THE PRESENCE OF SEVERAL GROWTH FACTORS

DURING THE STAGES OF FRACTURE REPAIR

By

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In memory of my father's struggle with alzheimers disease
(1975-1985)

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ABSTRACT

An animal model has been developed to study repair of a tibial fracture. The model provides both reproducible and quantifiable results. Using this model, the fracture repair process has been divided into four stages. The duration of each stage is consistently reproducible. Stage 1 termed "the inflammatory response stage", lasts from immediately post-fracture until day 2 post-fracture. Histologically, this stage was marked by the formation of a haematoma, and transformation of monocytes to macrophages. Stage 2, termed the "periosteal-fibrous callus stage", lasted from days 2 to 5 post-fracture. Histologically, this stage was marked by cellular activity in the cambial layer of the periosteum and the accumulation of fibroblast-like mesenchymal cells. Stage 3, termed the "chondrogenic stage", lasted from days 5 to 9 post-fracture. During this stage, the fibrous callus was transformed into cartilage. Stage 4, termed the "endochondral stage", lasted from days 9 to 21 post-fracture. During this stage, the cartilaginous callus was replaced by new bone.

A new tissue processing method was devised which preserves tissue architecture and antigenic sites on growth factors during decalcification. Using this new method four growth factors were localized during the repair process. PDGF was visualized in macrophages in close proximity to the periosteum during stage 1. aFGF was visualized in cells of the expanding cambial layer and was associated with a rapid increase in the population of fibroblast-like mesenchymal cells during stage 2. IGF-I was visualized in young chondroblasts at the edge of the cartilage mass replacing the fibrous callus during stage 3. TGF-β_1 was visualized in calcified matrix producing chondrocytes at the edge of ossification fronts replacing the cartilage callus during stage 4.

mRNA to aFGF was present in tissues harvested from the fracture site during the repair process. This supported the hypothesis that aFGF is produced in situ during fracture repair. A model was proposed to explain the relationship between the cellular activity in tissues (replication,matrix production) occurring during each stage of fracture repair, and the particular growth factor present at each stage.

ABBREVIATIONS

aFGF	acidic fibroblast growth factor
bFGF	basic fibroblast growth factor
DAB	diaminobenzadine
dCTP	deoxycytosine triphosphate
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EDTA	ethylenediamine tetracetic acid

EGF	epidermal growth factor
FGF	fibroblast growth factor
HBGF	heparin-binding growth factor
HBQ	Hall-Brunt-Quadruple stain
IGF	insulin-like growth factor
LMP	low melting point
MMLV	mouse mammary leukemia virus
mRNA	messenger ribonucleic acid
NGF	nerve growth factor
NRK	normal rat kidney

PBS	phosphate buffered saline
PCR	polymerize chain reaction
PDGF	platelet-derived growth factor
PLP	periodate-lysine-paraformaldehyde
PMSF	phenylmethylsulfonyl flouride
-r	receptor
RNA	ribonucleic acid
SGF	sarcoma growth factor
SRE	serum response element
SRF	serum response factor

TBS	tris buffered saline
TGF	transforming growth factor

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Chapter 1

1.1 The Discovery of Growth Factors.

The first "growth factor" to be discovered was nerve growth factor (NGF). Bueker (1948) conducted experiments involving the implantation of small pieces of tumor in the hind limb field of the embryonic chick to study the developmental response of the lumbo-sacral nervous system. He observed that mouse sarcomas 37 and 180 produce an agent which encouraged the growth of spinal ganglia. Levi-Montalcini et al. (1954) placed small fragments of mouse sarcomas 37 and 180 1-4 mm from spinal or sympathetic ganglia of a chick embryo in hanging drop tissue cultures. A significant number of nerve fibers which grew radially in all directions and formed a dense halo around the ganglion were reported. They concluded that the mouse sarcomas produced a diffusible agent which strongly promoted the nerve fiber outgrowth of ganglia. Cohen and Levi-Montalcini (1957) later purified and characterized a protein with nerve growth-promoting activities from mouse sarcoma 180. That protein was NGF.

While working with snake venom preparations, used as a source for phosphodiesterase for their NGF experiments, Cohen (1959) noticed that snake venom itself was a natural source of NGF. Cohen observed that mouse submaxillary gland was also a rich source of phosphodiesterase and soon was able to isolate NGF. However, when NGF was purified from mouse submaxillary

glands, Cohen was also able to isolate and purify another growth factor which had quite different effects from NGF. This new growth factor, after partial purification was injected into new-born mice. The injections caused a number of gross anatomical changes which included early eyelid opening (seven days instead of the average fourteen days), early tooth eruption (six-seven days instead of the average eight to ten days), and a pronounced stunting of the test animals with inhibition of hair growth. This activity was confined only to extracts of adult male mouse submaxillary gland (Cohen, 1962). Further experiments led Dr. Cohen to suggest that the new protein affected mostly epidermal cellular activity, thus the title "epidermal growth factor " (EGF) was given to this protein.

Insulin-like growth factor (IGF) was characterized as a component of plasma with three distinct characteristics: (i) it stimulated sulfate incorporation into chondroitin sulfate in cartilage, (ii) it had insulin-like activity (not neutralized by anti-insulin serum) in adipose tissue, and (iii) it caused increased thymidine incorporation into DNA in various tissues (Baxter, 1986).

In 1956, Salmon and Daughaday performed experiments to study the effects of the removal of the pituitary gland on S^{35} uptake to form chondroitin sulfate in costal cartilage. The uptake of S^{35} into cartilage was stimulated in hypophysectomized rats injected with growth hormone, or after incubation of the tissue in normal rat serum. Incubation of this tissue in medium with added growth hormone did not enhance S^{35} uptake. They concluded that normal serum contained a "sulfation factor" which increased uptake of sulfate by cartilage.

Further experiments (Salmon and Daughaday, 1957) showed that this sulfation factor was present in the serum of normal rats and also appeared in the plasma of hypophysectomized rats treated with growth hormone. Froesch et al. (1963) conducted experiments using serum as a stimulator of adipose tissue metabolism. It was believed that insulin was the major stimulator of adipose tissue metabolism. By using antibodies to insulin they were able to block this insulin stimulation response. However, insulin-blocked serum still stimulated adipose tissue metabolism. They concluded that serum contained an "insulin-like activity" in addition to the insulin already present in serum.

In 1972, Pierson and Temin used chick embryo fibroblasts in cell culture as a model for studying protein components of serum. They isolated and partially purified a low molecular weight fraction from calf serum that stimulated DNA synthesis of cultured chick embryo fibroblasts and also had non-supressible insulin-like activity. In 1976, Rinderknecht and Humbel determined the amino-terminal sequences of two polypeptides with non-suppressible insulin-like and cell growth promoting activities in human serum. These polypeptides were named "insulin-like growth factors".

Ross and Glomset (1973) while studying lesion formation on the walls of arteries, observed that the focal accumulations of smooth muscle cells from the intimal wall of the artery was a fundamental part of atherogenesis. Using tissue cultures of smooth muscle cells derived from the thoracic aorta of macaques (M. nemestrina), experiments were conducted in an attempt to identify growth

promoting substances present in various serum fractions. Later, Ross et al. (1974) observed that dialyzed serum from clotted monkey blood, "blood serum" promoted the proliferation of these cultured smooth muscle cells. Dialyzed serum prepared from platelet poor plasma ("plasma serum") was, however, much less effective in stimulating the proliferation of smooth muscle cells. Addition of platelets and calcium to platelet-poor plasma increased the activity of the plasma serum to the same level as that of the blood serum. They concluded that much of the growth-promoting activity of dialyzed serum was directly or indirectly derived from platelets. Ross et al. (1974) suggested the name "platelet derived factor", for this growth factor (PDGF) present in platelets which stimulated the otherwise nonproliferative cultured smooth muscle cells to proliferate.

Gospodarowicz (1974) applied extracts of different organ tissues to cultures of resting 3T3 fibroblasts. He found that brain or pituitary extracts stimulated DNA synthesis and cell division and suggested that there was a "fibroblast growth factor" (FGF) present in the brain or pituitary extracts. Gospodarowicz and Morgan (1974) observed that when 3T3 cells were grown in the presence of these extracts, the cells became rounded and went through one or two cycles of division with no sign of contact inhibition. Gospodarowicz (1975) purified from bovine pituitary gland an FGF responsible for the control of 3T3 cell division in tissue culture. It was distinct from all other previously described pituitary hormones and had its most potent mitogenic effect on 3T3 fibroblasts. In 1978, Gospodarowicz et al. isolated two forms of FGF from bovine brain and found that they had a mitogenic

effect on a wide variety of mesoderm-derived cells.

DeLarco and Todaro (1978) while working with murine virus-transformed mouse fibroblasts noted that these cells produced polypeptide growth factors and released them into serum-free medium. These sarcoma growth factors (SGF) were found to be potent mitogens: they caused overgrowth, morphological transformation of normal fibroblasts, and also strongly stimulated anchorage-independent growth of cells that would not multiply and form colonies in soft agar in their absence. From these observations they concluded that these SGF's were a new class of polypeptide tropic factors that conferred on fibroblasts in vitro, properties associated with the transformed phenotype. (One of these SGF's was later determined to be an EGF-like molecule).

Cultures of non-neoplastic rat kidney fibroblasts (NRK) became the standard test cells for assaying for the presence of SGF. A positive test was considered the production of the transformed phenotype in these NRK cells. Roberts et al. (1981) reported non-neoplastic tissues of the adult mouse including submandibular gland, kidney, liver, muscle, heart, and brain contained this SGF. SGF was characterized as an acid-stable peptide requiring disulfide bonds for activity, with low molecular weight (600-10,000 daltons). Assoian et al. (1983) reported the presence of SGF in platelets. SGF began to be referred to as "transforming growth factor beta" (TGF-ß).

In contrast to TGF-ß's ability to stimulate overgrowth and transformation of phenotype in NRK cells, Holley et al. (1980) reported that TGF-ß was an

inhibitor to the replication of epithelial cell cultures derived from African green monkey kidney. They reported that TGF-ß reversibly arrested the growth of these cells in the G_1 phase of the cell cycle. This inhibitory response became a second assay for the presence of TGF-ß. From these contradicting results of stimulation and inhibition, Roberts et al. (1985) concluded that TGF-ß was a bifunctional signal molecule.

Some growth factors (EGF, TGF-ß) were originally isolated from neoplastic cells grown in culture and appeared to stimulate cells in an autocrine fashion. Other growth factors (FGF) were isolated from normal tissues. While still other growth factors (IGF, PDGF) were isolated from serum and platelets and appear to function in a paracrine fashion. Regardless of their origins, all of these growth factors have one important common property, they stimulate cell division in normal as well as neoplastic cell types. However, there are some growth factors which display another important property, that is, they stimulate cellular differentiation. Some growth factors (TGF-ß) terminate cell division and stimulate undifferentiated cells to become a differentiated cell type.

The field of growth factor research has expanded rapidly over the past 20 years. With the advent of molecular biology techniques such as polymerase chain reaction (PCR) and improved protein purification techniques, a growing number of growth factors have been discovered. Yet considering the vast numbers of different types of cells in the bodies of higher animals, a relatively small number of growth factors appears to serve in different combinations, to selectively

regulate the proliferation of each of the many types of cells (Alberts et al., 1989).

To try to organize this collection of growth factors, researchers have divided growth factors into catagories called families. Each growth factor family contains several members with a high degree of sequence homology in their amino acid composition. A list of some growth factor families and their members is presented in Table 1.

The growth factors listed in the table and outlined above can be described as "skeletal growth factors" because they effect mainly skeletal tissues. Other tissues such as heamatopoietic and lymphoid also produce their own growth factors, but these other growth factors will not be discussed here. What follows is a general outline of the structures of growth factors and how they communicate their message to cell nuclei. Following this the relationship of growth factors to bone physiology and fracture repair will be discussed.

1.1.1 The Structures of Several Skeletal Growth Factors

Growth factors are small peptides that in their active forms, range in size from 53 amino acids for EGF (Scott et al., 1983; Gray et al., 1983) to 112 amino acids for TGF-ß (Derynck et al., 1985). They are generally derived from larger precursor molecules. The EGF precursor is 1217 amino acids long (Gray et al., 1983, Scott et al., 1983) and TGF-$ß_1$ has 391 amino acids (Derynck et al., 1985).

PDGF is a glycosylated two chain polypeptide with an isoelectric point (pI) of 9.8 and a molecular weight of 30,000 daltons (Deuel et al., 1981; Heldin et al., 1979). It has eight half cysteine residues all apparently linked by disulfide bonds (Antoniades et al., 1979). PDGF is composed of two disulfide-linked chains (A and B) with approximately 60% sequence identity. All three possible combinations of A and B chains exist naturally (Kaslawkas et al., 1988).

TGF-ß is composed of 112 amino acids and is structured as two 12,500 dalton subunits held together by disulfide bonds. The 112 amino acid peptide contains nine cysteine residues (Derynck et al., 1985). Three forms of TGF-ß are obtained by alternating the chains of the two dimers. TGF-$ß_1$ is a disulfide-linked homodimer consisting of two $ß_1$ subunits. TGF-$ß_2$ is a disulfide-linked homodimer consisting of two $ß_2$ subunits. The third, TGF-$ß_{1.2}$ is composed of one $ß_1$ subunit and one $ß_2$ subunit (Cheifetz et al., 1987). The $ß_1$ and $ß_2$ subunits are closely related; they share 70% amino acid sequence identity in their N-terminal halves. TGF-$ß_3$, has been reported and it shares approximately 80% homology with TGF-$ß_1$ and $ß_2$ (Teh Dijke et al., 1988). TGF-$ß_4$ mRNA and protein have been demonstrated in developing cartilage and heart tissues (Jakowlew et al., 1991). TGF-$ß_5$ mRNA was isolated by Kondaiah et al. (1990). In addition to its mature protein length of 112 amino acids with 9 cysteines, it had 76,66,69, and 72% sequence homology to TGF-$ß_1$-$ß_4$ respectively.

IGF is composed of two members, IGF-I and IGF-II, both structurally homologous to insulin (Zapf et al., 1986). IGF-I is a basic, single chain peptide

composed of 70 amino acids and has a molecular weight of 7649 daltons (Baxter, 1986; Rinderknecht and Humbel, 1978a). IGF-II is a slightly acidic peptide composed of 67 amino acids and weighing 7471 daltons. The amino acid sequence of IGF-II is 62% homologous with that of IGF-I (Rindenknecht and Humbel, 1978b).

Acidic Fibroblast Growth Factor (aFGF) and basic Fibroblast Growth Factor (bFGF) are members of the heparin-binding growth factor (HBGF) family (Burgess and Maciag, 1989). aFGF has an acidic pI of 5-6 and is composed of 140 amino acids (Thomas et al., 1980; Esch et al., 1985), whereas, bFGF has a basic pI of 9-10 and is composed of 146 amino acids (Lemmon et al., 1982; Esch et al., 1985). The amino acid sequences are 50% homologous. The main difference is in the affinity for heparin, which is much higher for bFGF than for aFGF (Vigny et al., 1988).

1.1.2 Binding to Growth Factor Receptors

The many intrachain disulfide bonds and the properties of the R-groups on the amino acids give each growth factor a final conformation unique to its family. This allows growth factor receptors located on cell surfaces to distinguish among classes of growth factors. For example, the monomeric EGF molecule has a structural shape similar to that of a mitten grasping an object.

The interaction between EGF and its receptor (EGF-r) is thought to take place through the hollow area of the mitten (Kohda et al., 1988) via hydrogen bonding.

It is important to distinguish between the amino acids required for the folding and assembly of the three-dimensional structure, and those amino acids required to activate the growth factor receptor. IGF-I receptors identify and bind to the specific amino acid sequence (Phe_{23}-Tyr_{24}-Phe_{25}) or FYF of the IGF-I molecule (Cascieri et al., 1988). The peptide sequence (Arg_{259}- Gly_{260}-Asp_{261}-Leu_{262}) or RGDL is conserved in TGF-ß and is thought to perform this function also (Derynck et al., 1988).

1.1.3 Structure of Growth Factor Receptors

The PDGF receptor (PDGF-r) consists of an extracellular ligand domain, a single membrane-spanning segment, and an intracellular tyrosine kinase domain with associated intrinsic kinase activity. It has a distinctive pattern of cysteine spacing within its extracellular domain that includes amino acid sequences characteristic of the immunoglobulin gene (Heldin and Westermark, 1989).

Cross-competition experiments with AA, AB, and BB forms of PDGF revealed the existence of two different PDGF-r types: type A PDGF-r binds all three dimeric forms of PDGF; type B PDGF-r binds PDGF-BB with high affinity, AB with lower affinity, but does not bind PDGF-AA (Heldin et al., 1988a).

Studies using the three dimeric forms of PDGF to stimulate thymidine incorporation into human fibroblasts suggested that type B receptor plays a major role in mediating the mitogenic response of PDGF (Heldin et al., 1988b).

The IGF-I-r is a heterotetramer composed of two disulfide-linked "ab" dimers. The "a" subunit contains the growth factor binding site. The "b" subunit contains the membrane spanning segment as well as an intrinsic tyrosine-specific protein kinase activity (Tollefsen and Thompson, 1988).

The FGF-r is a 135 kDa phosphoprotein which also has intrinsic tyrosine kinase activity (Huang and Huang, 1986). Its exact structure has not yet been elucidated, however it does have extracellular, membrane spanning, and intracellular segments.

Two distinct types of TGF-ß receptors (TGF-ß-r) have been distinguished as a result of structural analysis by ^{125}I-TGF-ß peptide mapping. Type I receptors are composed of a 280 kDa receptor believed to be a disulfide linked glycosylated receptor complex (Massague, 1985). Type II TGF-ß receptors are composed of 85-110 kDa receptor form. This second receptor type does not appear to be a disulfide linked receptor complex (Cheifetz et al., 1986).

The 280 kDa type I receptor displays high affinity for both TGF-$ß_1$ and TGF-$ß_2$. In contrast to this, the 85 kDa type II receptor has high affinity for TGF-$ß_1$, but lower affinity for TGF-$ß_2$. Cheifetz et al. (1987) suggested that the existence of distinct forms of TGF-ß that interact differently with a family of TGF-ß-r's may provide flexibility to the regulation of tissue growth and differentiation by the

TGF-ß system.

Recently it has been found that the binding of PDGF-BB to purified B-receptor induces dimerization of the receptor in a concentration dependent manner. Dimerization is closely associated with activation of the receptor kinase (Heldin and Westermark, 1989). It is possible that other growth factors with tyrosine kinase activity (i.e.FGF and IGF-I) may also oligomerize in response to binding of their growth factors.

The phenomenon known as down-regulation reflects the ability of cells stimulated by PDGF to regulate the distribution of their receptors via selective internalization and intracellular degradation. Down-regulation of these receptors lasts as long as high concentrations of their ligands are present in the medium and is slowly reversed, upon removal of ligands, mainly by de novo receptor synthesis. Down-regulation by their growth factor ligand molecules is probably a cellular mechanism regulating the cell's responsiveness to these growth factors (Schlessinger, 1986).

Friesel and Maciag (1988) demonstrated that FGF and its receptor were rapidly internalized and directed to a lysosomal cellular compartment where the FGF molecule was slowly degraded. Nilsson et al. (1983) observed that PDGF and its receptor were internalized via coated regions of the plasma membrane and carried to lysosomes for degradation. Some receptor recycling back to the plasma membrane also occurred.

EGF-r mutants which lack tyrosine kinase activity internalize the receptors

normally, yet the receptors do not undergo ligand-stimulated degradation. Thus, tyrosine kinase activity may affect receptor recycling and intracellular routing and/or receptor degradation (Backer et al., 1989). TGF-ß-r is not subject to down-regulation by high levels of TGF-ß and lacks it's own intrinsic tyrosine kinase activity (Fanger et al., 1985). Perhaps this lack of tyrosine kinase activity is why TGF-ß-r is not down-regulated.

1.1.4 Intracellular Events and Genetics Involved in GF Stimulation

Intracellular events initiated as a result of growth factor stimulation include rapid ionic changes, protein phosphorylation, membrane lipid metabolism changes, and production of secondary messengers. It is the diffusion of secondary messengers which enables a signal to propagate rapidly throughout the cell. Two major signal pathways for growth factor receptors have been deduced. One employs the hydrolization of the membrane-bound lipid phosphatidylinositol. The other employs the phosphorylation of a protein substrate by the tyrosine kinase portion of the growth factor receptor (Berridge, 1985). The exact degree to which these two pathways contribute to the overall cellular response has not been determined (Berridge, 1985).

The tyrosine kinase activity of the growth factor receptors has been hypothesized to be involved in the phosphorylation of protein substrates.

Magnaldo et al. (1986) reported that the FGF-r signaling pathway is not mediated through the breakdown of inositol lipid and protein kinase C as in the PDGF-r signal pathway. Stimulation of the FGF-r activates a tyrosine kinase activity in vivo. This leads to the appearance and phosphorylation of a 90 kDa protein substrate (Coughlin et al., 1988). A possible role for this 90 kDa substrate in signal transduction is suggested by the rapid phosphorylation of this protein upon FGF stimulation and its rapid disappearance upon FGF removal. It has also been reported (Frackelton et al., 1984; Ek and Heldin, 1984) that PDGF-r contains tyrosine kinase activity and phosphorylates several proteins in the cytoplasm.

By regulating gene expression within their target cells, growth factors regulate differentiation and morphogenesis. A common set of genes (including the proto-oncogenes c-myc, c-fos, and c-jun), are expressed in response to numerous growth factors. It is possible that these proto-oncogenes are prototypes of a class of genes whose products behave as intracellular mediators of the growth factor response (Mercola and Stiles, 1988).

These early response genes encode for nucleoprotein transcription factors. The transcription factors bind upstream to the promoter sequences of other genes involved in cell replication. When c-fos is transcribed, it produces the nuclear phosphoprotein fos (Curran et al., 1984). This phosphoprotein is known to be associated with the transcriptional complexes of other genes (Sambucetti and Curran, 1986). c-jun encodes for the nuclear protein AP-1, which is thought to perform a function similar to fos (Bohmann et al., 1987). The transcription of

these proto-oncogenes is triggered by several second messenger systems. Many contain a sequence binding site for a protein factor found in nuclear extracts from a variety of cell types. This DNA sequence binding site is called the serum response element (SRE) (Gilman, 1988). It is believed that protein serum response factors (SRF) within the nucleus are recipients of the incoming signals from the second messengers. When stimulated, these proteins attach to the SRE and initiate transcription of the proto-oncogenes (Treisman, 1987).

