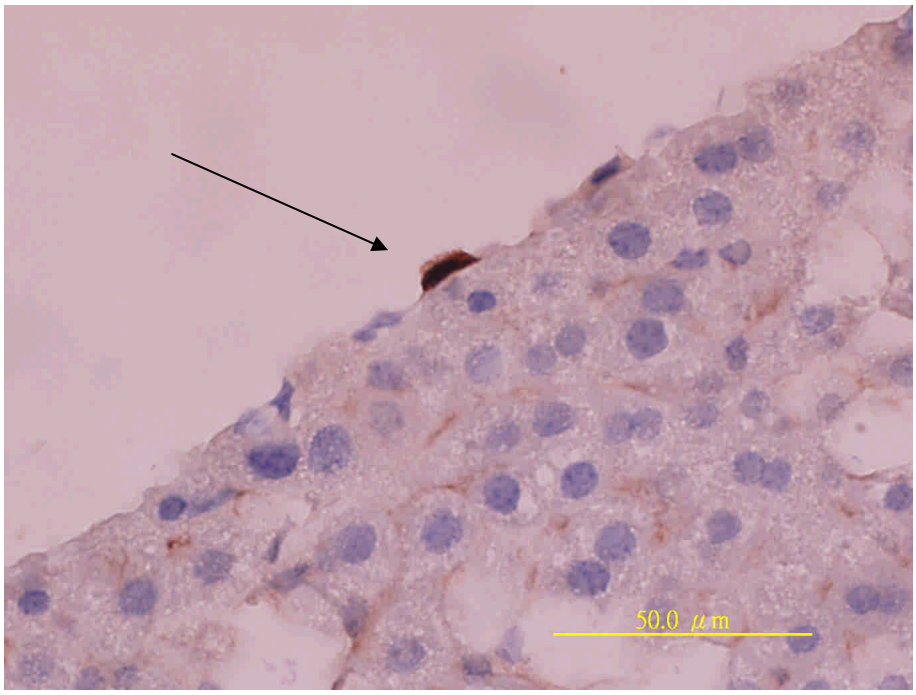
Figure 2



A





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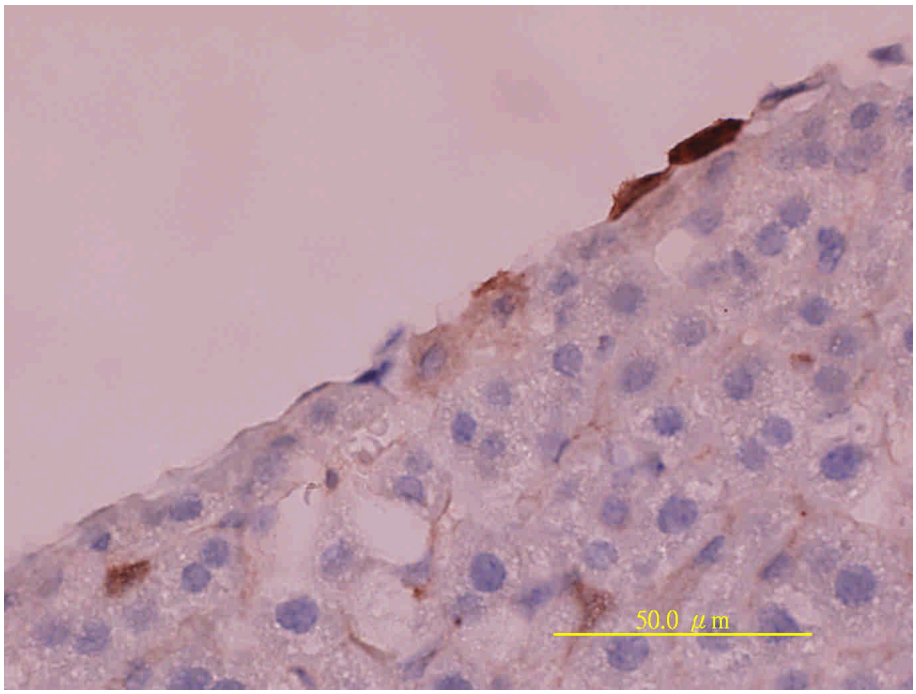

