Cell proliferation rates in an artificial tissue-engineered environment

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Abstract. Worldwide, and particularly in Europe, Japan and the USA, cardiovascular disease is a major killer. It can be treated using tissue or organ transplant surgery, but donor organs may be scarce. Tissue engineering is the integration of engineering principles and biology to produce satisfactory synthetic replacement body parts, using viable cells in a suitable matrix, for regenerative medicine. The aim of this study was to measure and compare cell proliferation kinetics after different time intervals of myofibroblasts in a synthetic matrix, thus to be able to deduce the period that a transplanted-cell population can be expected to survive in a tissue-engineered environment. Porcine aortic wall cells were grown in a porous sponge scaffold, that later could be fashioned into aortic or heart valve substitutes. Freshly acquired cells were seeded on identical sponges and were grown under normal culture conditions for a period of 4 weeks. Seeding concentration was a million cells per sponge. Cells progressively populated the sponges, both covering the surface and infiltrating the depth of the matrix, via sponge pores. Samples were taken at 1 week and at 4 weeks, and the rate of cell proliferation was determined by the metaphase arrest technique. Specimens were also taken for light and electron microscopy to determine whether these transplanted cells were capable of synthesizing their own extracellular matrix.

INTRODUCTION

This study is preliminary to constructing tissue-engineered aortic arch and aortic ventricular trileaflet valve replacements, and reports successful laboratory synthesis of a suitable bio-engineered component consisting of mesenchymal cells populating a sponge matrix. Ideally, a perfect tissue-engineered graft would be surgically inserted into the required body position, and gradually the matrix would be integrated by influx of host cells (Hench & Polak 2002). The transplanted matrix cells would have to support the repair until this occurred. Myofibroblasts are a conditionally renewing cell population, able to proliferate, but not perpetually. In life, fibroblast-like cell types have a finite number of cell cycles available to them (Baum & Arpey 2005) and, if the construct described here is to be an acceptable model for grafts, the cell cycle parameters of its constituent cell population need to have been evaluated. Here, rates of cell proliferation in the

matrix have been measured in two sets of samples, after 1 week and after 4 weeks in culture. It has been found that, when compared, there was no appreciable difference in these cells' propagation capabilities after this time period. Histology and ultrastructure of the cells have been examined, revealing that they actively produce both collagen and elastin into the extracellular matrix.

MATERIALS AND METHODS

Here, flat, but nevertheless three-dimensional tissues have been built from porcine aortic wall myofibroblasts grown in a collagen sponge matrix, 5-cm diameter, 4-mm depth, with irregular connecting pores $58 \pm 24 \mu\text{m}$ diameter (Perouse SARL, Lyon, France). Aortas were collected fresh from the abattoir, cells were disassociated from other aortic tissues and were grown for 2 weeks in culture to expand cell numbers. At this point, they were counted and were seeded on sponges (10^6 per sponge) then grown under normal culture conditions for a period of 4 weeks. At week 1 and week 4 the metaphase arrest technique was performed on sets of six identical sponges/cells, five test categories treated with $10 \mu\text{M}$ vincristine sulphate, and one non-vincristine-treated control category. Exposure was stopped at progressive half-hour intervals (five sponges per time point) by rinsing the sponges and fixing these proto-tissues in neutral buffered formalin. Specimens were prepared for light microscopy in the normal way, and were embedded in paraffin wax; sections were cut at $4 \mu\text{m}$. For histology of the metaphase arrests, sections were stained with haematoxylin and eosin (H&E). Vincristine interrupts the cell cycle at metaphase as it destroys the microtubular cytoplasmic apparatus requisite for telophase to proceed; at light microscope level these arrested metaphase chromosomes are clearly visible in tissue sections. For each time point, metaphases in the samples were counted, as was the total number of cells (at least 2000 cells per section). The metaphase index was then calculated as the percentage of metaphases to the total number of cells; these were plotted against time (in 0.5-h increments) after vincristine treatment.

Of non-vincristine-treated specimens, samples were taken for light microscopy, as described above. For normal histology, sections were stained with H with van Gieson's stain to demonstrate collagen and by Verhoeff's procedure to demonstrate elastin. Further samples were taken for transmission electron microscopy. These were fixed in 2% glutaraldehyde and osmium tetroxide, dehydrated in graded alcohols and were embedded in araldite. Sections were cut at approximately 100 nm, and were stained with uranyl acetate and lead citrate. Specimens were examined under a Philips 110 electron microscope.

RESULTS

During the 4-week period, as the cultures became more densely inhabited, there was progressive population of the matrices by interdigitating spindle-shaped cells orientating their long axes to, and covering the surface of the sponge. They formed layers at depths of four to six cells beneath the sponge surface (Fig. 1), penetrating the latticed architecture. At greater depths, lower cell density was observed (Fig. 2).

In the metaphase arrest study, the slope of the line – metaphase arrest to time – is a measure of the rate that cells entered into mitosis, r_M , that is of course equal to the rate of cell birth, K_B (Aherne *et al.* 1977) (Table 1) (Figs 3 and 4).

Figure 1. Porcine aortic wall cells in the porous matrix. Transmission electron micrograph of cells proximal to the surface of the construct. At this level, cells arranged themselves with their long axes aligned to each other and to the surface of the sponge. Little collagen is visible, but for small numbers of strands between the cells (original magnification 5 K).

Figure 2. Metaphase arrest sample after 2.5-h exposure to vincristine sulphate. Light micrograph of an area deep within the sponge matrix. Cells are not close together. Arrested metaphases are easily visible (original magnification $\times 100$).