A negative feedback mechanism is associated with these proto-oncogenes. For example, c-fos promoter activity is repressed by fos protein. This accounts for the dramatic decrease in the transcriptional rate of the c-fos gene after peak induction. It is thought that the fos protein may displace the SRF from the c-fos SRE (Sassone-Cors et al., 1988).

The proto-oncogenes c-myc and c-fos have important roles in cells which are involved in the cell cycle. c-myc gene expression is highest in proliferating cells and its expression is rapidly reduced when cells are induced to differentiate (Gonda and Metcalf, 1984; Reitsma et al., 1983). Generally, when cells cease growing and begin to differentiate, c-myc expression falls and c-fos expression increases (Kaslawkas et al., 1988). Sustained, unregulated expression of induced c-myc inhibits cellular differentiation in a dose-dependent manner in mouse erythroleukemia cells. These types of experiments support the hypothesis that c-myc drives proliferation, while its absence allows differentiation to occur. There is evidence that two different c-myc mRNA degration pathways are utilized, one

in growing cells and one in differentiating cells. In growing cells, c-myc mRNA is degraded slowly, while in differentiating cells it is degraded rapidly (Swartwout and Kinniburgh, 1989). The observation of c-fos expression in studies of in vitro differentiation systems suggests a correlation between c-fos expression and cellular differentiation (Muller et al., 1984).

1.1.5 Transcriptional Control of Growth Factors

TGF-ß provides some interesting insights into the transcriptional control of growth factors. TGF-ß proteins are frequently associated with the same cell type as their mRNA. However, in some cases, TGF-ß proteins can be localized to cells where mRNA to TGF-ß is not produced. Pelton et al. (1991) suggested there may be some pattern to the transcription, translation, and secretion of TGF-ß. In situ hybridization experiments have been used to study TGF-ß mRNA expression in embryonic development. Hybridization designates which cells and tissues synthesize mRNA transcripts for a given gene, however these studies cannot determine if the mRNA's are translated into proteins. The relative amount of mRNA may not reflect similar amounts of protein (Pelton et al., 1991). Lehnert and Akhurst (1988) used in situ hybridization to probe for TGF-$ß_i$ mRNA in mouse embryos. Strong signals for TGF-$ß_1$ mRNA were detected in epithelium at sites undergoing morphogenesis. TGF-$ß_1$ protein was not detected

in the epithelium, but was detected in underlying mesoderm. Lehnert and Akhurst (1988) suggested that the absence of TGF-$ß_1$ protein in the epithelium might be explained if the TGF-$ß_1$ precursor protein was not recognized by the antibodies, or the newly synthesized TGF-$ß_1$ protein was very rapidly transported from the epithelium to the underlying mesoderm. Akhurst et al. (1990) while studying the embryonic formation of heart valves in mice, found that high levels of TGF-ß mRNA in cardiac valves correlated with times of mesenchymal and endothelial cell growth and tissue movement. In contrast, intense staining with TGF-ß antibodies did not appear until later stages of embryogenesis and continued to increase until shortly after birth. Their results suggested a lack of correlation between TGF-ß protein and mRNA levels in embryonic tissue. In adult tissue, very low transcription levels may be translated to give much higher levels of protein.

Miller et al. (1990) examined the in vivo expression of mammalian TGF-ß in adult mouse tissues and organs. Northern blot analysis revealed that a single mRNA transcript for TGF-$ß_1$ and TGF-$ß_3$ was expressed in the adult mouse, but, four TGF-$ß_2$ transcripts of distinctly different sizes were expressed. No attempt was made to determine how many of these TGF-$ß_2$ mRNA's were translated into functional proteins.

In some instances, TGF-ß mRNA is produced but no TGF-ß protein is expressed by the cell. Assoian et al. (1987), observed in alveolar macrophages that there was minimal TGF-ß protein secretion in unactivated monocytes, even

though TGF-ß mRNA was expressed in these cells at a level similar to that present in activated macrophages which secrete TGF-ß. Van Obberghen-Schilling et al. (1988) reported that TGF-$ß_1$ increased steady-state levels of its own message in several types of normal and transformed cells grown in culture. Accumulation of TGF-$ß_1$ mRNA could be detected by Northern Blot analysis within three hours of addition of the TGF-$ß_1$ peptide to the cultured cells. Enhanced TGF-$ß_1$ mRNA levels persisted as long as TGF-$ß_1$ was present in the culture medium, suggesting an autocrine form of stimulation.

1.2 Growth Factors in Bone and Cartilage

Bone itself acts as a reservoir of non-collagenous extracellular proteins which include growth factor molecules such as FGF (Hauschka et al., 1986), TGF-ß (Seyedin et al., 1985), and IGF (Frolik et al., 1988). In which cells and under what conditions these growth factors are produced in vivo is only beginning to be established. A review of each of these growth factors and some of the findings in relation to bone physiology follows.

1.2.1 Insulin-like Growth Factor

In vitro studies performed on chondrocytes isolated from embryonic tissue have demonstrated that IGF-II is a potent stimulator of chondrocyte proliferation. The same studies performed on chondrocytes isolated from adult tissue demonstrated that IGF-I was a more potent stimulator of chondrocyte proliferation (Vetter et al., 1986). Bohme et al. (1992), while studying chick embryo chondrocyte cultures, observed that IGF-I administration triggered cell proliferation and increased matrix deposition.

With respect to bone, Schmid et al. (1984) noted that IGF-I enhanced the differentiation of osteoblast-like cells into osteoblasts. Hock et al. (1988), substantiated this observation by recording that the addition of IGF-I to cultures of 21 day-old fetal rat calvaria, increased DNA, collagen, and noncollagen matrix synthesis. Mohan et al. (1988) reported that human osteoblast-like cells in culture produce both IGF-I and IGF-II. Ernst and Froesch (1988) reported that osteoblast-like cell cultures synthesize IGF-I in response to stimulation by growth hormone.

Two approaches have been used to study the effects of IGF in vivo. One approach was to study the administration of IGF by injection into a rat. The second approach involved administering the hormone insulin into a blood vessel in a rat limb. Using the former approach, Schoenle et al. (1985), reported a pronounced insulin-like effect on glucose homeostasis and metabolism as well as

an increase in body weight and tibial epiphyseal width. Using the latter approach, Alarid et al. (1992) explored the possibility that insulin stimulated cartilage growth in hypophysectomized rats via local production of IGF-I. Infusion of insulin into the hindlimb of a rat significantly increased the width of the epiphyseal plate of the infused limb. Immunohistochemical analysis with an antiserum to IGF-I showed that the growth response was accompanied by the accumulation of IGF-I in the chondrocytes in the tibial plate. When the optimum dose of insulin was infused with antiserum to IGF-I, the growth response was completely nullified.

1.2.2 Transforming Growth Factor ß

TGF-ß is a multifunctional peptide that controls proliferation, differentiation, and other functions in many cell types including bone and cartilage cells. TGF-ß increases DNA synthesis by growth plate chondrocytes in cell culture (O'Keefe et al., 1988). Frazer et al. (1991) and Villiger and Lotz (1992) observed that cultured chondrocytes release TGF-$ß_1$, -$ß_2$, and -$ß_3$ into culture media. The chondrocyte conditioned media contained active TGF-ß and larger quantities of TGF-ß in a latent form. It was also suggested that the TGF-ß produced in latent form might be a way of storing the cytokine for future use. Thorp et al. (1992) studied the immunolocalization of TGF-$ß_1$, -$ß_2$, and -$ß_3$ in the growth plate, epiphysis, and metaphysis of the tibiotarsus of three-week-old chicks. The

different TGF-ß isoforms were immunohistochemically detected in hypertrophic chondrocytes, chondroclasts, osteoblasts, and osteoclasts. They suggested that TGF-ß plays a role in the coupling of new bone formation to bone and cartilage matrix resorption.

TGF-ß is mitogenic for fetal rat calvariae in serum-free organ culture (Centrella et al., 1986). Centrella et al. (1988) studied the effect of varying the dosage of TGF-ß administered to osteoblast-enriched cell cultures. They found that low TGF-ß doses increase DNA synthesis. Higher levels were less mitogenic, but, stimulated collagen production, and decreased alkaline phosphatase activity. Using tissue cultures of osteoblast-like cells derived from pericranial periosteum of 21-day-old fetal rats, Hock et al. (1990) studied the effects of administration of recombinant TGF-$ß_1$. TGF-$ß_1$ was a potent mitogen on cells of the periosteum and presumably increased the number of osteoblast precursors. It also had specific stimulatory effects on bone matrix formation and inhibitory effects on osteoclast differentiation. Robey et al. (1987) using Northern blot analysis, observed the presence of TGF-ß mRNA in developing bone tissue and fetal bone-forming cells. They suggested that because TGF-ß was synthesized and secreted by metabolically-labelled bone cells in vitro, TGF-ß may have an autocrine function in bone cell metabolism. In vivo studies have been performed by Noda and Camilliere (1989). In their experiments, TGF-ß was injected directly into the periostea of parietal bones in neonatal rats. The result was that TGF-ß stimulated the formation of periosteal woven bone in a dose-dependent manner.

In addition, TGF-ß's effects were localized at the site of injection. Joyce et al. (1990c) studied the effect of exogenous TGF-$ß_1$ injected into the femoral periosteum of newborn Long-Evans rats. They found proliferation of mesenchymal cells within the cambial layer of the periosteum after two, 200 ng injections. After further injections, chondrocytes were seen and further injections resulted in an even larger cartilaginous mass. After cessation of the injections, endochondral ossification occurred and the cartilage was replaced by bone.

1.2.3 Fibroblast Growth Factor

FGF stimulates proliferation of low-density primary cultures of rabbit costal chondrocytes (Kato and Gospodarowiez, 1984) and cultures of rabbit articular chondrocytes (Jones and Addison, 1975). It also stabilizes the chondrocyte phenotype once cultures become confluent (Kato and Gospodarowiez, 1985). Trippel et al. (1992) studied the interaction of bFGF with cultures of chondrocytes derived from bovine growth plates. They detected two distinct classes of FGF binding sites on these growth plate chondrocytes and suggested that bFGF participated in the regulation of skeletal growth at the growth plate.

Canalis and Raisz (1980) demonstrated that the major effect of FGF on rat calvaria was to increase DNA synthesis and stimulate the proliferation of periosteal fibroblasts. Globus et al. (1988) studied the effects of FGF on

osteoblast-like cells derived from central bone explants of fetal calf calvaria. They reported that FGF was mitogenic for bone cells in vitro. FGF increased both the rate of bone cell proliferation and the final density at confluence. Canalis et al. (1988) reported that bFGF stimulated cell replication in cultures of rat calvaria. The stimulatory effect was greatest in the osteoprogenitor-cell-rich periosteal layer. bFGF also had a direct inhibitory effect on collagen synthesis. McCarthy et al. (1989) observed that both aFGF and bFGF were mitogenic for bone cell populations with fewer osteoblastic characteristics. bFGF was intrinsically 10-fold more stimulatory than aFGF. Both FGF's decreased alkaline phosphatase activity. Fu et al. (1991) investigated the immunoreactivity of aFGF during progressive stages of development in rat embryos. The most intense immunohistochemical staining for aFGF occurred in ossification centers therefore suggesting aFGF may play a role in stimulating chondroblast and osteoblast proliferation.

In vivo studies have concentrated on FGF administration in response to injury. Gospodarowiez et al. (1975) treated the amputated stumps of adult frogs with FGF. An agar pellet containing 100μg of FGF was placed over the stump and beginning five days later, 10μg of FGF in 50μl of saline was injected into the stump at 48 h intervals. This resulted in large deposits of undifferentiated cells resembling a regeneration blastema. In some test animals, differentiation of large masses of chondrocytes and muscle fibers was observed. Wellmitz et al. (1980) tested the effectiveness of bovine brain derived FGF extract on articular

cartilage regeneration in rabbits which had received a knee-joint injury in a standardized manner. They applied 2 mg and 1 mg FGF extract dissolved at 1mg/ml saline on days 5 and 7 post-operation, respectively. This caused the stimulation of proliferation and differentiation of chondrocytes in injured articular cartilage.

1.2.4 Platelet-derived Growth Factor

Bowen-Pope et al. (1985) observed PDGF-r were expressed only on "connective tissue" cell types such as articular chondrocytes. Wroblewski and Edwall (1992) administered PDGF to cultures of rat rib growth plate chondrocytes which stimulated DNA synthesis in a dose dependent manner and caused an increase in alkaline phosphatase activity.

Canalis (1981) studied the effects of PDGF on cultures of fetal rat calvariae. His studies indicated that PDGF stimulated DNA synthesis in bone as well as non-specific protein synthesis in short-term cultures. Canalis et al. (1989) quantified these observations by noting that PDGF stimulated DNA synthesis primarily in the fibroblast and precursor-cell-rich periosteum of rat calvariae. In addition, they supported an earlier report by Tashjian et al. (1982) in which it was reported that PDGF stimulated bone resorption primarily by stimulating prostaglandin E production. Pfeilschifter et al. (1992) found differential effects on

the stimulatory activity of the three isoforms of PDGF (AA, AB, BB) on fetal rat calvaria cultures. PDGF was found to be the most potent, PDGF-AB less potent, and PDGF-AA least potent in stimulating of DNA synthesis.

PDGF or PDGF-like molecules have been isolated from bone matrix (Hauschka et al., 1986) and osteosarcoma cells (Heldin et al., 1986). Rydziel et al. (1992) extracted and quantified PDGF-AA in serum-free culture medium conditioned by fetal rat osteoblast-enriched cells. This was conclusive evidence that osteoblast cells synthesize their own PDGF in an autocrine fashion.

Howes et al. (1988) studied the effect of PDGF on cartilage and bone formation using an in vivo model. They subcutaneously implanted demineralized bone matrix supplemented with PDGF into rats. It was noted that, in older rats, the PDGF-supplemented treatment increased production of mRNA for collagen II, alkaline phosphatase activity, and the calcium content of the implant. Joyce et al. (1990a) observed the effect of PDGF in vivo using exogenous PDGF injected under the periosteum of the femur of new born rats. In their model, the periosteum thickness doubled after fourteen injections of 200 ng PDGF per injection.

1.2.5 Summary of Growth Factors and Skeletal Tissues

In vitro studies have demonstrated IGF's ability to stimulate replication and

matrix formation by chondrocytes, cellular differentiation, and matrix production by osteoblast-like cells. Also, osteoblast-like cells can be induced to produce and secrete IGF into their culture media. In vivo studies suggest that IGF can produce an insulin-effect on soft tissues (i.e. adipose), and a growth hormone effect on skeletal growth (i.e. femoral growth plate).

In vitro research on the effects of TGF-ß demonstrated it's ability to stimulate DNA synthesis in chondrocyte cultures. These cultures also secrete TGF-ß into their culture media and respond to it, suggesting a possible autocrine function. TGF-ß is mitogenic for bone cell cultures and, depending on the dosage, stimulates bone matrix production. In vivo administration of TGF-ß has demonstrated it's ability to stimulate bone formation when injected into the periostea of bones.

In vitro investigation of FGF has revealed that it is a stimulator of chondrocyte and osteoblast-like cell replication. With respect to osteoblast-like cell cultures, FGF was found to inhibit the expression of differentiated cell markers (i.e. alkaline phosphatase activity). In vivo administration of FGF demonstrated it's ability to stimulate the replication of undifferentiated mesenchymal cells which later become cartilage and bone.

In vitro administration of PDGF to cultures of chondrocytes or osteoblast-like cells resulted in DNA synthesis. Cultures of osteoblast-like cells were also found to synthesize PDGF and respond to it, suggesting a possible autocrine role for PDGF in bone cells. In vivo administration of PDGF has demonstrated it's ability

to stimulate cell division in the periostea of long bones.

1.3 Cellular Events in Fracture Repair

The development of various kinds of connective tissue cells and the differentiation of cartilage and bone occurs in fracture healing in a standard pattern similar to fetal osteogenesis. In fracture repair and in fetal osteogenesis, a model of the bone is first formed from fibrous connective tissue and cartilage. The model is then invaded by blood vessels, perivascular connective tissue cells, and osteoblasts from periosteum and endosteum, and is finally replaced by new bone (Urist and McLean, 1953).

The response to fracture produces a well-defined cascade of tissue responses [requiring much energy in the form of carbohydrate metabolism (Kuhlman and Bakowski, 1975)] which are designed to remove tissue debris, re-establish the vascular supply, and produce new skeletal matrices. The fracture initially disrupts the local blood supply, causing haemorrhage, anoxia, death of cells, and an aseptic inflammatory response (Simmons, 1985). A clot soon forms between the bone ends. Polymorphs, histiocytes, and mast cells soon make their appearance and the process of clearing debris begins (McKibbin, 1978). A fibroblast-rich granulation tissue forms when the clot is very large (Simmons, 1985).

This inflammatory response is believed to initiate the repair response i.e.

proliferation-differentiation of pluripotent stem cells. Hulth (1980) postulated that the damaged tissue at a fracture site stimulated (possibly via molecular determinants) the structural genes of undifferentiated cells to produce mRNA and proteins for either fibrous tissue and/or bone formation.

Traditionally, the repair process has been described in terms of the organization of the fracture haematoma. The haematoma is invaded by fibrovascular tissue which replaces the clot and lays down collagen fibers and matrix which will later become mineralized to form the woven bone of the primary callus. The most active cell division is seen in the forefront of this invasion, leaving the more mature tissue closer to the bone ends to differentiate. The peripheral region of the callus is where the first new bone is seen anchoring the newly formed mass of callus to the bone ends (McKibbin, 1978). It is also in this peripheral zone that alkaline phosphatase activity becomes detectable and increases in intensity during the remainder of the osteogenic phase (Raekallio and Makinen, 1969).

From a cellular perspective, fracture healing initially requires the proliferation of cells with a chondrogenic potential, then population of the fracture site with these cells. These chondrogenic and osteogenic elements are drawn from cells resident around the fracture site, while others, like osteoclasts, are drawn from monocyte-macrophage cells (Simmons, 1985). The cambial layer of the periosteum is the primary cell surface for the external callus. The periosteal cell has the potential to become a chondroblast or an osteoblast (Kernek and Wray,

1973).

The periosteum consists of an outer fibrous layer and an inner cambial layer (McKibbin, 1978). The first evidence of cell division occurs within 24 h after fracture and is seen in the periosteum and tissues immediately around it. The requirement for cellular proliferation of cells with chondrogenic potential is met initially by the proliferation of undifferentiated stem cells within the cambial layer of the periosteum and endosteum (Simmons, 1985).

Two theories (McKibbin, 1978) have arisen to explain the origin of osteoproginator cells in the early fracture callus. One theory suggests that the repair tissue arises from specialized cells with a predetermined commitment to bone formation. These are the osteoprogenitor cells which occur in close association with the bone surface. As these cells proliferate, the fibrous periosteum becomes pushed away from the bone to produce two advancing collars of callus, which eventually fuse with one another. The other theory holds that the repair tissue arises from activity of previously uncommitted fibroblasts [polymorphic mesenchymal cells of Brighton and Hunt (1991)] which develop the power of osteogenesis if given the appropriate environmental stimulus. Thus, the reparative tissues arise from the surrounding soft tissues and not from the bone itself (McKibbin, 1978).

Low oxygen pressure gives rise to cartilage being the first skeletal tissue formed (Girgis and Pritchard, 1958). The formation of a fibrous, fibrocartilaginous, or cartilage model is always a preliminary step of the process of

bone repair. The periosteum is the tissue from which cartilage initially appears (Cohen, 1956). The size and shape of the cartilage callus conforms to the proportions of the fracture gap and the displacement of bone ends. In displaced fractures of the shaft of long bones, the defect becomes filled with a fibrocartilaginous callus which re-establishes the continuity of the original bone. An irregular type of endochondral ossification and replacement of the cartilage by bone follows by means of a special design of construction. In cross-section, this resembles the process of forming a fixed-arch-bridge (Urist and Johnson, 1943). New bone grows out upon the surface of the model enveloping the cartilage to form an arch of new bone over the fracture gap. The new bone grows through and replaces the cartilage centripetally toward the fracture gap. The space between the fracture ends becomes filled with new bone which grows inside the arch in the final stage of healing (Urist and McLean, 1953). The formation of bone and replacement of cartilage coincides with revascularization of the cartilage callus and increased oxygen availability. This is an important event since bone formation involves increased oxygen consumption (Penttinen, 1972). The cartilage of the fracture callus is replaced endochondrally by lamellar bone (Simmons, 1985).

Once the fracture has been bridged satisfactorily, it is necessary for the newly formed bone to adapt to its new function. The process essentially involves simultaneous bone removal and replacement through the respective activities of osteoclasts and osteoblasts together with accompanying blood vessels (McKibbin,

1978).

1.3.1 Growth Factors and Fracture Repair

The effect of the administration of aFGF on normal fracture healing was examined in a rat fracture model by Jingushi et al. (1990). aFGF, in 1μg doses, was injected into the fracture site over a nine day period. The resulting calluses were significantly larger than control calluses. Histologically they demonstrated a marked increase in the size of the cartilaginous soft callus and concluded that aFGF injections induced cartilage enlargement.

Joyce et al. (1990a) observed the effect of PDGF both on early fracture calluses in vitro and in vivo using exogenous PDGF injected under the periosteum of the femur of newborn Long-Evans rats. In fracture callus organ cultures, PDGF at doses of 10 ng/ml stimulated a dose dependent five fold increase in thymidine incorporation. These experiments suggest a role for PDGF in the initiation of fracture repair by stimulating mesenchymal cell proliferation and the synthesis of intramembranous bone.

Using immunohistochemical and recombinant DNA techniques, Joyce et al. (1990b) demonstrated TGF-ß in regions of cell differentiation and proliferation during fracture repair. This established TGF-ß as a growth factor locally synthesized by cells in response to fracture. TGF-ß was added to fracture calluses

maintained in organ culture. Results included inhibition of type II collagen and cartilage specific proteoglycan during early fracture repair, and increased expression of type I collagen and osteonectin genes later during repair. These findings suggested that TGF-ß was synthesized in the fracture callus and initiated and regulated critical events during fracture repair.

