GOAL:
To determine the effect of dynamic fluid pressure on periosteal cell proliferation.

HYPOTHESIS:
Dynamic fluid pressure will stimulate periosteal cell proliferation in vitro.

RATIONALE:
Mechanical factors are known to be important in normal cartilage development, maintenance, and outcome of repair techniques. Thus, mechanical stimuli can be considered an essential component of joint homeostasis. However, the effects on periosteal cartilage formation in vitro have not been studied and how cartilage metabolism or repair / regeneration are affected remains to be fully understood. It is essential that we understand the role of mechanical factors in regulating these processes.

METHODS:
Periosteal explants were cultured in the presence or absence of dynamic fluid pressure. The effect on cell proliferation was determined using $^3$H-thymidine uptake studies, autoradiography, Proliferating Cell Nuclear Antigen immunostaining, cell proliferation blocking assays and total DNA measurements.
CHAPTER
5

PERIOSTEUM Responds TO Dynamic Fluid Pressure
By proliferating in vitro
INTRODUCTION

The present decade has witnessed an explosion of interest in the field of cartilage repair, as evidenced by the widespread coverage devoted to this topic in scientific publications and the lay press. To better understand the factors and conditions regulating the basic process of cartilage formation requires good *in vitro* and *in vivo* models for studying chondrogenesis, as well as methods for simulating mechanical factors.

Periosteum provides a source of undifferentiated chondrocyte precursor cells for fracture healing that can also be used for cartilage repair. In both processes, the quantity of cartilage that can be produced is related to the number of available stem cells. Optimal cartilage repair or fracture healing requires increasing the quantity as well as the quality of cartilage produced. The quantity of cartilage is believed to be limited by the number of chondrocyte precursors in the cambium layer, which can be increased by stimulating cell division in this population.

The periosteal organ culture model, which has been described for studying the process of cartilage formation in whole periosteal explants suspended in agarose, mimics the events during periosteal chondrogenesis *in vivo*. Prior to expression of the cartilage phenotype in transplanted or cultured periosteum, DNA synthesis and cell proliferation are the first events that occur during periosteal chondrogenesis. Expanding the chondrogenic cell population will increase the quantity of cartilage produced. In periosteal chondrogenesis, the total cell count in the cambium layer of the periosteum, where the chondrocyte precursors are believed to reside, is the rate limiting factor determining chondrogenic potential of periosteum and its diminution with age. As the first stage of periosteal chondrogenesis is cell proliferation, we need to understand how this can be stimulated.

Klein-Nulend *et al.* have shown that low-level oscillations in hydrostatic pressure (13 kPa at 0.3 Hz) increased periosteal and periachondrial cell density, while Lafeber *et al.*, have shown that chondrocytes from normal or osteoarthritic cartilage in culture respond to such a mechanical stimulus by alteration of their metabolic rate and increased proteoglycan synthesis.

As the pressures are dynamic rather than static, a term such as *dynamic fluid pressure* (DFP) might be preferable. Such dynamic fluid pressures have been recorded in synovial fluid during joint motion and gait.
The effects of experimentally applied dynamic fluid pressure have been studied and found by many investigators to regulate (i.e. stimulate) cellular activity in cartilage and bone. It is recognized that mechanical stimuli are important during the development, homeostasis, repair and degeneration of cartilage. Biological repair of cartilage is enhanced by continuous passive motion (CPM) of the joint postoperatively. This is true for cartilage regeneration by transplantation of periosteum, which contains undifferentiated mesenchymal stem cells with osteochondrogenic potential. The important role of mechanical factors is illustrated by the finding that continuous passive motion of a joint into which periosteum has been transplanted greatly increases the quantity and quality of the cartilage produced by the periosteum, as compared to that formed in immobilized joints.

Movement of a joint causes sinusoidal oscillations in the synovial fluid pressure. The beneficial effects of joint motion on repair of damaged cartilage might be explained in part by the contribution of dynamic fluid pressure. At this time there is no published information on the effect of dynamic fluid pressure on periosteal chondrogenesis. However, it is clear that periosteum has a chondrogenic potential, which is influenced by mechanical factors, and motion alters the phenotypic expression of the chondrocyte precursor cells in the periosteum. The major limitation with periosteal transplantation is the age-related decline in periosteal chondrogenic potential which is examined in Chapter 8.

