CHAPTER FOUR

The Possible Clinical Exploitation of Adipogenic Healing:

Preliminary Studies
4.1. Introduction

4.1.a. Fat as a Surgical Tool

4.1.a.i. Loss of Tissue and General Surgical Solutions

4.1.a.i.1. Prosthetic Devices

Trauma, destructive infections and the surgical management of tumours often results in significant loss of tissue. Mechanical appliances are sometimes used to try and reconstruct these lost tissues, examples of which are breast implants and acrylic facial prostheses. The cosmetic illusion of these appliances is, however, often incomplete and there are also occasions of appliance failure with attendant clinical problems (Converse, 1977; McGregor and McGregor, 1995).

4.1.a.i.2. Flaps and Grafts

An alternative is to reconstruct using tissues harvested from elsewhere in the patient. This can be achieved by using either flaps or grafts. Flaps are supplied with defined major blood vessels and can be repositioned by rotating the tissue into a new site, sometimes called a "pedicle flap". In a "free flap", the major vessels supplying the flap are cut and rejoined to other major vessels close to the receptor site. A variant of the free flap is the "microvascular free flap", in which vessels of about 1mm diameter in the bed of the acceptor site are joined to small vessels in the flap. Grafts are different, in that no major vessels are involved and the tissue is simply repositioned to its new site. The vascular supply to this initially ischaemic tissue is established by spontaneous microvascular anastomosis in the early life of the graft (Converse, 1977; McGregor and McGregor, 1995).

4.1.b. Applications of Fat in Surgery
4.2 Possible Clinical Exploitation of Adipogenic Healing

4.1.b.i. Tissue Reconstruction

Gross facial and other abnormalities are occasionally seen as congenital defects. The correction of such deformities with fat flaps or grafts has been widely practiced. Similarly, tissue lost due to trauma or the surgical management of tumours is also often replaced using fat flaps or grafts. Fat is also used in other surgical procedures to control bone formation, obliterate sinuses, prevent gustatory sweating or reduce the impact of scars after laminectomy (Converse, 1977; Krauss, 1999; McGregor and McGregor, 1995; Mordick et al., 1992).

4.1.b.ii. Elective Cosmetic Surgery

Breast, gluteal, lip and penile augmentation is carried out with fat tissue as flaps (Mordick et al., 1992) or alternatively in grafts (Alter, 1998; Bircoll, 1988; Krauss, 1999; Mordick et al., 1992; Pinski and Roenigk, 1992).

4.1.b.iii. Replacement of Subcutaneous Fat

Subcutaneous fat is an important component of normal skin and the absence of this tissue results in significant changes in the appearance and texture of skin. An important part of the deformity accompanying post-surgical or traumatic scarring is due to the altered mechanical properties of skin where seams of dense collagenous tissue replace subcutaneous fat. In addition to this, severe crush injuries, burns, linear morphea, expression lines, acne scars and discoid lupus erythematosus scars result in altered skin shape and texture. The replacement of this subcutaneous fat in Dermal-fat or fat grafts and flaps is an important objective in many surgical procedures aimed at the rehabilitation of these patients (McGregor and McGregor, 1995; Pinski and Roenigk, 1992).

4.1.c. Problems Encountered by Surgeons in the Use of Fat

4.1.c.i. Harvesting Fat for Surgical Use

Not surprisingly, fat transplanted from cadavers or unrelated individuals is rejected by the new host, with the use of immuno-suppressive drugs probably not being justifiable for reconstructive
4.3 Possible Clinical Exploitation of Adipogenic Healing

surgery alone (Converse, 1977; Pohl and Uebel, 1985). For this reason, fat used in surgical
procedures is harvested from the patient's own body. The two methods for harvesting fat are to
either excise fat from a donor site, or alternatively to collect fat by liposuction and inject the
resulting tissue slurry into the recipient site (Bircoll, 1987; Converse, 1977). Excised fat may be
applied as either a graft or flap, while fat obtained by liposuction is always applied as a graft.
Liposuction derived fatty material has the two advantages of both minimizing the extent of donor
site trauma and allowing precise inflation and contouring by injection.

4.1.c.ii. Loss of Fat Volume, Necrosis and Cyst Formation

Loss of fat volume after auto-transplantation is one of the most significant problems for surgeons in
fat transplantation. Flaps and grafts of fatty tissue may also fail to survive transplantation, resulting
in cystic degeneration and sometimes the rejection of necrotic tissue masses (Converse, 1977;
Mandrekas et al., 1998).

Both loss of volume and cystic degeneration appear to be related to the degree of trauma
experienced by the tissue during harvesting and reimplantation. Contributing to this is the highly
lobular structure of fat, making it very difficult to handle the tissue without it falling apart into
separate poorly cohesive masses. This is reflected by the significantly reduced stability and greater
fibrosis of fat obtained by liposuction as compared with fat obtained by excision (Billings and May,
1989; Fagrell et al., 1996; Ullmann et al., 1998).

A further factor contributing to this reduction in volume appears to be the efficiency of
vascularization, as vascularized fat flaps are more stable than grafts (Goldberg et al., 1993;
Mordick et al., 1992) and pre-surgical abrasion of underlying muscle tissue appears to improve
volume retention after grafting (Samdal et al., 1992).
4.4 Possible Clinical Exploitation of Adipogenic Healing

4.1.c.iii. Survival of Adipose Tissue Transplants is Enhanced by Dermal Connective Tissue

Apart from careful handling of fatty tissue, one way to minimize trauma during harvesting and placement of fat is to collect the fatty tissue on a fibrous base. This is done by first raising the superficial dermis with a dermatome and then removing the underlying subcutaneous fat attached to the remaining fibrous dermis. Common sites for harvesting such dermis-fat grafts are the abdomen and buttocks. It also seems likely that such fat-dermis grafts more easily establish microvascular anastomosis through the dermal vasculature than with vessels in the less mechanically stable free fatty surface of fat grafts (Converse, 1977; Mordick et al., 1992; Sihota et al., 1994). It is possible that improved vascularization through the dermal component of such grafts explains the counterintuitive observation that thicker grafts in the order of 20mm thickness are more successful than 10mm thick grafts (Sihota et al., 1994). Further difficulties with such grafts, however, are the occasional formation of epidermoid cysts from epithelial rests and infections with contaminating surface bacteria (Davis et al., 1995; Sihota et al., 1994). Also, there is the discomfort and eventual unsightly defect in the donor site (Baker, 1986).

4.1.d. Adipogenesis and Adipogenic Healing

4.1.d.i. Adipogenesis is Normally Limited to the Neonate. Limiting Surgical Use of Fat

A limitation upon the surgical exploitation of fat has been that adipose tissue formation is largely confined to neonatal development (3.1. a.i.). Because of this, fat tissue harvested from donor sites can not be replaced while the regeneration of adipose tissue after loss of tissue is not possible.

4.1.d.ii. Possible Surgical Use of Adipose Tissue Formed During Adipogenic Healing

If it were possible to generate sheets of fatty tissue of a reproducible size, shape and viability, surgeons could plan and perform procedures to restore tissues with fat in the clinical settings outlined above.
4.5 Possible Clinical Exploitation of Adipogenic Healing

This may have particular application if the fat were ensheathed in a fibrous capsule, which would mimic the normal relationship between the dermis and subcutaneous fat, greatly facilitating reconstruction of subcutaneous fat. Apart from more closely simulating the normal structure of skin, fatty tissue enclosed with in a fibrous sheath would be expected to have the improved survival seen in dermis-fat auto-transplants without the disadvantages of epidermoid cyst formation, infection by contaminating skin bacteria or donor site morbidity (Baker, 1986; Converse, 1977; Mordick et al., 1992; Sihota et al., 1994).

Alternatively, surgeons would benefit from a technology, which could generate new fatty tissue of the correct size and shape in the site requiring reconstruction, thus avoiding transplantation.

There are several difficulties which arise from implantation of non-resorbable prosthetic devices including: foreign body reactions, infection, incorrect tissue texture, failure to grow and accommodate during childhood and failure to heal in sites of previous scarring or radiotherapy (Davis et al., 1995). These would be largely overcome by implantation of devices in which fatty tissue is allowed to form before resorption of the device.

4.1.e. The Need to Investigate the Possible Clinical Value of Adipogenic Healing

As described in 3.4.g, the biology of traumatic lipomas in humans is highly suggestive of adipogenic healing. Supporting this, is that adipose precursor cells can be isolated and stimulated into adipocytic differentiation even from elderly people (Kirkland and Dobson, 1997).

It is possible that the adipogenic healing described in Chapter 3 could be exploited to reconstruct fatty tissue. This Chapter describes preliminary studies investigating the viability of transplanted newly formed fat tissue in mice as well as the possible occurrence of adipogenic healing in baboons, a widely accepted primate model (Green et al., 2000; Lewis et al., 1986; 1991; Lewis and Soderstrom, 1993).
4.2. Materials and Methods

4.2.a. Materials

The surgical materials, drugs and reagents used for histology were obtained from the same sources as indicated in 2.2.a. and 3.2.a. Tissue transplantation in Balb/C mice was approved by Westmead Hospital Animal Care and Ethics Committee; Implantation of the devices in baboons was approved by Central Sydney Health Service and Animal Ethics Committee.

4.2.b. Methods

4.2.b.i. Auto-Transplantation Experiments in Mice

4.2.b.1.1. Surgical Procedures

12 female mice aged between 6 to 10 weeks received nylon mesh tube implants as described in 3.2.b. After 6 weeks, the nylon tubes and their adipogenic contents were removed from the wound bed in 10 of these mice and auto-transplanted into new but identical contra-lateral sites.