1.4 Experimental Plan

Recent investigations have shown that skeletal tissue is a rich source of growth factors with important effects on bone formation. While in some instances, growth factors act as systemic agents (PDGF in platelets), an increasing body of evidence suggests that they are also produced by skeletal tissue for local action. Although a great deal of information about the influence of growth factors on bone metabolism has accumulated, the specific roles of growth factors in the physiologic regulation of bone remodeling and pathogenesis remains obscure. What is clear is that growth factors are important local regulators of bone metabolism (Raisz, 1988).

Bone is not static, but a dynamic living organ undergoing continuous remodelling throughout life. The basic mechanisms involved in the maintenance of bone is the remodeling process. This continuous turnover of bone is essential for bone repair. Bone resorption and bone formation are the two main events of

this remodeling process. Many local factors are implicated in bone formation and turnover (Bleicher, 1988).

The process of repairing a bone fracture presents an ideal situation where the activities of growth factors in cell recruitment, cell replication, and cellular differentiation can be studied. The fact that several growth factors are readily isolated from bone matrix strongly suggests a role for them in bone physiology. Therefore, it was proposed that the growth factors found in abundance in bone matrix (i.e. PDGF, TGF-β_1, FGF, IGF) are involved in the process of fracture repair. Many of these growth factors are produced by skeletal tissue in vitro. Therefore, the hypothesis was expanded to include the theory that many of these growth factors are produced locally during the repair process to act as local stimulators of this process. The following set of experiments were devised to test this hypothesis:

1.) A reproducible animal fracture repair model (male CD-1 mouse) was devised that would facilitate the study of the stages of fracture repair histologically. The model had to involve reproducible fractures in order to distinguish specific and quantifiable stages of fracture.

2.) An immunohistochemical method which preserves the growth factor antigens in bone following decalcification had to be devised. Existing fixation and decalcification methods seriously compromised both tissue integrity and antigenic

site preservation.

3.) The distribution of growth factors FGF, IGF, PDGF, and TGF-ß were studied using commercially available polyclonal antibodies during the stages of fracture. These antibodies were directed against human and in some cases bovine growth factors. The excellent preservation of proteins provided by the newly devised immunohistochemical method, and the closely conserved growth factor amino acid sequences in mammals, allowed the polyclonal antibodies to be effective. These commercial antibodies were accompanied by literature stating the ability to recognize growth factors cross species with no loss in specificity of the antibody.

4.) As the research project progressed, it was suggested that the immunohistochemically detected growth factors (PDGF, FGF, IGF-I, and TGF-ß) were being produced locally during fracture repair. To test for the local production of growth factors at or near the fracture site, two 20 base primers were made to the mRNA of mouse aFGF. The two 20 base primers, one 5'-3' and the second 3'-5' to the mRNA were constructed using cDNA sequence information found in the Gene Bank. RNA would be harvested and separated using phenol extraction. Using PCR technology, cDNA to the mRNA in the collected samples would be made and probed with the mouse aFGF primers.

Table 1. Growth factor families (Mercola and Stiles, 1988)

Family	Members
Epidermal growth factor	EGF TGF-α VGF
Insulin-like growth factor	IGF-I IGF-II Relaxin
Transforming growth factor-ß	TGF-$ß_{1-5}$ TGF-$ß_{1.2}$ Inhibin-A Inhibin-B Activin-A Activin-AB Mullerian inhibiting substance
Heparin-binding growth factors	Acidic FGF Basic FGF Products of int-2, hst Kaposi's sarcoma proto-oncogenes
Platelet-derived growth factor	PDGF-A PDGF-B PDGF-AB

Chapter 2: A fracture repair model suitable to study the stages of fracture repair histologically.

2.1 Introduction

Numerous methods have been devised to induce fractures in animals and thus allow study of the fracture repair process. These techniques suffered from several liabilities. One was the lack of reproducibility. Another was the lack of convenience. A third was the production of a fracture which did not resemble a natural fracture and thus gave a distorted picture of the processes involved in fracture repair.

The first liability is the lack of reproducibility. Hyldebrant et al. (1974) used digital pressure to produce a fracture in mice. While this method may fracture long bones in mice, it certainly is not practical for larger animals. The method itself suffers from too many uncontrollable variables. For example, the fracture pattern may not be consistent. A spiral fracture heals differently than a sharp transverse fracture. The amount of pressure needed to digitally produce the fracture is difficult to control and the fractures may be of varying dispiacement. Too much pressure may produce a large displacement between the fracture ends. Fractures with large displacements require more time to heal than those with minimal displacement.

A second liability is the lack of convenience. Jackson et al. (1970) designed a pneumatic punch press which standardized the amount of pressure necessary to produce a fracture. The problem was that the apparatus was so big that two people were needed to operate it. The size of the apparatus made it impractical to study fracture repair in small animals like mice.

A third liability was to expose a long bone surgically and then cut the bone with a saw (Yamagishi and Yoshimura, 1955). This surgical method introduces it's own problems. A skill in operating techniques and possibly a second body would be required. The amount of time to operate may be longer than a more efficient method. Thirdly, a cut in the epidermis may expose the animal to infectious agents thus requiring antibiotic therapy, which is costly and lengthens the time of healing.

Fractures produced naturally never contain smooth edges between the bone ends. Natural fractures have jagged edges which abut and grind against each other. Thus the inflammatory response between the cut surface and the natural fracture surface may be different. The amount of soft tissue damage around the fracture site may be different between natural and saw cut fractures. A difference in soft tissue damage may express itself in different degrees of inflammatory response and in degree of cell recruitment from the soft tissues. In the last thirty years there have been several suggestions for quantification of the fracture repair process. Early descriptions of the repair process centered on the analogy of the production of a blastema (Pritchard, 1963). Later, this analogy was replaced by

the division of the repair process into two phases, the phase of inflammation and the phase of osteogenic repair (McKibbin, 1978). Later still, these two phases were subdivided into four stages which were immediate injury response, intramembranous bone formation, chondrogenesis, and endochondral ossification (Joyce et al., 1990b).

A number of different animals have been used as models for the study of fracture repair; including rats (Mizuno et al., 1990), rabbits (Ketenjian and Arsenis, 1975), guinea pigs (Murakami and Kowalewski, 1966), sheep (McKibbin, 1978), dogs (Schenk and Willenegger, 1967), and mice (Hyldebrant et al., 1974). A comprehensive review of these animal models has been published by Asshurst (1992).

I have developed a simple closed fracture repair model which results in reproducible fractures, a precise time scale for the duration of the stages of the repair process, and a histological quantification of the repair process. This method is not only reproducible and convenient, it does not involve any surgical methods for the actual fracture other than the administration of an anesthetic.

2.2 Materials and Methods

CD-1 mice were obtained from Charles River Laboratories, St. Constant, Quebec, Canada. Only male mice were used in this study so that variables such as calcium flux from hormonal cycles would not be a factor in fracture repair. The mice were maintained in a temperature controlled room at a temperature of 22^0 C, fed a diet consisting of a commercial food stuff (Purina Rodent Lab Chow) and water, and maintained on a 12 h light schedule.

To examine the effects of fracture repair, the tibia of the mouse was selected as the site to induce the fracture. The tibia was chosen for two reasons; the tibia is a long bone, and it is easy to manipulate in a mouse. A custom designed fracture apparatus (Figures 1a and 1b) was constructed to induce the fractures. The dimensions of the apparatus were as follows: base 30 cm long and 12 cm wide, height 8 cm. The base was constructed of polished hardwood. Attached to this base was a brass fulcrum and a brass lever. The apparatus operated by swinging the lever down over the fulcrum. Contact with the tibia was made by two forks on the lever which were on either side of the fulcrum. This device was constructed with the principle of using three points at which contact with the shaft of the tibia would be made. One of the points of contact was the fork on either side of the lever. Force was transmitted to these forks as the lever was swung. The fulcrum was the third point of contact with the shaft of the tibia. The fulcrum produced an equal and opposite shearing force at this point on the tibia.

Several modifications over a period of several months were necessary. Initially, the surfaces of the forks and fulcrum were too sharp. This resulted in puncturing the skin which exposed the animal to the risk of infection. A second problem involved displacement of the lever. If the lever moved too far past the point at which the fracture occurred, the fracture ends were greatly displaced. To solve this problem, an adjustable stand was placed on the platform of the fracture apparatus and directly in line with the fulcrum. By adjusting this stand, displacement of the lever could be reduced to the point at which the fracture was induced. The result of all these modifications was a fracture apparatus which produced a closed, reproducible fracture with initial minimal displacement to the mouse tibia.

The oral anaesthetic Metofane (CDMV, St. Hyacinth, Quebec, Canada) was used to anaesthetize the mice after which one hind leg was shaved immediately before fracturing to clearly expose the site where the fracture would be produced. While under anaesthetic the mouse stayed unconscious for 12-15 seconds. The mouse was then reanaesthetized and placed on an adjustable pillow support (a plastic bag filled with fish tank gravel) adjacent to the fracture apparatus. The shaved leg was placed across the fulcrum of the apparatus and the lever brought down to produce the fracture. The cortical bone composing the tibia is of a brittle nature, therefore, minimal force was applied to the lever; the fracture produced was both heard and felt. The mouse was then placed in a clean cage alone and allowed to recover. A sham control was also conducted in which mice

were put through the same procedure except that no fracture was produced. The tibiae in the right hind limb of one hundred mice were fractured to test reproducibility of the fracture repair model. The treated mice were killed at 24 h intervals from 0 to 21 days post-fracture. They were euthanized via an overdose of Metofane followed by cervical extension. The fractured limb was removed, X-rayed, fixed in neutral buffered formalin, and decalcified in 10% acetic acid. For histological studies, the tissues were cut at 5μm and stained according to the Hall-Brunt-quadruple (HBQ) staining procedure (Hall, 1986). The HBQ staining procedure uses the histological stains, direct red, alcian blue, Mayer's hematoxylin, and celestine blue B, to provide excellent contrast between bone, cartilage, and connective tissue.

2.3 Results

Within the first hour post-fracture the treated mice were observed moving around inside the cages. Comparison of these mice with unfractured control mice revealed no observable differences in the recovery time from the anaesthetic. The fractured mice were observed to be weight bearing on the fractured limb within 24 h post-fracture. Dietary and sleeping habits appeared identical to those of the control mice.

X-rays taken of the fracture site (Figure 2), indicated that the fractures produced were complete and that the bone ends showed excellent alignment during the early stages of fracture repair. There was minimal displacement of the fracture ends during the initial stages of fracture repair. The excellent alignment and minimal displacement enhanced the preservation of delicate structures such as the fibrous layer of the periosteum. This intact fibrous layer was able to perform the function of a make-shift brace across the fracture site and to act as a scaffold for cells to bridge the fracture site.

Histologically, the fracture repair process was divided into four stages. There was minimal variation in the timing of the transition from stage to stage (no more than 24 h). Stage one (Figure 3a,b) was labelled the inflammatory response stage. This stage occurred from immediately post-fracture to 2 days post-fracture. Histologically this stage was marked by the formation of a haematoma, the influx of polymorphonuclear leukocytes and monocytes into the fracture site, and the

transformation of many monocytes into macrophages.

Stage two, or the periosteal-fibrous callus stage (Figure 4a,b) occurred from 2 days post-fracture to 5 days post-fracture. In the early part of this stage, cellular activity could be visualized in the cambial layer of the periosteum and this layer expanded dramatically throughout this stage. Concomitant with the expansion of the cambial layer, was the appearance of fibroblast-like mesenchymal cells. Their number also expanded and these cells formed the first bridge across the fracture site and formed a fibrous fracture callus.

Stage three (Figure 5a,b), the chondrogenic stage, was characterized by chondrification of the fibroblast-like mesenchymal cells. This stage occurred from 5 days post-fracture to 9 days post-fracture.

Stage four (Figure 6a,b) was termed the endochondral stage. It consisted of the replacement of the cartilage callus by new trabecular bone through the process of endochondral ossification. This stage occurred from 9 days post-fracture to its completion approximately 18 days post-fracture. The fracture was completely replaced by new trabecular bone within three weeks post-fracture. The stages of fracture repair with their times of duration and a brief histological description of each stage are found in Table 2.

2.4 Discussion

Several important objectives were attained with this fracture repair model. The fracture apparatus was small, portable, and easily operated by one person. The fracture induced was a sharp completely closed fracture, providing excellent alignment during the early stages of fracture repair. The closed fracture minimized discomfort and allowed the animal to use the limb early in the post-fracture period.

Displacement of the bone ends was evident during the latter stages of repair. It is believed that the latter displacement may result from several causes. During the middle to later stages of repair, the test animal was noticable more active. The increased movement of the fractured limb may have led to greater displacement of the bone ends. During the later stages of repair, cartilage and bone replace the fibroblast-like mesenchymal cells. This replacement of the fibrous callus by a cartilaginous one causes an expansion in the size of the callus, thus increasing displacement of the fracture ends.

Several other methods have been devised to produce fractures in animal models. Some are cumbersome and labour intensive as with the pneumatic press (Jackson et al., 1970). This method required surgery and the insertion of a medullary wire through the femoral head even before the fracture could be induced. This introduced the added variable of the risk of sepsis. The present model requires no surgical manipulation of the fracture site and its closed fracture

negates the variable of sepsis.

Some methods produced an unrealistic fracture, as with sawing bones in half (Yamagishi and Yoshimura, 1955). This technique also required surgery to expose the tibia and insertion of stainless steel wires to provide support. Of 250 experimental animals treated this way, 40 developed complications including post-operative infections, malnutrition, unexpected local injuries, and mechanical failure of the fixation. The present fracture model resulted in no infections, malnutrition, or unexpected local injuries to the mice.

Other methods suffer from a lack of reproducibility as with those fractures produced by digital pressure (Hyldebrandt et al., 1974). This technique was performed to produce a mid-shaft fracture of the tibia. However, other researchers have tried this method and found that the effort required to produce these fractures was uncontrollable, the fractures produced varied in pattern and site as well as in degree of displacement and soft tissue damage (Jackson et al., 1970). The advantages of the present fracture method are that fractures are controlled and that the pattern and site of fractures are constant. As well, the degree of displacement is minimal initially and soft tissue damage appears to be minimal.

The length of the repair process is relatively short; the entire repair process is completed within three weeks post-fracture. This is a much shorter time span than required for larger animals such as sheep and rabbits. It has been reported that cartilage remains in the fracture site of rabbit tibiae for up to four weeks

post-fracture (Asshurst, 1992). In mice therefore, the amount of time between production of a fracture and collection of experimental data is relatively short and makes the mouse an excellent model for fracture repair studies.

Early studies attempting to quantify fracture repair compared the repair process to the production of a blastema. The blastema grew centripetally into the injured region as necrotic tissue and the blood clot were removed. The blastema consisted of fibroblasts, osteogenic cells, and blood vessels. The fibroblasts come from the periosteum as well as from extraperiosteal connective tissue, while osteoprogenitor cells came from the cambial layer of the periosteum and marrow (Pritchard, 1963). This early quantification of the repair process was vague in its description of the histological activities taking place during fracture repair. With the present fracture model, a more detailed histological description of the activity of the cells in the repair process was possible and this gave a more detailed description of how the transition was made from one repair stage to the next. For example, the repair process could be divided into four distinct stages and a histological continuum was observed between each stage; ie stage 2 involved expansion of a population of fibroblast-like mesenchymal cells which formed the first bridge over the fracture site and in stage 3, these fibroblast-like mesenchymal cells became transformed into chondroblasts and chondrocytes.

A later quantification of fracture repair suggested that the process could be subdivided into a sequence of phases which overlap. The first phase was the phase of inflammation, then the osteogenic repair phase, and finally the

remodelling phase (McKibbin, 1978). This description was detailed, but several distinct histological activities were grouped together and this masked the detail of these stages during the repair process. For example, during the osteogenic phase of repair, the occurrence of fibrovascular tissue, chondrogenesis, and osteogenesis were all clumped together. With the present model, it is quite evident that these stages are distinct and follow a pattern of appearance during the repair process.

A recent characterization of the fracture repair process has been reported using a rat model. Four distinct stages were characterized by cellular features, extracellular matrix, and time of appearance of the tissues during fracture healing (Joyce et al., 1990b). The stages described are very similar to those of the present model, with the exception of stage 2. According to their observations, new bone forms from osteoblasts located adjacent to the fracture site and below the proliferating periosteal cells. In the present model, the lack of histological staining for calcified matrix during the initial stages of repair indicated there was no intramembranous bone formation (all bone deposition followed a cartilage intermediate). The study of Joyce et al. (1990b) did not assign time periods for the appearance and duration of each stage of the repair process, something my study was able to do precisely.

The choice of the mouse as the animal model is recommended because mice are among the least expensive test animals to maintain. They require less space for housing than larger animals. Their dietary requirements are less in terms of cost than for larger animals. They are also relatively inexpensive to purchase, and

breeding colonies can be readily maintained.

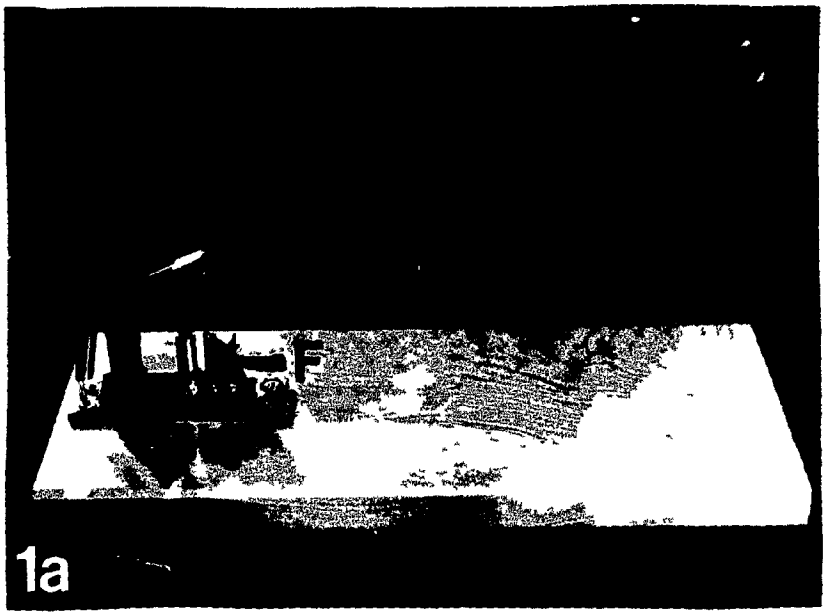
The mice used in this experiment were males, 4-6 months old. Males were chosen because their skeletons suffer less from calcium flux than do females. The 4-6 month age was chosen because at this age the mice had reached maturity (A.M. Taylor, pers. comm.) and had not yet started to suffer skeletal loss due to aging. Using this fracture apparatus and the mouse as the test animal, very precise time intervals (within 24 h) could be assigned to the stages of fracture repair and these stages themselves were easily distinguishable histologically. This will be important for researchers who want to explore the effects of substances applied to stimulate and/or alter the repair process. For example, researchers have contemplated the effects of certain biochemicals such as growth factors, on fracture repair (Joyce et al., 1990b). With a model as precise as this one, the effects of the addition of exogenous growth factors would be easy to monitor and relate to precise stages of the repair process. In addition, other experiments could be performed using this standard to study the affects on the duration and sequence of the fracture repair stages to diet, aging, gender, and pathological conditions such as diabetes.

However, there are limitations to the comparison of fracture repair information obtained from rodents to human fracture repair. Mouse cortical bone is not Haversian in nature, and as a result, differs from human cortical bone. This appears to be important in terms of bone remodelling. Rodent cortical bone remodelling occurs primarily along the endosteal and periosteal surfaces (Kelly et

al., 1990). This is of importance particularly during the remodelling stage of fracture repair. The small size of mouse bones makes them poor candidates for the study of internal fixation and how it affects fracture repair. However, the response to bone fracture produces the same end result, a healed fracture. Rodents tend to produce more cartilage during repair than do larger animals but the end result is still the same.

Figure 1a: A custom designed fracture apparatus which can be operated by one person. The dimensions are 30 cm long and 12 cm wide at the base and 7 cm in height. The tibia is placed across the fulcrum (F) at the point marked by the arrow.

Figure 1b: Alternative side view of the fracture apparatus which shows the braces (arrow heads), one on either side of the lever, which swing down with the lever handle and break the tibia over the fulcrum.


F
1a

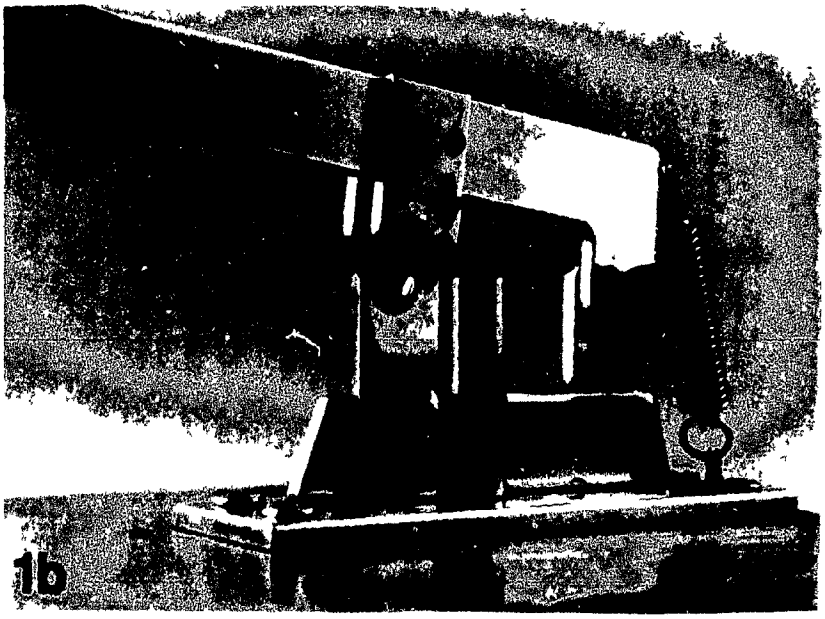

1b

Figure 2: On the left is the fractured tibia with an arrow indicating the fracture site. On the right is an unfractured tibia (2x).


