Endothelium and spontaneous angiogenesis

It was important that only rapidly proliferating cells were used for each experiment, to ensure consistent speed in network formation. Monitoring of vigour of a cell culture was important, with a cell doubling time during this proliferative phase being between twenty four and forty-eight hours (Figure 8-A,B,C) until confluence was achieved (Figure 8-D). Confluent endothelial cultures were passed by dispersion in trypsin/EDTA (Figure 8-E,F,G) and divided by placement into new single use flasks of double the surface area. After sufficient numbers have been cultured, excess cells were stored in liquid nitrogen until required. On thawing, these cells had a vital recovery rate of 60% ensuring a near confluent 25cm² flask. Two such confluent cultures were needed for each experiment.

The vigour of the endothelial cell culture was also assessed by the capacity to undergo spontaneous angiogenesis in vitro. This involves network formation of cellular cords arising from confluent monolayers of endothelial cells which were grown on unmodified tissue culture plastic (Figure 9-A-D). The monolayer has a characteristic cobble-stone appearance. Cells were allowed to proliferate rapidly to confluence in medium with a high level of endothelial growth factor. Then at confluence, the cells were fed with fresh medium and then all further feeding was withdrawn.

The cells form processes towards each other and eventually form cell to cell contacts. Once 4-5 cells are connected a branched network is established with elongated and bipolar cells with multicellular retraction. Cords form which later become free floating, with contracting multicellular junctions, attachment to the flask base is at intermittent points only. After maturation the original endothelial cells were usually found at the intersection of the three tubes in a capillary network of multiple layers. Remodelling occurred over the next week with new branches appearing, others contracting and points of attachment changing.

This model is inconvenient for experimental use since it takes many days to form, initially occurring in the central area between confluent and thinner seeding and only occurs in 50% of cultures. The network is not robust due to its intermittent attachments and could not withstand rinsing.
Figure 9: Spontaneous angiogenesis *in vitro* of human umbilical endothelial cells.

These figures show spontaneous angiogenesis achieved after rapid proliferation followed by starvation and stress. These particular networks took 10 days to form and as can be seen have a number of dead cells floating above the network (Figure A). The network was composed of free floating cellular cords, attached to the flask base by attenuated fibres. At low power the problems with poor light distribution can be seen (Figure A). At medium power the networks are easily visible, the branches are thin with cells aligned along them(Figure B,C). At high power the network junctions have multicellular clumps, with cells in a bipolar arrangement along the connecting branches (Figure D). These network were very fragile and on rinsing with PBS to remove the floating cells the networks were destroyed.

A: 5x low power magnification (note problems of uneven light distribution)

B and C: 10x medium power magnification

D: 20x high power magnification
Results

Optimisation of experimental conditions

Forming gels with rat collagen type I (0.1mg/ml) proved unpredictable and difficult, so Matrigel was used, as described earlier and proved satisfactory for the period of experimentation.

The logistics of this technique required only individual cultures to be maintained at any one time. The medium, trypsin/EDTA and PBS were warmed to 37°C in the incubator over a 30 minute period. Simultaneously, the Matrigel which had been thawed for at least 12 hours and stored at 4°C, was plated out in the previously cooled plates and then allowed to set at 37°C in the same incubator for 30 minutes.

The cells were checked for confluence and health, then passaged. An accurate count was made using a haemocytometer. The count was repeated, when cell distribution was uneven or an adjustment in cell concentration was necessary, until an even cell concentration of 80,000/ml was achieved. The volume needed for the ideal number of cells was calculated from the known cell concentration and the cells were plated onto the Matrigel and network formation was allowed to proceed for 6 hours. Initial problems with consistency of cell numbers especially in the second seeding which was even more critical than the first, with only a margin of error of +/- 500 cells per well, were overcome by using one designated calibrated pipette for all cell volume measuring.

The plates were examined 1 hour into network formation to check cell concentration and distribution. After a further 5 hours initial networks were observed (Figure 10-A-M) and concurrent controls were further viewed over the experimental period.

The majority of endothelial cells adhered rapidly to the Matrigel by 15 minute after seeding (Figure 10-A). At thirty minutes, the cells were rounded and adherent in a pattern still discernible 4 hours later (Figure 10-B). By one hour (Figure 10-C) the cells had assumed a more characteristic form, being more spindle shape, elongating, aligning and beginning to associate with each other. Over the next hour migration decreased, along with increased elongation and flattening of the endothelial cells (Figure 10-D,E) and a distinctive tessellated pattern emerged.
Figure 10: HUVEC network formation under ideal experimental conditions.

This sequence of photographs shows the typical pattern of network formation and maturation. Within 30 minutes from seeding the endothelial cells have adhered to the flask base, the cells are separate and spherical (Figure B). By one hour the cells assume a more polygonal characteristic shape, are elongated and spindle shaped and begin to align and associate with neighbouring cells (Figure C). Over the next hour the cells progressively elongate and flatten with the beginnings of a distinctive tessellated pattern emerging. By two hours gross migration is complete and the resultant network pattern easily discernible (Figure E). By four hours the network is well formed but the branches are still not streamlined (see arrows Figure I compared to Figure M). By six hours the network is relatively mature, with little change in the previous 30 minutes, the branches are thin and the junctions are multicellular clumps, while cells are in a bipolar arrangement along the branches (Figure M). The Matrigel is also beginning to be pulled up by the cells. By eight hours the network is much more open and the branches appear thinner and less numerous (Figure N). By eighteen hours the junctions are obviously multicellular and the branches thinner and longer with the size of the tessellation greatly increased. Distortion of the Matrigel is present (Figure O). By forty two hours the network is beginning to denature with increasing numbers of dead cells present and branches appear attenuated and some actually broken (compare arrows in Figure O and P).

A: 0 hours  
B: 0.5 hours  
C: 1.0 hours  
D: 1.5 hours  
E: 2.0 hours  
F: 2.5 hours  
G: 3.0 hours  
H: 3.5 hours  

Continued on second page

I: 4.0 hours  
J: 4.5 hours  
K: 5.0 hours  
L: 5.5 hours  
M: 6.0 hours  
N: 8.0 hours  
O: 18 hours  
P: 42 hours
Results

By two hours post-seeding (Figure 10-E), all gross migration was completed, with only minor modification remaining. The two hour network pattern was still recognisable six hours later. Hence a two hour time period for observing the second seeding, was felt to be long enough to show any initial preferential migration due to the possible influence of the original basement membrane or Matrigel, yet short enough to avoid any modification of this influence.

Over the next 4 hours the cells assumed a more flattened and distended appearance along the forming branches, while the islands of endothelial cells became clumped together and raised (Figure 10-E-M). All single bipolar cells rested on a single matrix line which passed directly beneath the long axis of the cell. Multicellular cords revealed an underlying line of basement membrane and assumed bipolar formation on contact with it. Isolated cells did not alter shape or move unless contacting the matrix line. By 6 hours the networks appeared relatively stable.

After 6 hours the network continued more slowly to mature, with constant remodelling resulting in some lines constricting, while others opened. Reorganisation and distortion of the underlying Matrigel was observed, with all the matrix lines being straight. The interconnections became thinner, lengthened or shortened and changed angulation to other matrix lines or cords in close proximity. There were a diminished number of matrix lines between the islands, which were in turn contracting and becoming increasingly raised from the well base. The spaces between the branches and clumps of cells became greater. The Matrigel could be seen to be organised underneath the endothelial cells and was pulled up from the base eventually causing perforations in the gel enlarging all the time, leaving the original plastic surface exposed.

By 20-24 hours the networks had pulled nearly all the basement membrane gel into islands on which the cells rested. Each remained connected by narrow straight bands of matrix cables elevated above the base of the well. Along the whole length of these cables cell migration occurred. These networks had become too fragile to resist repeated rinses and re-seeding necessary for the experimentation, hence 18 hours old networks were used. By 42 hours the cells appeared distressed and the number of dead cells present had greatly increased.
Six hour endothelial cell networks

At 6 hours the HUVEC networks appeared to be random, the branches or interconnections between islands of cells, were thin, with cells elongated along their length. The branches and cells appeared raised from the surrounding Matrigel, with the Matrigel in the immediate vicinity appearing re-organised by the cells. There was evidence of traction centres and traction forces on the gel, which as the network ages continued to contract until the gel separated from the underlying base (Figure 11).