The purpose of the present investigation was to test the hypothesis that cell proliferation in whole periosteal explants is stimulated by dynamic fluid pressure (DFP). If correct, this would explain, in part, the previously reported association between the cyclical changes in intra-articular pressure and the increased number of chondrocytes in periosteal grafts or transplants exposed to CPM. It might also be useful as a method to expand the stem cell pool (i.e. chondrocyte precursor population) and maintain differentiation and therefore help to reverse the age-related decline in the capacity of periosteum for cartilage repair and improve the pre implantation culture conditions.
Harvesting of periosteal explants and tissue cultures:

Four hundred and fifty two periosteal explants were harvested from the proximal medial tibias of 60, immature (two-month old) male New Zealand White rabbits, as described in Appendix A109,228,229. The culture conditions were as reported in the periosteal explant model by O’Driscoll et al detail of which is provided in Appendix B. To act as a positive control for the experiments on blocking the proliferative activity, TGF-ß1 was added to both the agarose gel and the fluid culture medium. This was done in a concentration of 10 ng/ml228, for the first two days of culture, unless otherwise mentioned. All explants were cultured in standard 48 well flat-bottom plates maintained at 37°C, 100% humidity and 5% CO₂ mixed with 95% air.

Dynamic Fluid Pressure apparatus:

Dynamic fluid pressure was applied to periosteal explants based on the validation experiments in Chapter 4, using machine number 1 described above, and explained in more detail in Appendix C (figure 5.1)159,167,284,285,312.
A pneumatically driven membrane chamber was used to create dynamic pressurization in the gas phase within the attached pressure chamber, in which the culture plates rest. The pressure oscillated between $0$ and $13 \pm 2$ kPa at a frequency of $0.3$ Hz, based on previous publications by other authors. The rate of pressure rise produced by the system was $33$ kPa/sec.

**DNA synthesis measured by $^{3}$H-thymidine uptake:**
To quantify cell proliferation we evaluated DNA synthesis by measuring $^{3}$H-thymidine uptake as described in Appendix D.

**Autoradiography and Immunostaining:**
The location of proliferative activity as determined from DNA synthesis was visualized by $^{3}$H-thymidine autoradiography on 40 explants that were cultured for 1, 2, 3, 4, 5, 6, 7, and 14 days as described in Appendix E. Histomorphometric analyses of autoradiograms were performed using a previously published counting technique. Photomicrographs of the autoradiographs were taken at 200x magnification. The total number of both labeled and unlabeled cells were recorded using a calibrated rectangular grid laid over the fibrous and cambium layers of the periosteal explants. Counts were performed twice and averaged. The reproducibility of this technique was confirmed previously as well as in this study. The accuracy was validated by the finding that the values for cell density in the cambium layer as well as for thickness of the layers were similar to those previously reported by Gallay. As a second indicator of proliferative activity we performed immunostaining with an antibody to Proliferating Cell Nuclear Antigen on paraffin embedded sections of 8 explants cultured for 1 or 4 days with or without DFP (Appendix E).

**Data Analyses:**
All data are presented as means $\pm 1$ standard deviation (SD). All experiments were designed to control for the donor rabbit such that comparisons between experimental and control groups were paired to donor rabbit. We used a sample size for each group of $n = 16$, to yield an 80% chance of detecting a change in the means $\geq 0.75$ standard deviations. This is considered to be between a medium and large effect size. Analyses were performed by repeated measures analysis of variance and
post-hoc testing using Duncan’s New Multiple Range Test, with a 95% confidence interval. Scintillation counts have been rounded off to two digits of accuracy.