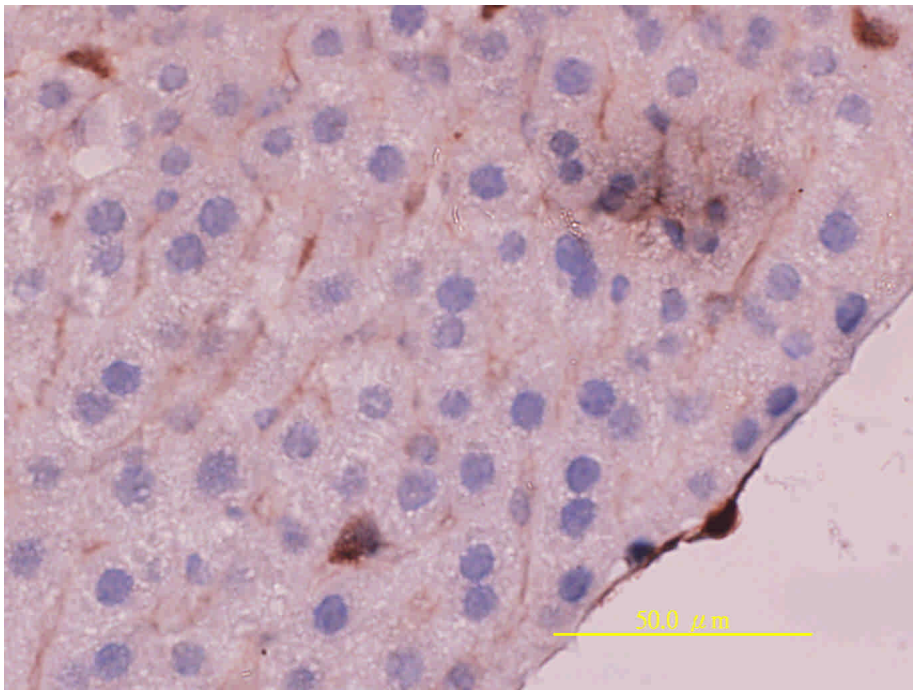

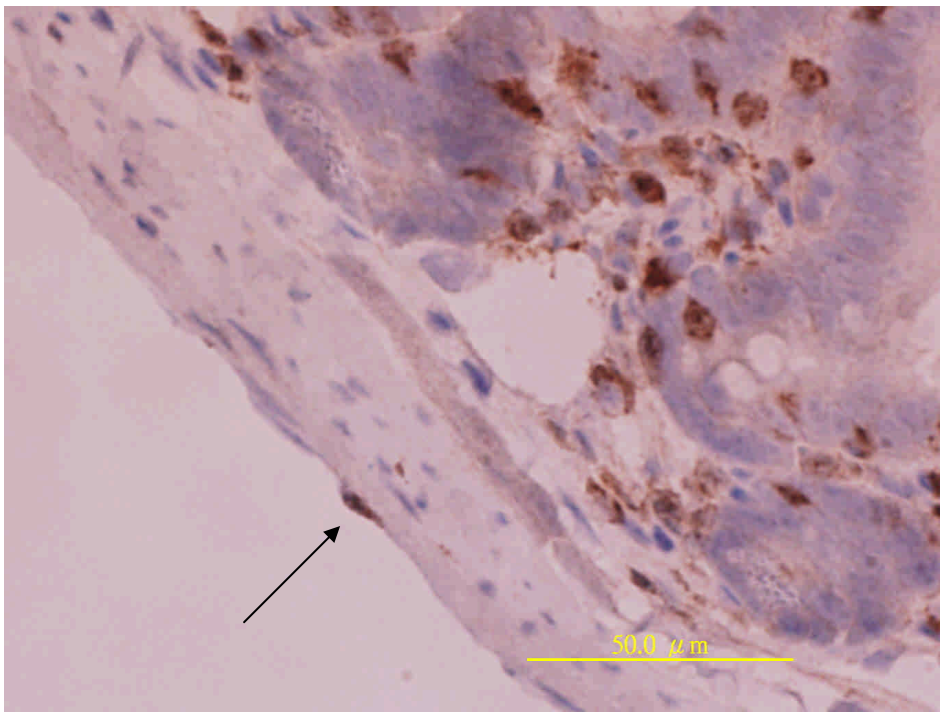

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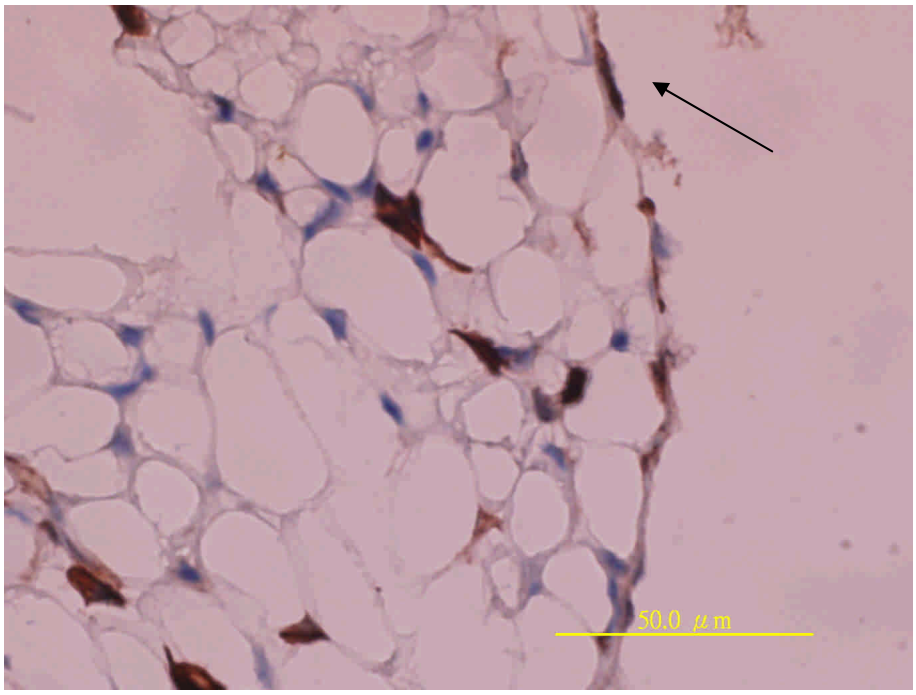
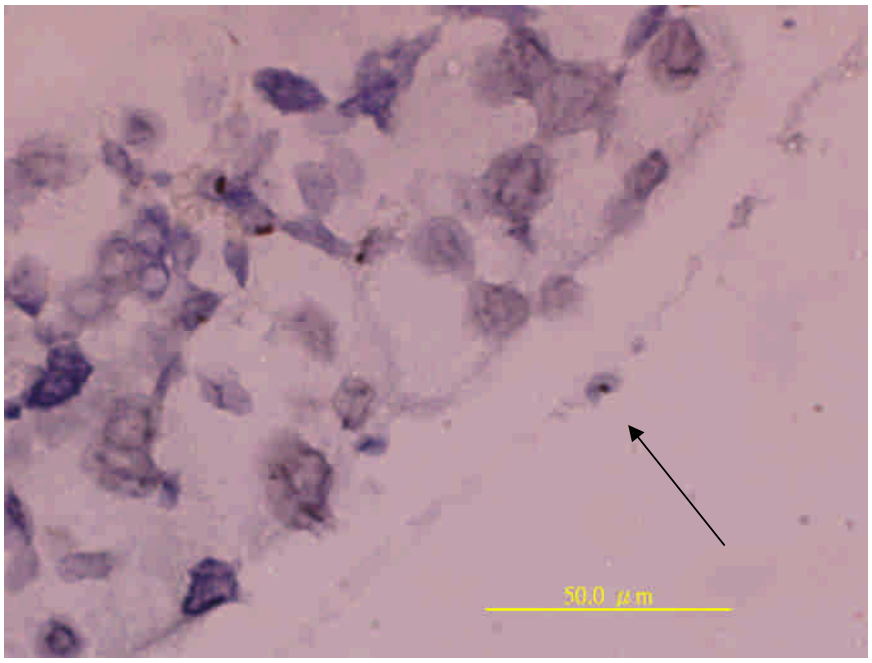
	D