Eighteen hour endothelial cell networks

By eighteen hours the networks are considered, according to literature, to be mature with the networks consisting of vascular tubes (Kubota et al., 1994; Grant et al., 1994) and are considered ideal to investigate the influence of mature networks on re-vascularization. The networks are unique, the branches are thin and appear to be strung and held up by the multicellular junctions which are raised higher than the branches. The Matrigel can be seen to be pulled up by the cells towards the network, despite using double the amount of gel than for a typical six hour network, areas of denuded well base appeared (demonstrated in Figure 12-A & B). The gaps between the branches are large with fewer attachments to the flask floor than at six hours.
**Figure 11:** Six hour HUVEC network formation.

Comparing these photographs, each network is random and unique, with each network being composed of numerous branches with bipolar cells aligned along them and multicellular clumps at the branch junctions. The junctions appear to be lifting up from the base of the flask carrying the Matrigel with them.

Each network displays a varying degree of effect on the Matrigel with significant changes observed in figures B, D and especially F (arrows depict areas where the Matrigel has been drawn up and organised). Other networks appear to have very little effect on the Matrigel. This effect on the gel appears to demonstrate the presence of traction centres and traction fields.
Results

To determine whether the original basement membrane influenced re-vascularization, the endothelial cells comprising the vascular networks needed to be killed quickly to prevent any release of hydrolytic enzymes by the dying cells, thus ensuring the basement membrane and Matrigel remained intact. The Matrigel would then support a second seeding of cells capable of network formation. Various solutions including metabolic inhibitors were tried at 37°C to kill the cells. Most of the cocktails tried, for example: 2% Lignocaine, Xylocaine and diaminobenzamidine; sodium azide at 0.1-0.2% or iodoacetamide 10mM and 0.2% wt/vol sodium azide, took more then fifteen minutes to kill the cells and most had irreversible effects on the Matrigel, causing any re-seeded cells to die before network formation could commence. Local anaesthetic agents 3% Cistanest and Octapressin were used with the aim of detaching the cells but caused the Matrigel to instantly disintegrate.

Eventually hypertonic saline (0.5g Na Cl / 20ml water) and hypotonic water were tried. Both effectively killed the endothelial cells rapidly, but the hypertonic saline resulted in more damage to the Matrigel. Graduated concentrations from 100% medium to 100% sterile water, then up to 100% medium, were used at two minute intervals while maintaining all solutions at 37°C. The water still resulted in the Matrigel becoming more granular and the resultant change in light scatter produced less clear images of the network in the second re-seeding. A high degree of organisation of the initial network and gel resulted in more distortion after exposure to the hypotonic water.

Relocation of field to be observed

After network formation, ideal locations in the tube networks were located for observation of a second seeding. Unfortunately an ideal initial network did not necessarily locate an area for a good second network formation. An area chosen for good contrast at six hours, was not always the best area for photography once the cells had been killed and re-seeded.

Difficulties were met in the location of a reproducible area of observation. Various methods of relocating the chosen field were tried including: inscribing the base of the culture chambers prior to seeding and imprinting three marks around a central field for location. Culture chambers pre-
marked with a grid proved better, but the focus level of the grid was so
different to the cells that light became defracted while travelling up through
the base of the well resulting in a distorted image of the network. Finally,
the cultures to be tested were located using a template of the 24 well plate
fixed to the microscope stage.

Although the original network pattern was discernible after killing and re-
seeding the precision of relocation was not sufficient to monitor more than
one field at a time during each experiment. This field was photographed,
exposed to water, then the osmolarity returned to normal and a second
seeding took place. Relocation was directed using the template and fine
adjustment done manually. After this second seeding the plate was left in
position for the remainder of the experiment. The necessity of early fixation
of the field to be observed often resulted in the area being followed not
being the best field on the plate at the end of the experiment, with frequent
localised areas of over and under seeding being unpredictable.

**Experimental results for re-seeding 18 hour cell networks**

Cell networks of 18 hours, were considered the latest time that the network
could withstand exposure to hypotonic water and re-seeding without
undergoing severe damage. It was felt that 18 hour networks would
represent true vascular tubes. Controls were run concurrently with each
eighteen hour experiment as described in Materials and Methods. The
results were all identical for five consecutive runs. The network became
raised above the base of the well and the Matrigel pulled up with it. On re-
seeding, the new cells settled at the lowest point being on the well base
and remained there forming a typical monolayer population unable to
migrate up towards the network of basement membrane. Further, no new
networks formed (Figure 12).

Different focal levels demonstrated the height of the network from the base.
In Figure 12-A the image focused on the network while the Figure 12-B it
is focused on the re-seeded cells.

Because of the failure of the re-seeding to form a second network after the
original network was allowed to mature for 18 hours, relatively stable six
hour networks were used.
Figure 12: Eighteen hour re-seeded endothelial cell networks.

These photographs show a typical eighteen hour network which is raised significantly from the well base, demonstrated by using the different focal levels of each of these images, "A" is focused on the network while "B" is focused on the well base. The branches are thin and fragile, appearing as if held up by the multicellular junctions which are raised higher than the branches. The Matrigel can be seen to be pulled up by the cells towards the network, leaving areas of denuded well base, despite using double the amount of Matrigel than for a typical six hour network. On re-seeding, the second phase of cells settled on the well base and remained there forming a typical monolayer population unable to migrate up towards the network. No new networks formed.

A: 18 hour network after exposure to water and re-seeded with an ideal number of endothelial cells, focused on the original network.

B: Identical field as above, focused on the well base.
**Results**

*Experimental results for re-seeding 6 hour cell networks*

Six hours after seeding the original networks were killed and re-seeded, taking up to 1 hour, the second network was then observed at 15 minute intervals over the next 2 hours, then hourly for the next 4 hours. Unfortunately photographs could not be taken automatically due to the necessity for fine adjustment of the focus for each picture. To ensure that the second network was viable and had similar characteristics to the original, the maturation of the second network was observed up to 42 hours after seeding. As Figure 13 shows maturation occurred in a similar manner to the previous network, with cells aligning and forming a tessellated pattern by four hours, then remodelling to form thinner branches and more open networks over the next 4 hours. The replacing networks were still viable 18 hours after seeding, although the branches appeared attenuated.

For each 6 hour experiment the experimental period took 14 hours, while the 18 hour experiments spanned over 24 hours. This allowed only one experiment to be completed in a 24 hour period. For each experiment two confluent cultures were needed, one for each seeding for good network formation and all three controls were run concurrently.
Figure 13: Maturation of re-seeded HUVEC networks.

Photographs show the normal maturation of a six hour network exposed to hypotonic shock and re-seeded following procedures described in Materials and Methods. At the time of re-seeding the previous network is easily visible and all re-seeded cells are still in suspension and out of focus (Figure A). By 2 hours the cells have settled and started to migrate towards the original network, but a number of cells still remain random in the areas between the branches. The individual migrating cells are elongated, spindle shaped and appearing to pull towards each other. Some cells remain spherical and separate (Figure B). By four hours a tessellation pattern is visible with branches appearing ill formed, with some cells still disassociated with the forming network (Figure C). By six hours the branches are streamlined, the cells all appear associated with the new network (Figure D). The network continues to mature and consolidate over the next two hours with the matrix lines straightening (see arrows Figure D compared to E). The network continues to mature up to 18 hours post re-seeding, when the network is still intact but becoming more attenuated with fewer branches and the Matrigel well raised up from the well base (Figure F). Maturation of the second network was similar to maturation of the original network.

Time of re-seeding

A: At time of re-seeding

B: 2 hours post re-seeding

C: 4 hours post re-seeding

D: 6 hours post re-seeding (arrow shows branch still not straight)

E: 8 hours post re-seeding (arrow shows branch now straight)

F: 18 hours post re-seeding
Experimental results for re-seeding 6 hour cell networks

(Figures 14 & Table 1)

Re-seeding of initial six hour networks after killing with hypotonic water was repeated over 30 times. Concurrent with each experiment all three controls were run to maintain consistent technique and effectiveness of the procedures. As mentioned above the Matrigel appeared granular and more fragile after hypotonic lysis of the endothelial cells. The new networks underwent the same maturation changes as the original 6 hour network with increasing spaces forming and diminishing numbers of branches which become raised from the well (Figure 13).

Results can be seen summarised in Table 1. In 25 of the 30 cases, the second seeding appeared to settle randomly on the Matrigel and within 30 minutes the cells showed a tendency to migrate towards the initial network. This migration was as rapid as in initial network formation. The cells then aligned and formed a new network with branches superimposed on the original tracks. Additional branches were present, proving that the Matrigel is still capable of supporting network formation throughout the plate even after exposure to the experimental condition previously described (Figure 14).