Briefly, mice for autotransplantation were anaesthetised and surgical areas shaved and disinfected as described earlier (3.2.b.i). 1cm vertical midline skin incisions were made over the base of tail to expose the underlying musculature on both sides of incisions. The implanted tubes were readily identified by the silk sutures which had been placed during the original surgery. Tubes were carefully separated from the wound bed using a scalpel and immediately placed into a muscle space created on the opposite side of the tail base by blunt dissection. The tubes were then secured into their new locations with two interrupted sutures each using 4-0 silk suture material. The original wound beds were cleaned and the muscle fibres sutured together. Skin wounds were then closed with continuous silk sutures. 2 mice were not treated in this way as controls for the adipogenic process, already established in Chapter 3.
4.7 Possible Clinical Exploitation of Adipogenic Healing

4.2.b.i.2. Tissue Harvesting and Processing from Auto-Transplanted Mice

Five auto-transplanted mice and 1 control animal were sacrificed by CO₂ asphyxiation 4 weeks after auto-transplantation, while the remaining 5 auto-transplanted and control mice were sacrificed in the same way at the 6 week time point. Tissues were immersed in formaldehyde (10%) for overnight fixation, and 4 μm paraffin sections were prepared as described in 2.2.b.v. Sections were stained with haematoxylin and eosin for histological assessment.

4.2.b.ii. Experiments with Baboons

4.2.b.ii.1. Preparation of Drum Implants

Net-wells for 24 well tissue culture plates were used to construct mesh chambers for implantation into baboons. These chambers measured 6 mm x 16 mm, and were prepared by modification and assembly of two opposing netwells.

Briefly, an acrylic dental bur and handpiece was used to reduce the height of net-wells to from 2 mm to 3 mm, leaving the mesh sheets attached and the base of the wells undamaged. 1 mm diameter slots were prepared in the side walls of net-wells before washing with water and securing the cut surfaces of the net-wells together with the same super-glue material used in the construction of nylon mesh implants (3.2.a.i). This created drum-shaped implants with nylon mesh forming the “skin” of drums, separated to a distance of approximately 6 mm by the plastic “sides” of the drums, with multiple small holes in the sides of these to facilitate the entry of blood into the chambers after implantation. These implants were then polished using an acrylic bur and washed thoroughly with pyrogen-free water before sterilisation with ethylene oxide.

4.2.b.ii.2. Other Implant Materials Used

In addition to the drum implants described in 4.2.b.ii.1, tube-shaped implants (3 mm x 7 mm), sterile surgical sponge material (10 mm x 10 mm), and gel-foam (10 mm x 10 mm) were implanted.
4.2.b.ii.3. Anaesthetic and Surgical Procedures of Baboons

Two female baboons, aged 5 and 8 years were used for this study. The first animal used received 4 implants on each side, being: one drum implant, one nylon mesh tube identical to those used in mice, one surgical sponge implant and one gelfoam implant. The results of this initial experiment lead to a modified procedure for the second animal, in which each side received two drum and surgical sponge implants only.

Anaesthesia was induced by intra-muscular administration of 1 ml Ketamine (100 mg/ml). Once anaesthetized, the surgical sites on the lateral thighs of both sides were shaved and disinfected with povidone-iodine solution (5%) followed by alcohol (70%). Anaesthesia was maintained by intravenous infusion of Ketamine (1 ml/kg/hr). In addition, 0.1 ml of Clonazepam (0.5 mg/ml) was given intravenously to reduce saliva secretion and 1 ml Maxolon (5 mg/ml) was injected intramuscularly as a muscle relaxant. The animals were then draped with sterile theatre drapes.

Surgery was performed on one side at a time. Briefly, two parallel skin incisions on each leg were made over the ischiofemoralis muscle, each measuring approximately 2 cm in length and being perpendicular to the muscle fibres. The underlying fascia was then dissected to expose the muscle fibres. Blunt dissection parallel to the Ischiofemoralis fibres was performed to create two spaces per incision and accommodate devices ranging in size from 3 mm x 7 mm to 6 mm x 16 mm. The drum implants, surgical sponge material, nylon mesh tubes or gelfoam were placed into these muscle spaces before closing the muscle tissue with interrupted 3-0 vicryl sutures. The overlying fascia and skin were then closed using by continuous subcutaneous sutures using the same material, burying knots so as to create a clean skin surface.

Both legs were dressed with iodine powder and surgical bands before returning animals to their cages. Baboons received intramuscular injections of temgesic (0.02 mg/ml) for analgesia immediately after surgery while the antibiotics penicillin (300mg) and tobramycin (20mg) were administered for 5 post-operative days.
4.2.b.ii.4. *Harvesting Implants from Baboons*

Tissues were collected after 2, 4, 6 and 8 weeks of the initial surgery. Briefly, animals were anaesthetized and surgical areas prepared as described in 4.2.b.ii.3. The implants were harvested from one surgical site in one leg of one baboon at each time point. After locating implants by blunt dissection, implants were carefully removed intact by dissection from wound beds. Each implant recovered was divided into two, with one half immersed in formaldehyde for paraffin histology (2.2.b.iv and 3.2.b.iii) and immunohistochemistry, while the second half was immersed in Karnovsky’s fixative for frozen sectioning and staining with Oil Red O. Surgical wounds were closed and the recovery of animals managed in the same way as described in 4.2.b.ii.3.

4.2.b.ii.5. *Lectin and Immuno-Histochemistry of Baboon Tissues*

Paraffin sections were prepared as described in 2.2.b.v. and 3.2.b.iii. Sections of tissues collected from all time points studied were stained for UEA-1 binding in order to identify endothelial cells. Briefly, sections of formalin fixed paraffin embedded tissue were de-paraffinized as described in 2.2.b.vii and UEA-1 histochemistry performed in a similar way to that described in 3.2.b.iv using biotin labelled UEA-1.

4.2.b.ii.6. *Frozen Sections and Oil Red O Staining*

Tissues were fixed overnight at 4°C in Karnovsky’s fixative comprised of paraformaldehyde (2%) with gluteraldehyde (2.5%) in PBS. After washing with PBS 3 times, tissues were incubated in a sucrose solution comprised of sucrose (30%) and MgCl₂ (2mM) in PBS, at 4°C over night. Tissues were then snap frozen in isopentane quenched liquid nitrogen for 5 minutes before preparation of 10 μm frozen sections. Frozen sections were then stained for Oil Red O labelling of triglyceride using the method described in 3.2.c.vii.

4.2.b.iii. 7. *Quantification of Vessel Numbers in Baboon Tissues*

Vessel numbers in baboon granulation tissue were counted in UEA-1 stained sections using methods almost identical to those described in 3.2.b.ix., with the exception that the only one
specimen was available for each time point in this experiment. Also, vessel size was not quantified because of the high degree of uniformity in size observed amongst vessels in the baboon tissues as well as the very restricted sample available.
4.3. Results

4.3.a. Mouse Auto-Transplantation Experiments

4.3.a.i. Necrosis was Common in Auto-Transplanted Mouse Adipogenic Implants

Control specimens not subjected to auto-transplantation did not reveal any signs of necrosis (Fig. 4.1), similar to the adipogenic tissues described in Chapter 3. Also similar to earlier observations, was the presence of occasional foci of lymphocyte infiltration while integration of the systemic lymphatic vasculature with that of lymph vessels in the adipogenic tissues was suggested by the fortuitous sectioning through an apparently dilated lymph vessel (Fig. 4.1).

In auto-transplanted tissues, however, necrosis was common and sometimes total. Of the five specimens collected at the 4 week time point, distinct large foci of necrosis were seen in 4 specimens, while in only one specimen no obviously necrotic foci were present. Foamy macrophages lined the cavities defined by the necrotic material, suggestive of phagocytosis of fatty necrotic tissue (Fig. 4.2). In some specimens, small foci of calcification were also noted, consistent with necrosis. These were often of a size similar to individual adipocytes (Fig. 4.2), suggestive of necrosis and calcification of individual cells.

Auto-transplanted tissues collected at 6 weeks were more variable, in that two specimens displayed total necrosis (Fig. 4.3), one specimen had a discrete focus of necrosis similar to those seen in 4 week specimens and two specimens appeared viable, but contained occasional discrete cyst-like structures (Fig. 4.4). The incidence of necrotic changes is summarised in Table 4.1.

4.3.a.ii. Despite necrosis, Significant Areas of Viable Auto-Transplanted Fat Were Present

Importantly, despite the presence of numerous necrotic foci, there were significant areas of apparently viable adipose tissue (Figs. 4.2, 4.3, 4.4). Also, one of the specimens collected at 4 weeks was apparently viable, while in two specimens collected at the 6 week time point small cystic structures were seen in the absence of obvious necrosis (Fig. 4.4).
Similar to controls (Fig. 4.1), foci of lymphocytic infiltration were common in these viable regions of adipose tissue, however, the intensity of diffuse infiltrates between individual adipocytes (Fig. 4.2) often seemed greater than that seen in controls. Very occasional multinucleated cells were seen, suggestive of a granulomatous inflammatory response (Fig. 4.2). A dense mononuclear inflammatory infiltrate was also apparent in the fibrotic capsule surrounding the nylon mesh material in many of these specimens (Fig. 4.2, 4.3), although this was not consistent in all specimens (Fig. 4.4). The survival or otherwise of tissues is summarised in Table 4.1.