Figure 3



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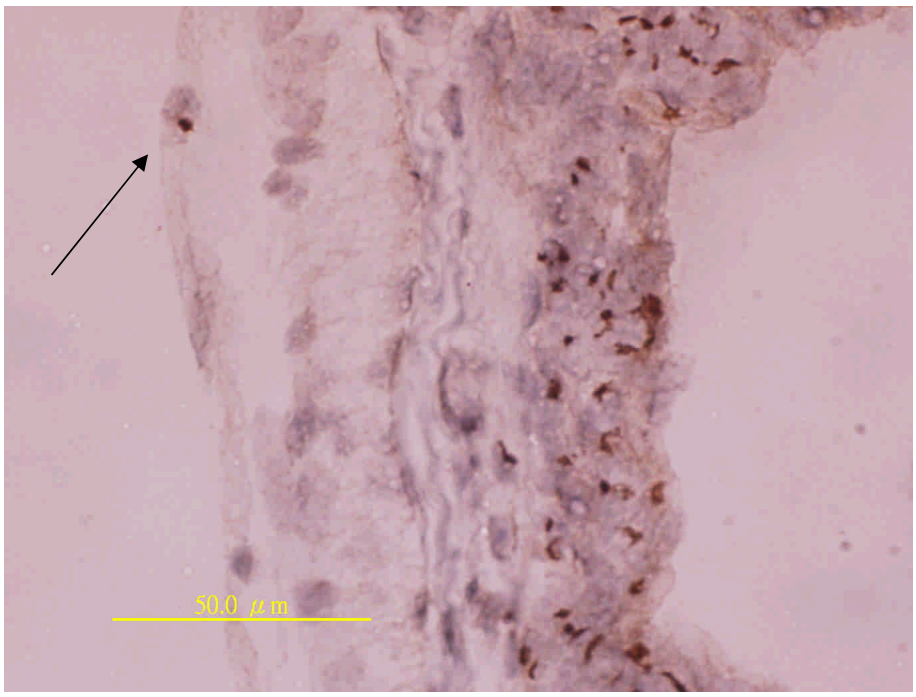