RESULTS

$^3$H-thymidine uptake:
$^3$H-thymidine uptake, which was measured on days 1, 3 and 5, reached a peak on day 3 in both the experimental and control groups, consistent with a previous report using this model\textsuperscript{197,200}. At each tested time point there was a significant increase in $^3$H-thymidine uptake ($p < 0.001$) in the experimental group exposed to dynamic fluid pressure (DFP) of $13 \pm 2$ kPa at 0.3 Hz, compared to the control group, that was cultured in a similar chamber at atmospheric pressure (figure 5.2). On day 1, there was a 60% increase in $^3$H-thymidine uptake in the DFP group over controls.

![Graph showing $^3$H-thymidine uptake over days 1, 3, and 5.](image)

Figure 5.2
The continuous application of dynamic fluid pressure at $13 \pm 2$ kPa, 0.3 Hz for 1, 3, or 5 days led to a two-fold increase in $^3$H-thymidine uptake under DFP compared to controls. The time course for DNA synthesis under the influence of DFP peaks on day 3, which is identical to that in the standard model. Explants at each time point were labeled with $^3$H-thymidine during the last 24 hours in culture. (Data are presented as means $\pm$ SD, $n=16$, ** = $p < 0.001$).
The results on day 3, when DNA synthesis is maximal, showed a 110% increase with DFP (97,000 ± 5,700 vs. 46,000 ± 6,000 DPM/µg DNA). A 40% stimulation of 3H-thymidine uptake with DFP was still present on day 5 (DFP: 60,000 ± 2,700 vs. controls: 45,000 ± 4,900 DPM/µg DNA).

DNA Content:
The DFP-induced increase in 3H-thymidine uptake was reflected by a higher DNA content in the DFP-treated explants when compared to the controls. In the control explants, the total amount of DNA decreases with time, presumably due to the trauma of explantation or adaptation to the culture environment. A similar observation has been described in other systems. There was also a decline in DNA content in the experimental DFP group, but less so than in the controls. The DNA content of the explants exposed to DFP was 60% higher than that of the controls on day 3 (DFP 5,700 ± 720 vs. control 3,700 ± 630 ng/mg wet weight) and 40% higher on day 5 (DFP 5,200 ± 490, control 3,700 ± 390 ng/mg wet weight) (p < 0.01; figure 5.3).

Figure 5.3
In adapting to the in vitro environment, the periosteal explants experience a decrease in total DNA. This decrease was however, significantly inhibited in the DFP group on days 3 and 5 compared to controls. (Data are presented as means ± SD, n=16, ** p <0.01).
Blocking of DNA Synthesis:

To confirm that the $^3$H-thymidine and DNA measurements actually represented stimulation of cell proliferation by DFP, DNA synthesis was blocked with aphidicolin, which inhibits DNA polymerase $^2$. DNA polymerase is essential for chromosomal DNA replication and cell division $^{146,165}$. We measured $^3$H-thymidine uptake in three different groups: a negative control (no DFP, no growth factor), a positive control (10 ng/ml TGF-$\beta_1$ - known to stimulate periosteal DNA synthesis $^{197,200}$), and the experimental DFP group (without TGF-$\beta_1$). Explants were cultured in the presence of 0, 0.5, 1.0, or 2.5$\mu$g per day of aphidicolin until day three when proliferation peaks. The explants were pooled after labeling with $^3$H-thymidine, and the pooled uptake measured on day 3. The negative control showed levels of $^3$H-thymidine uptake as expected in our standard model; i.e. addition of TGF-$\beta_1$ increased $^3$H-thymidine uptake significantly. DFP significantly increased $^3$H-thymidine uptake. In all groups, the response to aphidicolin was a dose-dependent inhibition of $^3$H-thymidine, confirming that the $^3$H-thymidine measurements truly represent DNA synthesis. Explants were pooled, columns represent values for whole groups, experiments were repeated and reproducibility was confirmed.