4.3.b. Implantation of Devices in Baboons

4.3.b.i. Only Drum Chambers Were Recoverable or Were Useful for Studying Events in Baboons

When implants were harvested for examination, it proved that the nylon tube implants identical to those used in mice were so small they could not be found in the tissues. Also, the gelfoam implants could not be identified, and this was assumed to reflect resorption of the material. For these reasons, only the sponge and drum chamber implants were recovered from baboons.

Also, when sponge material was examined, although a fibrotic sleeve was found surrounding the sponge material, granulation tissue was not found within the sponge itself, different to observations in mice. The failure of granulation tissue to grow into the sponge material coupled with the inability to locate either the small nylon mesh or gelfoam implants meant that only the drum implants were of value for studying changes in baboons.

4.3.b.ii. Granulation Tissue Formed in Drum Implants

Granulation tissue grew into drum implants in a similar way to that seen in mice (3.3.a.i). One difference, however, was that the drum implants were completely filled by new tissue at a much later time as compared with those used in mice, so that the fibrinous material was only completely replaced after 6 weeks of implantation.
4.3.b.iii. The Vascularity of Granulation Tissues in Baboons was Lower Than in Mice and Reduced Over Time

The vascularity of the granulation tissue formed in the drum implants placed in baboons appeared lower than that in mouse tissues (Figs. 4.5 and 3.4). Despite using essentially identical methods for quantification, this morphological appearance was supported by quantification of vessel profile number (Fig. 4.6 and 3.5). However, the same pattern of initially increasing vessel number followed by a significant reduction in vascularity and peaking at around the 4th post-surgical week, was very similar to changes seen in mice (Figs. 4.5 and 3.5). The extreme vascular dilation seen in mice (Fig. 3.11) was not, however, seen in baboons (Fig. 4.5).

4.3.b.iv. Adipose Tissue Did Not Form in Drum Implants in Baboons

An important difference between changes seen in baboons as compared with mice, was that the granulation tissue in baboons appeared to mature to scar tissue (Fig. 4.5) so that the pattern of adipogenic healing observed in mice was not replicated in baboons.

4.3.b.v. Occasional Oil Red O Positive Cells Were Present 4 Weeks After Placement of Drum Implants

Although no mature adipose tissue was found in the harvested baboon tissues, when these tissues were subjected to Oil Red O staining, occasional cells were found with discrete lipid vacuoles (Fig. 4.7). These cells did not have the clear nuclear indentation often characteristic of lipoblasts, but were only seen at the leading edge of the growing reparative granulation tissue at the 4 week time point. Also, these cells appeared to be smaller than the lipoblasts seen in mouse tissues in Chapter 3 (Fig. 3.12). The presence of these small, seemingly pre-lipoblastic cells suggested that in baboons, adipogenic healing may have commenced but been terminated at an early stage with subsequent healing by scarring.
Table 4.1. Table summarising the incidence of necrosis in auto-transplanted or control tissues in mice.

<table>
<thead>
<tr>
<th>Tissue Harvested</th>
<th>Mouse Specimen</th>
<th>No Necrosis</th>
<th>Foci of Necrosis</th>
<th>Cystic Change</th>
<th>Total Necrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>X</td>
<td></td>
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<tr>
<td>Auto-Transplant 1</td>
<td>X</td>
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<td>Auto-Transplant 2</td>
<td>X</td>
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<tr>
<td>4 Weeks</td>
<td>Auto-Transplant 3</td>
<td>X</td>
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<td>Auto-Transplant 4</td>
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<td>Auto-Transplant 5</td>
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<td>Control</td>
<td>X</td>
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<td>Auto-Transplant 1</td>
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<td>Auto-Transplant 2</td>
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<tr>
<td>6 Weeks</td>
<td>Auto-Transplant 3</td>
<td>X*</td>
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<td>X</td>
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<td></td>
<td>Auto-Transplant 4</td>
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</tr>
<tr>
<td></td>
<td>Auto-Transplant 5</td>
<td>X*</td>
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<td>X</td>
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</tbody>
</table>

Both control specimens showed no sign of necrosis, while foci of necrosis, cystic change or total necrosis was seen in all but one of the auto-transplanted specimens. In two specimens collected at the 6 week time point, no obvious necrosis was seen, although cystic change was present (X*), and it seems likely that this reflected the resolution of earlier episodes of localised necrosis.
Fig. 4.1. Photomicrographs of a control untransplanted mesh tube implant, collected 4 weeks after auto-transplantation of test implants (4.2.b.i.1). (A) At low magnification, adipose tissue (Ad) was found to occupy the lumen of the implant, while the location of the nylon mesh (N), adjacent fibrotic tissue (F), and muscle (M) was clearly seen. Foci of inflammatory cells were noted within the adipose tissue (arrows). (B) At higher magnification, these foci were found to consist of aggregates of lymphocytes (Ly) while an endothelium lined and apparently dilated lymphatic vessel filled with lymphocytes and lacking erythrocytes was seen (arrow). (C) The lymphocytic infiltrates were highly localised, although occasional lymphocytes and very minor infiltrates were present throughout the remaining adipose tissue (arrows). (H&E).

(Bar for A = 700 μm; B and C = 100 μm).
Fig. 4.2. Photomicrographs of a nylon mesh tube containing adipose tissue which was autotransplanted and then harvested for paraffin histology after 4 weeks recovery (4.2.b.i). (A) Adipose tissue (Ad) appeared to remain viable despite the presence of significant foci of frank necrosis (Nc). The necrotic areas were closely associated with the surface of the nylon mesh (N) while the surrounding muscle tissue receiving the auto-transplanted tissue (M) was apparent. Focal accumulations of inflammatory cells were seen throughout the adipose tissue (arrows). The location of high power micrographs within this specimen are indicated by rectangles labelled B to F. (B) At higher magnification, small calcific deposits were noted which had a shape suggestive of necrosis and calcification of isolated adipocytes (arrowheads). Diffuse infiltrates of lymphocytes were present scattered throughout the adipose tissue (arrows). (C) Examination the nylon mesh revealed a dense lymphocytic infiltrate (arrows) surrounding the nylon material (N). (D) In some areas, the diffuse lymphocytic infiltrate was more dense, so that individual adipocytes were separated by aggregates of lymphocytes (arrows). (E) The necrotic area was defined by a fibrotic capsule like structure which was heavily infiltrated with lymphocytes and lined by numerous cells with an appearance typical of foamy macrophages (arrows), suggestive of phagocytosis of large quantities of lipid laden material. (F) Although much of the necrotic material appeared to have been lost during sectioning, small aggregates of this material were seen and appeared to contain infiltrates of lymphocytes, PMN and occasional foamy macrophages (arrow). (H&E).

(Bar for A = 1,000 μm for A; 100 μm for B,C,D,E,F).
Fig. 4.3. Photomicrographs nylon mesh tubes containing adipose tissue which was autotransplanted and then harvested for paraffin histology after 6 weeks recovery from a mouse in which the tissue remained viable (A,B,C,D) and one in which there was total necrosis (E, F) (4.2.b.i.1). (A) Adipose tissue (Ad) was viable in some specimens, while the location of the nylon mesh (N), muscle tissue (M) and fibrous reaction to the nylon material (F) were clearly seen. Dense lymphocytic infiltrates were seen in places (arrow). (B) Examination at higher magnification of the nylon mesh (N) (rectangle in A) indicates the presence of a dense lymphocytic infiltrate while the adjacent muscle tissue (M) appeared to be comparatively uninvolved. (C) The dense lymphocytic infiltrate (Ly), indicated in the rectangle in A, was seen to contain very occasional multinucleated giant cells (arrow). (D) However, higher magnification of other areas of adipose tissue revealed only very few infiltrating lymphocytes (arrow). (E) In some specimens, no viable adipose tissue was found so that only necrotic debris was seen in the luminal space defined by the nylon mesh (N). (F) Examination of the necrotic material at higher magnification revealed the presence of numerous PMN and lymphocytes as well as foamy macrophages, sometimes containing condensed nuclear debris suggestive of phagocytosis of necrotic adipose and inflammatory cells (arrows). (H&E).

(Bars = 1000 \mu m for A and E; 100 \mu m for B, C, D and F).
Fig. 4.4. Photomicrographs of a nylon mesh tube containing adipose tissue which was auto-transplanted and then harvested for paraffin histology after 6 weeks recovery (4.2.b.i.1). (A) The nylon mesh material (N), fibrotic region (F) and adjacent muscle tissue (M) were readily recognised as was the adipose tissue (Ad) within the tube. Although no clear areas of necrosis were seen, numerous well defined cystic lesions were noted (arrows). (B) Examination of these cystic structures at higher magnification revealed that they were lined by a monolayer of flattened cells (arrows), some of which contained vacuoles. (C) Despite the apparent degeneration of adipose tissue in these regions, the inflammatory response to the nylon mesh (N) in the fibrous tissue (F) and adjacent muscle (M) was minimal in some regions, so that only limited lymphocytic infiltrates (small arrow heads) and occasional multinucleated macrophages (large arrow head) were present. (H&E).