In cases of the second seeding being very low the network superimposed on the previous network with no new branches and some of the original branches missing, the branches formed were no thicker than in the original network. If re-seeding was identical to the original seeding, the branches pre-existing appeared the same thickness and up to 5 new branches could be identified (Figure 14-B). As re-seeding numbers increased slightly more branches were formed and as the re-seeding number progressively increased so did the thickness of the branches, resulting in a grossly thickened network super-imposed on the original network, with the space between branches greatly reduced (Figure 14-A).

In three of the experiments the second seeding resulted in a slow migration with the second network not fully forming until much later. This seemed to occur in cases where the original 6 hour network was highly organised having raised the Matrigel up from the base of the well (Figure 11-B,D,F,G).
If re-seeding numbers were low as in two of the experiments, not all the original branches were re-formed.

Of the two experiments that showed no similarity, distortion of the Matrigel was significant and re-seeding numbers were low. The two experiments abandoned were due to the control for the second seeding not forming a cell network within the experimental period.

Computer-manipulated images helped to demonstrate more clearly the degree of superimposition. The first images (Figure 14 second page, left hand side) show the original image cleared of background detail and coloured red, superimposed under the untouched second network image still in black and white with 40% opacity. The second images (Figure 14 second page right hand side) demonstrate the original and second images with both backgrounds cleared, the original coloured red and the second coloured blue then superimposed as before at 50% opacity, with areas of purple indicating the degree of overlap. As can be seen significant areas of overlap are visible.
**Figure 14:** Original networks are shown together with the two hour re-seeded networks.

*Photographs on the first page* show the original networks on the left with the paired 2 hour old re-seeded networks on the right.

All networks are clearly unique and vary in number and thickness of branches. Although the field of view has altered minimally the original patterns can be identified in the second images. In example 'A' the second network had few extra branches but the branches present are greatly thickened (⇒). In 'B' the original network is clearly visible as indicated by '→', yet there are a number of extra branches (∧). Example C has a less distinctive pattern, the multicellular junctions are identified with '×' on each photograph and the original branches indicated by '∧', the second image is rotated slightly from the first. In example 'D' the original network is distinctive. Unfortunately the field is slightly shifted to the right and rotated, but the original branches are identified by '→' and junctions with '×'.

*On the second page, are the same images as on the first page,*

*On the left side:* the original images have the background cleared leaving the network alone. This was achieved using the computer image manipulation software, Adobe Photoshop, on a Power Macintosh computer. This image was coloured pale red and then the image of the second network floated on the manipulated original image until superimposed and then merged.

*On the right side:* the same images are both manipulated to remove the background detail; the second images were coloured blue and the original image coloured red. These images were then superimposed and merged. The areas appearing purple are the regions of superimposition.

**KEY**

- Branches present in the original and second networks are labelled with '→'
- Islands are indicated with '×'
- Extra branches are labelled '∧'
- Thickened branches labelled with '⇒'

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Table 1 Co-incidence of the re-seeded network with the original cell network.

Thirty two independent experiments are summarised:

<table>
<thead>
<tr>
<th>Abandoned</th>
<th>No Similarity</th>
<th>Slow Migration</th>
<th>Cooper</th>
<th>Variations from original network</th>
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<tr>
<td></td>
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<td></td>
<td>Abnormal Braches</td>
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<td>Extra Braches</td>
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<td>2</td>
<td>2</td>
<td>3</td>
<td>25</td>
<td>2</td>
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</tbody>
</table>

In 25 of these experiments carried out as detailed in Materials and Methods, the second seeding appeared to settle initially at random on the Matrigel, then within 30 minutes the cells showed a tendency to migrate rapidly towards the initial network. The cells aligned and appeared to form a new network superimposed on the original network. In addition to these superimposed branches, extra branches were present; the number depended on the re-seeding cell numbers. These extra branches proved that the Matrigel was still capable of supporting network formation. In heavier re-seeding, branches appeared thickened as well as increased in number.

Slow re-seeding occurred in highly organised original networks (3 experiments) and in the two that had no resemblance to the original networks, the Matrigel was distorted and re-seeding numbers were low. The two experiments abandoned were due to the second seeding control not forming a network.
Results

Controls

Controls for 6 hour networks

At least three controls were run concurrently with each six hour experimental run and each control was recorded separately on three consecutive occasions (Figure 15). For each experiment, three initial wells were seeded and one plated with Matrigel alone and covered with media. These were observed after the 6 hour period.

The best initial network was chosen for the experiment and re-seeded at the same time as,

- one culture was chosen for killing and no re-seeding,
- one culture was left undisturbed to continue maturation.
- the remaining unseeded well, was exposed to hypotonic conditions, returned to normal osmolarity and seeded for the first time.

At the end of each experiment, the original undisturbed well was observed for normal maturation (Figure 15-B,C). As can be seen little network modification had occurred, with the cells remaining healthy. The treated well showed no modification of the network pattern and the cells appeared dead (Figure 15-D, E). The control seeded at the same time as the re-seeding for the experiment, showed a normal rate of network formation as expected in the original seeding (Figure 15-A).

Similar controls were run on eighteen hour networks to ensure that a similar effect was experienced on a mature network as on six hour network. (Figure 16-A-E).

The Matrigel was capable of network formation after having been gelled for 18 hours, stored covered with medium in an incubator, then exposed to hypotonic conditions and returned to normal osmolarity, even though an obvious granular appearance was observed. The eighteen hour network in the second control matured at a normal rate indicating that suitable conditions remained throughout the experiment for normal network maintenance. The third control, the eighteen hour network, was exposed to the hypotonic conditions, then returned to normal osmolarity and observed two hours later. It showed an unmodified network and the cells appeared dead. Distortion of the Matrigel was significant throughout the experiment.
Results

Figure 15: Six hour controls.

These photographs show the controls run concurrently with each experiment to ensure a consistency in procedure and efficiency for each part of the experiment. Figure A shows a two hour network forming normally even though it had been seeded after the Matrigel had gelled and been covered with medium for six hours, then exposed to experimental hypotonic conditions and returned to normal osmolarity ready for seeding. This checked that the second seeding numbers were supportive for a second network to form. The second control (Figure B and C) shows a six hour network originally seeded at the same time as the experiment, but allowed to continue maturation undisturbed. As can be seen the network is still intact and very similar to the original six hour network. This proved that the experimental conditions allowed normal maturation to occur. The third control (Figure D and E) shows a six hour network seeded at the same time as the original network, allowed to mature for six hours, then exposed to the same hypotonic conditions as the experiment and returned to normal osmolarity and covered with medium for the two hour experimental period. This proved that the treatment was effective and recovery of the cells did not occur. As can be seen in the second image the cells are spherical, separated and appearing dead with no change to the network pattern.

A: Matrigel gelled for six hours, then exposed to water and re-seeded with an optimal number of cells
B: Endothelial cell network six hours old
C: Identical location observed two hours later
D: Endothelial cell network six hours old
E: Network exposed to water and observed two hours later
**Figure 16:** Eighteen hour controls.

These photographs show the same controls as for the six hour networks, run on mature 18 hour networks which are believed to have tube formation. The first image (Figure A) shows a two hour network formed on Matrigel that had been allowed to gel, covered with medium, stored in an incubator along with the 18 hour experiment, then exposed to hypotonic conditions concurrent with the experiment and seeded with an optimal cell number. The formation of a network showed that the gel although obviously now granular with stress lines easily visible, was still capable of supporting network formation and that the cell number for the second seeding had been correct for good network formation. The second control (Figure B and C) shows an eighteen hour network recorded then left undisturbed for another two hours. This proved that the conditions in the incubator allowed normal maturation to occur and the cells remained viable and the Matrigel intact. The third control (Figure D and E) shows the 18 hour network prior to exposure to hypotonic conditions and observed two hours later. The cells appear separated, spherical and dead in the second photograph and the network is unmodified suggesting the cells have not survived the hypotonic water. Distortion of the gel was clearly visible for this network with areas of denuded well-base obvious.