2

Figure 3a: Fractured tibia (T) 1 day post-fracture. The fracture (F) is clearly visible. Sections were stained with HBQ staining method. Bar = 192 μm

Figure 3b: High-power magnification of area in insert from Figure 3a. The site is composed of loose areolar connective tissue infiltrated by macrophages (m) and polymorphonuclear leukocytes (pmn). Bar = 21 μm


F
T
3

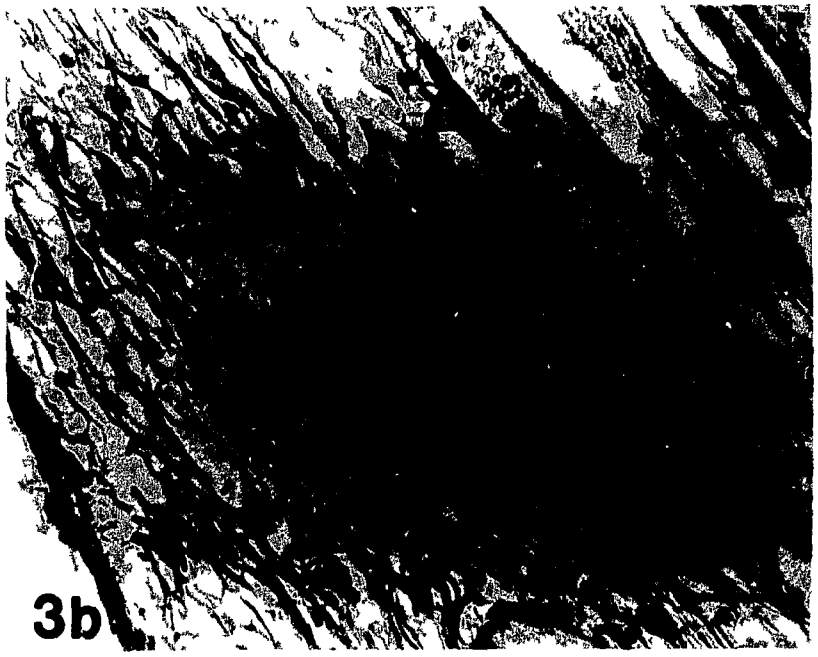

3b

Figure 4a: Fractured tibia 4 days post-fracture. The shaft of the tibia (T) is visible as is the fracture (F). Bar = 192 μm

Figure 4b: High-power magnification of area in insert from Figure 4a. The site is composed of layers of fibroblast-like mesenchymal cells (fm), an active cambial layer of the periosteum (cl), and the tibia (T) of the bone. Bar = 21 μm

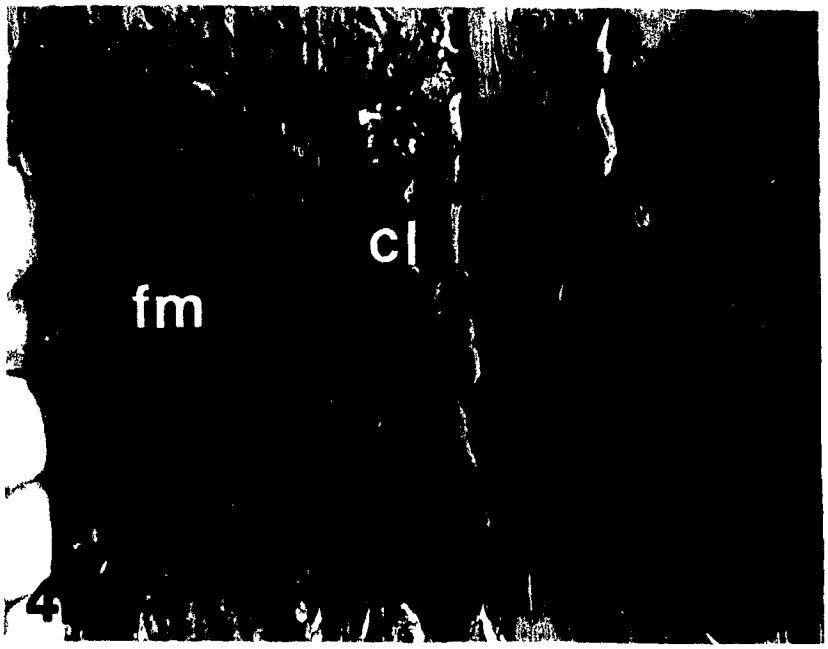
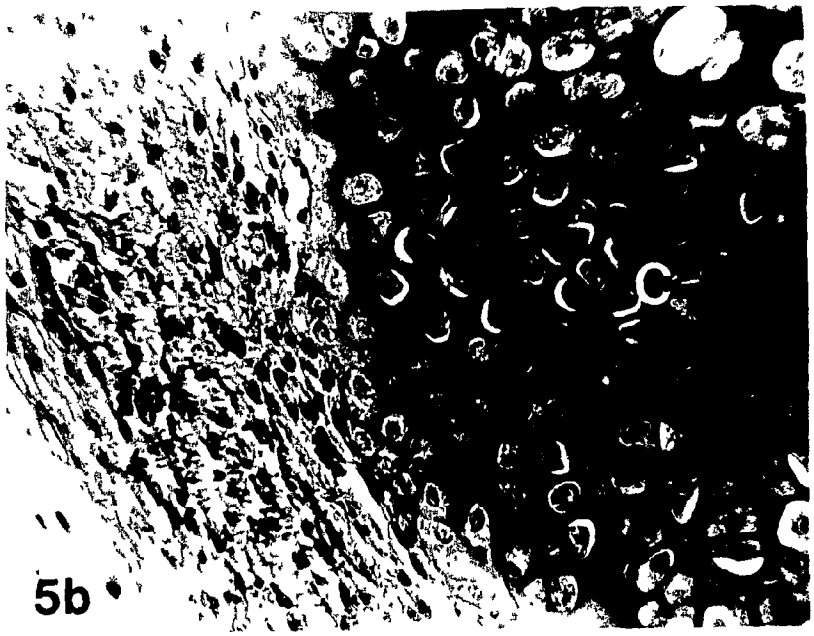

cl
fm

Figure 5a: Fractured tibia (T) 8 days post-fracture. The fracture (F) is visible. Bar = 192 μm

Figure 5b: High-power magnification of area in insert from Figure 5a. This site is composed of cartilage (C) and fibroblast-like mesenchymal cells (fm). Bar = 21 μm

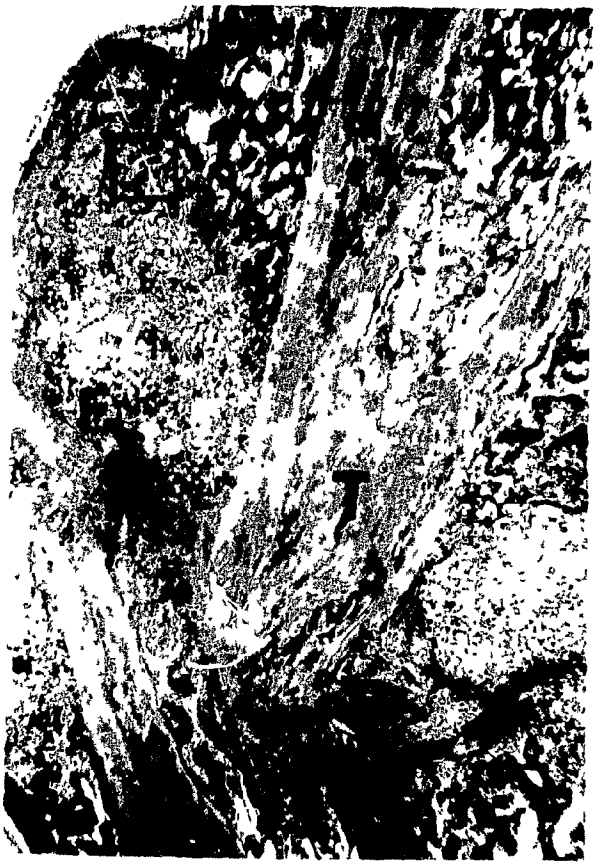
5a



5b

Figure 6a: Fractured tibia (T) 14 days post-fracture, the fracture (F) is also visible. Bar = 192 μm

Figure 6b: High-power magnification of area in insert from Figure 6a. The site is composed of new trabecular bone (tb) and cartilage undergoing endochondral ossification (oe). Bar = 21 μm



6b

Table 2 A summary of the duration and histological composition of each stage of fracture repair.

Stage	Time (Days post-fracture)	Histological Description
1	0-2	-haematoma, influx of polymorphonuclear leukocytes, monocytes, macrophages
2	2-5	-expansion of cambial layer of periosteum, appearance and expansion of population of fibroblast-like mesenchymal cells which eventually bridge fracture gap producing fibrous fracture callus
3	5-9	-replacement of the fibroblast-like mesenchymal cells by chondroblasts and cartilage, production of cartilaginous callus
4	9-21	-replacement of cartilage by osteoblasts and new trabecular bone, endochondral ossification produces bone callus, bone marrow infiltrates into new bone trabeculae

Chapter 3 An immunohistochemical method which preserves growth factor antigens in bone following decalcification.

3.1 Introduction

The use of conventional fixatives such as neutral buffered formalin (NBF) and Bouin's, followed by routine paraffin embedding of tissues destroys many delicate antigenic sites, while cryosectioning compromises morphological detail in the tissues (Stein et al., 1984). Many surface antigens are glycoproteins embedded in the lipid-containing plasma membrane and are especially susceptible to destabilization followed by denaturation and/or mobilization during tissue processing (Holgate et al., 1986).

Using frozen section immunohistochemistry, correlating morphologic and cytologic features with immunophenotype is difficult. Alternately, routine paraffin embedding destroys many delicate antigens that may be detected by frozen sectioning. Theoretically, plastic embedding should be superior to either frozen or paraffin sections for immunohistochemistry because of better antigenic and morphologic preservation. In practice, however, plastic embedding is limited by technical difficulties. Water soluble plastics such as glycol methacrylate apparently bind to amino-terminal portions of proteins during polymerization, masking antigenic epitopes. This masking may be reversed by trypsin or protease

treatment of sections, but proteolysis alters or destroys some antigens and compromises morphology (Casey et al., 1988).

Bone is an inherently difficult tissue to work with histologically. Most histological techniques require some form of decalcification before bone can be sectioned (Humason, 1979). Bone itself acts as a reservoir of non-collagenous extracellular proteins which include growth factors such as aFGF (Hauschka et al., 1986), TGF-ß (Seyedin et al., 1985), IGF-I and IGF-II (Frolik et al., 1988); see chapter 1.2. The identity of what types of cells and under what conditions these growth factors are produced in vivo is only beginning to be established.

I have modified several previously existing histological methods to form a new technique for immunohistochemically studying calcified tissue. This new method provides excellent tissue integrity to serially sectioned decalcified tissues. Attempts have been made to study growth factors immunohistochemically in bone but antibody staining has been weak. In addition, the decalcification and tissue processing methods previously employed seriously compromised tissue integrity. My technique maximises the preservation of tissue integrity and thus has allowed me to observe fine details (ie. collagen fibrils in cartilage), previously unattainable with existing paraffin embedding techniques. This increased preservation of detail has dramatically improved antibody staining results. Antibody staining is both distinct and specific.

There were several reasons why antibodies to aFGF were used to pioneer this technique and test for the preservation of antigenic sites on decalcified tissues.

aFGF can be extracted from bone matrix and immunohistochemical localization of aFGF in decalcified tissues had not previously been reported. Also, administration of aFGF to fractures stimulated fracture repair (Jingushi et al., 1990).

3.2 Materials and Methods

Polyclonal rabbit anti-bovine aFGF was obtained from Upstate Biotechnology (Lake Placid, NY). Biotinylated anti-rabbit IgG was purchased from Vector Laboratories (Burlingame,CA). Natural bovine aFGF (containing bovine serum albumin as a carrier protein) was purchased from R&D Systems (Minneapolis,MN).

3.2.1. Harvest Of Tissues

Male CD-1 mice (Charles River;Montreal,Quebec), 4-6 months old, were subjected to a reproducible closed fracture procedure using the custom designed fracture apparatus described in chapter 2. The right tibia was fractured on seventeen mice. Five of these fractured mice were used for histological processing and immunohistochemical localization of aFGF. The twelve other mice were used for the immunological detection of aFGF using Western blot analysis. At Day 5 post-fracture, these animals were euthanized and the fractured limbs removed. Fracture calluses at this stage of fracture repair consisted of the tibial shaft, bone marrow, proliferating cartilage-like cells of the cambial layer of the periosteum, and undifferentiated fibroblast-like mesenchymal cells (See Chapter 2). The twelve fractured mice for Western blot analysis had only the

fracture callus dissected and homogenized. The five mice for immunohistological study had their fractured tibia removed and placed in freshly prepared periodate-lysine-paraformaldehyde (PLP) fixative (2% paraformaldehyde containing 0.075 M lysine and 0.01 M sodium periodate solution) as previously described (McLean and Nakane, 1974). The fixed tissues were then placed in glass vials, placed in a rotating drum and left for 24 h at 5^0C.

3.2.2. Processing of tissues for Western blot

The twelve dissected calluses were placed in phosphate-buffered saline (PBS) pH 7.2 containing 1:1000 phenylmethylsulfonyl fluoride (PMSF) (Sigma; St. Louis, MO). They were then homogenized in a Kinematica tissue homogenizer (Brinkman Instruments; Westbury,NY) for 45 seconds. The homogenate was then spun for 20 minutes at 3000 rpm in a Beckman GP centrifuge (Beckman Instruments, Palo Alto, CA) to remove solid material. A separation column was constructed consisting of a Pharmacia column (40 cm long and 2 cm diameter) packed with G-200 sephadex (Pharmacia; Uppsala, Sweden). The column was standardized by passing proteins of known molecular weights down the column and collecting the eluent. The collected eluent was analyzed spectrophotometrically to determine which fractions contained the standard molecular weight proteins. The tissue homogenate was loaded into the column

and eluted with Tris buffer pH 8.0 containing PMSF. Only the eluted fractions containing proteins of less than 43 KDa were collected. This eluent was further concentrated using a Centricon-10 centrifuge concentrator (Amicon; Danvers, MA) which allowed proteins of less than 10 KDa to be removed from the solution. The retentate solution (containing the 43-10 KDa protein fractions) was further concentrated using a SpeedVac SVC 100 (Savant; Farmingdale, NY). The final volume of the retentate was 1.0 ml.

3.2.3. Decalcification of Tissues

After fixation, the five fractured tibiae were washed for 12 h intervals at 5^0C (in a rotating drum) in the following series of solutions: 0.01M PBS containing 5% glycerol, 0.01M PBS containing 10% glycerol, and 0.01M PBS containing 15% glycerol as previously mentioned (Mori et al., 1988).

The tissues were then suspended by thread in 155 ml of ethylene diamine tetraacetic acid glycerol (EDTA-G) solution at -4^0C [14.50g EDTA ($C_{10}H_{14}N_2O_8Na_2 \cdot 2H_2O$), 15 ml of glycerol, 85 ml distilled water, and solid sodium hydroxide (NaOH) added until a final pH of 7.3 was reached as previously described (Mori et al., 1988)]. A hole was pierced in the plastic lid of a 200 ml glass jar and the thread attached to the tissue suspended from it. This EDTA-G solution was replaced every 7 days. Progression of decalcification was checked by

piercing the tissue with a dissecting needle. Decalcification was complete after 16 days in the fracture tibia samples.

3.2.4. Washing of Decalcified Tissues

Glycerol was removed from the calcified tissues through lengthy washing. Tissues were washed with a solution containing 15% sucrose and 15% glycerol in PBS at -4^{0}C for 12 h. Following this, the washes alternated between 12 h under vacuum at 5^{0}C and 12 h in a rotating drum at 5^{0}C. The washing solutions were as follows: 20% sucrose and 10% glycerol in PBS, 20% sucrose and 5% glycerol in PBS, 20% sucrose in PBS, 10% sucrose in PBS, 5% sucrose in PBS, and 100% PBS.

3.2.5. Dehydration and Paraffin Embedding

The fractured tibiae were then dehydrated at 5^{0}C in a rotating drum through the following series of isopropanol solutions: 2 changes of 50:50 isopropanol/dH_2O for 1 h each, 1 change of 60:40 isopropanol/dH_2O for 1 h, 1 change of 70:30 isopropanol/dH_2O for 1 h, 1 change of 80:20 isopropanol/dH_2O

for 1 h, 1 change of 90:10 isopropanol/dH_2O for 1 h, 4 changes of 100% isopropanol for 1 h each, 1 change of 50:50 isopropanol/chloroform for 1 h, 1 change of 100% chloroform for 2 h at -20^{0}C. The tissues were then transferred to Paraplast X-Tra low melting point (LMP) paraffin mp 56^0 C (Fisher; Montreal, Quebec). Four changes of wax (1 h each) under vacuum were used before embedding in LMP paraffin.

3.2.6. Trimming of Blocks

The tissue blocks were trimmed down to the tissue surface. The block (tissue surface down) was placed in a dish of distilled water for 20 min, the tissue surface blot dried and placed in a freezer at -20^{0}C for 20 min. The block was then placed in a microtome and cut at a thickness of 5μm. Tissue sections were floated in a water bath at 48^{0}C and placed on poly-l-lysine coated glass slides. Sections of fracture callus were stained with the HBQ method. This method gives excellent distinction between bone, cartilage, and connective tissue (Hall, 1986).

3.2.7. Immunohistochemical Method

The paraffin sections were deparaffinized and stained by the avidin-biotin-

peroxidase complex (ABC) immunoperoxidase technique according to Hsu et al. (1981) with some modifications. Endogenous peroxidase activity was blocked by immersing the sections in 3% hydrogen peroxide for 5 min followed by washing in running water for 5 min and then flooding with PBS for 5 min. The sections were then incubated with 100% (v/v) normal goat serum for 30 min at room temperature to block nonspecific binding. The sections were drained well and then incubated with primary antibody diluted with PBS (containing 0.5% bovine serum albumin) in the ratio of 1:500 overnight at 4^0C. After washing with PBS, the sections were incubated with secondary antibody (biotinylated goat anti-rabbit IgG) diluted with PBS in the ratio of 1:200 for 30 min at room temperature. The sections were then washed with PBS and incubated with ABC Elite Kit PK-6100 (Vector; Burlingame, CA) for 30 min at room temperature. After washing with PBS, brown precipitate was produced by 2 min treatment with 3,3'-diaminobenzidine (DAB) (Sigma, St.Louis, MO) (1 DAB tablet dissolved in 20 ml 0.05M Tris-HCl buffer, pH 7.6, containing 0.005% hydrogen peroxide). As a positive control, natural bovine aFGF was added to an agarose gel along with a tissue homogenate of day 5 post-fracture calluses and run as a Western blot. As a negative control, PBS was substituted for the primary antibody in the immunohistological method.

3.2.8 Western Blot

This procedure was standard. The aFGF (15μl) and 45μl fracture callus retentate were electrophoresed on a 12.5% SDS PAGE gel. The protein was transferred to nitrocellulose paper with pore size 0.45μm (Schleicher & Schuell; Keene, NH) in transfer buffer (25 mM Tris, 192 mM glycine, pH 8.3, with 20% methanol).

Overnight transfer was performed in a Bio-Rad (Trans-Blot) transfer apparatus (30 mA constant current). Nonspecific binding was blocked by washing the nitrocellulose in 5% gelatin solution for 30 min. Nitrocellulose was washed in Tween-Tris buffered saline (TTBS) (10 mM Tris-HCl pH 7.4, 140 mM NaCl, 0.1% (v/v) Tween 20) then incubated with anti-aFGF antibody for 1.5 h at room temperature.

This was followed by washing in TTBS and incubation in biotinylated goat anti-rabbit secondary antibody for 30 min. After rinsing with TTBS, the nitrocellulose was incubated in avidin-biotin complex conjugated to alkaline phosphatase ABC-AP kit AK-5000 (Vector; Burlingame, CA). The nitrocellulose was then washed with TTBS containing a high salt concentration to remove nonspecific binding. A final rinse in TBS was followed by the production of brown precipitate using BCIP and NBT (Sigma; St. Louis, MO). The reaction was stopped by rinsing the nitrocellulose in distilled water. A flow diagram of the processing procedure, which allows stages in the procedure to be broken down into easy to follow steps,

into easy to follow steps, is presented as Table 3.

3.3. Results

3.3.1. Tissue Integrity:

The stage of fracture repair examined immunohistochemically was the second stage (See chapter 2 for details). The day 5 post-fracture callus tissue sections showed no evidence of tearing. The decalcified bone and loose fibroblast-like mesenchymal tissue remain intact. Also, there was no evidence of distortion due to shrinkage between the tibia, cambial layer of the periosteum, and fibroblast-like mesenchymal cells (Figures 7a and 7b).

3.3.2. Immunohistochemical studies:

The anti-bovine aFGF antibody recognized the pure bovine aFGF in lane 2 of the Western blot and also recognized a protein in the fracture callus retentate in lane 1. The fracture callus retentate band occurs close to the positively stained bovine aFGF band; difference in the molecular weights may be due to species variation (Figure 8).

When this antibody was applied to tissue sections for immunohistochemical analysis, cells in the expanded cambial layer of the periosteum adjacent to the fracture site stained intensely. The staining was very specific and confined to these

cambial layer cells in the Day 5 post-fracture callus (Figure 7c). The negative control contained no nonspecific staining (Figure 9).

3.4. Discussion

3.4.1. Effects of fixation:

Cell surface antigens are very delicate proteins which can be easily denatured during routine fixation and decalcification. The PLP fixative devised by McLean and Nakane (1974) links carbohydrate moieties without excessive binding to protein antigenic sites. Pollard et al. (1987) demonstrated optimal cell surface antigenic preservation on T-cells embedded in paraffin. They used a modified PLP fixative and processed the tissue samples at 4^0C through isopropanol, chloroform, to low melting point paraffin. However, Pollard et al. (1987) did not work with bone. Mori et al. (1988) demonstrated excellent retention of antigenicity for cell surface antigens on lymphocytes and macrophages when cryosectioning PLP fixed samples of destructive lesions from bone tissues. As a result of the work of Pollard et al. (1987) and Mori et al. (1988), the fracture calluses in my study were all fixed in PLP and processed at 5^0C using isopropanol as the dehydrating agent.

3.4.2. Effects of decalcification:

Cells in decalcified bone typically shrink, and many details of the bone matrix

become blurred because of swelling of the osteocollagenous fibers by the decalcifying agents, and processing procedures used to decalcify bone (Leeson et al., 1985). It has been reported that PLP fixed tissues give excellent preservation of tissue architecture (Collins et al., 1984). Mori et al. (1988) decalcified small bone samples at pH 7.1-7.4 and at a temperature of -4^{0}C. The calcium was removed from the bone in these samples with minimal damage to surface antigens. In the present study, problems arose when attempting to section Day 5 post-fracture calluses according to the method of Mori et al. (1988). The glycerol used to depress the freezing point of the decalcifying solution below 0^{0}C was not readily removed from muscle or dense bone. This problem was overcome by alternating washing in PBS at 5^{0}C with vacuum extraction of the glycerol into PBS at 5^{0}C. A gradation of the washing solutions from PBS-glycerol to PBS-sucrose to 100% PBS prevented cell rupturing due to osmotic shock.