(Bars for A = 750 μm, for B and C = 950 μm)
Fig. 4.5. Photomicrographs of UEA-1 lectin labelled paraffin sections of granulation tissue 2 (A), 4 (B), 6 (C) and 8 (D) weeks after implantation of drum implants. Blood vessels were identified as linear structures with positive UEA-1 binding (arrows). Inflammatory cells and fibroblasts occupied the space between blood vessels. Vascular granulation progressively replaced fibrinous matrix (Fn) in the chamber space, with complete filling seen by the 6th post-operative week. By the 8th post-operative week, granulation tissue had matured to scar tissue was (Sc) with very few vessels seen towards the centre of the chamber. Long arrows beneath the photomicrographs indicate the direction of cellular migration from the nylon mesh surface towards the centers of the implants. The changes seen were more characteristic of wound healing by scarring than of the adipogenic healing seen in mice. Also, blood vessels were comparatively small in diameter, and no large dilated vessels similar to those seen in mice were present. (Bars = 140µm).
4.20 Possible Clinical Exploitation of Adipogenic Healing

![Graph showing the mean number of vessels per field in the reparative granulation tissue found in baboon drum implants 2, 4, 6 and 8 weeks after implantation. The vessel profile number peaked by the 4th week after implantation (p < 0.05) and reduced to a low level at 8th post-surgical week (p < 0.05). This was similar as for tissue in mice, with the difference that the overall level of vascularity was much lower.](image-url)
Fig. 4.7. Photomicrographs of cells containing lipid droplets (arrows) as seen in frozen sections treated with Oil Red O. These cells were only seen in granulation tissue collected four weeks after implantation, and appeared only at the leading edge of the granulation tissue. These cells appeared to be smaller as compared with the lipoblasts found in mice while the lipid vacuoles did not seem to be as large or indent the nuclei (Fig.3.12). Also, there did not seem to be a clear relationship between these lipoblast like cells and capillaries. (Bars = 15 μm).
4.4. Discussion

4.4.a. Auto-transplantation of Adipogenic Tissue in Mice was Partially Successful

4.4.a.i. Re-implantation into The Equivalent Site of Adipogenesis was Considered Optimal

Since adipose tissue is often transplanted for clinical use (Converse, 1977; McGregor and McGregor, 1995), it seemed important to determine if adipose tissue formed during adipogenic healing would survive auto-transplantation. Re-implantation into sites similar to that within which the adipogenic tissue had formed was considered most likely to allow successful integration of the grafts, however, despite this, significant necrosis was seen. It is possible that had other perhaps more vascular sites been selected for re-implantation, then grafts would have been more successful. Nonetheless, the viability of some grafts was maintained, while in most re-implanted tissues, there were significant regions of viable adipose tissue at the time of tissue harvesting.

In many instances, necrosis was limited to specific domains within auto-transplants, and this may reflect the success of microvascular Anastomosis in some regions as compared with the failure of this in other micro-domains.

4.4.a.ii. Inflammatory or Immune Mechanisms May Have Contributed to Necrotic Changes

The pattern of necrosis appeared to differ significantly amongst specimens, so that in some cases, the entire implant was necrotic while in others only small focal regions were affected. Whether this reflected microvascular Anastomosis or not is unclear in the current study. However, since total necrosis was only seen in tissues collected after 6 weeks of recovery, it seems possible that in some implants, localised necrotic changes elicited further inflammatory changes in the surviving viable adipose tissue, with the result that necrotic areas propagated to involve the entire implant. Supporting this possibility was the presence of dense lymphocytic infiltrates throughout the adipose tissue and fibrous wall of implants, as well as occasional multinucleated giant cells characteristic of granulomatous inflammation.
The relative absence of such intense infiltrates of inflammatory cells in the more successful auto-transplants may reflect a comparatively more benign host response, although it is also possible that the inflammatory infiltrates were more responsive to the necrotic tissue than the cause of necrosis.

There are several mechanisms through which such "propagative inflammatory necrosis" could operate. For example, as discussed in Chapter 2, a variety of inflammatory cytokines including TNF, TGF-β and INF-γ are able to cause apoptosis in endothelium (Maier et al., 1995; Polunovsky et al., 1994; Robaye et al., 1991; Tsukada et al., 1995). It is possible that the local production of such cytokines by inflammatory and other cells could initiate localized endothelial apoptosis and cause further local necrosis. In addition to this, PMN and macrophages release a range of potentially toxic free radical and enzyme agents (Cotran et al., 1999), which could contribute to cellular death in tissues adjacent to initially small foci of necrosis. One difficulty with this interpretation, is that there do not appear to be any clear examples of such a "propagative necrotic" processes, other than in clostridial and other infections, where bacterial toxins cause expanding necrosis of the tissues (Cotran et al., 1999).

Calcification is seen in necrosis (Cotran et al., 1999) and the highly localized calcification of apparently isolated adipose cells also suggested that adipocyte death in auto-transplanted implants was not entirely due to microvascular events, which would affect wide regions of tissue. Instead, such highly localised events implied of necrosis by highly local inflammatory or immune factors.

4.4.a.iii. Lymphocyte Infiltration May Be Important in Adipogenic Healing

Although lymphocytic infiltrates were seen in the earlier work initially describing adipogenic healing (Chapter 3), the very much greater intensity of these infiltrates in auto-transplanted tissues drew more attention to the presence of these immune effector cells. Even in control tissues which were not auto-transplanted, lymphocytic infiltrates were sometimes very intense. In addition to this, the presence of dilated lymphatic channels containing lymphocytes was noted in one specimen,
both confirming the generation of new lymph vessels during adipogenic healing and what appeared to be lymphocyte recirculation through this newly formed tissue.

In as much as inflammatory and immune mechanism may have contributed to the necrosis of adipogenic tissue (4.4.a.ii), it is also possible that the lymphocyte population plays a critical role in the initial formation or maintenance of adipogenic tissues.

4.4.a.iv. Cyst Formation May Have Reflected the Final Stage of Fat Necrosis

Cystic degeneration is reported for auto-transplanted adipose tissue and constitutes a form of failure of such fat grafts (Mandrekas et al., 1998). In the current study, this was only seen in otherwise successful implants at the latter 6 week time point. This was interpreted as reflecting a successful host response to local necrotic changes with the eventual removal of necrotic cells, possibly by foamy macrophages which may exit the tissues via the lymphatics. The contents of the cysts were not determined in the current study, but it would be interesting to confirm or disprove these as triglyceride in Oil-Red O stains.

4.4.a.v. It May be Possible to Improve the Stability of Auto-Transplanted Adipogenic Tissue

From the above, it seems that microvascular and perhaps immune or inflammatory mechanisms determine if auto-transplanted adipogenic tissues are able to survive in mice. It is possible that the survival of such transplants could be improved through better surgical methodology, perhaps to re-establish microvascular anastomosis, or alternatively, through the use of anti-inflammatory drugs. Regardless, it seems clear that a more optimal clinical outcome would be achieved if auto-transplantation is not required and the new tissue is permitted to form in the site where it is desired.

4.4.a.vi. The Possible Advantage of Adipogenic Healing Over Other Methods for Inducing Adipogenesis

As discussed in 3.1.c, there are several recent reports of methods to form new adipose tissues in animals using agents such as Matrigel together with FGF (Kawaguchi et al., 1998) or IGF
impregnated beads (Yuksel et al., 2000) and in view of this, it seems reasonable to question whether adipogenic healing would have any possible advantages over the use of such agents to reconstruct tissues.

There seem to be several significant difficulties with the use of such agents to elicit adipogenesis. Firstly, cytokines administered to the tissues are rapidly degraded so that the expense of delivering them to the tissues in sufficient quantities to be effective may severely limit the use of such methods. Also, matrigel is a tumour cell line product (Baatout, 1997), so that this material may be potentially contaminated by infectious agents from animal or human products used to raise and maintain the cells secreting the material. Finally, even recombinant cytokine material may be contaminated with microbial or animal products, so that the risk of infection and or inflammation in response to such contaminants remains.

Since adipogenic healing appears to only require the implantation of a suitable sterile implant, these potential problems do not arise. Also, a range of potentially appropriate resorbable materials has been developed already (Shalaby, 1994), so that the clinical application of adipogenic healing may require only the selection of one of these already established materials for development of successful clinical appliances.

For these reasons, despite the limited survival of auto-transplanted adipogenic tissues, it seems reasonable to continue further investigations into the possible clinical value of this pattern of wound healing.

4.4.b. Scarring in Baboon Tissues

4.4.b.i. Events in Baboon Tissues Support the Suggestion that Adipogenic Changes in Mice are Reflective of Wound Healing

Adipose tissue did not form in baboon tissues, but was more typical of healing by scarring (Ferguson et al., 1996; Linares, 1996; Mutsaers et al., 1997). Despite this, early events were
essentially identical in baboon tissues as in mouse adipogenic implants, as in both cases fibrinous coagulum formed in the tissue space and was replaced by reparative granulation tissue Also, the vascularity of this granulation tissue rose and fell with much the same time course in both species, typical of wound healing (Cotran et al., 1999; Desmouliere et al., 1995; Greenhalgh, 1998; Linares, 1996; Mutsaers et al., 1997; Sandison, 1928)

If it is accepted that the scarring seen in baboon tissues reflected wound maturation as expected from the literature (Desmouliere et al., 1995; Ferguson et al., 1996; Linares, 1996; Mutsaers et al., 1997), then it seems reasonable to argue that the maturation of essentially similar granulation tissue in mice to adipose tissue is also a form of wound healing. The only difference between the two cases would then seem to be that instead of maturing to scar tissue, there is maturation to fat.

4.4.b.ii. Baboons Appeared to Display Aborted Adipogenesis

Although no mature fat was seen in baboon tissues, occasional small cells with triglyceride were found at the advancing edge of the invading granulation tissue at the 4th post surgical week. These cells were smaller than the lipoblasts seen in mice, and the lipid vacuoles appeared insufficiently developed to indent the nuclei. These cells were interpreted as being lipoblasts at a very early stage of development, in which triglyceride droplets had not had sufficient time to form the large masses required to compel cells into the classically lipoblastic shape seen in mouse tissues. Importantly, however, these cells were not seen at latter time points, suggesting that the requirements for further lipoblast and adipocyte differentiation were not satisfied.