C: Matrigel 18 hours old exposed to water and seeded with an ideal concentration of endothelial cells then allowed to network for two hours

D: Cell network 18 hours old

E: Identical field two hours later

F: Cell network 18 hours old prior to lysis

G: Identical field two hours after lysis
Results

Sections

Transverse sections of re-seeded 6 hour networks (Figure 17 A, B)

Two re-seeded networks were sectioned perpendicular to the Matrigel surface. The Matrigel appeared condensed underneath the endothelial cells which were still on the gel surface (Figure 17-A, arrows). The live cells were flattened and grouped in clumps of two or three, while some dead cells were observed floating off the gel surface. As the re-seeded network matured, to six hours old, the cells continued to pull up gel and form clumps becoming raised from the surrounding gel. There was evidence of tube formation commencing and the occasional invasion of the gel (Figure 17-B).

Sections parallel to gel surface, re-seeded 6 hour networks (Figure 17 C, D, E, F, G)

Dead cells can be observed detaching from the Matrigel surface, while live cells are flattened and attached to the gel surface and along pre-existing clefts present within the Matrigel. The cells send out cell processes, appearing to feel along the clefts past dead cells and eventually travel along the clefts. Tube formation is visible in Figure 17-C (T) four hours after re-seeding. In Figure 17-D the potential distance the cell process can extend is demonstrated with the cell process indicated “P” associated with the cell body labelled “L”. Figure 17-E, F, G are transverse sections 6 hours after re-seeding and show the intimate relation of live cells with cell processes to the gel surface, the clefts and to the dead cells.

Transmission electron microscopy (TEM) sections (Figure 18)

The TEM sections were taken at varying magnifications. Figures 18-A and B shows dead cells, present at two and six hours after the re-seeding of a six hour network, thus proving that lysis was complete. Figures 18-C and D show live cells, the first at two hours after re-seeding has cell processes appearing to extend towards the top of the photograph, possibly feeling for a path of least resistance, collagen fibril or chemical stimulus from a pre-existing cell, for example in Figure 18-B. At six hours after re-seeding the cell processes are extended uni-directionally towards a stimulus at the top of the photograph. These cell processes can be clearly seen in Figure 18-E extending along the cleft left in the Matrigel and towards neighbouring cells in Figure 18-F which shows the relations of the three cells.
Figure 17: Section of fixed networks stained with Toluidine Blue.

Photographs show (40x magnification) re-seeded networks of varying ages. The dead cells can be observed detaching from the Matrigel surface in all the sections, while live cells are flattened and attached to the gel surface (Figure A) and along pre-existing clefts present in the Matrigel after four hours post-re-seeding (Figure C & D). The cells appear to have sent out cell processes, feeling along the tunnels around the dead cells and eventually the whole cell travelled along the tubes. Tube formation is visible in Figure B (T) six hours after re-seeding. In Figure D the potential distance the cell process can extend is demonstrated with the cell process indicated “P” associated with the cell body labelled “L”. Figure E, F & G, show transverse section 6 hours after re-seeding, again the relation of live cells with cell processes to the surface and tunnels in the gel.

Key Magnification 40X

| L | - live cell |
| D | - dead cell |
| T | - tunnel    |
| P | - cell process |
| → | - Matrigel organised |

A and B show photographs of transverse sections.  
A : 2 hours after re-seeding a killed six hour network, cells are on the gel surface and dead cells are floating off.

B : 6 hours after re-seeding a killed six hour network. The relation of the live to dead cells is clearly visible and tunnel formation is commencing.

C and D show photographs of a section taken parallel to the gel surface 4 hours after re-seeding of a killed six hour network. 
The relation of the live to dead cells is visible and cell processes extending along the clefts in the Matrigel appear to feel past existing dead cells.

E: F; and G: are all 6 hours after re-seeding a previously killed six hour network. 
Dead cells are present trapped in the clefts of the Matrigel or lifting off the gel surface and the live cells are flattened on the gel surface or are extending cell processes along the clefts past dead cells.
Results

Figure 18 TEM section of re-seeded networks

This Figure shows electron-micrographs taken at various magnifications.

Figure A and B show dead cells, present at two and six hours after the re-seeding of a six hour network. Thus proving that the cells were indeed killed by the hypotonic treatment. Figure C and D show live cells the first at two hours after re-seeding with cell processes appearing to extend towards the top of the photograph, possibly feeling for a path of least resistance, collagen fibrils or chemical stimulus from a pre-existing cell as in Figure B. At six hours after re-seeding the cell processes are extended uni-directionally towards a possible stimulus at the top of the photograph (Figure D). These cell processes can be clearly seen in Figure E extending along the tunnel left in the Matrigel and towards neighbouring cells in Figure F which shows the relations of the three cells.

**Key**

L - live cell
D - dead cell
T - tunnel
P - cell process
→ - Matrigel organised
G - TEM grid line

A: 13200X (mag), 2 hour re-seeded network showing dead endothelial cell

B: 4400X (mag), 6 hour re-seeded network showing dead cells present with associated debris and live cell sending out a cell process.

C: 3300X (mag) 2 hours after re-seeding, live cell with processes extending from the cell in most directions

D: 5900X (mag) 6 hours after re-seeding, live cell has processes more uni-directional in response probably to a stimulus at the top of the photograph.

E: 7800X (mag) 2 hours after re-seeding, processes of a number of cells inter-meshing with one another.

F: 10400X (mag) 2 hours after re-seeding, showing the relations of processes of three cells intermingling.
Results

Effects of monoclonal antibodies

Effects of monoclonal antibodies to human collagen type IV

(Table 2 and Figures 19 & 21)

On network formation

When the medium used for the initial seeding was combined with 1/100 concentration antibody to human type IV collagen, the resultant six hour network appeared sparse and fragile with attenuated branches (Figure 19 A). By 12 hours the networks were intact but appeared less robust than in a standard network (Figure 19 B), especially compared to an initial seeding where the medium was diluted by a 20% concentration of PBS (Figure 19 E). The network formed in 80% medium/ 20% PBS appeared robust and fully developed at 12 hours. To ensure correct cell concentration and vitality, controls were always run concurrently with the experiment in progress.

Established networks

Monoclonal human type IV collagen antibody in a 1/100 final concentration appeared to have no effect on established six hour networks or on any further network maturation. The initial network illustrating this is thin and fragile.

Re-establishment of networks after simulated acute injury

When killed six hour networks were exposed to the same antibody for thirty minutes, then rinsed with PBS, the re-seeded cells formed a network unrelated to the original network (Figure 21-D). The second network formed and maturated in a normal fashion.
**Results**

**Effects of monoclonal antibodies to human Laminin**

*(Table 2 and Figures 20 & 22)*

**On network formation**

When initial networks were seeded in the presence of 1/100 final concentration of antibody to human laminin, the resultant formation at six hours was not a network but a disorganised sparse monolayer of endothelial cells with some cells elongated and flattened trying to join up with distant cells (Figure 20 A). Even after twelve hours no further maturation of a network had occurred (Figure 20 B).

**Established networks**

When established six hour networks were exposed to 1/100 final concentration antibody to human laminin, the network appeared after six hours further incubation to be poorly maintained and barely intact with attenuated branches, many discontinued. The maturation process appeared disrupted, with the spaces between the branches remaining the same (Figure 20 C, D).

**Re-establishment of networks after simulated acute inflammation**

Established networks were killed as in the previous experiment and then exposed to antibody to human laminin for thirty minutes, then rinsed with PBS and re-seeded as before with an optimum cell number. The Matrigel appeared to be much more granular and over the next two and six hours the photographs showed that although the cells migrated towards the original network pattern initially, the cells then continued to migrate, and again there was a failure to form any networks up to 12 hours after re-seeding (Figure 22).

A summary of the effects of antibodies to human laminin and human collagen type IV is shown in Table 2.
Table 2: Showing the effect of antibodies to basement membrane components on network formation and on established networks.

<table>
<thead>
<tr>
<th>Monoclonal antibody</th>
<th>Network formation</th>
<th>Effect on established network</th>
<th>Re-seeding of killed networks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibody to human type IV collagen</td>
<td>Delayed with attenuated structures formed (5/5 experiments)</td>
<td>No effect (5/5 experiments)</td>
<td>Good networks but unrelated to the original network. (5/5 experiments)</td>
</tr>
<tr>
<td>Antibody to human laminin</td>
<td>Complete Inhibition (3/3 experiments)</td>
<td>Some attenuation and inhibition of maturation (3/3 experiments)</td>
<td>Initial migration coincident with the original network but failure to form networks (5/5 experiments)</td>
</tr>
</tbody>
</table>

Monoclonal antibodies to human type IV collagen and human laminin were incubated at a 1/100 final concentration with developing, established (six hour) networks and killed then re-seeded six hour networks as described in Materials and Methods.