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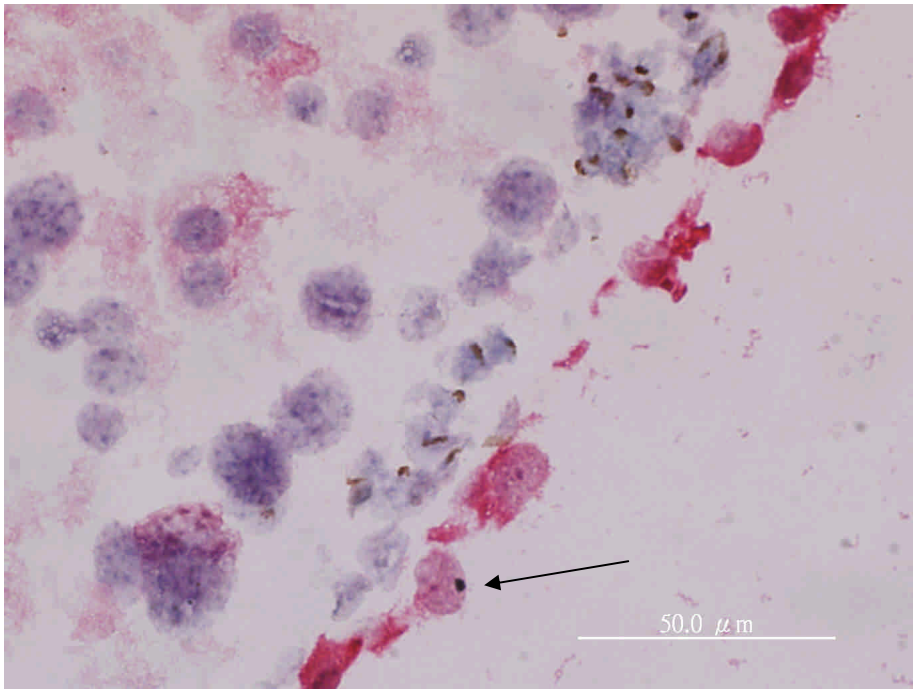
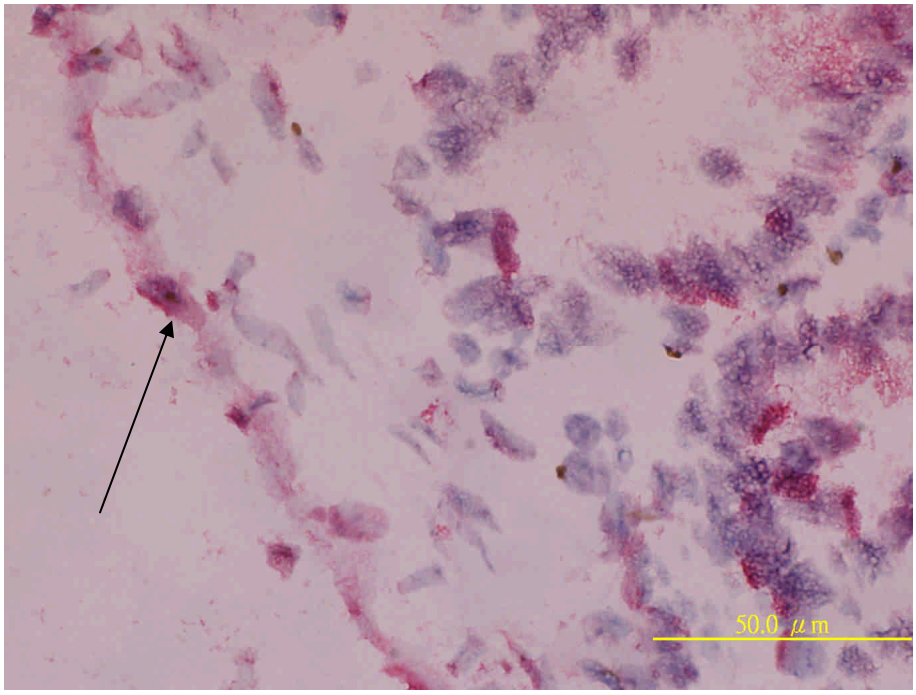
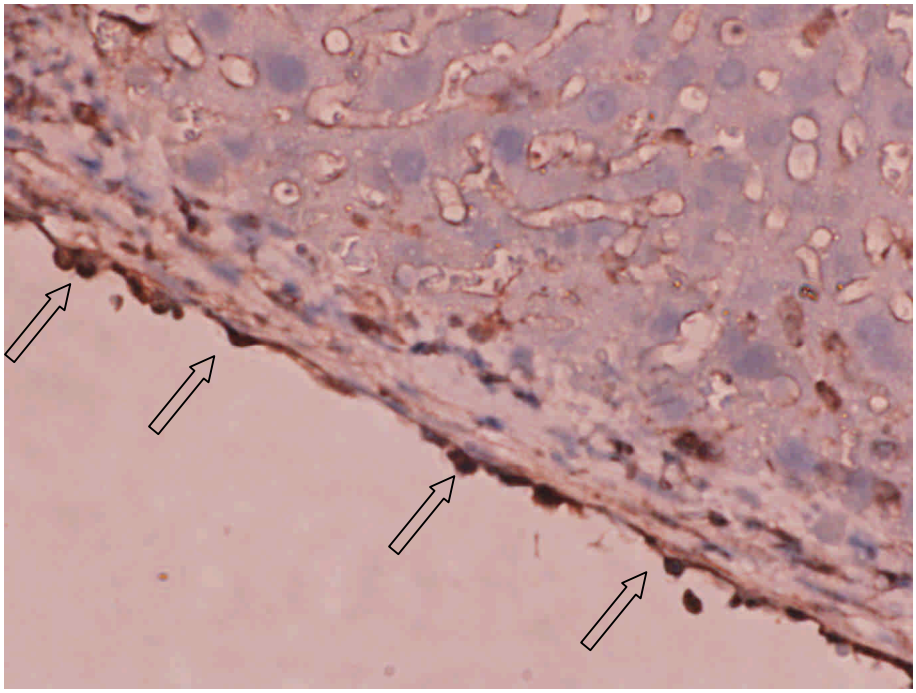