4.4.b.iii. Aborted Adipogenesis in Baboons May be Due to Reduced Vascularity, Low Body Fat, or an Excessive Inflammatory Response

As noted in Chapter 3, lipoblast differentiation appears to be intimately related to the development of the vasculature (Hausman and Richardson, 1983; Poissonnet et al., 1983; Wright and Hausman, 1990) while it was clear that in the case of baboon tissues, vessels were both much smaller and less frequent in granulation tissues. From this, it seems possible that adipogenesis was initiated in
baboon tissues, but that the vasculature was insufficient to support and maintain this. In this way, it is possible that if angiogenesis were increased in the baboon tissues, that there would also be successful adipogenesis.

It should also be noted that baboons are exceptionally lean animals, having very little body fat. For this reason, it may be necessary for baboons to be fed a high fat diet in order to achieve sufficient circulating triglyceride levels for adipogenic healing to occur.

Also, as discussed above in 4.4.a.ii, it is possible that inflammatory changes have a profound impact upon adipogenic healing. Nylon is a poorly bio-compatible material, and it is possible that the use of this material in baboons established an inflammatory environment inconsistent with adipogenic healing. It is possible that the use of more bio-compatible materials would have permitted adipogenesis to proceed to completion. With regard to this, it is interesting to note the intensely fibrotic response to surgical sponge material in baboons as compared with the much milder and adipogenic response seen in mice. This is consistent with the suggestion that baboons elicit a much stronger fibrotic inflammatory response to the same materials than do mice, and this may help to account for both the lower vascularity of baboon reparative tissue and the absence of clear adipogenic maturation.

From this, although no clear adipogenesis was seen in baboons, the current study can not exclude adipogenic healing from primates. Unfortunately, the time constraints imposed upon this thesis prevented further work characterising the changes seen in baboons or mice. The direction of further work required is described in Chapter 5.
CHAPTER FIVE

General Discussion
5.1 General Discussion

5.1. The Main Findings of Work in This Thesis

5.1.a. *The Fundamental Issue Addressed in This Thesis: Mechanisms for Formation of Large Scale Structures by Cells*

This thesis was intended to address the morphological aspects of overall question of how individual cells co-ordinate with each other to produce complex, large scale, multicellular structures. Vascular endothelium was considered to be particularly interesting in this regard as individual endothelial cells self-assemble into extensive and complex branching networks comprised of a monolayer of single cells and in which individual vessel segments must be spatially arranged so as to permit effective tissue perfusion. The possible role of endothelial cell apoptosis in such self-organising processes was the particular focus of the work described in Chapter 2 while in Chapter 3, an attempt is made to extend this question to organisation of tissues during adipogenesis.

5.1.b. *Intercellular Adhesion and Apoptosis in Lumen and Monolayer Formation*

An experimental model system was established in which HUVEC were deprived of matrix adhesion, but were able to contact each other. As expected from the literature, it was found that matrix deprivation caused endothelial apoptosis (Meredith *et al.*, 1993; Re *et al.*, 1994; Zoellner *et al.*, 1996). A novel finding, however, was that endothelial cells which were able to co-aggregate into clustered structures were able to escape apoptosis for some time, despite being deprived of matrix adhesion (Chapter 2). Further, it was found that inter-cellular contact alone was only able to inhibit endothelial apoptosis in matrix deprived HUVEC for a comparatively short time, and that the establishment of a free surface was also important to circumvent apoptosis, as cells effectively surrounded by their colleagues became apoptotic. The surviving surface cells acquired the flattened morphology and ultrastructural properties of differentiated capillary endothelial cells *in vivo*. The outcome of these events was the production of structures reminiscent of capillaries, suggesting that similar mechanisms may contribute to lumenisation and monolayer formation during vascular development. This supported the published role of apoptosis in lumen formation for cultured cells
in three-dimensional networks (Meyer et al., 1997), but also demonstrated independence from matrix adhesion of these events.

The long term survival of these clusters was, however, limited by the ultimate requirement of cells for an adhesive matrix, and this together with the above mentioned observations, revealed seemingly for the first time, a hierarchical series of signals inhibiting endothelial apoptosis.

It was concluded that: intercellular adhesion was a weak anti-apoptotic signal; establishment of a flattened monolayer was a further and more potent signal inhibiting endothelial apoptosis; and matrix adhesion was an even more effective anti-apoptotic signal for endothelium. In this way, the work described in Chapter 2 provided some insight into mechanisms through which the formation of large-scale vascular structures may be achieved by individual cells exploiting highly localised intercellular signals which inhibit, or perhaps in the case of cells trapped within clusters, activate apoptosis. Also, the role of such localised signals in control of capillary like differentiation was illustrated.

5.1.c. Adipogenic Healing May Account for the Self-Limiting Behavior of Common Adipose Tumours

Attempts were made to establish methods for investigation of events in vivo. To this end, a wound healing model was established, as outlined in Chapter 3 and Appendix A.3.a, and exploited to study the development of new tissue.

A disappointment was that apoptotic endothelial events were difficult to define, other than in terms of changes in overall tissue vascularity. However, a novel observation was the development of adipose tissue in the experimental lesions produced. This appeared to represent a previously unreported pattern of wound healing. Lipomas and traumatic lipomas are essentially self-limiting and this is difficult to explain in terms of normal tumour biology, where any neoplastic tissue has by
definition escaped growth control and proliferates without regard to the adjacent tissues. The observation of adipogenic healing suggests that these common fatty tumours are not neoplastic as is currently thought (Franz and Sharon, 1995), but that they are reactive lesions representing adipogenic healing in humans.

A close relationship between adipogenesis and angiogenesis has been reported by other workers (Bouloumiec et al., 1998; Crandall et al., 1997; Wright and Hausman, 1990) and appeared to be reflected by the close apposition of endothelial cells and lipoblasts in tissues described in Chapter 3. In this way, the findings of Chapter 3 revealed some potential dependence of lipoblastic cells upon vascular cells may be interpreted as an example of co-ordination between dissimilar cells in the formation of large scale structures. In addition, very significant changes in vascular structure were noted with development and maturation of the adipose tissue, raising the possibility of later exploiting this model system to study mechanisms for vascular dilation.

5.1.d. Auto-Transplantation of Tissues Obtained by Adipogenic Healing had Only Limited Success

Adipogenic healing observed in Chapter 3 raised the possibility that adipose tissue obtained in this way could be exploited for surgical reconstruction or tissue augmentation. Auto-transplantation of adipogenic implants was found to be variably successful in Chapter 4, with necrosis and cystic change occurring commonly in the transplanted tissue. Nonetheless, some adipose tissue remained viable after such treatment, suggesting that improved surgical technique may improve the outcome of such procedures.

Although some lymphocyte infiltrates were noted in Chapter 3, these appeared much more prominent after auto-transplantation, raising the possibility that immune or inflammatory mechanisms contributed to the demise of these tissues. In this way, it was also concluded that it may be possible to improve graft survival of these auto-transplanted tissues through modulation of the inflammatory or immune responses.
5.4 General Discussion

5.1.e. Baboons Had Very Little Sign of Adipogenic Healing and This May Reflect Dependence Upon Vascular Cells of Adipogenesis

To further assess the possible clinical value of adipogenic healing, a small experimental study was performed using baboons as a primate model, to determine if adipogenic healing occurred in this species and this is also described in Chapter 4. Despite the clear formation of reparative granulation tissue, the only sign of adipogenesis was the presence of some scattered cells with comparatively small droplets of lipid material in their cytoplasm. It was concluded that although adipogenesis may be initiated in baboons, that it is readily aborted, perhaps reflecting local inflammatory events or alternatively the very lean lipid metabolism in this species.

It was interesting to note that the seemingly non-adipogenic tissues of baboons were significantly less vascular as compared with the adipogenic tissue in mice. This supported the idea that vascular cells are important for lipoblast differentiation, and that co-ordinated function of these cell types is critical for the formation of adipose tissue.

5.2. Work Arising from That Described in This Thesis

5.2.a. Extension of This Morphological Study to The Molecular Level

The work described in this thesis is primarily morphological in nature, reflecting the requirement of initially characterizing the structural aspects of the HUVEC cluster and adipogenic healing models. Having now been established, it would now be interesting to continue study of these model systems to investigate the molecular mechanism responsible for controlling and mediating the morphological changes described in this thesis.

5.2.a.i. Role of The Extracellular Matrix in The Branching of Clusters

As mentioned above (5.1.b), endothelial cells in clusters acquired some of differentiated properties of capillaries (2.4.c.iv). However, the isolation of individual clusters meant that it was not possible to produce linear or branching structures, while no branching or sprouting between clusters was
observed. It seems likely that any attempt to form sprouts from such clusters would have resulted in fusion of adjacent structures due to the absence of any matrix attachment. This contrasts with endothelial cells grown on collagen coated microcarrier beads, which extend processes to form branches (Ingber and Folkman, 1989). From this, it appears that the extracellular matrix plays an indispensable role in the formation of branching functional vessels not only through the provision of a stable matrix, but also by permitting branch point formation.

It would be interesting to investigate the possible roles of individual components of the extracellular matrix in further differentiation of the endothelial cells and the construction of vascular networks using this model system. The suspension culture system has been used by others to illustrate the importance of the extracellular matrix for EC survival (Meredith et al., 1993; Re et al., 1994; Zoellner et al., 1996). However, this model has the advantage with regard to current “tube and branch forming” models (Jackson et al., 1994; Sweeney et al., 1998; Yang et al., 1999), in that extracellular matrix components can be added to the cells after the initial formation of lumenised and differentiated vessel like spheres. In this way, mechanisms for initial differentiation of the monolayer may be separable from those responsible for branch point formation and vessel extension.