3.4.3. Effects of paraffin embedding:

The method of Mori et al. (1988) using cryosectioning of the decalcified tissues still presented problems even after extensive washing of the decalcified bone with PBS. The cryostat used to section these tissues produced torn sections of irregular thickness. In addition, the sections routinely lifted off poly-l-lysine coated glass slides. Pollard et al. (1987) conducted an extensive study of the

effects dehydrating agents and processing temperatures have on the preservation of surface antigenic sites on T-cells. They found that, the use of isopropanol as a dehydrating agent, processing tissue at low temperatures, and infiltration with low melting point paraffin, vastly improved the preservation of surface antigenic sites over dehydrating in ethanol, processing tissues at room temperature, and embedding in higher temperature paraffin. I decided to infiltrate the decalcified bone with low melting point paraffin. The tissue specimens were all dehydrated through an ascending series of isopropanol solutions at 5^0C, cleared in chloroform at -20^0C, and vacuum infiltrated with low melting point paraffin. The use of a vacuum was found to be essential for the removal of glycerol from decalcified specimens and for the proper infiltration of the paraffin wax into bone.

An additional step involved trimming the paraffin block to expose the tissue surface and then placing this exposed surface in a petri-dish containing distilled water for a period of 20 min. Following this, the block was placed face down in another petri-dish and then inserted into a freezer for 20 min. This additional step significantly improved the ability of the embedded tissue to be serially sectioned. Omitting this step resulted in torn tissue sections. This procedure differs from the routine lowering of the tissue block surface with ice prior to cutting. The rehydration step had to be followed by freezing the rehydrated tissue. Omitting the freezing step resulted in the tissues becoming soggy and unable to cut.

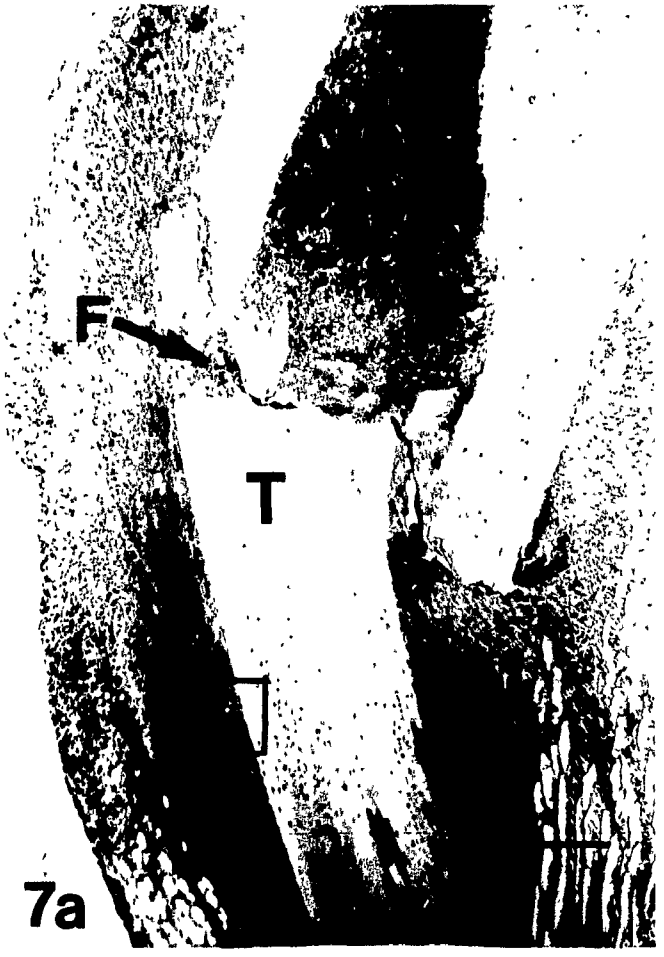
3.4.4. Ability of the method to immunohistochemically detect aFGF:

The present procedure for fixing, decalcifying, processing, and embedding calcified tissues clearly demonstrates the presence of aFGF in Day 5 post-fracture calluses. The immunohistochemical staining was both specific to the cells of the cambial layer and distinct. Other cell types present in the Day 5 post-fracture tissue sections were devoid of any DAB precipitate. These other types of cells included fibroblast-like mesenchymal cells, bone marrow cells, chondroblasts, macrophages, and osteocytes in the original bone.

Studies by Gospodarowicz et al. (1975) and Wellmitz et al. (1980) have shown that administration of aFGF to amputation stumps of adult frog's limbs and knee-joint injuries in rabbits, induce the proliferation of undifferentiated cells and chondrocytes. Jingushi et al. (1990) also administered aFGF into rat fractures and reported a marked increase in the size of the cartilaginous soft callus. These studies established aFGF as a marker for cell proliferation. Hauschka et al. (1986) have reported that aFGF can be isolated from bone matrix. It therefore appears that aFGF is a constitutive part of bone tissue. However, since only the cells of the expanded cambial layer stained positive for aFGF, it is possible that in response to injury, bone may produce significantly increased amounts of aFGF to induce the proliferation of cells involved in the early stages of fracture repair (i.e. fibroblast-like mesenchymal cells and chondrocytes). Because of the ability of the present technique to preserve tissue integrity and preserve aFGF

antigens, this method was used to detect other growth factors during the stages of fracture repair. IGF-I, TGF-β_1, and PDGF were successfully detected in the fracture site during the stages of fracture repair. The distribution of these other growth factors and their roles in fracture repair are described in Chapter 4.

Figure 7a: Fractured tibia 5 days post-fracture stained by the HBQ method. The shaft of the tibia (T) is visible as is the fracture (F). Bar = 192 μm



7a

Figure 7b: This is a higher-power photomicrograph of the area outlined in Figure 7a. The cells of the cambial layer (cl) of the periosteum and fibroblast-like mesenchymal cells (fm) are clearly visible. Bar = 21 μm

Figure 7c: Anti-aFGF antibodies were applied to a serially adjacent tissue section to the one stained with HBQ in Figure 7a. Photomicrographed under high power, positive anti-aFGF staining (arrow heads) in cells of the cambial layer was demonstrated. Bar = 21 μm

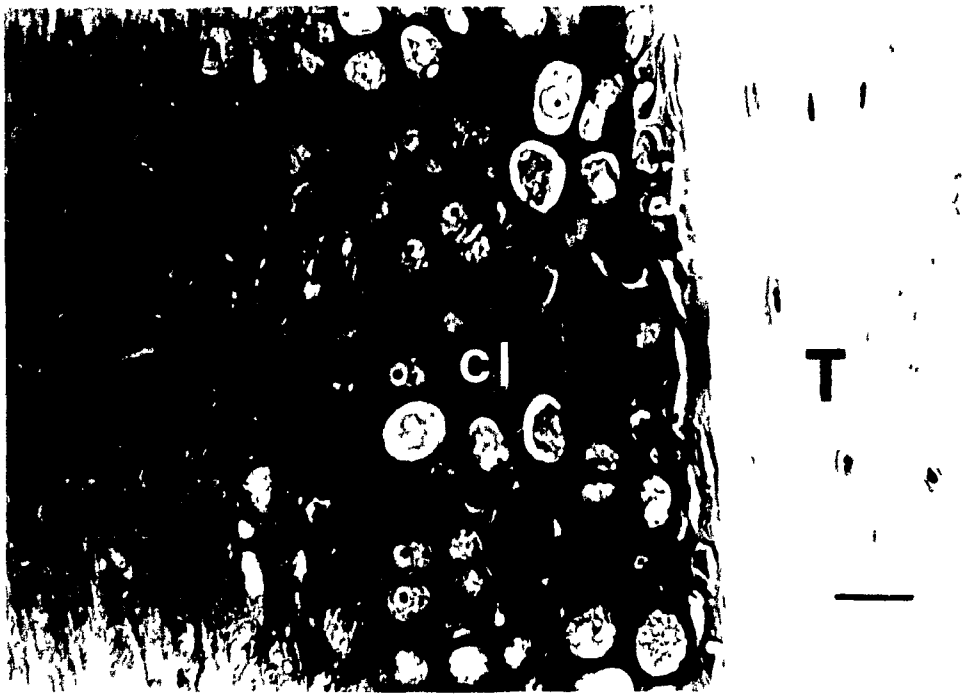
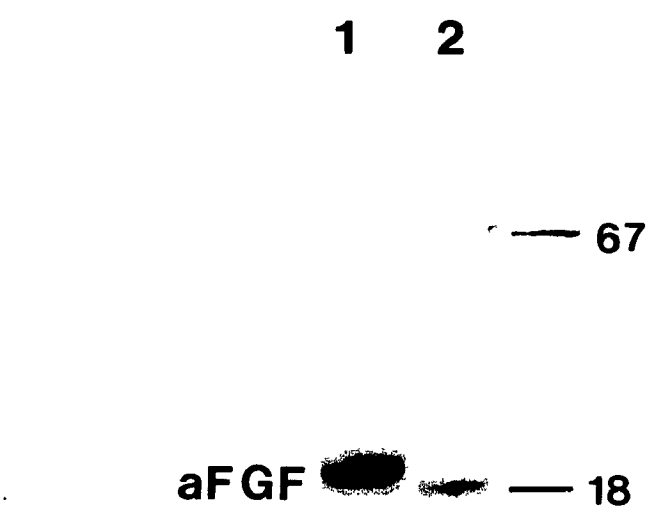

Cl
T

Figure 8: A Western blot probed with anti-bovine aFGF. Lane 1 contains retentate from day 5 post-fracture calluses. Lane 2 contains pure aFGF and bovine serum albumin as a carrier protein. Bars mark positions of 18 and 67 KDa molecular weight markers.



8

Figure 9: Negative control for immunohistochemical staining in Figure 7c. Same area on serially adjacent section is depicted.

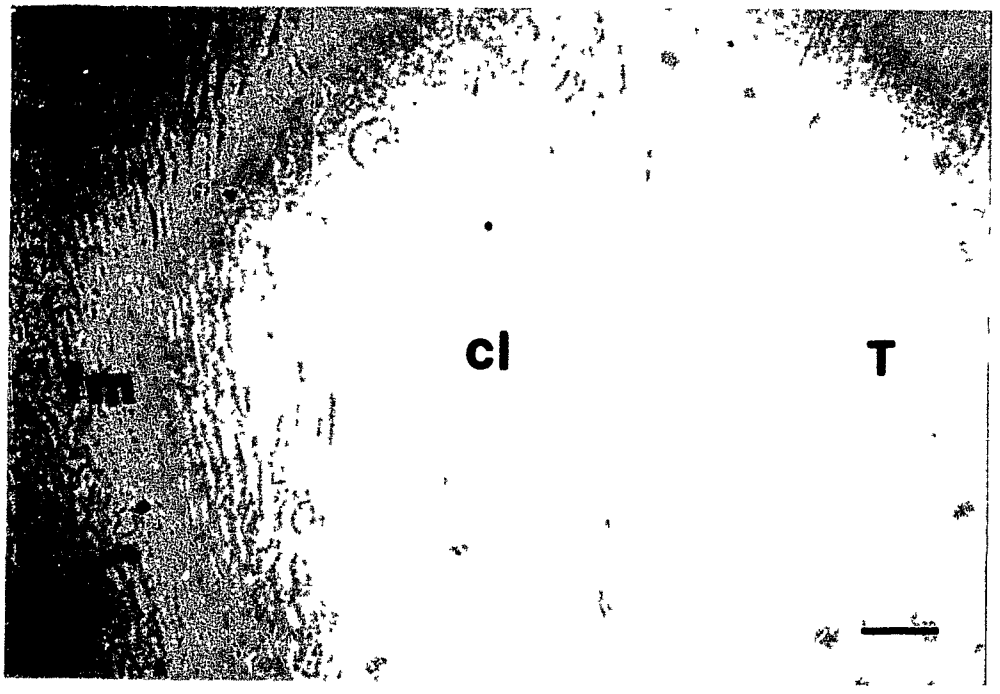

Cl
T

Table 3 A new tissue processing method which preserves tissue architecture and antigenic sites on growth factors.

Fix tissue at 5^0C

in PLP fixative

Calcified Tissues	Noncalcified Tissues
Wash in PBS pH 7.2/Glycerol	
Decalcify in EDTA-G at -4^0C	
Wash in PBS-Glycerol-Sucrose at 5^0C	

Wash in 100% PBS at 5^0C

Dehydrate through ascending

series of isopropyl alcohols at 5^0C

Clear in chloroform at -20^0C

Vacuum infiltration & embed in LMP paraffin at 56^0C

Trim block to tissue surface and

immerse tissue surface in a tray

of distilled water at room temperature

Place tissue block in freezer at -10^0C for 20 min

Cut tissue sections and float them on a water bath

Mount sections on poly-l-lysine coated slides

Chapter 4 Immunolocalization of Growth Factors During the Stages of Fracture Repair

4.1 Introduction

Inflammation is the initial response to any type of wound or infection. Its importance centers on its two principle functions, removal of cell debris and attraction of repair cells. Typically, the inflammatory phase consists of neutrophils, lymphocytes, macrophages, and fibroblasts (Boros, 1978). In relation to wound healing, the inflammatory response follows a time course where macrophages appear at the wound site, followed by fibroblasts (Ross and Benditt, 1961). This association, leads Leibovich and Ross (1975) to propose that macrophages stimulated the proliferation of fibroblasts. Using peritoneal macrophages isolated from Guinea pigs, they found that these macrophages produce and secrete a growth-promoting activity into culture medium (Leibovich and Ross, 1976). Subsequent investigations by other researchers demonstrated that macrophages can produce FGF, PDGF, and TGF-ß (Baird et al., 1985; Shimokado et al., 1985; Assoian et al., 1987). Roberts et al. (1986) studied wound repair by injecting TGF-ß subcutaneously into newborn mice, resulting in angiogenesis and activation of fibroblasts to produce collagen. Walsh et al. (1990) explored the activities of several growth factors produced by macrophages on

cultures of fibroblasts. They concluded that PDGF stimulated fibroblast proliferation while TGF-ß inhibited fibroblast growth.

In summary, in any type of wound repair, macrophages play a major role both in debridement and as a stimulator of cell proliferation. The major skeletal cell involved in proliferation is the fibroblast. The most potent macrophage derived stimulator of fibroblast proliferation is PDGF. These wound fibroblasts are not constrained to remain as fibroblasts, they are in fact capable of differentiating into other mesodermal cell types most notable, chondrocytes.

Fibroblasts which accumulate at sites of wound repair, and the fibroblast-like cells which appear following limb amputation in amphibians, share a common heritage, they are both derived from mesoderm. As such, they may be expected to share simular developmental pathways. Undifferentiated mesenchymal cells, obtained from limb buds of embryos, and grown in vitro, undergo transformation into chondrocytes (Ahrens et al., 1977). The reverse can also happen; cartilage cells can be isolated from human tissue and dedifferentiate into fibroblast-like cells in culture (Solursh, 1991).

It is believed that increasingly close contact between cells is one reason for this transformation of fibroblast-like mesenchymal cells to cartilage (Thorogood and Hinchliffe, 1975). High cell densities of mesenchymal cells in culture spontaneously form chondrogenic micromasses (Swalla et al., 1983). It is also believed that extracellular matrix plays a role in determining the differentiation of mesenchymal cells to chondrocytes (Solursh, 1989).

Therefore, it may be stated that wound fibroblasts can be induced to transform into chondroblasts under appropriate environmental conditions. This is important during limb regeneration and also during fracture repair. In both situations, transformation of fibroblast-like cells to cartilage-producing cells is essential for the repair process to continue.

The production of a calcified cartilage matrix is a necessary intermediary step in the transition from cartilage to bone during endochondral ossification. Calcification of cartilage, is followed by invasion of the calcified cartilage matrix by blood vessels and osteogenic cells which break down the calcified cartilage matrix. When the osteogenic cells and capillaries reach the interior of the calcified cartilage, they constitute a center of ossification. This means that bone formation beginning here will spread out to replace most of the remaining cartilage. In this process, proliferating osteogenic cells gather round the remnants of calcified cartilage and differentiate into osteoblasts that lay down new bone (Ham and Cormack, 1979). A major source of osteogenic cells is the periosteum. The periosteum is a membrane which covers the outer surface of a bone except where it articulates with other bones in joints. It has an outer fibrous layer and an inner osteogenic layer. When neither appositional growth nor resorption are occurring, it is referred to as a resting periosteum. At this stage, its outer layer consists of collagenic fibers and a few fibroblasts. The deeper osteogenic layer consists of a few pale, flattened, spindle-shaped cells lying on the outer surface of a bone (Ham and Cormack, 1979). Rhinelander (1974) reported that blood

vessels enter long bones via the periosteum.

Experiments involving the removal of this inner layer of osteogenic cells demonstrated their ability to differentiate directly into osteoblasts or chondrocytes if inoculated in vivo. These osteogenic cells retain their differential potential in vivo even after enzymic isolation, cell culture, subculturing, and freeze preservation (Nakahara et al., 1990).

Eyre-Brook (1983) reported that the periosteum is stimulated to form bone when it is surrounded by a haematoma. He also confined the osteogenic potential of the periosteum to the inner or cambial layer. In pathological situations such as fracture repair, the periosteum provides mechanical connection between the bone fragments.

I will now discuss the stages of fracture repair and the potential role of growth factors during these stages. As discussed in Chapter 1.2, aFGF, IGF-I, and TGF-β_1 are all present in bone matrix and active participants in bone and cartilage metabolism. They are, therefore, prime candidates for roles in mediating the cellular response to fracture repair. PDGF was also considered a possible participant because of its prominent role as an initiator of cell division in cells of mesodermal origin.

Macrophages are one of the major cell types present during the first stage of fracture repair (see Chapter 2). Following this appearance, fibroblast-like cells begin to accumulate in and around the fracture site. Toward the latter part of stage 1, cell division could be seen in the cambial layer of the periosteum. PDGF

is produced by macrophages and is a potent stimulator of cell division in cells of mesodermal origin (see Chapter 1.2.4). For these reasons, PDGF is likely to be involved in fracture repair and more specifically during the early stages of fracture repair.

As was stated in Chapter 3, aFGF was immunohistochemically detected in cells of the cambial layer of the periosteum. aFGF was chosen as a candidate for participation in fracture repair because it is a potent stimulator of cell division in fibroblasts and cells with chondroprogenitor and osteoprogenitor characteristics (see Chapter 1.2.3). The second stage of fracture repair involved replication of fibroblast-like cells and chondroprogenitor cells in the cambial layer of the periosteum (see Chapter 2). Therefore it was hypothesized that aFGF would be involved in fracture repair and so its presence was analyzed immunohistochemically.

The third stage of fracture repair involves chondrogenesis (see Chapter 2). This process involves both the production of cartilage matrix and proliferation of chondrocytes. As discussed in Chapter 1.2.1, IGF is a stimulator of both cartilage matrix production and chondrocyte proliferation. Therefore IGF-I was analyzed immunohistochemically during fracture repair.

The last stage of fracture repair is the production of bone through the process of endochondral ossification (see Chapter 2). TGF-$ß_1$ is a potent stimulator of cell division and matrix production in cells of chondrogenic and osteogenic origin (see Chapter 1.2.2). Therefore, TGF-$ß_1$ was studied immunohistochemically for a

role during fracture repair.

4.2 Materials and Methods

Polyclonal neutralizing anti-human PDGF and polyclonal anti-human TGF-$ß_1$ were obtained from R&D Systems (Minneapolis, MN). Polyclonal anti-bovine aFGF was obtained from Genzyme (Cambridge, MA). Polyclonal anti-human IGF-I was obtained from KabiGen (Stockholm, Sweden). Biotinylated rabbit anti-goat and biotinylated goat anti-rabbit secondary antibodies were obtained from Vector Laboratories (Burlingame, CA). Normal rabbit and normal goat serum were obtained from Vector Laboratories (Burlingame, CA). Hydrogen peroxide (H_2O_2) and Diaminobenzadine (DAB) were obtained from Sigma (St.Louis, MO).

Thirty-three male CD-1 mice were fractured according to the procedure outlined in chapter 2. Three mice were harvested per day from day-1 post-fracture to day-10 post-fracture and three more mice from day-14 post-fracture. The animals were anesthesized and euthanized. The fractured tibiae were removed, fixed, decalcified and processed to paraffin according to the procedure outlined in chapter 3. After embedding, the tissues were sectioned on a microtome at 5μm and analysed immunohistochemically for the presence of growth factors.

Modifications to the immunohistochemical procedure used in chapter 3 were as follows. Normal goat serum was used as a blocking agent when the primary antibody was raised in rabbit (anti-aFGF, anti-IGF-I, and anti-TGF-$ß_1$). Normal rabbit serum was used as a blocking agent when the primary antibody was raised

in goat (anti-PDGF). Negative controls consisted of performing the immunohistochemical method outlined in Chapter 3.2.7 but substituting PBS in place of the primary antibody.

Serially adjacent sections to those studied immunohistochemically were stained with the HBQ method (see Chapter 2.2). The HBQ staining procedure uses the histological stains, direct red, alcian blue, Mayer's hematoxylin, and celestian blue B, to provide excellent contrast between bone (stains red), cartilage (stains blue), and connective tissue.

Three specimens/day were immunohistochemically analysed for the presence of growth factors. Negative controls were conducted for every immunohistochemical procedure to detect any non-specific antibody staining which might be produced by chemicals used in the immunohistochemical procedure. Every negative control performed indicated that no nonspecific antibody staining was produced. A photomicrograph of a negative control is presented in Chapter 3 as Figure 9.

The change from one stage of fracture repair to the next is marked by a transition phase. For example, the cartilaginous stage starts at day 5 post-fracture and ends by day 9 post-fracture. At day 5, the cell population contains cartilaginous cells but also fibroblast-like mesenchymal cells, cambial layer cells, and some macrophages. At day 9, the cell population contains cartilaginous cells but also contains osteoblasts and osteoproginator cells. Therefore all of the principal cell types involved in fracture repair can be tested over this time span.

Because each stage of fracture repair covered several days, representative times during each stage were selected and analysed immunohistochemically for growth factors.