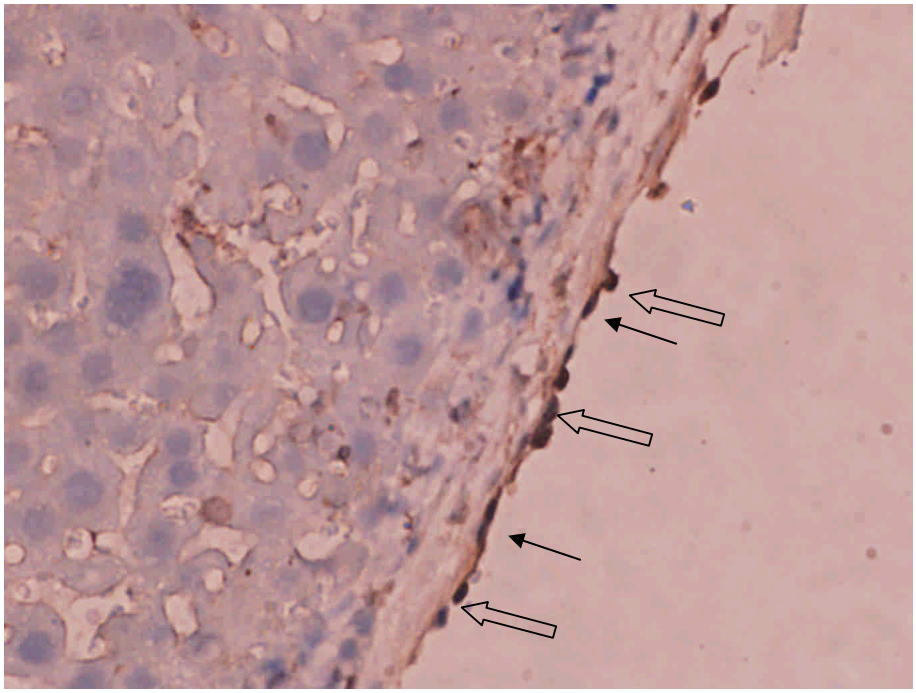
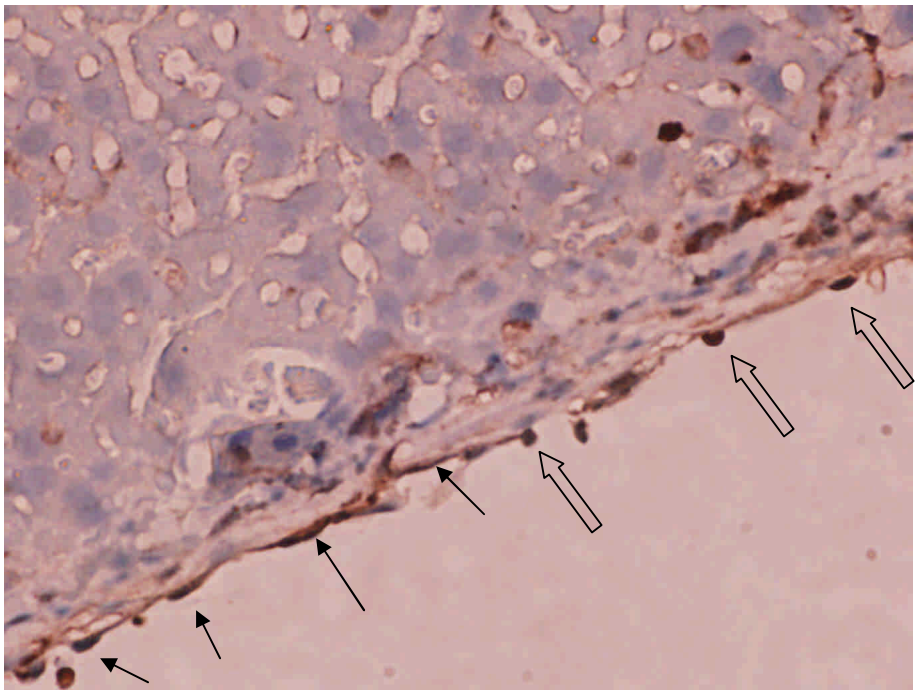
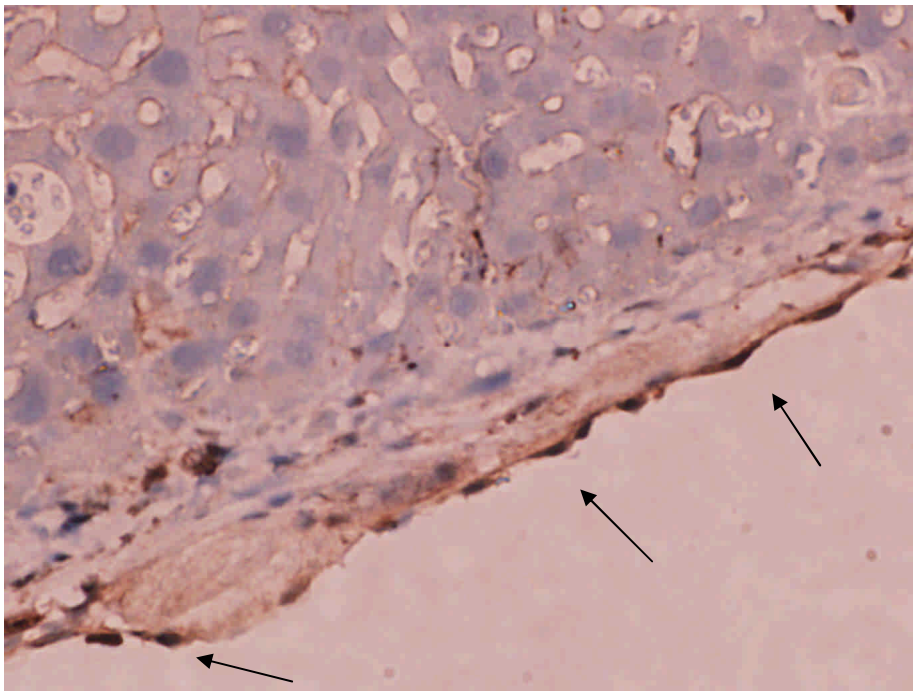
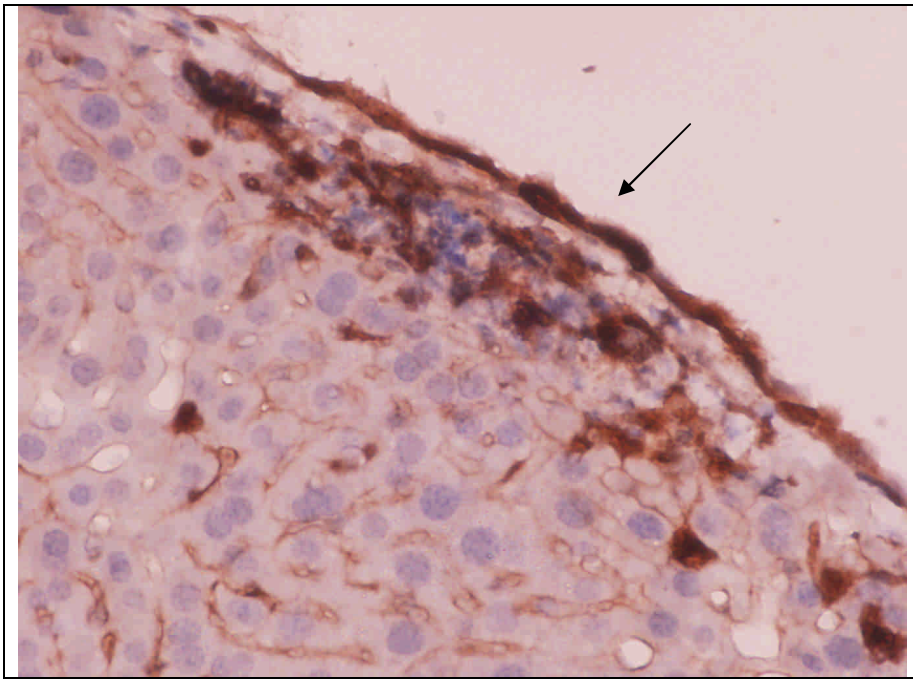
	C
	D