To address these questions, it would be interesting to perform a series of experiments in which individual matrix components or other stimuli are added to the clustered cells at different stages of development, and the effect on the shape and differentiation state of clusters determined. Also, these studies could be extended to the embedding of clusters already established in matrix-deprived culture into matrix material such as collagen gels, fibrin or Matrigel. Again, the effect of specific matrix or other stimuli added to such cultures of embedded clusters could be determined to define the role of such factors in endothelial differentiation.

Direct comparison of pre and post-embedded or branching cultures by molecular techniques such as subtractive hybridization or micro-array systems comparing mRNA expressed by such cultures
would be possible. This would provide insight into both the expression of specific proteins associated with these events as well as information about the proteins activated as intracellular second messengers during activation of these differentiation steps.

5.2.a.ii. Characterization of Mechanisms Involved with Lumen Formation and Monolayer Differentiation

The cluster model provides an opportunity to determine the second messengers involved with monolayer and lumen formation, independent of confounding effects imposed by matrix stimulation. The effects of agents inhibiting or stimulating specific second messenger pathways upon these events could be readily determined using the cluster model. There is an extensive literature detailing both second messenger pathways and the tools available for their investigation which is beyond the scope of this thesis (Kappel et al., 1999; Mariotti et al., 2000; Morandini et al., 1996; Mukhopadhyay et al., 1998; Risau and Flamme, 1995; Traub and Berk, 1998). However, through the use of agents such as PMA stimulating protein kinase C, Genestine inhibiting tyrosine kinases, or Vanadate inhibiting protein phosphatases, and investigation of specific transcription factors such as Myc, Fos or Jun (Cowan et al., 1998; Dike and Ingber, 1996; Lampugnani et al., 1990; Lu et al., 1996; Wyllie et al., 1992), it should be possible to learn more about the mechanisms responsible for these differentiation steps. Similarly, the cluster model can be exploited to investigate the role of apoptosis related genes in vessel differentiation (Dragovich et al., 1998; Harada-Shiba et al., 1998; Kim et al., 2000; Nunez et al., 1998). The ease with which paraffin or even frozen sections could be prepared of these clusters makes the use of immuno-histochemistry and in-situ-Reverse Transcription Polymerase Chain Reaction (In-situ RT-PCR) to determine the localized expression of specific proteins comparatively simple.
5.7 General Discussion

5.2.a.iii. Further Characterisation of the Expression of Intercellular Adhesion Molecules in Clusters

5.2.a.iii.1. Confocal Laser Scanning Microscopy and Transmission Electron Microscopy

Due to time limitations, it was not possible to more fully characterise the expression of intercellular adhesion molecules in clusters, although as described in A.4, attempts were made to establish the necessary methodology using CLSM. All three adhesion molecules are reported as having roles in tube formation using collagen gel systems (Ashton et al., 1999). Only VE-CAD is reported to have an influence upon tubes already formed (Bach et al., 1998), indicating that these adhesion molecules have distinct roles in vessel formation. It would be interesting to exploit the methods described in A.4 to determine the expression of Con-43, PECAM-1 and VE-CAD during cluster formation. Immuno-gold labelling would be valuable to determine the location or otherwise of these molecules at the focal adhesion points noted at early time points during cluster formation (2.3.c.iii.) Also, as described in 2.3.f, antibodies against these proteins reduced cluster formation, however, the effect of these upon the differentiation of cells in clusters already established was not determined. This would be interesting to determine in experiments where antibodies are added at defined time points after cluster formation and the resulting clusters assessed by TEM and CLSM.

5.2.a.iii.2. Western Blots, RT-PCR and In-Situ RT-PCR

Expression of the intercellular adhesion molecules at different stages of cluster formation could also be studied by Western blot (Gabriels and Paul, 1998; Goldberger et al., 1994; Schnittler et al., 1997). mRNA for these proteins could also be studied using Northern blots or RT-PCR (Bach et al., 1998; Goldberger et al., 1994).

5.2.a.iii.3. The Role of Cytoskeletal Proteins in Cluster Formation

Although initial contact of EC is established by adhesion molecules, more stable interactions require the further activity of the cytoskeletal structures, often associated with adhesion molecules (Freemont, 1998). For instance, VE-cadherin associates with plakoglobin, β-catenin and p120 in the cytoplasm. VE-cadherin preferentially associates with β-catenin and p120 in loosely confluent
monolayers of EC, while it is also associated with plackoglobin in tightly confluent EC (Lampugnani et al., 1997). Expression of plackoglobin and cytoskeleton alignment are reported to inhibit EC migration and tube formation (Dike et al., 1999; Nagashima et al., 1997). In the cluster model used in Chapter 2, prominent stress fibres were seen in the stretched cells which were protected from apoptosis and this supports the involvement of the cytoskeleton in differentiation of these cells. Changes in the shape of cells may result in altered growth signalling via the cytoskeleton (Huang et al., 1998). It would be an interesting addition to the data if the dynamics of the cytoskeletal proteins were characterised in this model using CLSM and Western blotting methods (Lampugnani et al., 1997).

5.2.a.iii.4. Adipose Transcription Factors and Regulatory Proteins

The transcription factors expressed in adipogenic tissues were not investigated due to time limitations. PPAR-γ and C/EBP are considered important regulators of adipogenesis (Gaskins et al., 1989; Lee et al., 1998; Spiegelman et al., 1997), however, due to the low frequency of adipose tissue hyperplasia in adults, studies of these factors are largely based on embryonic development or differentiation of fat cells in culture. The adipogenic healing model provides a convenient system to characterise expression of these and other factors during adipogenesis in adults. Interestingly, PPAR-γ is reported to regulate endothelial cell PAI expression in culture (Marx et al., 1999) and since angiogenesis and adipogenesis were associated in this model, studying endothelial cell specificity during adipogenesis may provide useful insight to obesity related vascular diseases. It would also be interesting to investigate the expression of cytokine or hormone factors thought to influence adipogenesis such as Leptin, FGF, PDGF, IGF-1, TNFα, GH, corticosteroids and thyroxin (Ailhaud, 1990, 1997; Chen et al., 2000; Choy et al., 2000; Guller et al., 1989; Hausman and Yu, 1998; Richelsen, 1997; Serrero et al., 1992). It would also be possible to determine the effect of these agents upon adipogenesis when administered locally or systemically.
5.9 General Discussion

5.2.b. The Use of Microvascular Endothelial Cells in Cluster Experiments

Endothelial cells are highly diverse cells, which display different characteristics in different vessels and tissues. Microvascular endothelial cells are different from macrovascular endothelial cells in many ways (Jackson and Nguyen, 1997; Stins et al., 1997; Wojta et al., 1993). Angiogenesis is largely a microvascular endothelial cell event, however, HUVEC are widely accepted for studies for in vitro angiogenesis (Bach et al., 1998; Bird et al., 1999; Esser et al., 1998; Pollman et al., 1999) primarily because of the ease with which these cells can be obtained in large numbers. Nonetheless, having now established the cluster model using readily accessible HUVEC, it would now be interesting to expand these studies into work with cultured microvascular cells. There would also appear to be more biological sense in using such microvascular cells for the study of these microvascular events.

5.2.c. The Origin of Lipoblasts

Embryonic adipose tissue originates from mesenchymal cells (Poissonnet et al., 1983; Poissonnet et al., 1988), however, the exact cell type responsible for the hyperplastic increase of adipose tissue is not defined. Pluripotent cells form the bone marrow can be induced to differentiate into adipocytes in vitro (Cui et al., 1997), while stromal vascular cells isolated from adult humans and animals may also differentiate into adipocytes in some culture conditions (Hausman et al., 1985; Sorisky, 1999). Because of the intimate relationship between adipogenesis and angiogenesis, some earlier researchers suggested that adipocytes may be derived from endothelial cells (Deslex et al., 1987; Hausman and Richardson, 1983; Poissonnet et al., 1983). In the adipogenic wound healing model described in Chapter 3, we speculated that adipogenic healing might be specifically related to healing in muscle tissue, suggesting that pre-adipocytic cells reside in muscle or its associated fascia and connective tissues. However, some researchers have found that marrow stromal or related cells serve as a source for the continual renewal of cells in a number of non-haematopoietic tissues (Pereira et al., 1998). Also, circulating EC progenitors have been reported to become incorporated into sites of active angiogenesis from the peripheral circulating blood (Asahara et al.,
1997). Although it seems unlikely that such circulating progenitor cells contributed to adipogenesis in Chapters 3 and 4, this possibility could not be excluded on the basis of the experiments performed. The methodology used by Pereira et al. (1998) could be applied to this question to exclude the presence of such circulating progenitor cells. In this model, male mouse marrow cells were infused into irradiated female mice and detecting by in-situ hybridization for the Y chromosome (Pereira et al., 1998). Similar experiments with male and female mice could be performed, and the presence or otherwise of Y Chromosome positive lipoblasts determined in adipogenic implants.

Tracing the origin of fat cells in vivo can be difficult, while the labeling of proliferating cells by Brdu or radioactive thymidine lacks cell specificity and the absence of specific antibodies recognizing mouse adipocytes further adds the difficulty of the task. To address this, it may be possible to locally apply cultured populations of cloned genetically labelled specific cell types such as fibroblasts, endothelial cells or marrow cells to the tissues at the time of adipogenic implantation, and so identify the presence or absence of these cells in the adipogenic tissues once established.