Table 1. Metaphase arrests and the metaphase arrest index

Time post-vincristine (h)	Arrested metaphases average per 2000 cells	Metaphase index
Week 1		
0.5	56	0.28
1.0	68	0.34
1.5	139	0.07
2.0	201	0.10
2.5	95	0.05
Week 4		
0.5	64	0.03
1.0	120	0.06
1.5	119	0.06
2.0	155	0.08
2.5	117	0.06

To derive the value of cell proliferation rate, cells/cell/h, in each category metaphase indices were plotted against their appropriate time points, relating to cells' exposure to vincristine sulphate.

Figure 3. Metaphase arrest curve for cells after 1 week of culture in the matrix. The slope of the line, r_M is equal to a cell production rate of 0.025 cells/cell/h, and thus an apparent cell cycle time of 27.7 h.

Figure 4. Metaphase arrest curve for cells after 4 weeks of culture in the matrix. The slope of the line, r_M is equal to a cell production rate of 0.02 cells/cell/h, and thus an apparent cell cycle time of 34.6 h.

In turn, r_M and K_B are equal to the value $\frac{\ln 2}{tC(a)}$

$tC(a)$ being the apparent cell cycle time.

Thus, after 1 week in the matrix, cell birth rate was 0.025 cells/cell/h.

Apparent cell cycle time being $\frac{0.693}{0.025} = 27.7 \text{ h}$

And after 4 weeks in the matrix, cell birth rate was 0.02 cells/cell/h.

The apparent cell cycle time was $\frac{0.693}{0.02} = 34.6 \text{ h}$

The potential of the cells to create their own extracellular matrix has been examined in this study, and these aortic wall myofibroblasts demonstrated their capability of producing copious amounts of banded collagen (Fig. 5) and elastin (Fig. 6). At the surface of the sponge, where cells were densely packed and layered, there was little evidence of production of extracellular constituents, but by electron microscopy, and staining tissue sections with van Gieson's stain for light microscopy, cells at deeper levels were shown to produce considerable quantities of porcine

Figure 5. Densely packed strands of banded collagen emerging from myofibroblasts. Electron micrograph of myofibroblasts deep within the construct. Densely packed strands of collagen are emergent from the cells (original magnification 5 K).

Figure 6. Myofibroblasts expressing elastin. Low power light micrograph of cells within the construct stained with Verhoeff's stain to demonstrate elastin. All cells appear to be associated with extracellular elastin (original magnification $\times 25$).

collagen. Staining with Verhoeff's compound revealed that the cells also produced elastin; electron microscopy confirmed the presence of secreted elastin fibres. These findings are consistent with myofibroblast behaviour for cardiovascular development in normal embryology and wound healing *in vivo*, productive cells being interspersed with generous quantities of newly formed extracellular matrix.

DISCUSSION

The sponge used here proved to be a compatible environment for the cells, and so can be considered as a suitable matrix for further investigation. If such or similar tissue-engineered materials are to be introduced into the human body, it is essential that the cell population growth parameters are known. Here, the values derived for apparent cell cycle times at 1 week in culture and 4 weeks in culture are not significantly different from each other. Further studies need to be performed with greater time spans and with various kinds of synthetic matrix. Although cell population kinetic measurements are well understood and used (particularly in gut and tumour

biology) (Sarraf & Bowen 1986; Sarraf *et al.* 1995; Mandir *et al.* 2005), bio-engineered tissues so far have been close-to-never evaluated in this way. This is a fertile and important area for tissue-engineering research – too little cell proliferation and the construct cells will die before native cells can take over; too much cell proliferation and we descend into areas of potential neoplasia as a result of engineered graft introduction.

In natural tissues, myofibroblasts produce collagen and elastin; these are essential extracellular fibres in cardiovascular organs. In this study, the myofibroblasts, to the best of our judgement (via examination of very numerous light and electron microscope sections), seem capable of each cell producing collagen and elastin simultaneously. The readiness with which the cells have proliferated and differentiated calls upon similar observations of mesenchymal stem cells (Otto & Rao 2004). Clearly, the cells used in our experiment are not stem cells, but they have shown themselves to be phenotype progenitors able to populate their environment.

In our group, work is in progress to simulate tensile forces over whole tissue-engineered cultures to mimic natural stresses and strains sustained by the cardiovascular system. In life, clearly, any implanted tissues must have equal mechanical strength and diversity to the original one. At our disposal we have a variety of types of bioreactor – culture force monitors (CFM) and tensioning-culture force monitors (t-CFM) (Eastwood *et al.* 1988a, 1998b). Tensile forces produced by unstressed cells in the cultures can be measured on the CFM, and, by using the t-CFM forces of appropriate levels, can be exerted on the cultures. Contractile curves of porcine aortic wall myofibroblasts show that they reach tensional homeostasis after 24 h (work in progress). Already we have, in our laboratory, constructed a proto-aorta which we were able to keep alive for 2 weeks (planned termination of the study) in our multicue bioreactor (McCulloch *et al.* 2004).

In conclusion, cell proliferation parameters have been measured in a cultured tissue-engineered matrix, which has been found to be a satisfactory model for cardiovascular tissue research. After 1 week in culture, porcine aortic myofibroblasts proliferated at a rate of 0.025 cells/cell/h, thus having an apparent cell cycle time of 27.7 h. After 4 weeks' culture in the matrix, the cell proliferation rate was 0.02 cells/cell/h, apparent cell cycle time 34.6 h. The cells were also found to readily synthesize extracellular matrix components, collagen and elastin. This is the first measurement of this nature for a tissue-engineered potential graft. Its importance lies in the necessity to be able to predict (by comparing parameters after different periods of time) how long cells of such a graft would be able to function without deterioration, after implant regenerative surgery.

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