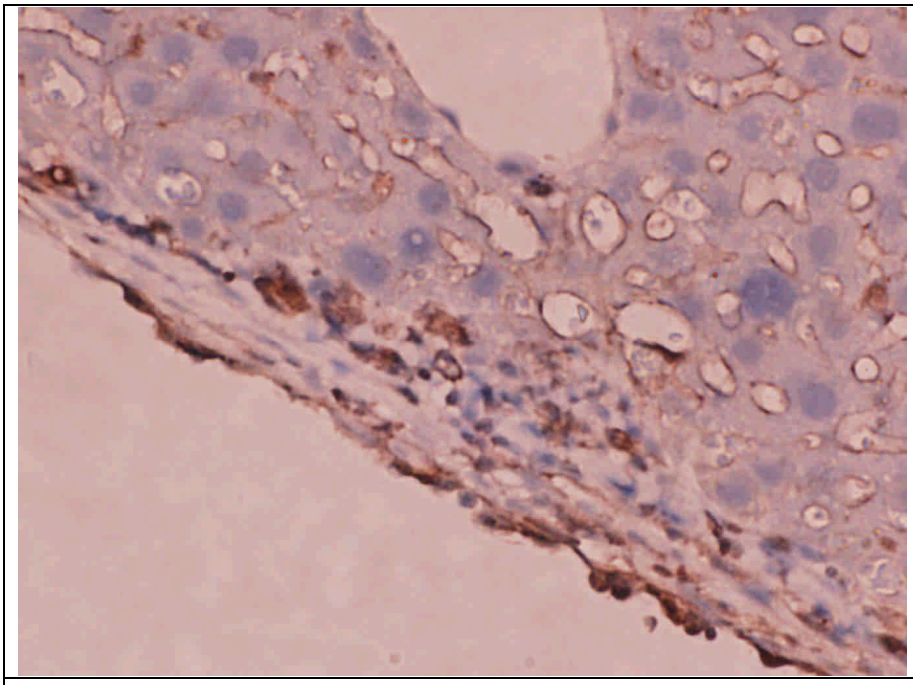
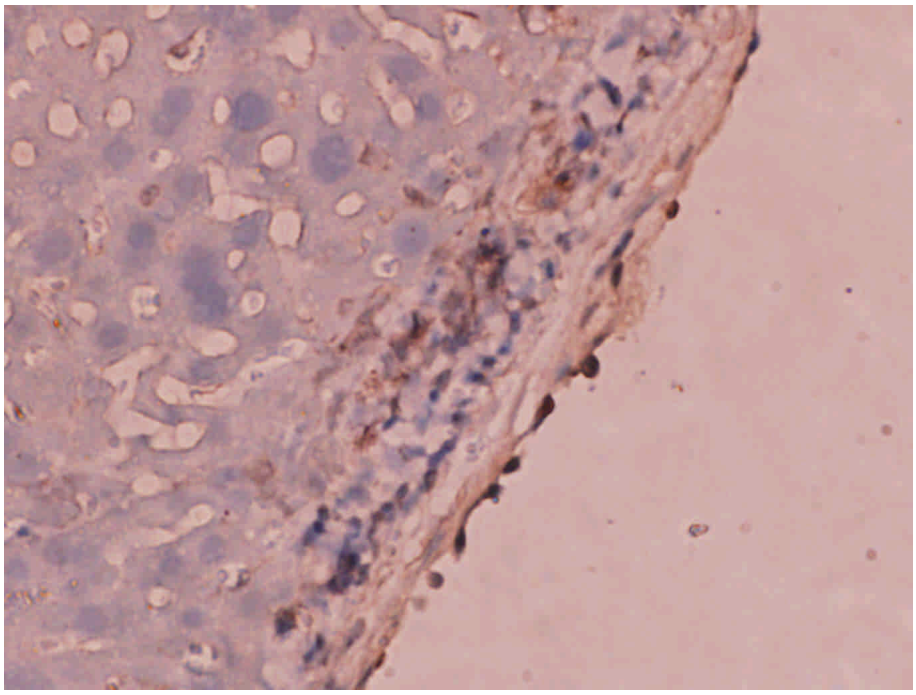
Figure 4