5.2.d. The Isolation Culture of Lipoblasts

Although pre-adipoblastic cells are isolated and cultured from a variety of tissues (Carraro et al., 1990; Chen et al., 1997; Dani, 1999; Hausman and Martin, 1989; Hentges and Hausman, 1989), the absence of a convenient source of tissues containing significant numbers of true lipoblasts has made the isolation and pure culture of these cells impossible. The adipogenic healing model described in Chapter 3 provides an opportunity to culture such cells in large numbers. Enzymatic disaggregation of the tissues should be possible to create a single cell suspension. The lipid vacuoles in these cells should make separation from contaminant cells by a step-density gradient comparatively simple. The identity of cells would be easily determined by examination for lipid vacuoles, although seeding cells for culture may be complicated by the tendency of such buoyant cells to float. Inverted culture using transwell devices could permit the seeding of the undersurface of transwells
with lipoblasts, which could then be used to study the effect of purified signals upon adipocyte differentiation as well as the molecular biology of this process using biologically relevant cells.

5.2.e. Co-Culture of Lipoblastic and Endothelial Cells

Since an association between adipogenesis and angiogenesis is postulated, it would be interesting to determine the effect of co-culture of lipoblastic cells isolated as indicated in 5.2.h. with endothelium. The synthesis and transfer of LPL for example may be studied using trans-well model systems, or alternatively, by direct co-culture. Dependence of differentiation upon soluble or contact dependent phenomena could be investigated as would the rate of differentiation of lipoblastic cells in response to endothelium pre-treated with a range of cytokine and hormone factors thought to regulate adipogenesis (Ailhaud, 1997; Chen et al., 2000; Hausman and Yu, 1998; Wabitsch et al., 1996). Culture of endothelium with different basal levels of proliferation or apoptosis could be performed by using culture media known to encourage endothelial growth (full growth medium), have little effect upon growth and strongly inhibit endothelial apoptosis (M199 with 20% FCS), inhibit apoptosis but not permit proliferation (M199 with 4% serum albumin) or permit significant endothelial apoptosis thus representing vascular regression (M199 alone) (Zoellner et al., 1996a). In this way, it would be further possible to investigate the overall question of how cells interact to produce complex tissues.

5.2.f. Characterization of Sub-Populations of Lymphocytes in Adipogenic Healing

The extent of lymphocytic infiltration in adipogenic tissues was surprising, and varied significantly with different specimens. It may be valuable to characterize the lymphocyte populations in these tissues over time, using both immuno-histochemical and FACS analysis methods. Isolation of these cells may permit study of the interactions between the specific lymphocyte population in these tissues and cultured lipoblasts and adipocytes. In this way, it may be possible to further define any possible role of the lymphocytic population in the necrosis seen during auto-transplantation.
5.12 General Discussion

5.2.g. Adipogenic Tissue Transplantation

The potential clinical application of adipogenic healing was discussed in Chapter 5, while preliminary experiments attempting to graft these tissues into new sites demonstrated only partial survival of these auto-transplants. Donor site morbidity and graft stability are the two major problems encountered in auto-transplantation of fat by surgeons (Converse, 1977; Mandrekas et al., 1998). The possibility that newly formed adipose tissue within a fibrous sleeve may have improved survival has been discussed (4.1.d.iv) as was the advantage of developing adipose tissue in the site where it is needed.

It is possible that the poor survival of auto-transplanted adipose tissue seen in Chapter 4 could be significantly improved if micro-surgical techniques were applied to establish re-anastomosis of any significant vessels in the capsule of these implants. This may not be practical in the case of mouse tissues, but would be perhaps possible in larger animals such as pigs or baboons (please see 5.2.k. below). Similarly, since the inflammatory and immune responses may have contributed to the degeneration of transplanted tissues, it would be interesting to determine the effect of anti-inflammatory drugs upon the survival of auto-transplanted adipogenic implants. Also, because of time constraints it was not possible to attempt transplant of adipose tissue at different stages of development, and this is an additional experiment which should be performed as a consequence of this thesis. Finally, for adipogenic healing to have significant clinical impact, it would be important to determine the adipogenic response to a variety of resorbable surgical implant materials (Park and Ward, 1995; Shalaby, 1994).

5.2.h. Adipogenic Healing in Large Animals and Primates

Baboons are an accepted nonhuman primate model for the study of human physiology including pregnancy and diet related adipose tissue development (Green et al., 2000; Lewis et al., 1989). In baboon granulation tissue collected from drum implants, no obvious fat tissue formation was seen although isolated lipid containing cells were present in granulation tissue four weeks after
implantation. It is possible that this could be improved by encouraging further angiogenesis in these tissues by using growth factors for endothelium incorporated into the implant materials. This is supported by the comparatively low levels of vascularity in baboon reparative tissue (4.3.b.iii) as compared with that seen mouse experiments (3.3.a.iii). As discussed in Chapter 3, adipogenesis is closely related to angiogenesis (Bouloumie et al., 1998; Crandall et al., 1997; Wright and Hausman, 1990), while lipid filling of adipocytes is related to an increase in vessel diameter (Hausman and Richardson, 1983). Vascularization of the tissue may be promoted by many angiogenic growth factors. As discussed in Chapter 1, VEGF, PDGF, TGF-β and aFGF are potent angiogenic stimulators in vitro and in vivo (1.2.c.i, 1.2.c.iii-v). aFGF also has profound effect on stimulating adipose precursor cells (Teichert-Kuliszewska et al., 1992). PDGF and aFGF are reported to induce de novo adipogenesis in mice when co-injected with Matrigel (Kawaguchi et al., 1998) while Insulin like growth factor is another angiogenic growth factor also inducing adipogenesis (Yuksel et al., 2000). These growth factors can be delivered locally by using microspheres or osmotic pumps for continuos administration to improve angiogenesis (Ogawa et al., 1990; Yuksel et al., 2000).

Also, because baboons are exceptionally lean animals, it is possible that adipogenic healing was aborted due to a lack of circulating triglyceride. This may be circumvented by performing experiments in animals receiving a high fat diet or alternatively, by working in animals with much more extensive fat deposits such as pigs.

An excessive inflammatory reaction was observed in tissues collected from baboon drum and polyvinyl sponge implants, as well as mouse autotransplantation tissues. Further, there were foci of dense lymphocytic infiltrates in non-transplanted mouse adipogenic implants which seemed even more prominent after auto-transplantation. This suggested that the response to the implant material as well as perhaps the material from any necrotic fat may have either directly damaged adipose tissue or alternatively, reduced vessel number with the effect that adipose tissue either failed to form or became necrotic. This may also be overcome by using more biocompatible materials. Alternatively, anti-inflammatory drugs may be administrated either locally or systemically. Some
5.14 General Discussion

of these may have additional effects upon adipogenesis. For example, glucocorticoids are
generally used as a postoperative anti-inflammatory drugs, but also have a potent pro-adipogenic
effect when used locally (Hauner et al., 1987).

With regard to baboons, it is important to note that similar to rodents, adipose tissue formation
occurs primarily before weaning. However, in adolescent female baboons (2-5 yeas of age),
omentumal fat depots are reported to increase due to an increase in the fat cell number while S-V cells
isolated from baboons are able to differentiate to adipocytes in culture (Lewis and Soderstrom,
1993). If it is accepted that lipomas may represent adipogenic healing, it is noteworthy that these
lesions also are seen in non-human primates including baboons (Fiori et al., 1994; Seibold and
Wolf, 1973). For these reasons, it seems reasonable to continue work with baboons to determine if
they are capable of a clearly adipogenic response.

In mice, due to the relatively small size of the musculature, implants could not be buried in single
muscles and instead, several muscle bundles were dissected to accommodate mesh tubes. This may
have facilitated the involvement of connective tissue cells from the fascial sheath during wound
repair. From this, it is possible that the critical cells for adipogenesis did not come from the
musculature, but were primarily derived from the fascial sheath. If this is the case, then perhaps
implantation into the fascial plane in baboons may improve the adipogenic response.
Appendix
A.1. Introduction

Throughout the experimental period of this thesis, work was carried out which was either preliminary to that described in detail in earlier chapters, or alternatively was unsuccessful. In order to maintain the continuity and focus upon the more important aspects of the thesis, these preliminary or unsuccessful experiments were not described in the earlier Chapters. This appendix describes this work in the form of additional brief notes which can be read in conjunction with the relevant thesis chapters, or alternatively, may be read in isolation.

A.2. Materials Used in Work Described in the Appendix and not Indicated in Earlier Chapters of this Thesis

Goat anti-rabbit FITC conjugate and propidium iodide (PI) were from Sigma (St. Louis, USA). 4 μm pore size net-wells were from Costar (MA, USA). BrdU was purchased from Amersham Pharmacia Biotech (Buckinghamshire, England). Mouse anti-BrdU and rabbit anti-mouse biotin conjugates were from DAKO (Glostrup, Denmark). Streptavidin-peroxidase-conjugate was from Biosource (CA, USA). Other reagents were from the same sources described in 2.2.a.i –ii and 3.2.a.i.

A.3. Development of Surgical Implantation Procedures for Mice

A.3.a. Optimization of Anaesthetic Methods

Mice were initially anaesthetized with recommended doses of Ketamin and Rompom of 150 mg/kg and 10 mg/kg body weight respectively. However, this resulted in an unacceptably high rate of mortality approximating 30%. A series of experiments was performed in which the anaesthetic dose was reduced to lower levels, but where effective anaesthesia was still achieved as evidenced by the absence of movement or reflexes. The concentrations of drugs at which anaesthesia was still effective but the
survival rate approached 100% were 85 mg/kg of Ketamine and 4 mg/kg of Rompom. Anaesthesia lasted from 20 to 30 minutes, which was more than sufficient time for the minor surgical procedures involved.