Figure 5.4

$^3$H-Thymidine uptake was measured in three groups: a negative control (no added growth factors, no DFP), a positive control (10 ng/ml TGF-$\beta_1$), and the experimental DFP group. For each group, a dose response effect was noted to aphidicolin. Explants were cultured for 3 days (when proliferation peaks) with and without aphidicolin, which blocks DNA polymerase and thus DNA synthesis. The controls showed levels of $^3$H-thymidine uptake as expected in our standard model; i.e. addition of TGF-$\beta_1$ increased $^3$H-thymidine uptake significantly. DFP significantly increased $^3$H-thymidine uptake. In all groups, the response to aphidicolin was a dose-dependent inhibition of $^3$H-thymidine, confirming that the $^3$H-thymidine measurements truly represent DNA synthesis. Explants were pooled, columns represent values for whole groups, experiments were repeated and reproducibility was confirmed.
Periosteum responds to dynamic fluid pressure by proliferating in vitro.

Table 5.1
Total DNA (ng/mg wet weight) while proliferation was blocked.

<table>
<thead>
<tr>
<th>Aphidicolin (µg/ml)</th>
<th>Negative control a</th>
<th>Positive control b</th>
<th>Dynamic fluid pressure c</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>820</td>
<td>1,000</td>
<td>1,800</td>
</tr>
<tr>
<td>0.5</td>
<td>150</td>
<td>320</td>
<td>580</td>
</tr>
<tr>
<td>1</td>
<td>100</td>
<td>220</td>
<td>100</td>
</tr>
<tr>
<td>2.5</td>
<td>30</td>
<td>130</td>
<td>210</td>
</tr>
</tbody>
</table>

a Without transforming growth factor β1 (TGF-β1). b Treated with TGF-β1. c From days 0 to 3; without TGF-β1.

and positive controls showed levels of thymidine uptake as expected in our standard model; i.e., the addition of TGF-β1 increased 3H-thymidine uptake (11,000 vs. 6,700 DPM/mg wet weight) (figure 5.4, table 5.1). In both the positive and negative controls the inhibition of 3H-thymidine uptake with aphidicolin was dose-dependent, ranging from 80% using 0.5µg to 95% using 2.5µg aphidicolin. These data confirm that indeed 3H-thymidine uptake represents cell proliferation in this system. In the experimental group, DFP increased thymidine uptake and, as expected, this increase was inhibited in the same dose dependent manner by blocking proliferation with aphidicolin (0.5µg: -82%, 1µg: -91%, 2.5µg: -94% inhibition respectively). The approximately 95% inhibition of 3H-thymidine uptake in all groups indicates that DNA synthesis could be virtually stopped. The total amount of DNA in the DFP group was higher than that in the other groups and this, too, could be blocked with aphidicolin which led to a moderate decrease in total DNA (control group: 820 vs. 150 ng/mg wet weight with 0.5µg of aphidicolin, TGF-β1 group: 1,000 vs. 320 ng/mg wet weight, DFP group: 1,800 vs. 580 ng/mg wet weight)(table 5.1). These data indicate that dynamic fluid pressure does indeed stimulate DNA synthesis and cell proliferation. Alternatively the difference in the total amount of DNA between the TGF-β and the DFP group might be interpreted as an effect on apoptosis, in which DFP helps retain cell viability in this in vitro setting, as well as a direct stimulation of periosteal cell proliferation.
3H-thymidine Autoradiography and PCNA Immunostaining:

Periosteum contains two discrete layers: the cambium layer (inner), which is believed to contain the undifferentiated mesenchymal cells, and the fibrous (outer) layer. To identify the location of cells that are stimulated to proliferate we performed autoradiography with 3H-thymidine, incorporated during DNA synthesis and immunostaining with proliferating cell nuclear antigen (PCNA, which appears at the G1/S boundary in the cell cycle during proliferation). The autoradiographic sections (figure 5.5) showed that on day 1 a small number of cells were proliferating (fibrous layer: 1% ± 1%, cambium layer: 1% ± 1%). The proportion of labeled cells increased in the fibrous layer on days 2 and 3 (35% ± 8% and 33% ± 7% respectively), but did not rise substantially in the cambium layer (4% ± 6 and 4% ± 5% respectively). The percentage of labeled cells in the fibrous layer peaked at 45% ± 9% after 4 days in culture, at which time the cambium cells were just showing a rise in proliferative activity (16% ± 11%). Proliferation in the cambium layer peaked 48 hours later on day 6, at 18% ± 4% of the total cells being labeled. This difference in timing of the peak activities in the fibrous and cambium layers was statistically significant (p < 0.03) (figure 5.6). This time difference of 24 - 48
Periosteum responds to dynamic fluid pressure by proliferating in vitro