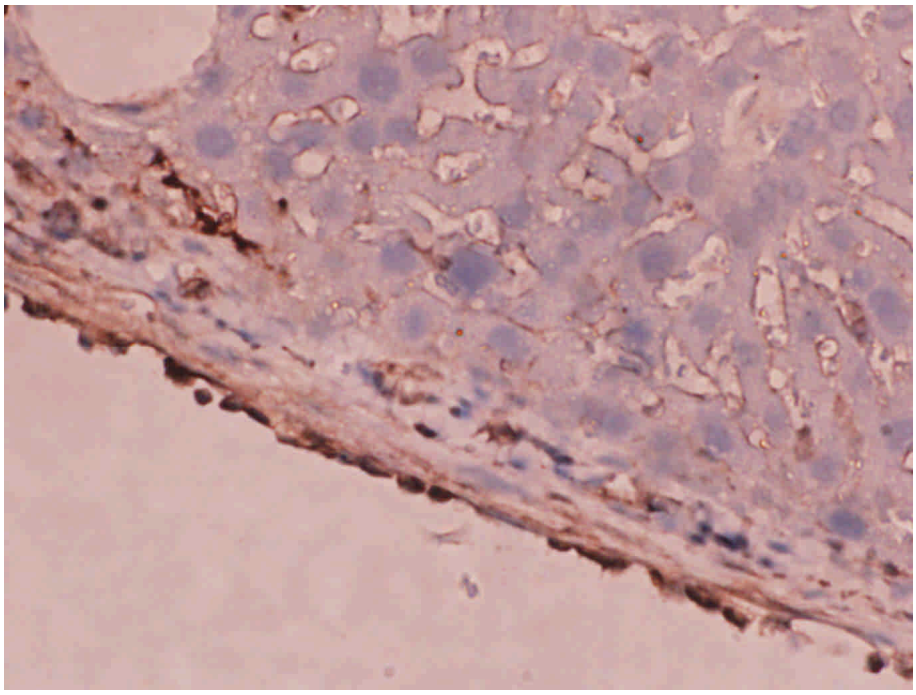
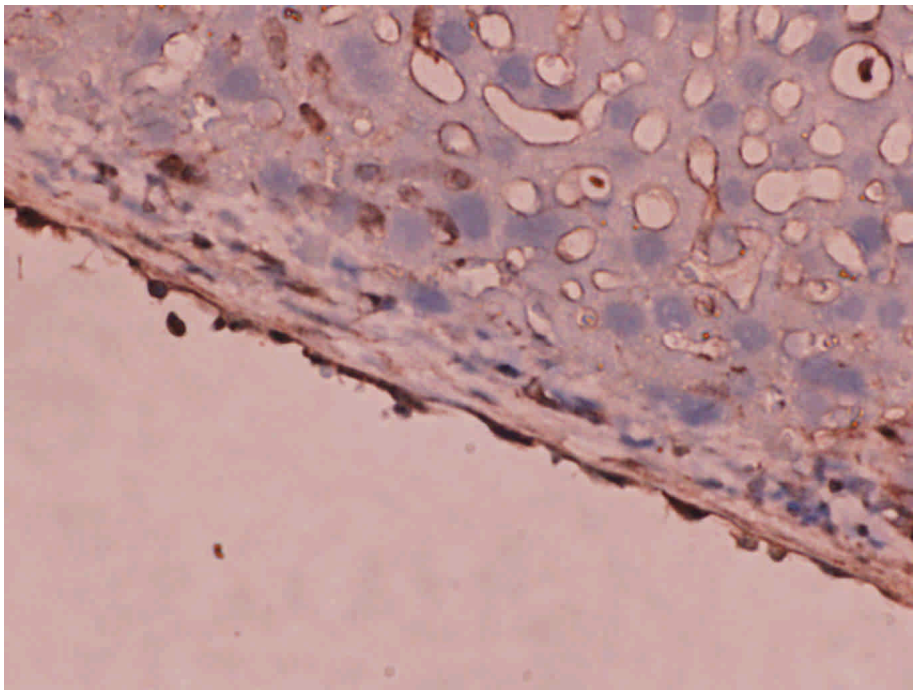
	1 A many round cells attched on wound surface
	2 B ○○ a few round cells flattened