PDGF was tested in this manner for days 1-5 post-fracture; aFGF was tested for days 3-10 post-fracture; IGF-I was tested for days 3-10 post-fracture and day-14 post-fracture; and TGF-β_1 was tested for days 8-10 and day 14 post-fracture. Because of the known actions of individual growth factors discussed earlier in this section, each growth factor was not tested over the entire repair period.

4.3 Results

At day 1 post-fracture, many monocytes are present in the fracture site, but few had transformed into macrophages. It is not until day 2 post-fracture, that the proportion of macrophages increases relative to the proportion of their precursor cells, the monocytes. By day 3 post-fracture, there is a noticeable decline in the number of macrophages which continues during successive days post-fracture. Monocytes appear to be centered in the fracture site and associated with the haematoma. Macrophages, on the other hand, occur in increasing numbers away from the fracture site.

The first growth factor identified during this stage 1 of fracture repair, was PDGF. At 2 days post-fracture, many macrophages appear at the periphery of the fracture site, in close proximity to the periosteum. Polyclonal antibodies to PDGF applied to tissue sections from this stage of the repair process (day 2 post-fracture) produced positive staining on macrophages indicating the presence of PDGF (Figures 10 a,b,c.). In the monocytes at day 1, no positive staining occurs for PDGF. In tissue sections from day 3 post-fracture, there was a noticeable reduction in the number of macrophages staining positive for PDGF. The largest number of PDGF producing macrophages coincides with initiation of cell division in the cambial layer of the periosteum. Other cell types present during the time period where antibodies to PDGF were applied, included monocytes, fibroblast-like mesenchymal cells, polymorphonuclear leukocytes, bone marrow cells, and

osteocytes from preexisting bone. None of these other cell types tested positive for the presence of PDGF (see Table 4a and Figures 10 a,b,c.).

The second growth factor demonstrated immunohistochemically during the fracture repair process was aFGF. Positive staining with polyclonal antibodies to aFGF was confined to cells of the expanding cambial layer of the periosteum. Even though cellular activity could be observed in the cambial layer at day 2 post-fracture, positive staining for aFGF was not detectable until day 3 post-fracture which coincided with stage 2 of the repair process (see Chapter 2). From day 3 to day 5 post-fracture, the cambial layer expands as a result of an increased cell population. There is a difference between the phenotypic shape of the day 2 post-fracture cambial layer cells and the day 3 post-fracture cambial layer cells. The day 2 cells were flattened and fibroblast-like in appearance. The day 3 cambial layer cells became rounded and began producing a cartilaginous matrix (as indicated by light alcian blue staining of the matrix). Beyond day 5 post-fracture, the cambial layer became indistinguishable from the cartilage tissue which was transforming the fracture callus at this point. The number of cells staining positively for aFGF increased from day 3 post-fracture to day 5 post-fracture. The aFGF positive cells in the cambial layer at day 3 post-fracture are presented in Figures 11 a,b,c. The aFGF positive cells in the cambial layer at day 5 post-fracture are presented in Figures 12 a,b,c. Other cell types present during the second stage of fracture repair included fibroblast-like mesenchymal cells, macrophages, bone marrow cells, endothelial cells, osteoblasts, and osteocytes

from pre-existing bone. No aFGF staining was detected on these cells or on chondroblasts, chondrocytes, or osteoblasts of later stages of repair (see Table 4b and Figures 11 and 12).

During the third stage of fracture repair (see Chapter 2), cartilage replaces the fibrous fracture callus. This process occurs from day 5 post-fracture to day 9 post-fracture. An essential feature of this process is the transformation of fibroblast-like mesenchymal cells into chondroblasts, an event similar to the transformation of wound fibroblast like cells to chondrocytes during limb regeneration in amphibians (Ahrens et al., 1977). The number of fibroblast-like mesenchymal cells increases from day 3 to day 5 post-fracture at which time cell density appears to reach a maximum. Starting at day 5 post-fracture, the fibroblast-like mesenchymal cells at the center of the cell mass became rounded and started producing a cartilaginous matrix (as signified by light alcian blue staining). As a cartilage mass formed and began to spread out, chondrocytes on the inside of the cartilage mass stained intensely blue indicating the presence of a much greater amount of chondroitin sulfate. Young chondroblasts at the periphery of the mass stain light blue. The cartilage mass is most noticable from day 7 to day 9 post-fracture. Young chondroblasts at the edge of the cartilage mass during this time period stain positively for IGF-I (see Figures 13 a,b,c). Other cell types present during this third stage of repair include fibroblast-like mesenchymal cells, endothelial cells, mature chondrocytes, bone marrow cells, and osteocytes from pre-existing bone. None of these other cells stained positive for IGF-I. Also in

tests of tissue sections from stages 1, 2 and 4 (day 3-9 post-fracture), where other cell types included macrophages, cambial layer cells, and osteoblasts, no positive staining for IGF-I was observed (see Table 4c and Figures 13 a,b,c.).

The fourth growth factor immunohistochemically detected was TGF-β_1. During the fourth stage of fracture repair (see Chapter 2), the cartilage mass was infiltrated by blood vessels and osteogenic cells and the cartilage was replaced through endochondral ossification. This occurred over a time frame from 9 to 20 days post-fracture. Actually, some transformation of cartilage to bone occurred at the very edge of the cartilage callus as early as day 7 post-fracture, but the process takes approximately two days to reach the cartilage mass in the fracture site. The cell types most conspicuous during this fourth stage of repair are chondrocytes and osteoblasts. Using direct red (a histological stain) the calcium in bone matrix stains dark red. Chondrocytes, at the edge of the ossification front infiltrating the cartilage mass, transform into calcified matrix-producing cells. This was signified by the appearance of a red-stained rim within the chondrocyte lacunae. This marked a transition of chondrocyte function from maintaining a proteoglycan matrix to producing a calcified matrix similar to that found in bone.

When polyclonal antibodies to TGF-β_1 were applied to tissue sections from this fourth stage, intense positive staining for the presence of TGF-β_1 occurred in the calcified matrix-producing cells at the edge of the ossification front (Figures 14 a,b,c). Other cell types present during this stage of fracture repair were bone marrow cells, osteocytes, and endothelial cells. None of these other cell types

stained positive for the presence of TGF-β_1. Also, other cell types from stages 2 and 3 (days 5-14 post-fracture) were tested for the presence of TGF-β_1. These other cell types; macrophages, fibroblast-like mesenchymal cells, chondroblasts, and cambial layer cells produced no positive staining for TGF-β_1 (see Table 4d and Figures 14 a,b,c.). Table 5 summarizes the immunoreactive cell types for each growth factor. The relative distribution of the growth factors with respect to days post-fracture and stage of fracture repair is presented in Figure 15. Figure 16 is a graphical representation of growth factor distribution, showing the pattern of localization within major cell types during stages of repair.

4.4 Discussion

It cannot be stressed enough, that in order to detect these four growth factors immunohistochemically, much time was spent developing new histological methods. Initially, the fractured tibiae were fixed in NBF and decalcified in 10% acetic acid (common procedures in the field). This method proved very unsatisfactory for immunohistochemical detection of growth factors or for the preservation of tissue integrity. Shrinkage of tissue and destruction of antigenic sites occurred. To improve the preservation of antigenic sites for immunohistochemical studies, tissues were fixed in PLP and decalcified in EDTA-G at low temperature (Mori et al., 1988). However, this method in its original form stated that all tissues treated this way should be cryosectioned. Cryosectioning was minimally successful on tissues from the later stages of fracture repair and not effective on tissues from earlier stages. The sections obtained were of irregular thickness, poor tissue integrity, and routinely lifted off glass slides during immunoperoxidase staining. To counteract these problems, I limited the amount of time the tissue sections were exposed to peroxide, and decreased incubation time in the primary antibody. The changes resulted in weak and very nonspecific antibody staining. I then decided to use the same fixative and decalcification methods (Mori et al., 1988) but process the tissues in a gentle dehydrating solution (Pollard et al., 1987) and vacuum infiltrate with low melting point paraffin wax. The results were remarkable. Tissue integrity was preserved. Previously unsectionable tissues such as day 1 post-fracture provided serial

sections (see Figure 3a from chapter 2).

Macrophages play a prominent role during the inflammatory response to injury. Their percursor cells, monocytes, first appear in the haematoma but transform into macrophages within hours of arrival at the fracture site. These macrophages contain cloudy cytoplasm, an indication they are phagocytosing dead and damaged tissue (A.Covert, pers. comm.). Away from the fracture site, macrophages can be seen in close proximity to the periosteum. As the literature states, macrophages are potent sources of growth factors. In particular, TGF-ß is secreted where collagen matrix production and secretion are important. FGF is secreted where fibroblast proliferation but not connective tissue matrix production is important. PDGF is secreted where fibroblast proliferation and the production of connective tissue matrix is important (Kovacs, 1991).

PDGF staining occurs at 2 days post-fracture in migrating and phagocytosing macrophages. PDGF is a potent stimulator of cell division in cells of mesenchymal origin (Bowen-Pope et al., 1985). Therefore, PDGF is an ideal candidate for the initiator of wound repair and in particular, fracture repair. In addition to the macrophage activity of removing dead cells, production of PDGF by macrophages can initiate the repair process which involves stimulating cell division as well as collagen production. Leibovich and Ross (1975) reported that first macrophages appear at a wound site and are followed by a proliferation of fibroblasts. Joyce et al. (1990a) observed that PDGF injections stimulated cell division in the periosteum. From my observations, both of these activities occur

during the initial response to fracture. PDGF may act as a key which unlocks cell division in these fibroblast-like mesenchymal cells, and in the cells of the cambial layer of the periosteum. Once cellular division has been initiated, other growth factors may provide the fuel to keep cell division going.

During the response to inflammation a large population of fibroblast-like mesenchymal cells accumulates. A similar response occurs toward the end of stage 1 and continuing through stage 2 of fracture repair (see chapter 2). After the appearance of a large number of macrophages during stage 1, these fibroblast-like mesenchymal cells begin to increase in number. A most dramatic increase in their number coincides with cellular activity in the cambial layer of the periosteum (stage 2). By day 5 post-fracture this large population of fibroblast-like mesenchymal cells forms the first bridge across the fracture gap.

The activity of the cambial layer of the periosteum is of key importance. With the decline in macrophages, a potent source of growth factors has been removed. Based on my observations, the loss in PDGF is replaced by a growth factor produced by the cells of the cambial layer. During this stage of the fracture repair process, cell replication is the major cellular activity (hence an important reason for searching for aFGF during this stage of repair). Cell replication is necessary to build-up a large population of fibroblast-like cells to allow the fracture gap to be bridged. Positive staining for aFGF occurs in these cells of the cambial layer for as long as the cambial layer is distinct (prior to chondrification, or third stage of fracture repair). As Kovacs (1991) and others

have noted, FGF is a potent stimulator of fibroblast proliferation. FGF has been found to stimulate cellular activity within the periosteum and has a direct inhibitory effect on collagen synthesis (Canalis et al., 1988). With the bridging of the fracture gap, a scaffolding is in place for the third stage of fracture repair, the chondrification stage.

During the third stage of fracture repair, chondrogenesis occurs and the fibrous callus, consisting of fibroblast-like mesenchymal cells and the cells of the cambial layer, is replaced by cartilage. These fibroblast-like mesenchymal cells resemble wound fibroblasts and are capable of undergoing chondrification (Ahrens et al., 1977). One of the key events in chondrogenesis of these mesenchymal cells is increasing close contact between neighbouring cells i.e. high density. By day 6 post-fracture, the fracture site is densely populated with fibroblast-like mesenchymal cells. Many of these cells can be observed rounding-up and starting to take on a chondroblast phenotype. Alcian blue is a histochemical stain which reacts with chondroitin sulfate to produce a blue stain in cartilage (the deeper the blue stain, the more matrix present). Cartilage appears in small amounts on day 6 post-fracture and increases until the fracture callus is totally chondrified by day 9 post-fracture. The cartilage mass increases significantly at day 8 post-fracture. At this time there are many young chondroblasts at the edge of the cartilage mass which stain positively for IGF-I. These young chondroblasts are actively involved in the synthesis of new cartilage matrix. The chondroblasts stain faintly with alcian blue, which indicates the

secretion of relatively small amounts of matrix components, including chondroitin sulfate. A different growth factor is necessary at this point; one which primarily stimulates matrix production instead of cell replication. When polyclonal antibodies to IGF-I were applied to tissue where chondrogenesis was occurring, young chondroblasts stained positively for IGF-I.

IGF-I was first characterized by its ability to stimulate S^{35} uptake by costal chondrocytes in culture (Salmon and Daughaday, 1956). It has since been found to stimulate chondrocyte proliferation and matrix production (Bohme et al., 1992). Therefore, IGF-I may be the growth factor responsible for the production of cartilage matrix during the chondrification of the fracture callus (stage 3 of fracture repair). This cartilaginous callus is only a temporary state because by day 9 post-fracture, the cartilaginous callus starts undergoing replacement by new bone through endochondral ossification.

At the conclusion of the chondrogenic phase of fracture repair and prior to endochondral ossification, the cartilage martix undergoes changes. Mineral crystals appear in the mitochondria of chondrocytes, calcium-phospholipid-phosphate is produced, and submicroscopic membrane-bound vesicles appear in the cartilage matrix (Martin and Matthews, 1970; Boskey and Posner, 1976; Anderson, 1980). In short, the mature cartilage becomes calcified. Following calcification of the cartilage matrix, blood vessels and osteogenic cells invade the cartilage mass and the process of endochondral ossification occurs (Ham and Cormack, 1979). Starting at approximately day 9 post-fracture and continuing to day 21 post-

fracture, endochondral ossification occurs. As bone replaces mature cartilage matrix in the callus, chondrocytes can be observed producing a calcified rim around their lacunae. These calcified lacunae appear at the edge of the ossification front where new bone is forming and capillaries are infiltrating the cartilage mass. These calcified matrix-producing cells appear to signal a change or transformation in cell type and matrix production. Intense TGF-$ß_1$ staining occurs in these calcified matrix-producing chondrocytes.

TGF-$ß_1$ was originally characterized by its ability to induce a transformed phenotype (DeLarco and Todaro, 1978). Chondrocyte transformation is not a new concept; Richman and Diewert (1988) observed transformation of chondrocytes into osteoprogenitor cells and osteoblasts. Frazer et al. (1991) observed that cultured chondrocytes release TGF-$ß_1$ into their culture media. TGF-$ß_1$ is a multifunctional peptide that controls proliferation and differentiation in bone cells. Perhaps TGF-$ß_1$ is produced at this stage of the repair process for three reasons. It may signal a transformation in cell and matrix type in the calcifying chondrocyte. It may stimulate the differentiation of invading osteoprogenitor cells into osteoblast-like cells. It may also stimulate the proliferation of these osteoprogenitor/osteoblast-like cells.

A review of Table 5 demonstrates the principle that for every stage of the repair process (see also Figures 15 and 16), there is a certain cell type which produces a growth factor. The cell types producing the growth factors are transient members of the repair process. It also appears they are effective only

during the stage in which they appear. Macrophages appear in stage 1 and produce PDGF. Their numbers drop-off substantially beyond stage 1 as does their ability to further produce growth factors. When the population of macrophages declines, the loss of their growth factor stimulation is replaced by another group of cells producing another growth factor, reflecting changing environmental conditions within the healing process at the fracture site. With most of the cellular debris removed by the phagocytizing macrophages, cell replication and the production of new tissue can begin in earnest. It seems logical to build a sufficent population of flexible fibroblast-like cells to bridge the opposite ends of the fracture site. Therefore, the cells of the cambial layer produce aFGF to stimulate cell replication in these fibroblast-like mesenchymal cells.

With the opposite ends of the fracture secured by the fibroblast-like mesenchymal cells, activity of the cells in the cambial layer in stimulating fibroblast-like mesenchymal cell replication is not necessary. The stimulatory activity of the cells of the cambial layer can be replaced by a different growth factor and a different cell type to produce it. Chondrogenesis, the next process to occur, required fibroblast-like mesenchymal cells to be replaced by cartilage-producing cells. Matrix synthesis becomes of paramount importance and, the chondroblasts are the source of this synthesizing activity. A growth factor which stimulates cartilage matrix production would be most appropriate at this point. Fortunately, such a growth factor exists; it is IGF-I. Chondroblasts produce IGF-I and respond to it in an autocrine manner. When cartilage matrix production is

complete, IGF-I synthesis and chondroblast activity ceases.

With invasion of the mature cartilage by blood vessels and osteoprogenitor cells, transformation of cartilage to bone begins. The site where this transformation takes place is at the edge of the cartilage mass along the ossification front. What is needed is a growth factor which signals this transformation to chondrocytes within the cartilage mass and initiates the transformation in chondrocyte activity from maintaining a cartilage matrix to producing a calcified cartilage matrix. Perhaps TGF-$ß_1$ is produced for this purpose and chondrocytes at the edges of the cartilage mass produce it until the ossification front moves past them and they become enclosed in new bone. TGF-$ß_1$ production ceases in these former chondrocytes and is initiated by chondrocytes where the ossification front is now located. With complete replacement of cartilage by new bone, TGF-$ß_1$ production ceases and no chondrocytes remain because the healing process is complete.

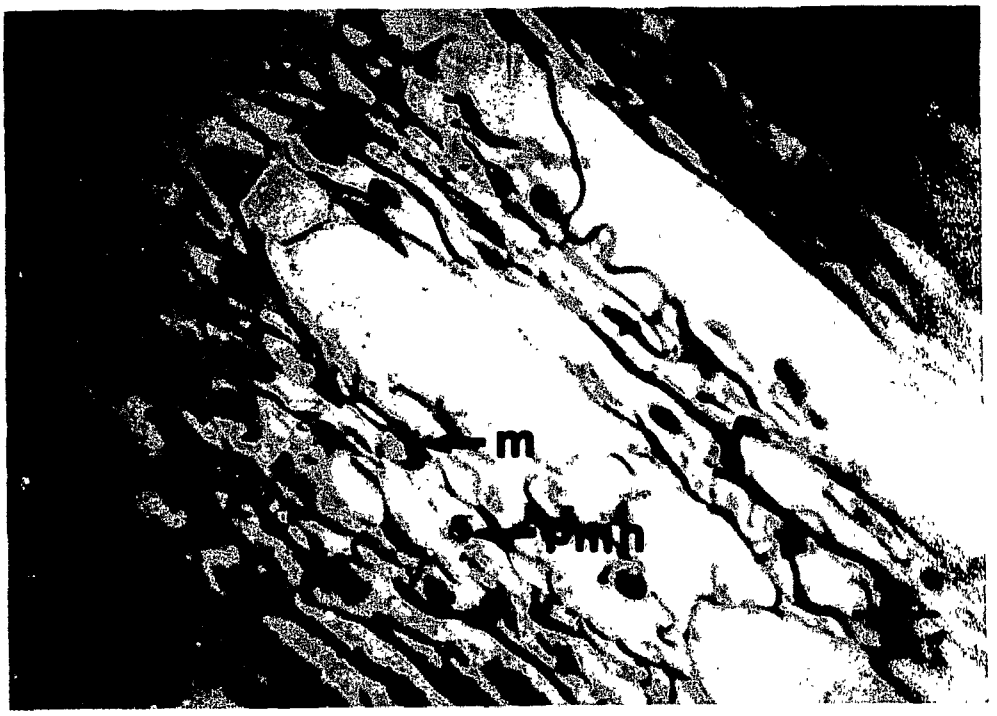
Figure 15 is a theoretical diagram depicting the above mentioned sequence of events. A comparison of the activity of the four growth factors during the stages of fracture repair demonstrates a relationship as a series of peaks. Each growth factor is responsible for the initiation and/or progression of a stage of the repair process.