Six hours after initial seeding in the presence of anti-type IV collagen attenuated networks had formed. On further maturation the networks appeared almost normal. Whereas six hours after incubation with anti-laminin the cells failed to form any networks, remaining in a monolayer of disorganised elongated and flattened cells, with no network formation up to 12 hours later.

When six hour established networks were exposed separately to each antibody, the anti-type IV collagen had little effect on maturation, whereas the anti-laminin appeared to disrupt the maturation process, leaving the network barely intact with attenuated branches, many being discontinued. The spaces between the branches remained static due to lack of remodelling.

Culture wells containing networks killed at six hours after initial seeding were incubated with either anti-type IV collagen or anti-laminin. These were washed out prior to re-seeding. The anti-type IV collagen treated cultures formed an attenuated second network uncoordinated with the original network. Anti-laminin prevented the formation of a second network, with the cells initially migrating towards the original basement membrane, then continuing to migrate in a completely different pattern, but remaining as a monolayer of elongated cells.
**Results**

**Figure 19:** Effect of monoclonal antibodies to human type IV collagen on network formation and on established networks.

Medium used for seeding the initial networks was diluted to a 1/100 final concentration of anti-type IV collagen. The resultant six hour network appeared sparse and fragile with attenuated branches (Figure A). By 12 hours the networks were intact but appeared marginally less robust than in a standard network (Figure B), and especially compared to an initial seeding where the medium was diluted by a 20% concentration PBS (Figure E), producing a network that appeared robust and fully developed at 12 hours.

Medium containing 1/100 final concentration of monoclonal human type IV collagen antibody was added to an established six hour network (Figure C). Two hours later there appeared to be no obvious effect on established networks or on any further network maturation. In the Figure illustrating this, the initial network is thin and fragile.

A: Networks six hours after initially seeding with monoclonal antibody to human collagen type IV.

B: Networks twelve hours after initially seeding with monoclonal antibody to human collagen type IV.

C: Network six hours old prior exposure to monoclonal antibody to human collagen type IV for 6 hours.

D: Network six hours old exposed to monoclonal antibody to human collagen type IV for 6 hours.

E: Networks six hours after initially seeding with 20% PBS.
**Figure 20:** Effect of monoclonal antibodies to human laminin on network formation and on established networks.

Cells were initially seeded in the presence of 1/100 concentration of anti-human laminin. After six hours, only a disorganised monolayer of endothelial cells with some cells elongated and flattened (Figure A) had formed. Even after twelve hours, no further maturation of a network had occurred (Figure B).

When established six hour networks were exposed to 1/100 concentration antibody to human laminin, the networks appeared to be poorly maintained and barely intact with attenuated branches, many being discontinued after six hours further incubation. The maturation process appeared to be disrupted, with the spaces between the branches remaining constant (Figure C, D).

A: Six hours after seeding in the presence of anti-laminin, no networks have formed.

B: After 12 hours there is still no evidence of network formation.

C: Photograph of a six hour cell network immediately after the addition of anti-laminin.

D: The identical field after two hours of exposure to anti-laminin. The network appears poorly maintained with some branches attenuated.
Figure 21: Effect of monoclonal antibody to human collagen type IV on re-establishment of networks after simulated acute injury.

Killed six hour networks were exposed to the anti-type IV collagen for thirty minutes, and thoroughly rinsed with PBS as described in the Methods. The re-seeded cells formed a network which was unrelated to the original network (Figure D). The second network formed and matured in a normal fashion. All the photographs were taken after re-seeding with the plate remaining undisturbed on the microscope platform.

A: Cell network at six hours, killed, then exposed to anti-human collagen type IV for 30 minutes, followed by rinsing with PBS and re-seeding with an ideal number of endothelial cells. Initial re-seeding time 0.

B: Identical field 1 hour after re-seeding.

C: Identical field 2 hours after re-seeding.

D: Identical field 6 hours after re-seeding.

E: Identical field 18 hours after re-seeding.
**Figure 22:** Effect of monoclonal antibody to human laminin on the re-establishment of a network after simulated acute injury.

Established six hour networks were killed as described in Methods. The networks were exposed to anti-laminin for thirty minutes, rinsed thoroughly with PBS and re-seeded with an optimum cell number. The Matrigel appeared more granular and over the next two and six hours the cells migrated towards the original network pattern initially, then continued to migrate, with a failure to form any networks up to 12 hours after re-seeding.

A: Cell network six hours old, exposed to water, then human laminin antibody for 30 minutes, followed by rinsing with PBS and re-seeding with an ideal number of endothelial cells.

B: Identical field 1 hour after re-seeding.

C: Identical field 2 hours after re-seeding.

D: Identical field 6 hours after re-seeding.
Results

Analysis of the results

The human umbilical vein is a non-branching, large vessel with a broad intimal surface. Cannulation, flushing, and recovery of effluents are technically uncomplicated. However, although the material is readily available "premortem", many variables such as foetal distress, maternal anaesthesia and anoxia interval during transportation to the laboratory, can affect the viability of isolated cells.

By using collagenase rather than trypsin (which attacks cell membranes), Jaffe and co-workers (1972) were able to obtain endothelial cells which grew to confluence in primary cultures. Collagenase loosens patches of intimal lining which can then be dislodged. In the present study, control of digestion time and concentration of collagenase proved critical for successful cell isolation.

Endothelial cells tend to form a monolayer with a cobble-stone appearance in vitro. Spontaneous angiogenesis required the endothelial cells to be healthy and rapidly multiplying in the presence of endothelial cell growth factor in agreement with Folkman and Haudenschild (1980). The cells achieved confluence rapidly and were then starved as previously described. Starvation and lack of space, caused the cells to become stressed, and stimulated the cells to secrete basement membrane constituents which in turn, stimulated tube formation.

Adhesive tendrils are formed beneath the monolayer of cells. These tendrils lengthen, organise, and cells accumulate along them, eventually lumens form in the tendrils and hollow tubular networks are established. The tendrils are elevated within the medium. Features of the networks formed agreed with other authors as described earlier (Folkman and Haudenschild, 1980) and modified as Iruela-Arispe et al (1991) demonstrated. Network formation was unpredictable taking up to two weeks and proved too fragile and unpredictable for experimental use.

When human umbilical vein endothelial cells are plated on Matrigel, cell attachment occurred within thirty minutes, the cells then migrated rapidly, establishing a pattern within two hours that was still recognisable 5 hours later. After rapid migration during the first two hours, cells elongated, flattened and began to associate with one another to form a distinctive tessellated pattern. After two hours, only minor changes occurred,
Results

suggesting that two hours was an ideal period to observe the initial preferential migration of the cells on re-seeding, before any modification had occurred. Over the following four hours the cells flattened more, branches formed, straightened and thinned, while islands of multicellular clumps became raised up from the well base, pulling the Matrigel up. As the network matured further, the voids between branches increased in area and the branches reduced in number and lifted higher.

At six hours the HUVEC networks appeared random, the branches or interconnections between the islands of cells were thin, with cells elongated along their length. The branches and cells appeared raised from the surrounding Matrigel, with the Matrigel in the immediate vicinity appearing re-organised by the cell, with evidence of traction centres and traction fields existing. The networks appeared relatively mature and capable of some manipulation.

By 18 hours the networks had pulled up significantly and the Matrigel has been drawn up from the base leaving areas denuded of gel. The literature suggests that only at 18 hours do the networks contain hollow tubes.

There was no previous literature to guide the strategy for rapid killing of cells without damage to the basement membranes or Matrigel. The use of purified water proved adequate, with careful control of the temperature and using graduated dilutions of medium. The Matrigel was disrupted slightly during hypotonic treatment, becoming more granular, but was still capable of supporting the formation of a second network.

Relocation of the identical field after lysis and re-seeding proved difficult. Locating the whole well using a template stuck to the microscope platform proved adequate, but subsequent computer-manipulated superimpositions would have been easier if a more accurate technique could be found.

Re-seeding the 18 hour networks resulted in the re-seeded cells collecting at the base of the denuded voids between the network branches and these were unable to contact any Matrigel to migrate up towards the original network. The amount that the branches had pulled away from the well base was easily demonstrated using different levels of focus. Because of this, it was not possible to re-establish a network despite increasing the amount of gel up to three times that used in six hour experiments. Experiments were abandoned for 18 hour networks and carried out on relatively mature six
hours networks. Re-seeded networks formed and matured in a similar manner to the original networks surviving for up to 42 hours after seeding.