Figures 5.6 and 5.7:

There was a significant temporospatial difference in proliferative response between the periosteal cambium and fibrous layers. Under the influence of DFP, the proliferation rate increased significantly. The peak in fibrous layer response occurs 24-48 hours prior to that in the cambium layer where the chondrocyte precursors reside.

Figure 5.6:

Proliferating cell nuclear antigen (PCNA) immunostaining clearly visualized the difference in proliferative activity between the unstimulated controls and the dynamic fluid pressure (DFP) group. Proliferating cells take up the PCNA stain (arrows) and are localized in the cambium and sub-cambial fibrous layer.

Hours would represent 1 - 2 cell cycles. The increased proliferative activity with DFP, as evidenced by $^3$H-thymidine uptake, was confirmed with greater PCNA immunostaining in the DFP than control explants (figure 5.7). The percentage of PCNA positive (i.e. proliferating) cells in the experimental group (with DFP, no growth factors) on day 4 was 21% ± 3% versus 9% ± 4% in the controls (no DFP, no growth factors) (p < 0.03).
DISCUSSION

The study presented in this chapter has shown that cell proliferation during periosteal cartilage formation can be stimulated by dynamic fluid pressure (DFP), while the total amount of DNA is better retained in the presence of a mechanical stimulus. The autoradiographs visualized initial proliferative activity in the fibrous layer of the periosteum. Twenty-four - forty-eight hours later similar activity was seen in the cambium layer. Furthermore, they demonstrated the presence of proliferating cells in the cambium layer, which contains the chondrocyte precursors. This was confirmed by PCNA immunophotomicrographs demonstrating an increase under DFP.

The observation that periosteal cells respond to mechanical stimulation by proliferating in vitro is important for several reasons. First, the ultimate goal of cartilage repair research is to optimize the quantity, as well as the quality, of cartilage produced. Quantity is limited by the number of chondrogenic cells, which for periosteum corresponds with the number of cells in the cambium layer\(^{113}\).

As further discussed in Chapter 8, the decline in the total cell count in the cambium layer with age is the major cause for age-dependent decrease in chondrogenic potential of the periosteum\(^{220,228,232}\).

During the early stages of periosteal cartilage formation, proliferation occurs prior to cartilage matrix synthesis, the two stages being discretely separated. These and other data suggest the cell differentiation might need to be preceded by proliferation\(^{25,28,29}\). Indeed, in an experiment described in Chapter 7, we found suggestion of a permissive relation between periosteal cell proliferation and differentiation. Cell proliferation precedes differentiation so that the stem cell population is at least partially retained, as demonstrated by previous data by Ito\(^{144,145}\), the proliferative activity increased in the cambium layer cell region where the morphological transition from flat to round cells occur, while it diminished during enlargement of round cells.

Potentially, one of the most important observations from this study is the temporal change in geographical distribution of cell proliferation seen by autoradiography and immunostaining with PCNA. There was a consistent time-dependent pattern, with cell proliferation first in the fibrous layer, followed 24 to 48 hours later by proliferation in the cambium layer. These findings suggest either a paracrine signaling mechanism between
the cells in these two layers of the periosteum, or recruitment / migration of proliferating cells from the fibrous to the cambium layer. We believe it most likely to represent evidence suggesting intercellular signaling between the two layers. Paracrine signaling mechanisms almost certainly exist within the periosteum, and this observation may provide a clue as to how periosteal cells respond to mechanical stimulation. Specifically, periosteum must contain cells with mechanoreceptors, and cell division in the fibrous layer is associated with endogenous expression of chondrogenic cytokines that induce the chondrocyte precursors in the cambium layer to divide and differentiate. The evidence for this claim includes the fact that the extent to which periosteum is induced to form cartilage in a fracture is determined by motion occurring at the fracture site^{47,48,286,287}. Periosteal chondrogenesis also is induced by physical intervention, such as when it is separated from the underlying bone by a blood clot^{264,267} and by transplantation into a joint^{86,229,274}. In each case, mechanical factors are present.