Figure 10a: Low-power photomicrograph of histological section from a day 2 post-fracture tibia. Note the tibia (T), and loose areolar connective tissue (CT). Bar = 192 μm

10a

Figure 10b: High-power photomicrograph of area in insert from Figure 10a. The tissue type present is mainly areolar connective tissue. Note the many macrophages (m). Also present are polymorphonuclear leukocytes and the tibial shaft. Bar = 21 μm

Figure 10c: Serial adjacent section to the slide in Figure 10b with polyclonal antibodies to PDGF applied to it. Note positive staining of the antibody only on macrophages (arrow heads). Bar = 21 μm


m
pmn

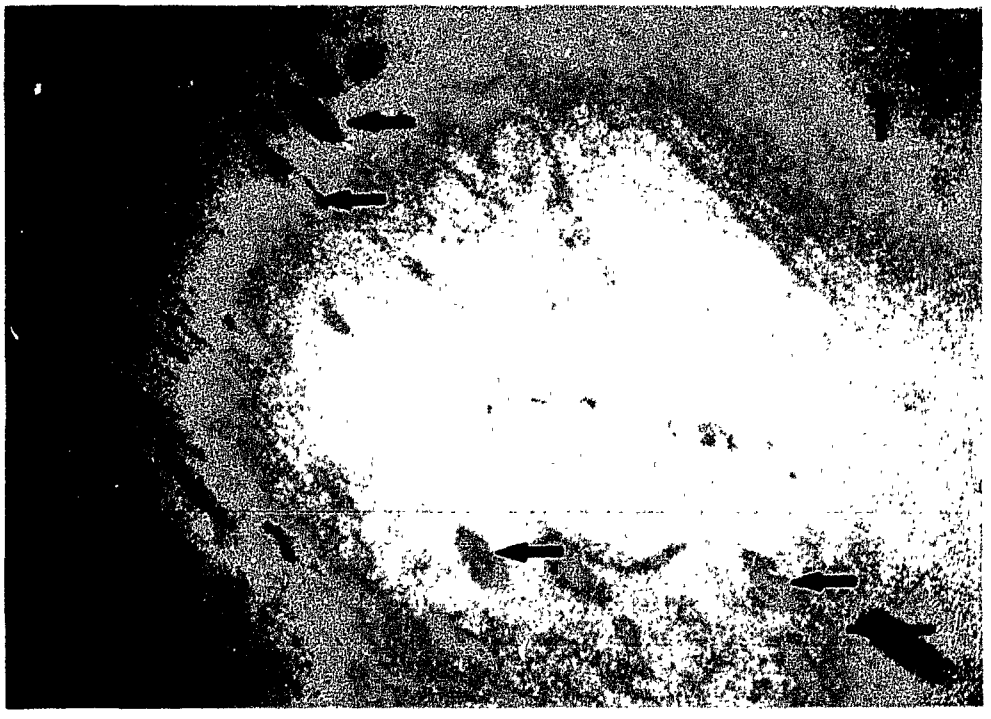
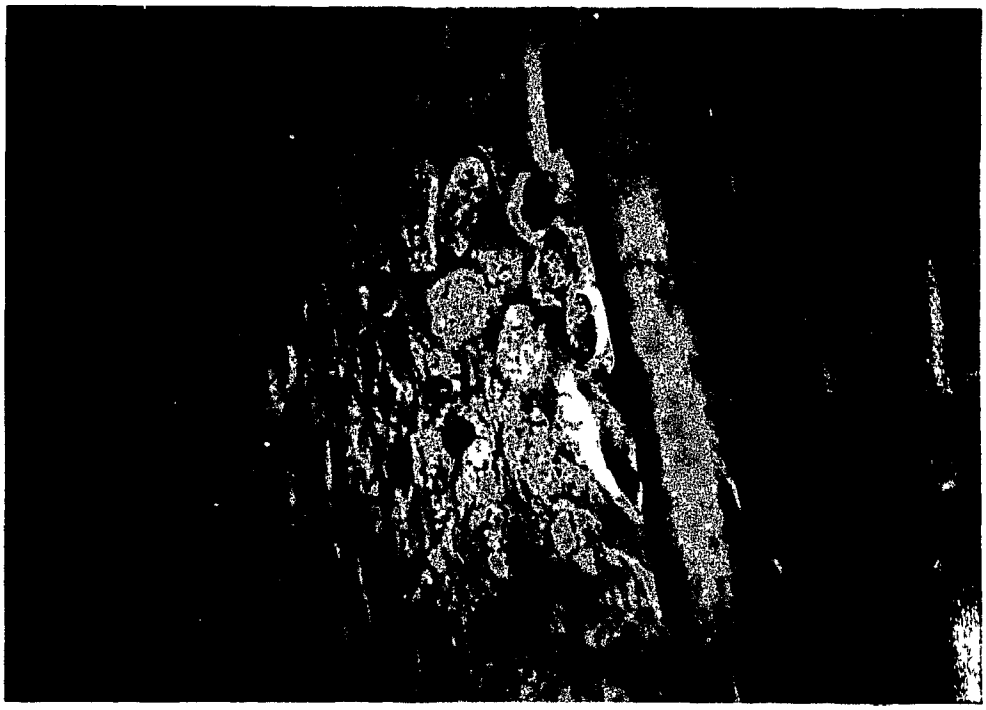


Figure 11a: This is a low-power photomicrograph of the fracture site at day 3 post-fracture. Note cellular activity in the cambial layer (cl) of the periosteum. Bar = 192 μm

Figure 11b: This is a high-power photomicrograph of area in insert from Figure 11a. To the right of the cambial layer is the tibia and to the left is a growing population of fibroblast-like mesenchymal cells. Also present in the field of view are osteocytes in pre-existing bone (opb). Bar = 21μm

Figure 11c: Serial section to the slide in Figure 11b with polyclonal antibodies to aFGF applied to it. Note only the cells of the cambial layer stain positive (arrow heads). Bar = 21μm



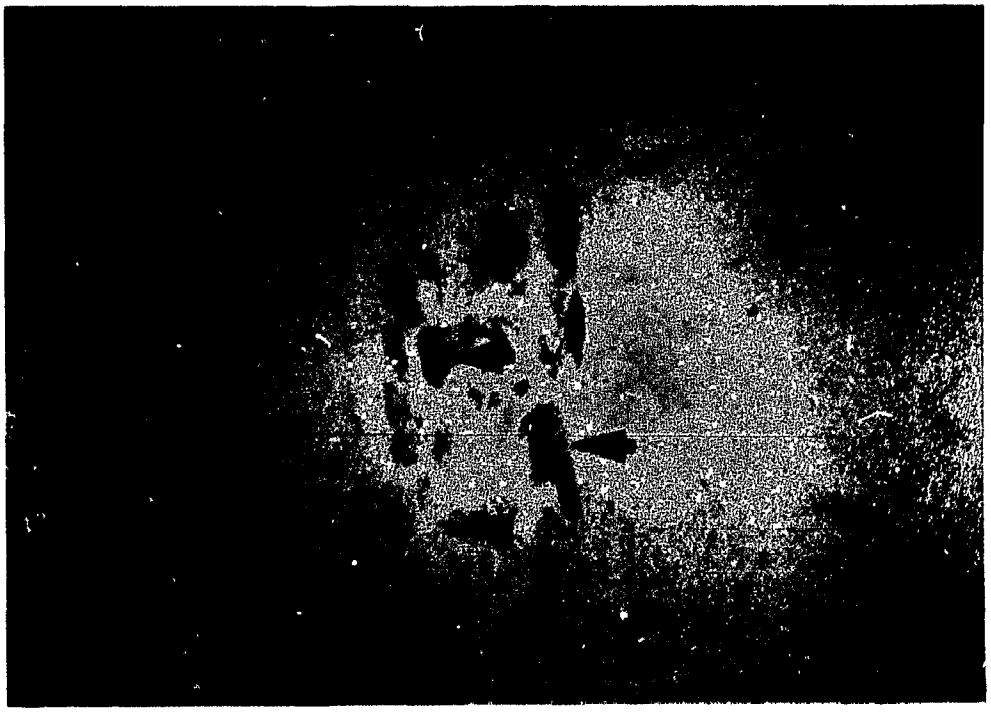


Figure 12a: This is a low-power photomicrograph of the fracture site at day 5 post-fracture. Note the increased population of fibroblast-like mesenchymal cells. Bar = 192 μm

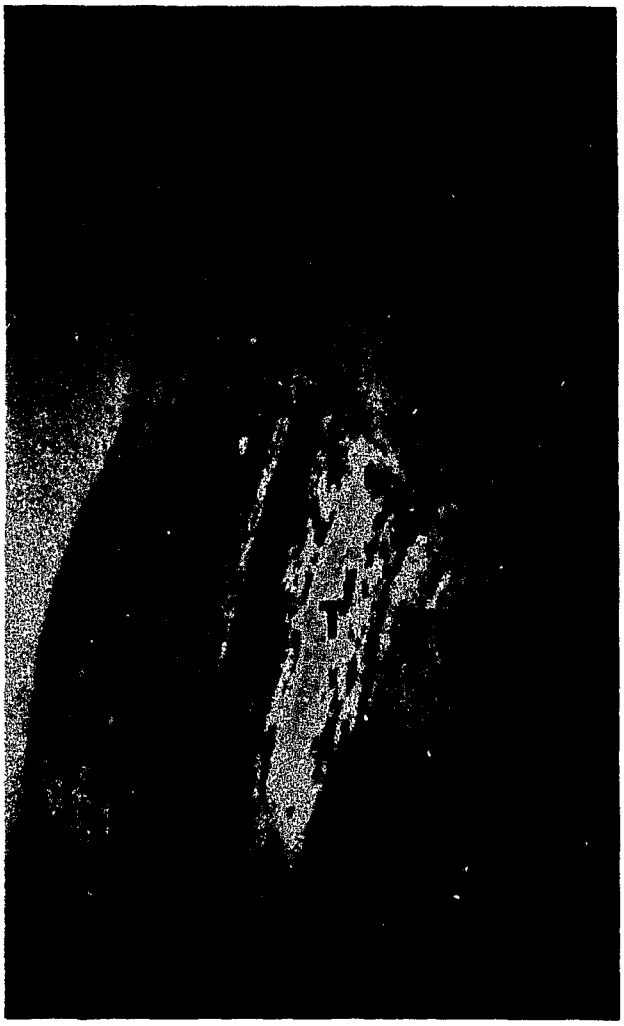
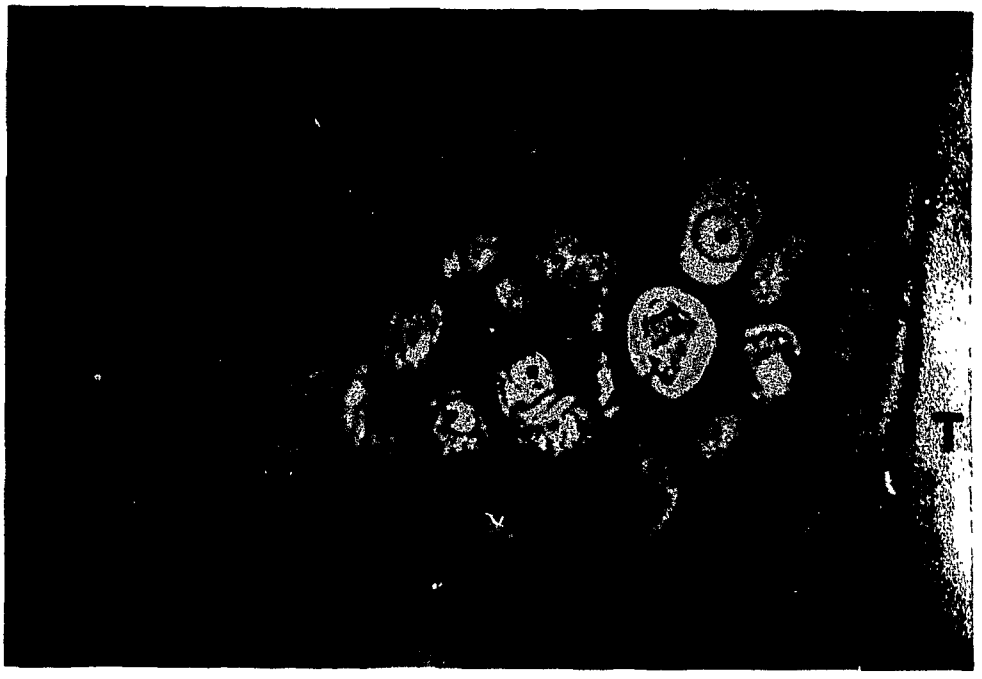


Figure 12b: This is a high-power photomicrograph of area in insert from Figure 12a. Note the dramatic increase in the size of the cambial layer. Bar = 21 μm

Figure 12c: Serial section to the slide in Figure 12b. Note the intense staining of the cells of the cambial layer with antibodies to aFGF (arrow heads). Note absence of staining in pre-existing osteocytes of the tibial shaft and fibroblast-like mesenchymal cells. Bar = 21 μm


T

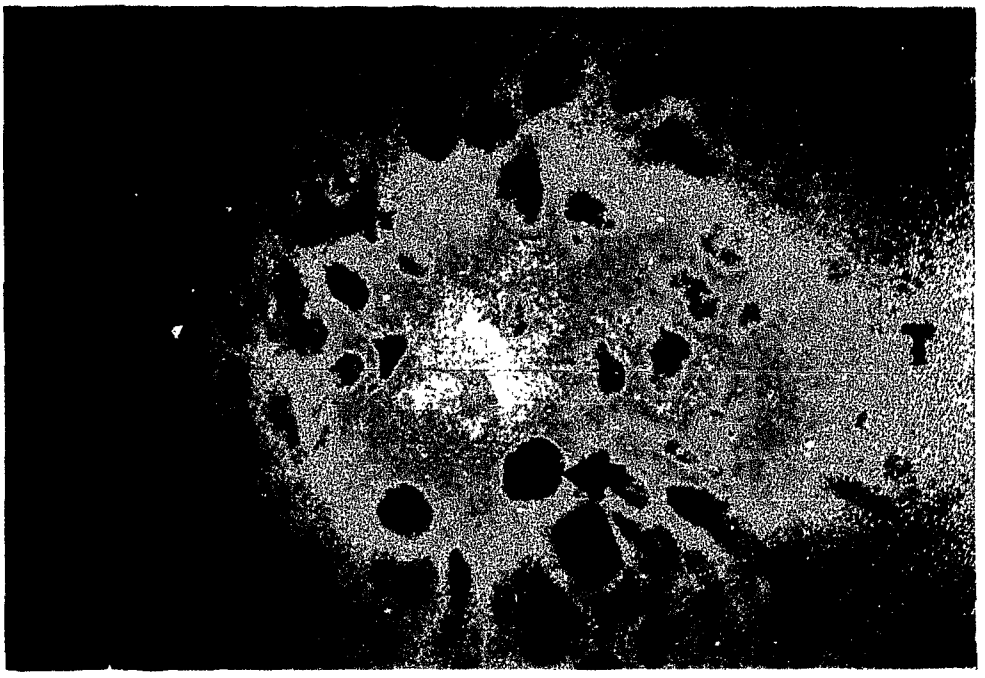
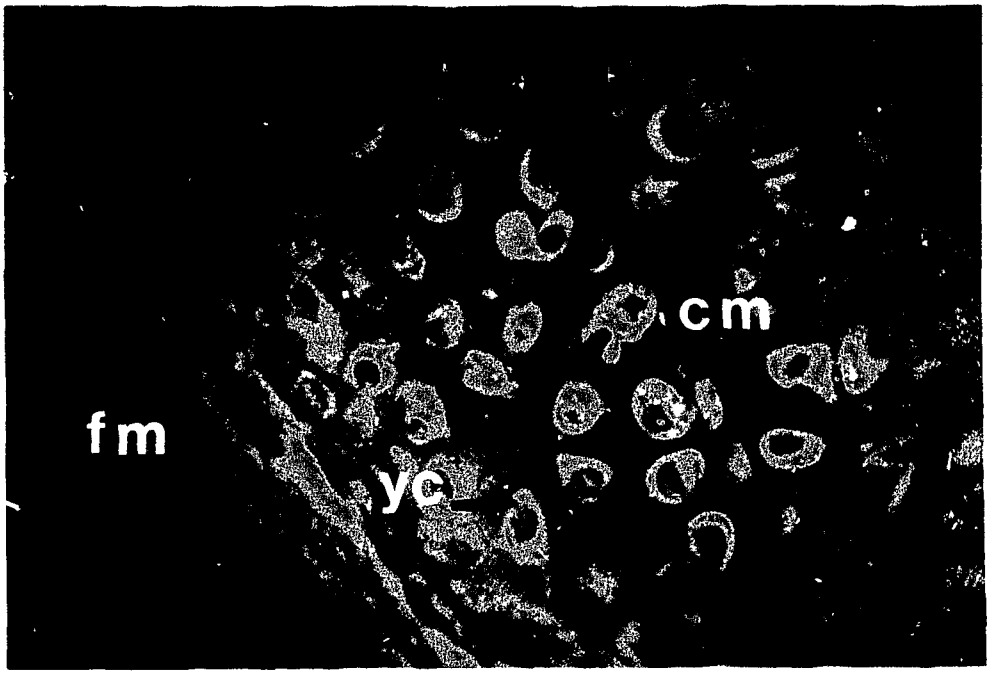

T

Figure 13a: This is a low-power photomicrograph of a fracture site at day 8 post-fracture. Note formation of a cartilage mass (cm). Bar = 192 μm

Figure 13b: This is a high-power photomicrograph of area in insert from Figure 13a. The area highlighted is the edge of the cartilage mass. Note young chondroblasts (yc) at the periphery of the cartilage mass which stain weakly with the histochemical stain. Bar = 21 μm

Figure 13c: Serial adjacent section to Figure 13b with polyclonal antibodies to IGF-I applied to it. Note the chondroblasts at the periphery of the cartilage mass stain positive for IGF-I (arrow heads). Note absence of staining on chondrocytes in the cartilage mass and fibroblast-like mesenchymal cells. Bar = 21 μm


cm
fm
yc

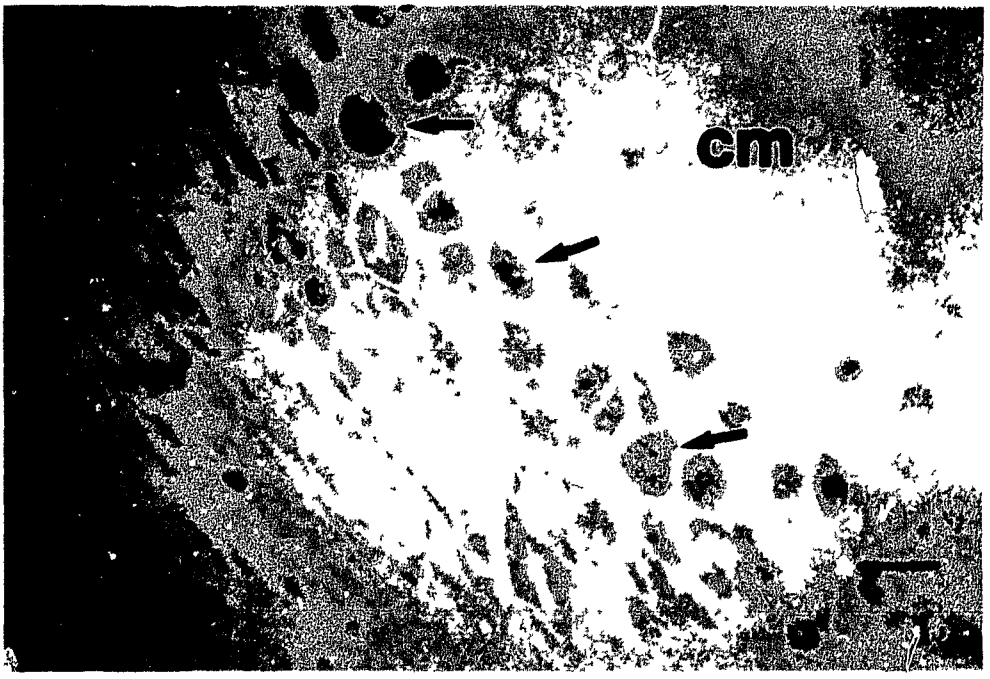

cm

Figure 14-1: This is a low-power photomicrograph of a fracture site at 14 days post-fracture. Note new bone trabeculae (bt) are infiltrating and replacing the cartilaginous mass. Bar = 192 μm

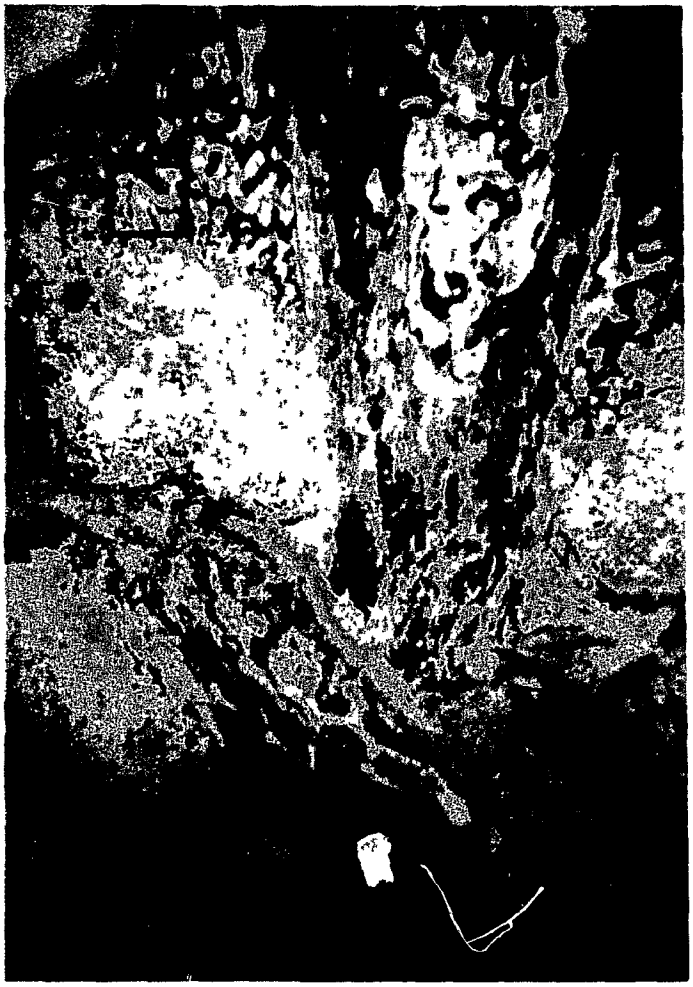
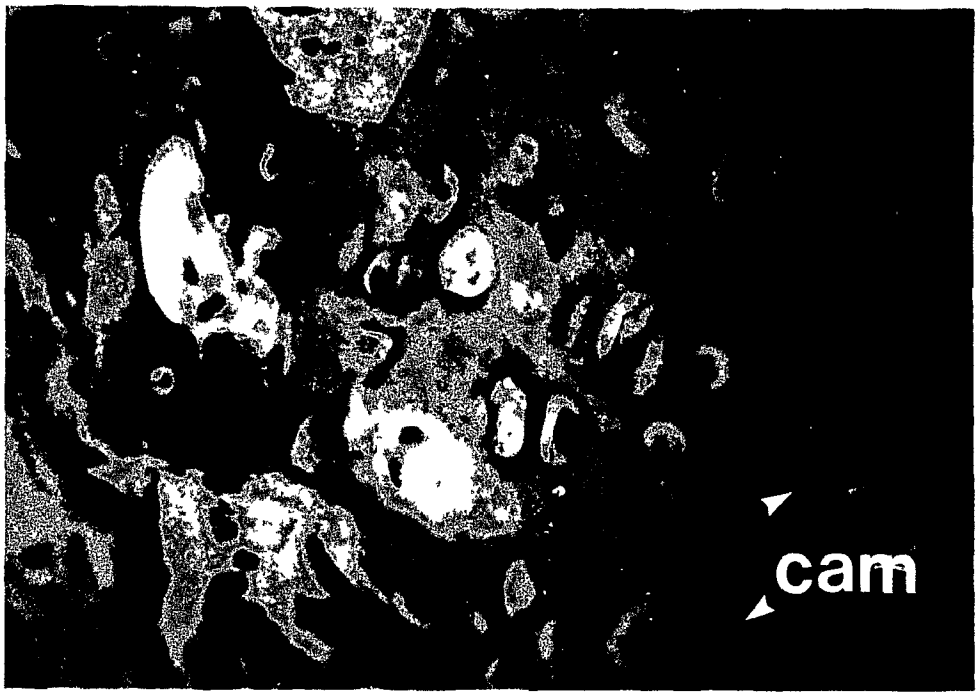


Figure 14b: This is a high-power photomicrograph of area in insert from Figure 14a. The area outlined is the periphery of one of the remaining cartilaginous masses. Note the ossification front (of) moving from left to right. Also note that chondrocytes at the edge of the ossification front are producing a red rim inside their lacunae signifying the production of a calcified matrix (cam). Bar = 21 μm

Figure 14c: Serial adjacent section to Figure 14b with polyclonal antibodies to TGF-$ß_1$ applied to it. Note the positive staining calcified chondrocytes (cc) at the edge of the ossification front (arrow heads). Note absence of staining on chondrocytes in cartilage mass. Bar = 21 μm


cam

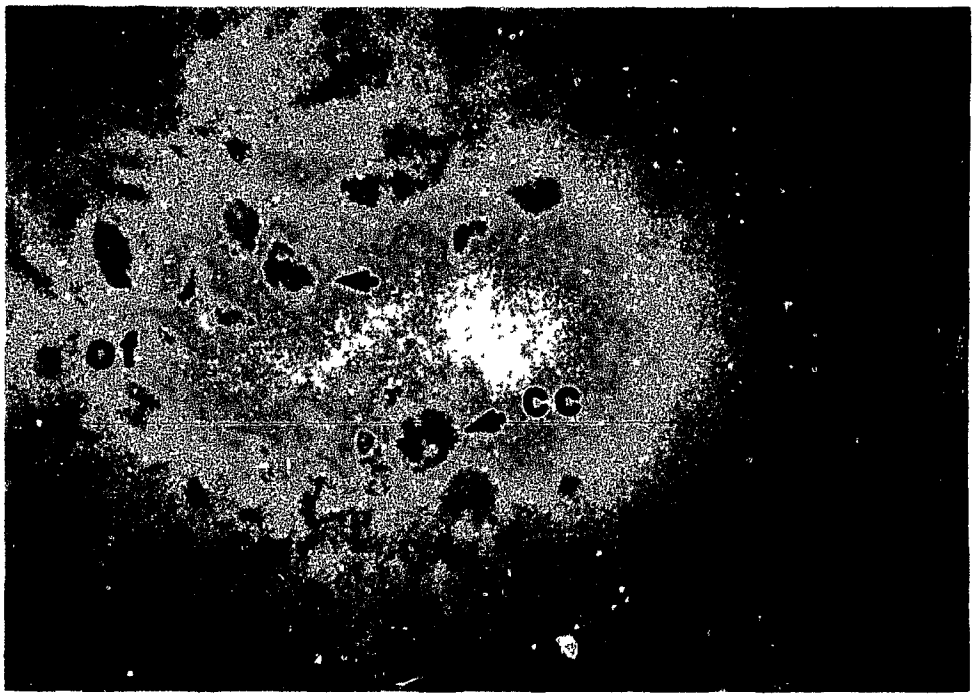

cc

Figure 15: Diagram of relative distribution of growth factors versus days post-fracture.