Re-seeded six hour experiments demonstrated the predictable nature of network formation. The majority of the second networks formed in a pattern superimposed on the original networks, with some extra and thicker branches present in good re-seeding, while lower re-seeding numbers produce superimposed networks minus some branches with the branches present no thicker than the original. Of the two experiments that were not similar, the Matrigel appeared slightly altered and cell viability questionable although the controls were adequate. Where migration was slow, the six hour network appeared well organised and the Matrigel was pulled up from the well base.

The 18 hour networks and 6 hour networks both demonstrated the same responses during the control experiments, which were run to ensure the consistency and efficiency of the experimental procedures. This suggests that the substitution of the relatively mature six network for the 18 hour established network is justified, with the six hour network representative of the old network which is known to have tube formation and resemble angiogenesis in vitro (Kubota et al., 1988; Vernon et al., 1992).

Relating the formation of the cell network to angiogenesis in vitro requires the observation of the in vitro network in three dimensions. In sections stained with Toluidine Blue, six hour networks, even two hours after re-seeding, showed that the cells remained on the gel surface as a planar network with only minimal invasion and only six hours after re-seeding were tunnels commenced. In the sections cut parallel to the planar network, the Matrigel had been pulled up under the cells and the sections consisted of Matrigel with live cells flattened and adherent to the gel surface, and along the cleft surfaces, with dead cells floating away. Endothelial cells appeared to extend cell processes over significant distances, seeming to feel their way past the dead cells caught in clefts in the gel. Eventually the cells travelled down these clefts in the gel surface.

The electron micrographs proved the presence of dead cells in the re-seeded networks and the close association of the live cells to them. The live cells initially send out cell processes in all directions, then eventually become more directional possibly due to the identification of other stimuli, contacting extracellular matrix collagen fibrils or paths of least resistance.
Results

The cell processes can be seen to feel their way along the clefts past dead cells remaining in the clefts of the gel.

The second networks appear closely related to the original networks in all periods after re-seeding.

To demonstrate the relative importance of the identification of the basement membrane or the organisation of the Matrigel to guide the second network formation, antibodies to human laminin and type IV collagen were used. The results were interpreted as indicating that laminin was essential for initial tube formation and on re-seeding the cells appeared to identify the original network, but then continued to migrate further, never forming a proper network. Laminin appeared also to be essential for the maintenance of networks once formed.

The anti-type IV collagen on the other hand allowed initial network formation although it was somewhat retarded; network formation was observed by 12 hours after seeding. On re-seeding type IV collagen appeared to be essential for the identification of the initial network with a second network forming uncoordinated with the original network. Because the anti-laminin did not prevent the identification of the original network we can assume that it was not the size of the antibody manifest as steric hindrance that restricted the identification of the original network.

The findings from this model are consistent with known observations that de novo synthesis of type IV collagen and laminin is essential for tube formation and that the endothelial cells appear to influence the extracellular matrix, physically via the existence of traction centres and traction fields. In the initial network formation these traction fields alter the extracellular matrix forming collagen cables that the cells appear to migrate along to form a network. Re-vascularization appears to utilise the previous network basement membrane, rather than the reorganisation of extracellular matrix to act as a template for a network to form, since in the presence of anti-type IV collagen although the matrix cables still exist the new network formed unrelated to the original template.
General Discussion

The objective of this study was to develop a model to enable the investigation of the role of the previous basement membrane on revascularization. It was motivated by the evidence that acute injury of the vascular supply was almost unavoidable in present orthodontic therapy. In particular, work described in the study has the potential to develop an understanding of the importance of the maintenance of the original vascular basement membrane by using minimal forces in orthodontic manipulation, hence minimal basement membrane damage, for the maximum re-establishment of the original blood supply.

Growth and migration of endothelial cells in vitro are similarly associated with both deposition and degradation of basement membrane components (Kalebic et al., 1983, Madri and Stenn, 1982). Both cellular migration and replication are important in angiogenesis (Sholley, et al., 1977). On initial seeding of an extracellular matrix such as Matrigel, the cells appear to reorganise and commence the synthesis of specific proteins required for the differentiation phenotype of the cells (Madri et al., 1983; Kubota et al., 1988; Grant et al., 1991; 1993). During the first two hours, mechanical tension interactions between cultured endothelial cells and the extracellular matrix attachment points may be central to the capillary organisation process (Inger and Folkman, 1989).

The findings related to initial network formation reported in the present study, correspond to those of Lawley and Kubota, (1989). Mechanical tension of the gel must be overcome to allow the cells to migrate extensively over the matrix surface, permitting increased cell-cell contact and alignment end-end and side-side which promotes network formation. Cytoplasmic extensions meet and merge, to form a primordial honeycomb network. In the present study migration slowed by two hours, (not 4-6) and endothelial cells began to interact, with the future network easily visible. This variation in the timing of the sequence could be due to a number of variables, for example, cell density, gel viscosity or concentration of medium components.

By 6 hours lateral association was visible between the cells and some remodelling of the matrix was observed. In some regions, the cells
General Discussion

appeared to have slightly penetrated the surface. After 8 hours, a honeycomb effect is present (Lawley and Kubota, 1989).

Using observation of the sections stained with Toluidine Blue, cells appeared to rest on the surface of the Matrigel with no burrowing or invasion observed until a considerable period of time had elapsed after seeding. This is in agreement with Kubota et al (1988), Grant et al (1989) and Kinsella et al (1992) although in these papers no relevant photographs were published. Sections of networks re-seeded for two hours showed the commencement of tunnel formation and sections taken parallel to the network surface demonstrated the presence of clefts in the gel, with viable cells sending out processes past dead cells trapped within these clefts.

Eighteen hour networks were considered to contain lumen as indicated by Kubota et al., (1988) and Vernon et al., (1992). The more established the network the more the tubes pulled up away from the floor of the well, eventually pulling the Matrigel clear from the plastic. After 72 hours the cells tend to detach.

**Tube formation around fibrils from ECM**

Collagen fibrils have been found in the lumen of networks. It has been proposed that when cells make contact with the apical side of endothelium, the collagen fibrils stimulate and provide a template for vascular tube formation (Ingber and Folkman, 1989; Jackson and Jenkins, 1991).

The orientation of the endothelial cells on Matrigel appears to depend on the reorganisation of the gel into a network of narrow tracks or cables. When endothelial cells contact the matrical network the cells assume elongated, bipolar shapes and migrate along the aligned matrix, and with time, colonisation corresponds to the network of cellular cords.

Hudlicka et al., 1996 suggested that during sprouting there is interaction between the endothelial cells, pericytes and fibroblasts, with the collagen fibres serving as a scaffolding for new sprouts. Pericytes appear in the vicinity of the sprouts, possibly moving there by migration within the basement membrane which separates the pericytes from the endothelial cells at this stage. This interstitial and cellular scaffolding guides the sprouts to adjacent endothelial cells, leading to the closure of a new capillary loop and lateral anastomoses.
Re-seeding

As discussed previously, the re-seeded network appeared to have a preference and mark resemblance in form to the original basement membrane template left intact after simulated acute injury. There appears to be no literature of cell culture systems that have examined the nature of a second seeding after simulated acute injury. However, animal models and grafting experiments support this finding (see literature review).

In the present study transmission electron microscopy demonstrated that lumen formation had commenced by two hours after re-seeding a six hour network. The re-seeded cells extended processes in all directions until an apparent stimulus was detected. Cells migrated either towards a dead cell or around a dead cell along a previous network cable. Other investigators have used higher magnification and observed that the wall of the structures is composed of 3 or 4 endothelial cells which are tightly associated via junctional complexes and spiral around each other (Kubota et al., 1988; Grant et al., 1991).

Antibodies and basement membrane synthesis

When antibodies to human type IV collagen and human laminin are used the importance of each to the identification of the original network by chemical rather than physical factors can be investigated and findings in the present study support the findings by Kubota et al (1986) and Graf et al (1987).

Laminin appears to be the principle factor in control of tube formation. Anti-laminin at a titre of 1:50 allowed attachment but not tube formation in agreement with Kubota et al., (1988) and Lawley and Kubota (1989), while anti-collagen type IV had less of an inhibitory effect used at a titre of 1:4-1:10 (Kubota et al, 1988; Lawley and Kubota, 1989).