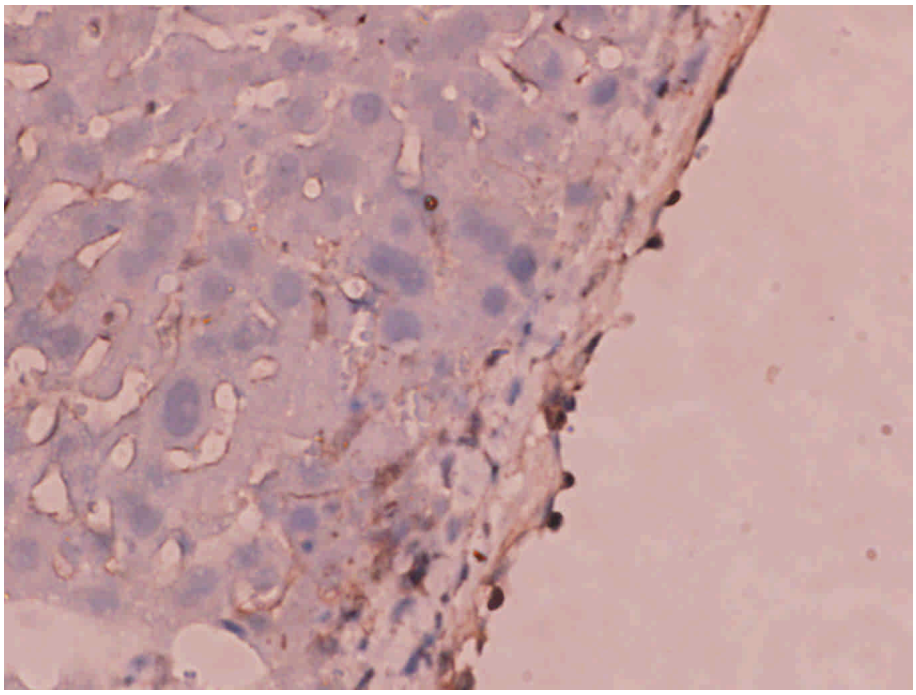
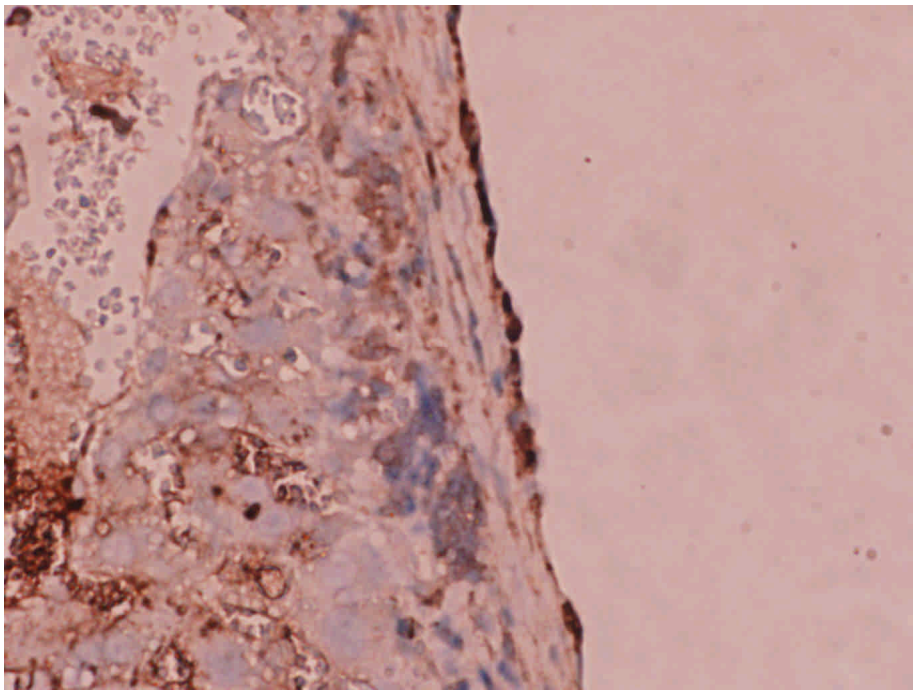
	3 C Partly round cells, partly flattened cells
	4 D Mainly flattened cells ○

	5 *<u>moderate background on canaliculi</u>* E bone marrow cells formed continuous layer

For reference　　請協助看看有否較好的 picture, 可以取代 Picture A,B,C,D

	antiGFP 1 A
	1 OK A ○○ round cells

	1 venule background A
	3-4 severe background on venule C ? D ?

	1-2 very mild background B ○
	4 severe background E Continuous layer