The alternative explanation for this observation could be that proliferating cells from the fibrous layer are recruited or migrate into the subcambial fibrous region or cambium layer, comparable to the lymphocyte recruitment seen in inflammatory response and similar effects in regulation of hematopoiesis in bone marrow. These two hypotheses could be investigated by performing in-situ hybridization studies early during periosteal chondrogenesis, to look for expression of mitogenic and / or chondrogenic factors in the fibrous layer.

It would be understandable if the cells responsive to mechanical stimulation reside in the fibrous layer of the periosteum. The fibrous layer of the periosteum contains cells dispersed within a meshwork of collagen and elastin fibers. Periosteum contains 2.1% elastin by weight^{271}, which provides it with mechanical properties including its significant contraction following elevation or stripping. The cambium layer, on the other hand, is densely cellular with little extra cellular matrix. It would therefore be plausible that the cells responsive to mechanical stimuli might reside in the fibrous layer.

The data in this chapter show that after one or two cell cycles of proliferative activity in the fibrous layer, proliferation is induced in the cambium layer, where cartilage formation eventually takes place. Uptake of $^{3}$H-thymidine as well as PCNA intensity are more obvious in the group stimulated with dynamic fluid pressure, which is what would be expected in
response to a mechanical stimulus. This result is consistent with published reports showing that mechanical stimulation by cyclical pressure oscillations, such as those seen during CPM of a joint, enhance cell proliferation. The mechanism of action of mechanical stimuli on chondrocyte precursor cells is unknown. So far there have been no previous studies into the response of periosteum to mechanical stimulation in vitro. However, the observation that these cells are capable of responding to a mechanical stimulus is important. Other authors have shown stimulatory effects of dynamic fluid pressure on cartilage, chick calvariae and bone metabolism in vitro, positive effects of continuous passive motion on periosteal chondrogenesis and cartilage repair in vivo, and partial restoration of cartilage in osteoarthritis with joint distraction. Collectively, these indicate that dynamic fluid pressure acts as a mechanical stimulus to influence matrix synthesis and cell proliferation. Mechanical stimuli are of paramount importance for development, maintenance and repair of the musculoskeletal system.

In summary, it can be concluded that dynamic fluid pressure (DFP) stimulates cell proliferation in periosteum in vitro. These observations are important for several reasons. First, they show that it is possible to study the effects of mechanical stimuli on periosteum in a controlled in vitro environment, and indeed there is an effect. Second, a rate-limiting factor in periosteal chondrogenesis is the number of starting cells, and this can be increased by DFP. Increased proliferation is seen in the cambium layer as well as the fibrous layer. These findings direct us toward seeking a paracrine regulation process or the induction of cell migration from the fibrous to cambium layer. Such paracrine regulation would control an initial response to mechanical stimulation by cells in the fibrous layer, releasing growth factors that induce undifferentiated chondrocyte precursor cells in the cambium layer to divide and differentiate into chondrocytes. Cell proliferation in the early stages of cartilage formation is stimulated by mechanical factors.

These findings provide a possible explanation for the increase in cartilage repair tissue seen in joints subjected to continuous passive motion postoperatively. This increases our understanding of the process of periosteal chondrogenesis, which plays a role in both cartilage repair and fracture healing. It has recently been determined that the physiological explanation for delayed fracture healing with age is a reduction in the amount of carti-
laginous callus produced at the fracture site. As most of this callus is formed by periosteum, the implications for understanding how to increase the number of chondrocyte precursors extends beyond cartilage repair to fracture healing as well. Finally, this work forms the basis for further detailed investigation of the biological response of cartilage repair tissues to mechanical stimulation in which we hope to determine the effect of DFP on differentiation and cartilage formation as well as the role of timing and magnitude of the stimulus.