A.3.b. Development of the Mesh Tube Implant Methodology

As mentioned earlier (2.4.f), this model was established with the initial intention of studying endothelial cell apoptosis during wound maturation. It was intended to establish a model in which the original margins of wounds could be clearly identified, and in which the size of wounds was identical for each animal. Early experiments involved the implantation of silicone cylinders filled with fibrin gels. These cylinders were prepared so that one or both ends were open and it was thought that granulation tissue would grow into these tubes to replace the fibrinous material at a quantifiable and predictable rate. By compelling the tissue to grow from clearly defined tube openings, it was thought that it would be possible to establish a precise system for defining discrete zones of wound healing. These silicone tubes were placed in a variety of sites including sub-cutaneous and intramuscular locations. Empty silicone tubes were also placed, as were metal and glass tubes of similar size. In all of these experiments, fibrotic encapsulation occurred around the implants while the tube lumina remained essentially empty, except for occasional heavy infiltrates of inflammatory cells. Importantly, the objective of compelling granulation tissue to grow from clearly defined starting sites was not fulfilled. This appeared to reflect insufficient access of the vascular tissues to the wound space. Mesh tube implants were prepared in an attempt to increase the access of vascular cells, but despite this, attempts to establish unambiguous granulation tissue formation in sub-cutaneous sites using these implants was still unsuccessful. Despite these disappointing results, granulation tissue was observed growing into implants placed intramuscularly and this defined the wound healing model eventually used in Chapter 3.
A.4. Preparation of HUVEC Clusters for Confocal Laser Scanning Microscopy

A.4.a. Development of Methodology for Confocal Laser Scanning Microscopy of HUVEC Clusters

HUVEC cultured in bacterial culture dishes for from 0 to 24 hours as described in 2.2.b.iii, were harvested by gentle washing with M199 and pelleting by centrifugation. Early experiments with clusters air dried or fixed to slides indicated that clusters were too fragile to survive such processing with their three-dimensional relationships intact. Instead, an alternative method using agarose suspension was developed. Briefly, cell pellets were fixed in formaldehyde (10%) for 10 minutes and washed in PBS by gently removing the fixative and replacing this with fresh PBS. The pellets were then resuspended in agarose gel (1%) heated to 60°C, and pipetted into 4 μm pore size net-wells to set at room temperature over a period of at least 10 min. 200 μl volumes of UEA-I (0.01 mg/ml) were applied to the tops of these gels and the net-wells placed on to the tops of centrifuge tubes containing tissue paper in such a way that the capillary action of the paper drew the fluid through and into the gels (Fig. A.1). Gels were then washed with PBS by applying 1 ml volumes to the gels and allowing this to be drawn through into the underlying tissue paper. The gels were then further incubated with rabbit anti Ulex lectin-I antibody (0.01 mg/ml) and after a further wash subsequently with goat anti rabbit FITC (0.01 mg/ml). All three antibodies were diluted in PBS with FCS (10%) and Tween 20 (0.5%) and applied to the gel for 45 minutes at room temperature. Finally, the gels were treated with propidium iodide (2 μg/ml) for 45 minutes and washed extensively with distilled water in the dark. Gels were removed from net-wells prior to cutting into smaller flat pieces and transfer to glass slides. Coverslips were then applied with gentle pressure and sealed using nail varnish. Controls consisted of cells not exposed to the primary lectin label. Cells were observed using a Biorad confocal microscope.
A.4. Appendix

A.4.b. The Experimental Objectives of Con-Focal Microscopic Studies Were Not Achieved

Fig. A.2 shows the result of such an experiment in which UEA-1 was found to label cells diffusely rather than in a membrane specific manner. Propidium iodide did, however, clearly localise to nuclear material. The original intention of the con-focal microscopic studies was to further characterise the patterns of adopted by endothelium in the clusters described in Chapter 2. Since, however, proper membrane localisation was not achieved using UEA-1, this experimental objective was not achieved. Unfortunately, there was insufficient time to pursue other alternative studies using this methodology, to characterise the distribution of intercellular adhesion molecules such as PECAM-1, VE-CAD or Con-43, or alternatively using non-specific fluorescent membrane dyes such as STYO-22.

Nonetheless, despite this disappointing outcome, the location of condensed propidium iodide labelled particles as seen by con-focal microscopy was supportive of observations made in Chapter 2 (Fig. A.2).

A.5. BrdU Labelling of Cells and Tissues

A.5.a. BrdU Labelling of Re-seeded Clustered HUVEC

A.5.a.i. Methodology for BrdU Labelling of Re-seeded Clustered HUVEC

Cells were collected 4 hours, 24 hours and 48 hours after culture on non-adherent bacterial culture plates as described in 2.2.b.iii, and replated onto a gelatin coated tissue culture cover slips for further culture in full EC growth medium for 24 hours. Cells were re-fed with fresh medium containing Brdu (20 ng/ml) and incubated at 37°C for 30 minutes. Coverslips were then washed twice with PBS and immersed in 1.5 mM HCl for 20 minutes before neutralizing the acid by immersion of coverslips in borax buffer (0.5%) for 20 minutes. The coverslips were then incubated with biotin labeled mouse anti Brdu (1mg/ml) for 1 hour, followed by washing with NaCl (0.9%) and incubation with a rabbit anti
mouse streptavidin-peroxidase conjugate. Color was developed by immersing coverslips in DAB solution as described in 2.2.b.vii. Cells were counter stained with haematoxylin and coverslipped before examination by light microscopy.

A.5.a.ii. BrdU Labelling Confirmed that HUVEC from Replated Clusters Were Capable of DNA Synthesis

Nuclear labelling was observed in all cultures of re-plated clustered cells (Fig. A. 3), confirming the morphological observations described in Chapter 2 indicating the viability of cells surviving matrix deprived culture in clusters. This work was not included with the data shown in Chapter 2, as the viability of cells seemed self-evident on the basis of mono-layer formation alone, and these experiments with BrdU did not seem to add significantly to the obvious morphological observations.

A.5.b. BrdU Labelling of Mouse Granulation Tissue

A.5.b.i. Methodology for Labelling Mouse Tissues With BrdU

Mice were implanted with mesh tubes as described in 3.2.b.ii. 3 of these mice received intra-peritoneal BrdU (200 µg/kg) injections for 3 consecutive days starting from the 10th day after implantation surgery while 2 mice received Brdu 8 weeks after surgery. The mice were sacrificed four days after their first injection by CO₂ asphyxiation and granulation tissue from mesh tubes was collected for the preparation of paraffin sections as described in 2.2.b.v. Paraffin sections were deparaffinised and rehydrated as previously described (2.2.b.viii) and BrdU labelling detected as described above (A5.a.i).

A.5.b.ii BrdU Labelling of Mouse Tissues Indicated Proliferation of Granulation Tissue But Was Insufficiently Strong to Confirm Proliferation of Lipoblastic Cells

The objective of this study was to demonstrate BrdU positive lipoblasts, confirming their origin by cell proliferation. However, the only BrdU positive cells found were very occasional cells in the peripheral
regions of the granulation tissues. It was clear that the dosage of BrdU used was insufficient to perform this experiment properly. Unfortunately, limitations of carrying out further such experiments using more frequent administrations of BrdU at higher concentrations were not possible due to time constraints. An alternative approach may have been to use an osmotic pump to apply this nuclear label (Breider et al., 1999). The minimal observation of some positive cells in granulation tissues did, at least, support the assumption made in Chapter 3 that the tissue in tube lumens was proliferative in origin.
Fig. A.1. Cell staining unit used for preparation of the clusters for confocal microscopy described in A.4. Antibody and wash solution applied to the top of the apparatus directly onto the agarose gel material containing cells was soaked down into the underlying tissue paper by capillary action, creating a form of "flow cell" for labelling suspended experimental cells.
Fig. A.2. CLSM image of HUVEC as single cells and clusters labelled with *UEA-I* and PI. These cells were collected 0 (A), 12(B, C) and 24 (D, E) Hr after matrix deprivation (2.2.b.iii). (A) At 0 Hr, HUVEC displayed large visicular nuclei (arrows) and had plentiful of cytoplasm (arrowheads). (C, D) Different sections of a cluster 12hr after matrix deprivation, (C, D) single cells were small and condensed (open stars) while the nuclear fragmentation typical of apoptosis was seen (arrows). This contrasted with cells in small clusters which appeared non-apoptotic (stars). (D, E) Two different sections of a cluster 24 Hr after matrix deprivation, cells in the periphery of the clusters had nuclear material which was elongated (arrows), while apoptotic particles were mostly centrally located (arrows plus open stars). These finding were largely consistent with the result of TEM.

(Bars in A and B = 10 μm; Bars in C-E = 5 μm)
Fig. A.3. Photomicrograph of HUVEC initially in clusters labelled for BrdU after re-seeding onto an adherent gelatine matrix (2.2.b.iii). HUVEC were re-plated on gelatin coated coverslips after 24 Hr of matrix deprivation, and incubated in normal culture medium for 24 Hr. Proliferating cells were clearly labelled by BrdU (arrows), while non-proliferating cells did not up-take BrdU (arrowheads).

(Bar = 10 μm).
References
R.1 References


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R.2 References


R.3 References


R.5 References


R.11 References


R.24 References


References


R.27 References


R.28 References