Collagen type IV, not laminin seemed important for the identification of the previous basement membrane by the new HUVECs by chemical recognition rather than physical stimulation. Since anti-laminin caused no inhibition and is a similar molecular size to anti-type IV collagen, steric hindrance was eliminated.
Limitations of planar angiogenesis *in vitro*

The interpretation of the findings and extrapolation to the tissue of the periodontal attachment is clearly restricted by the fidelity of the model system. One of the most difficult aspects of *in vitro* studies is the extrapolation of the results to the *in vivo* situation where the environment totally changes. Even when cells are cultured in collagen gel, the environment bears little similarity to the dense and specialised extracellular matrix seen in vivo. That the environment can readily influence cells *in vitro*, is seen from the different appearance of cells when cultured is the presence or absence of collagen gels (Hay, 1982).

Lack of standardisation of culture methods makes literature comparison of network formation difficult. The culture technique used may select for a particular sub-population of endothelial cells, which is not typical of endothelial cells *in vivo*, while the properties of the cells may change with the number of passages (Murphy and Daniel., 1987). For example, cultures grown for 34 cumulative population doublings (Maclag *et al.*, 1982), can occur at various passage levels depending on split ratios used (Madri *et al.*, 1980) which vary per author from 1:5 (Maclag *et al.*, 1982) to 1:10 (Madri and Williams, 1983). Unfortunately these articles did not specify number of passages that had accumulated before cell use in the experiments.

The behaviour of endothelial cells in planar culture systems has been referred to as angiogenesis *in vitro*, largely on the basis of the network-like organization of the cells. It is apparent, however, that planar models exhibit characteristics that are atypical of angiogenesis or neovascularization *in vivo*. For example, planar networks of cells form by tessellation that is simultaneous (more or less) throughout a field of pre-positioned endothelial cells, a process that differs from the growth of angiogenic sprouts that arborize by multiple levels of branching *in vivo*.

Endothelial cells within planar networks frequently enfold the supportive scaffold of extracellular matrix and assume tubular shapes that may resemble capillaries although the morphogenesis of vascular lumens *in vivo* is poorly understood. It has been proposed that cords of extracellular matrix may mediate capillary tubulogenesis by acting as mandrels around which the endothelial cell could wrap. But matrical mandrels have not been found *in vivo* and clearance of mandrel material from endothelial cell tubes *in vitro*, which is necessary to establish a functional lumen, is uncommon.
A better simulation of angiogenesis with respect to invasion of extracellular matrix and formation of lumens occurs in cultures of monolayer endothelial cells which penetrate thick substrates of gelled type I collagen in response to phorbol esters or polypeptide growth factors. The endothelial cells invaginate and form branched tubes with patent, fluid filled lumens, a process that resembles tubulogenesis during the early stages of sprout formation in vivo.

Contact with malleable extracellular matrix in vitro causes a variety of cell types to exhibit characteristics of differentiation that include decreases in proliferation, elevated expression of cell-specific gene products, and appropriate responses to molecular signals. Endothelial cells cultured on extracellular matrix increase the expression of molecules contributing to traction-mediated tessellation. This may or may not be an indicator of vascular differentiation.

Two dimensional models demonstrate migration, alignment and differentiation, but not degradation and invasion, proliferation or basement membrane synthesis (Jackson and Jenkins, 1991; Sage and Vernon, 1994). Nehl (1994) co-cultivated endothelial cells and fibroblasts on a fibrin matrix and observed that the fibroblasts rapidly invaded the matrix, but endothelial cells did not.

Traction-mediated contraction of type I collagen gels and invasion of collagen by endothelial cells in vitro is stimulated by basic fibroblast growth factor and vascular endothelial growth factor which also enhance angiogenic sprouting in vivo by enhancing the degradation of the extracellular matrix by the endothelial cells (proteolysis) essential to angiogenesis, by increasing the synthesis of plasminogen activator, collagenase and other proteases.

Three-dimensional models show variations from two dimensional ones. Two dimensional networks involve cell elongation, anastomoses and branching without the requirement for transcription and translation but require post-translational events, while a more complex three-dimensional process resulting in a capillary network containing lumens requires both transcriptional and post-translational events (Zimrin, Villeponteau and Maciag, 1995). Endothelial cell proliferation is unaffected by aFGF in 3-dimensional culture, while in 2-dimensional culture it is actively stimulated (Williams 1993). TGFβ inhibits endothelial cell proliferation in two
dimensional culture but has similar effect to aFGF in three dimensional culture (Goto et al., 1993).

One main deficiency of an in vitro cell culture system is the absence of the microvasculature. Whether the Matrigel-induced differentiation of endothelial cells in vitro closely resembles events in vivo is not entirely clear. The tubes resemble nascent capillaries, but it is not known whether they have the same biochemical and functional characteristics. The rapidity with which the cellular remodelling takes place gives an insight into wound healing and angiogenesis (Lawley and Kubota, 1989).

The exact mechanism of angiogenesis is still unknown. Data relating to the determination of vascular regeneration as a critical component of tissue repair will provide insight into the outcome of the reparative process, particularly in the periodontal ligament. Regrowth along predetermined pathways would facilitate the restoration of a functional ligament, whereas a random angiogenic and repair process would lead to scar tissue formation with loss of function and potential outcomes of ankylosis or pathological resorption.

The model used in the present study helps to show the relations between the original basement membrane network and the newly established network after simulated acute injury. The basement membranes in vitro and in vivo are ultrastructurally similar, indicating a good model (Kramer et al., 1984).

**Further research**

The use of a three dimensional model such as the collagen gel sandwich (Delvos et al., 1982; Montesano, Orci and Vassalli, 1983) for investigating original basement membrane template properties would provide more accurate information relating to angiogenesis in vitro. The use of a larger number of antibody experimental runs especially the collagen type IV antibody would provide more assessment of the antibodies effects. Combining the two antibodies to see if complete tube formation is blocked and the use of other combinations of antibodies to components such as fibronectin and other basement membrane products to assess the relative contribution to identification of the original network basement membrane.
Conclusions

From the present study the conclusions drawn are:

- The vascular model present is useful to provide information on re-vascularization, but only a two dimensional network forms in the time period studied. The use of six hour established networks was representative of mature eighteen hour networks.

- In this two dimensional study, the original basement membrane template appears to influence the re-vascularization pattern formed on re-seeding. Re-seeding numbers are critical for consistent results.

- The networks appears to form on the surface of Matrigel only invading after a significant period of time.

- On experimental hypotonic shock the cells are killed and the gel remains able to support a new network.

- The re-seeded cells rest on the gel surface, elongate, flattened and send out processes initially in all directions, later becoming unidirectional toward a stimulus either chemical or physical.

- The recognition of the previous basement membrane template appears chemical rather than physical. Identification of human collagen type IV in the original basement membrane is essential for replication of the original template, whereas laminin is essential for tube formation.
Appendix 1

Cell culture techniques

Endothelial cell extraction from human umbilical cord veins

Each cord requires:

- 5 mg Collagenase in 10 mls Hank's solution.
- 3 ml of 0.2% gelatin solution at room temperature.
- 3 ml Human Umbilical Vein Endothelial Cell Growth Media at 37°C.
- 40 ml Hank's Buffered Salts Solution at 37°C.
- 50 ml Cord Buffers at 37°C.
- 2 x 14g cannula.
- 2 x back flow stoppers.
- 4 x 7.5 x 7.5 cm sterile gauze swabs.
- 2 x sterile umbilical cord clamps.
- 0.2 µm Acrodisc filter.
- Sterile scissors and fine tooth forceps.
- Sterile underlay.
- Sterile gloves.
- Sterile beaker.
- 50 ml syringe.
- 30 ml syringe.
- 10 ml syringe.
- 50 ml centrifuge tube.
- 25 cm² culture flask.
- 500 ml Phosphate Buffered Saline (PBS) at 37°C.
- 2 x sterile gallipots.

Method:

1. Wipe down the lamina flow hood with ethanol and place sterile underlay in hood.

2. On the underlay set out: sterile syringes, gauzes, cannulas, back flow stoppers, scissors, forceps, filter and beaker.

3. Pour cord buffer into a sterile galipot and draw up into a 50 ml syringe and place on underlay.

4. In a sterile galipot containing the collagenase add 10 mls of Hank’s Buffer salts solution, mix and draw this up into a 10ml syringe and place on underlay (keep at 37°C).

5. Draw up 30 ml of Hank’s Buffered Salts Solution and place on the underlay.

6. Pre-coat flask with gelatin and set aside.
7. Open the cord container with sterile gloves and using gauze and the cord buffers in the galipot, wipe down the cord to remove excessive blood.

8. Check the cord for needle marks and previous clamp marks and discard these sections. Section the cord into two or three parts for easier perfusion control. Cannulate the vein at one end and secure with a cord clamp.

9. Attach the 50ml syringe containing 25ml of cord buffers and wash out the vein until the buffer runs clear. Cannulate the other end and secure with a cord clamp.

10. Wash out vein with the remaining cord buffers. Back flow stopper one end and fit the filter to the other end.

11. Attach the 10 ml syringe containing the collagenase and Hank's Buffered Salts Solution, and fill the vein until under high pressure, then remove the filter and syringe, and place the second back flow stopper. If there are any areas of hernia discard this part of the vein and recannulate.

12. Place the entire cord in a beaker and add the 500 ml of PBS at 37°C, and incubate for 15 minutes (any longer than 15 minutes may result in smooth muscle contamination).

13. Remove the cord from the beaker and gently massage to dislodge loosened endothelial cells.

14. On one end, place the 50 ml syringe that previously contained the cord buffers and then remove the stopper from the other end. Draw back the 50 ml syringe. (Place 5 mls foetal calf serum in base of centrifuge tube). Massage as flushing.

15. Flush the vein from the free end with Hank’s Buffered Salts Solution so that it is added to the collagenase in the 50 ml syringe.

16. Spin the collected solution for 10 minutes at 1,000 rpm to pellet the cells in the 50 ml centrifuge tube.

17. Discard supernatant and resuspend the cells in 3 ml of culture media. Discard the gelatin in the 25 cm² flask.

18. Place cells in 25 cm² flask and label as P0 HUVEC, date and incubate at 37°C and 5 % CO₂ atmosphere.

19. At 18 hours draw off the supernatant and any red blood cells, rinse off with M199 and replace with fresh media.

20. Passage when confluent at approximately three days into a gelatin pre-coated 75 cm² flask and label as P1 HUVEC, dated and incubate at 37°C and 5% CO₂ atmosphere.

21. Repeat the entire process from three or more cords if possible.
Method for subculturing human endothelial cells

1. Flat culture flasks are removed from the incubator and placed under the lamina flow hood, a class 3 biological chamber.

2. The growth medium is aspirated from the flask.

3. The flask is gently rinsed with PBS to remove all floating cells and media.

4. Add 5ml trypsin/EDTA to the cells and place flask in the incubator at 37°C and 5% CO₂ atmosphere for 3 minutes.

5. The solution is vigorously pipetted to dislodge the cells from the flask base.

6. The 5 ml of Trypsin/EDTA containing the cells is diluted with 5 mls of medium and then transferred by pipette to a centrifuge tube and a further 5ml of culture medium is used to rinse the flask.

7. The centrifuge is counter-balanced and spun at 1,000 rpm for 10 minutes.

8. The 10 ml of supernatant is removed and usually 15 ml of growth medium is added for a 75 cm² flask.

9. The contents are again vigorously pipetted and a cell count made using a haemocytometer.

10. The fluid volume is adjusted to give a cell count of 85,000 cells/ml.

11. This is then passed into pre-coated gelatin flasks at a rate of 3 ml per 25 cm² and the flasks labelled with the date, passage number and cell type.

12. This is incubated at 37°C at 5% CO₂ atmosphere.

13. Change the media in the flask one day after the initial culture.

14. Change the media as required, usually twice a week.
Direct immunofluorescence

Direct immunofluorescence was performed using fluorescein-conjugated *Ulex europaeus* I (UEA I-FITC; Sigma).

Endothelial cells were washed twice in PBS.

Fixed in 100% methanol for 5 minutes at -20°C.

After two further washes in PBS,

UEA I-FITC (1:50 dilution) was added for 30 minute at room temperature.

Cells are washed three times with PBS and mounted in glycerol: PBS (1:1) under glass coverslips for fluorescence microscopy.

**Endothelial cell viability**

HUVEC were seeded at normal density for 24 hours, test agents were added in fresh medium then washed twice in HBSS and dispersed from the wells using 0.05% trypsin (Difo, Detroit, MI) / 0.02% EDTA (Sigma). Once lifted off the trypsin was neutralised with FCS and an equal volume of 0.1% Trypan Blue (Koch-Light Laboratories Ltd., Colnbrook, Berks U K,) in 0.1M PBS. Both viable and nonviable (stained) cells were counted using an Improved Neubauer haemocytometer.
Thin coating procedures

1. Thaw Matrigel at 4°C (overnight and keep the product on ice before use). Using cooled pipettes, mix the Matrigel to homogeneity.

2. Keep culture plate on ice prior to use but free of moisture.

3. Add the Matrigel to the plates to be coated at a volume of 25 microlitres per 1 cm².

4. Warm plates at 37°C for 30 minutes before use.

Karnovsky's fixative

Dissolve at 70°C: (if higher if may start to break down)

100ml PBS

4.00g Paraformaldehyde powder on foil.

Check pH is 7.4

To make 50 mls fixative mix:

• 29.7ml of the 4% Paraformaldehyde in PBS (2.5% paraformaldehyde)

• 17.8 ml PBS

• 2.5 ml Glutaraldehyde 50% (Sigma chemical company St Louis USA) (2.5% Glutaraldehyde)
Cell culture recipes

Medium 199 with Earle's Salts
Dissolve in 900 ml sterile water:
• 1 sachet of M199 with Earle's salts.
• 29 ml of 7.5% Bicarbonate of Soda solution.
• 20 ml penicillin / streptomycin.
  Adjust to pH 7.2, volume to 1 litre and sterile filter. Store at 2 - 7°C.

Hank's Buffered Salts
Dissolve in water to make 1 litre:
• 1 sachet of Hank's salts. (Sigma USA)
• 0.35 grams NaHCO₃.
  Adjust to pH 7.2. Sterile filter and store 2-7°C.

Cord Buffers
Dissolve in 3 litres of distilled water:
• 8.182 grams NaCL
• 0.298 grams KCL
• 1.982 grams Glucose
• 0.006 grams KH₂PO₄
• 0.115 grams Na₂HPO₄
  Adjust to pH 7.2 and sterile filter. Store at 2-7°C.
Appendix 1

Vascular endothelial cell growth media
To make 100 ml combine:
- 86 ml liquid media 199 with Earle’s salts and penicillin/streptomycin.
- 20 ml foetal calf serum
- 1.0 ml endothelial cell growth supplement (10mg/ml of media for networking, 3mg/ml media for culturing).
- 1.0 ml heparin sodium at 50μg/ml.
- 2.0 ml sodium pyruvate
- 1.0 ml fungizone 250μg/ml (Multicell Trace BioScience PTY LTD Australia)

Sterile filter and store at 2-7°C.
Glutamine can be added weekly at a rate of 2 ml / 100 ml of media to increase media shelf life.

Gelatin solution
1. Dissolve in water to make 1 litre:
   - 2.0 grams of gelatin. (Cytosystems PTY NSW)
2. Microwave on high for 10 minutes.
4. Aliquot at 5 ml and store at -20°C.

Glutamine
Dissolve in M199 with Earle’s Salts to make 100 ml.
- 1.5 grams Glutamine.
- Sterile filter
  Aliquot at 5 ml and store at -20°C.
This can be added weekly at a rate of 2 ml / 100 ml of media to increase media shelf life.

Antibiotics
Combine in equal amounts:
Penicillin at 5000 IU/ml.
Streptomycin at 5000 IU/ml.
Trypsin / EDTA solution

To make EDTA stock combine
- 200mg Ethylene Diaminetetraacetic Acid
- 100 ml PBS = 0.2% stock.

Stock EDTA/Trypsin solution combine
- 50 mg Trypsin
- 10 ml EDTA 0.2% stock
- 90 ml PBS

Makes 100 ml. Sterile filter and store at 2-7°C.

For working concentration combine
- 20 ml trypsin / EDTA
- 80 ml sterile PBS.

Phosphate Buffered Saline (PBS)

Dissolve in water to make 1 litre.
- 10 phosphate buffered saline tablets.

Adjust to pH 7.2. and sterile filter. Autoclave. Store at 2-7°C.
Appendix 2

Poster presentations of this research

1997 Faculty of Dentistry Research Day

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(Abbreviations as per Index Medicus)


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