15

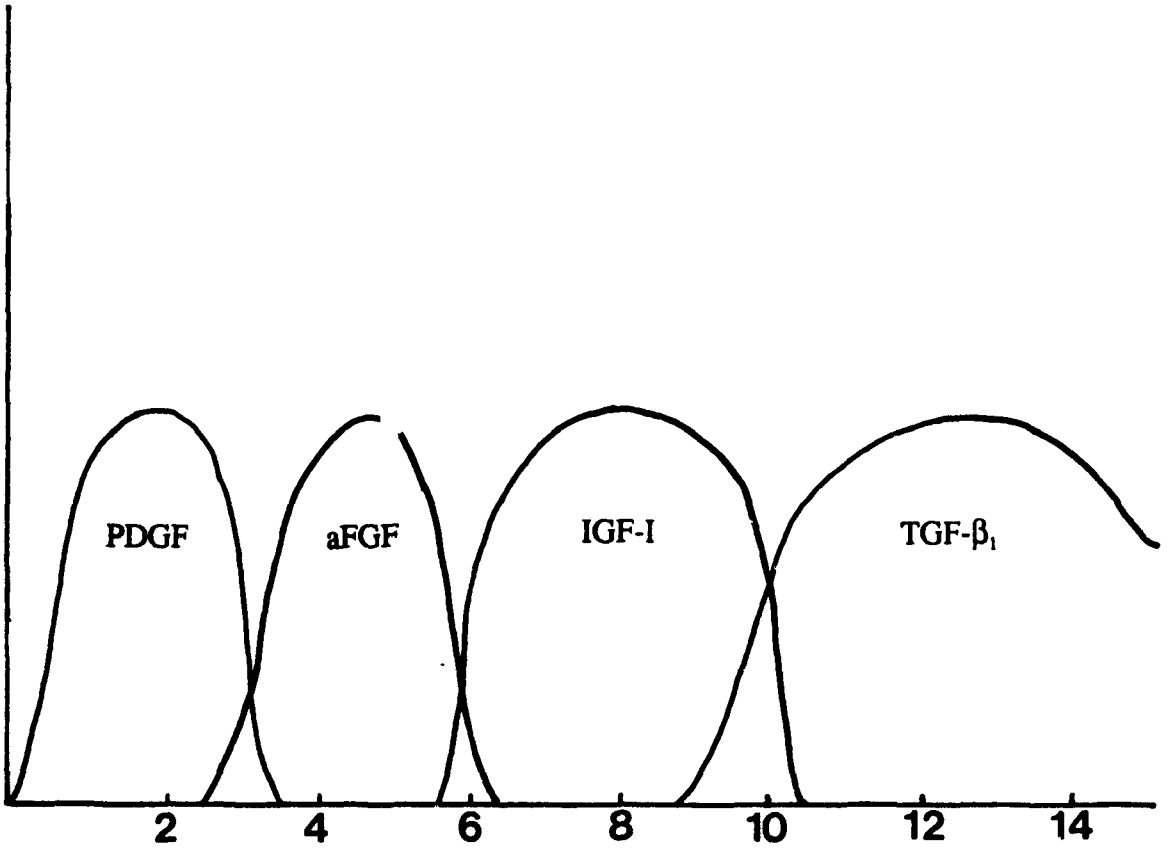

PDGF
aFGF
IGF-I
TGF-β1
2
4
6
8
10
12
14
Days post-fracture
Stage 1
Stage 2
Stage 3
Stage 4

Figure 16: Graphical representation of growth factor distribution showing the pattern of localization within major cell types during stages of repair.

16

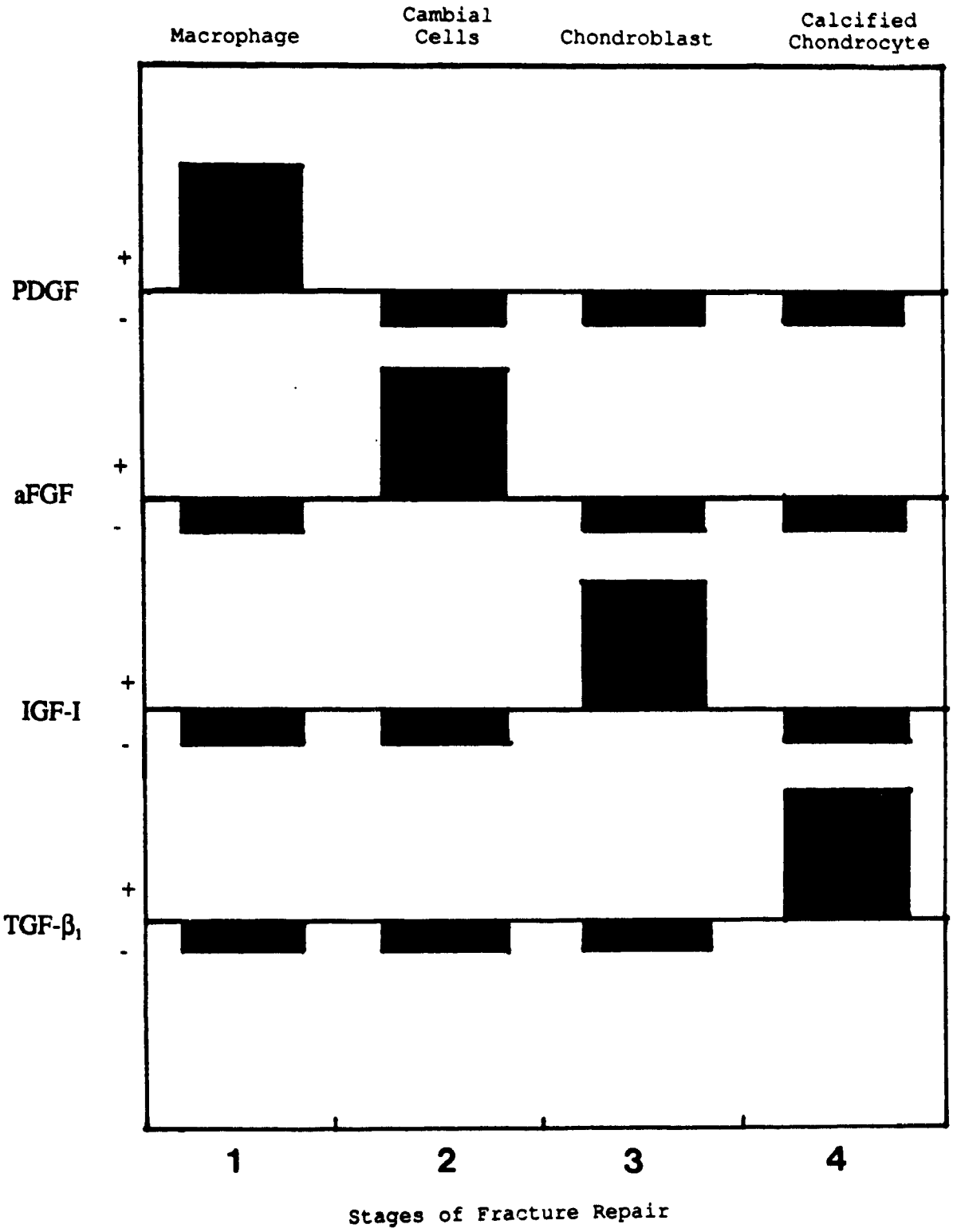

Macrophage
Cambial Cells
Chondroblast
Calcified Chondrocyte
PDGF
aFGF
IGF-I
TGF-β1
+
-
1
2
3
4
Stages of Fracture Repair

Table 4a Cell types present during Stage 1 of fracture repair and presence or absence of PDGF.

	Cell Types Present					
PDGF	Mono-cyte	macro-phage	Fibro-blast mesench-ymal	Poly-morpho-nuclear leuko-cyte	Bone marrow	Pre-existing osteocyte
	- ve	+ ve	- ve	- ve	- ve	- ve

Table 4b Cell types present during stage 2 of fracture repair and presence or absence of aFGF.

	Cell Types Present						
aFGF	Macro -phage	cambial layer	fibro-blast mesench -ymal	bone marrow	endo-thelial	Pre-existing osteo-cyte	osteo-blast
	- ve	+ ve	- ve	- ve	- ve	- ve	- ve

Table 4c. Cell types present during stage 3 of fracture repair and presence or absence of IGF-I.

	Cell Types Present						
IGF-I	Fibro-blast mesench-ymal	chondro-blast	chondro-cyte	endo-thelial	bone marrow	pre-existing osteo-cytes	osteo-blasts
	- ve	+ ve	- ve	- ve	- ve	- ve	- ve

Table 4d. Cell types present during stage 4 of fracture repair and presence or absence of TGF-$ß_1$.

	Cell Types Present					
TGF-$ß_1$	chondro-cytes	calcified chondro-cytes	osteo-cytes	osteo-blasts	endo-thelial	bone marrow
	- ve	+ ve	- ve	- ve	- ve	- ve

Table 5 Growth factors and their immunohistochemically positive cells at the four stages of fracture repair.

Growth Factor	Macro-phages	Cambial Layer Cells	Fibroblast mesenchymal cells	Chondro-blast	Osteo-blast
Stage 1					
PDGF	+ ve	- ve	- ve	not present[a]	not present
Stage 2					
aFGF	- ve	+ ve	- ve	-ve	not present
Stage 3					
IGF-I	not present	- ve	- ve	+ ve	- ve
Stage 4					
TGF-β_1	not present	- ve	- ve	- ve	+ ve

[a] Not present means that the cell type was not present at that stage.

Chapter 5 Detection of mRNA to aFGF

5.1 Introduction

aFGF was detected immunohistochemically in cells of the cambial layer during stage 2 of the fracture repair process (see Chapters 2 and 4). Therefore, I proposed the hypothesis that aFGF was produced by the cambial layer cells for local use in stimulating cell replication in cambial layer cells and fibroblast-like mesenchymal cells. To test this hypothesis, I used molecular biology techniques to detect mRNA to aFGF in tissue harvested from the fracture site. I chose not to make primers to PDGF or IGF-I because only the sequences of a few exons to mouse mRNA for these growth factors are known. I chose not to make primers to TGF-$ß_1$ because of the high sequence homology between TGF-$ß_{1-3}$.

Two metastatic cell lines, one established from medullablastoma and one from rhabdomyosarcoma, produce mRNA to aFGF (Lobb et al., 1986). Non-metastatic cell lines i.e. cultured human vascular smooth muscle cells, also express mRNA to aFGF (Winkles et al., 1987). Burgess and Maciag (1989) observed that most tissues contain significant concentrations of FGF (40-120 pm/g) but that only low levels of mRNA were observed in situ. To them this observation emphasized the degree of technical difficulty in obtaining mRNA to aFGF from tissue. With this in mind, polymerase chain reaction (PCR) was employed to detect aFGF mRNA in the present study. PCR uses several steps to amplify initially small amounts of

DNA or RNA. For mRNA amplification, the mRNA is reverse transcribed to produce complementary (cDNA). This cDNA operates as a template strand; primers which are short lengths of cDNA are added to the reaction. By selecting appropriate pairs of primers, millions of copies of a particular DNA sequence can be made in a few hours [Each cycle contains three steps; annealing of primers to the DNA template, extension of the copy DNA, and strand separation (Wright and Wynford-Thomas, 1990).]. This DNA can be visualized on a size-fractionated electrophoresis gel.

The cDNA sequence for mouse aFGF was published by Herbert et al. (1990). Two, twenty base primers were made, one 5' and one 3' to the cDNA. By surgically removing the tissue in the fracture site from day 1 to day 10 and day 14 post-fracture, the presence of mRNA to murine aFGF could be detected and thus aFGF could be said to be produced locally in the fracture site.

5.2 Materials and Methods

5.2.1 RNA Preparation

Male CD-1 mice 4-6 months of age were fractured according to the procedure described in Chapter 2. Three mice were collected each day from day 1 to day 10 post-fracture and three mice were harvested on day 14 post-fracture. RNA extracted from the three samples per day was pooled. The extraction procedure consisted of removing muscle from around the tibia and cutting the tibia approximately 2 mm above and below the fracture site. The tissues in the 4 mm spread across the fracture site were snap frozen in isopropanol (approximately -50^{0}C).

The following procedures were performed to remove RNAase as a possible contaminant. All glassware and surgical tools used to harvest tissue from the fracture site were baked at 300^{0}F for 12 h prior to use. The tissue homogenizor was soaked in diethyl pyrocarbonate (DEPC)-treated water for 1 h prior to use. The fractured tibiae themselves were repeatedly dipped in DEPC -treated water while fracture site tissues were being excised.

Deoxynucleotide triphosphates (dNTPs) and random hexanucleotide primers pd (N6), were purchased from Pharmacia Fine Chemicals (Dorval, Quebec, Canada). Mouse mammary leukemia virus (MMLV) reverse transcriptase was obtained from Bethesda Research Laboratories/Life Technologies (Burlington,

Ontario, Canada). Taq DNA polymerase was purchased from Promega (Madison, WI). All other chemicals were from Sigma Chemical Co. (St. Louis, MO).

Oligodeoxynucleotide primers were synthesized at the Gene Probe Laboratory (Halifax, N.S., Canada). The 5' and 3' twenty base primers to mouse aFGF were chosen from the published cDNA to mouse mRNA described by Herbert et al. (1990) [Gene bank accession no. M30641]. The primers were as follows: primer 1, 5'-CTG AAG GGG AGA TCA CAA CC -3' complementary to cDNA nucleotides 4 to 24; and primer 2, 5'-GTC AGA AGA CAC CGG GAG GG -3', corresponding to nucleotides 445 to 465. The predicted size of the amplified product was 461 nucleotides. Herbert et al. (1990) published the cDNA to the complete mouse aFGF mRNA sequence. The primers I chose were to the 5' and 3' ends of the cDNA and most likely span at least one intron. This is an important precaution to ensure genomic aFGF is not amplified as a PCR product.

RNA was prepared from harvested fracture sites (calluses) by the acid-guanidinium-phenol-chloroform method of Chomczynski and Sacchi (1987). Tissues were snap frozen in isopropanol, super cooled on dry ice. They were then homogenized with a Kinematica high speed tissue homogenizor (Beckmann Instruments; Westbury, NY) containing guanidium thiocyanate, and extracted with phenol-chloroform. RNA remaining in the aqueous phase was precipitated with isopropanol and then ethanol at -20^{0}C. The final pellet of RNA was dissolved in water, quantitated by reading the optical density at 260 nm and stored at -20^{0}C.

5.2.2 cDNA Preparation

Total cellular RNA (5μg in 25μl) was converted to cDNA by reverse transcription using MMLV reverse transcriptase. Specifically, 5μg total RNA in 10μl sterile H_2O were heated at 90^{0}C for 10 min, quick chilled on ice for 5 min, then added to the reverse transcription reaction mix in 1.8-ml microfuge tubes. Each reaction contained 5μl of a 5-fold concentrated buffer [250 mM Tris-HCl (pH 8.3), 375 mM KCl, and 15 mM $MgCl_2$], 100μM of each of the four dNTP's and ^{32}P deoxycytosine triphosphate (dCTP) added to the mix of dNTP's for autoradiographic exposure, 1 nmol/μL random hexamer primers , 0.01 M dithiothreitol, 40 U RNasin, 200 U MMLV reverse transcriptase, and 5μg RNA in a total volume of 25μl. The reactions were incubated for 10 min at 23^{0}C, followed by 3 h at 42 ^{0}C, and finally 7 min at 95 ^{0}C. They were then quick-chilled on ice and stored at -20C.

The PCR reaction mixtures (25μl final volume) contained 100μM of each of the four dNTP's, 1.25 U Taq polymerase, 2.5μl of a 10-fold concentrated reaction buffer (500 mM KCl and 100 mM Tris-HCl, pH 9.0), 15 mM $MgCl_2$, 1% Triton X-100), 50 pmol of each primer, and 5μl reverse transcription reaction. The reaction mixtures were overlain with 100μl of light mineral oil and incubated in an Ericomp single block thermal cycler (Ericomp, Inc., San Diego, CA). After an initial denaturation for 2 min at 94 ^{0}C, the samples were amplified for 40 cycles, each consisting of annealing at 65 ^{0}C for 1 min, elongation at 72 ^{0}C for 2 min, and

denaturation at 94 ^{0}C for 1 min. A second series of reactions were carried out using primers to actin. Two actin primers (a kind gift from Dr. Paul Murphy) were added to a second reaction mixture and PCR products were produced.

A 2% agarose gel was run loaded with both sets of PCR products and containing 0.1μg/ml ethedium bromide. The gel was washed in TBS, then exposed for autoradiography on Kodak X-Omat AR x-ray film (Eastman Kodak, Rochester, NY) at -70^{0}C for 12 h. Bands were scanned with an Abaton 300-GS scanner (Everex Systems, Inc., Fremont, CA), and the resulting digitized image was analyzed with Scan Analysis software (Biosoft, Ferguson, MO) on an Apple MacIntosh IIsi computer (Apple Computer, Inc., Cupertino, CA).

5.3 Results

The total RNA harvested from the tissue of the fracture sites yielded distinct and intense 28 and 18 S bands when examined electrophoretically (see Figure 17). This signified that the total RNA harvested was of high quality and not degraded.

The PCR reaction products for each sample were run on an agarose gel; the result is presented as Figure 18. Product shows up as a single band at approximately 460 base pairs. Some nonspecific banding is seen in lanes 4 to 11, but these bands are very weak. In the lower half of Figure 18 are lanes where the PCR actin products are located. The relative weakness of the 460 base pair band in lane 7 can be explained as a result of a lower concentration of total RNA as starting material for the PCR reaction (the intensity of the actin band indicates total RNA present in the sample).

The autoradiograph of the PCR products was measured with a densitometer; the results are depicted in Table 6. Using the actin product as a control of how much total RNA was originally in the sample, the relative amount of aFGF PCR product was calculated. The results are depicted in the histogram as part of Table 6. From the histogram, it can be observed that the relative amount of PCR amplified aFGF mRNA increases from day 1 to day 10 post-fracture (this time frame includes stages 1-3 as outlined in Chapter 2). Beyond day 10, the amount of aFGF mRNA began to decrease but by day 14 there was still more mRNA than from days 1-5 post-fracture. Surprisingly, aFGF mRNA is detected in the

tissues of the fracture site at day 1 post-fracture but the protein is not detected immunohistochemically until day 3 post-fracture (see Chapter 4). Also surprising, is the observation that aFGF mRNA is present beyond day 5 post-fracture while the aFGF protein cannot be detected beyond day 5 post-fracture (see Chapter 4). Not only is mRNA to aFGF present beyond day 5 post-fracture, its concentration increases until it reaches a peak at day 10 post-fracture. Table 7 displays a comparison between the distribution of mRNA and protein to aFGF over the four stages of fracture repair.

5.4 Discussion

In order to discuss the experimental results, I will present some background information on the molecular biology of aFGF. Jaye et al. (1986) isolated cDNA clones encoding human aFGF from a human cDNA brain stem library. Southern blots suggested a single copy of the aFGF gene, and that it was located on chromosome 5 at bands 5q31.3 to 33.2. The aFGF gene produces a mRNA transcript of 4.8 kilobases. Chiu et al. (1990), also worked with a human brain stem cDNA library, and observed that alternative splicing generated at least two different forms of aFGF mRNA. The difference occurs in the 5'-untranslated regions, these transcripts may result from the usage of alternative promoters. Further investigations suggested that there are at least two additional exons coding for 5'-untranslated sequences residing further upstream.

Crumley et al. (1990) isolated two cDNA clones encoding human aFGF which represented the utilization of alternative upstream exons in aFGF mRNA. Isolation and sequence analysis of genomic clones spanning the first coding exon and each of the upstream sequences confirmed that the divergent 5' sequences are separate exons, spliced alternatively to the first coding exon 34 nucleotides upstream of the initiator AUG codon. The presence of 5' untranslated exons located far from the coding region raised new questions of how aFGF transcription was initiated and controlled.

Large nuclear proteins must possess a signal sequence to pass through nuclear

pores (Newmeyer and Forbes, 1988). Imamura et al. (1990) observed that the amino acid sequences 21 to 27 (NYKKPKL) were similar to nuclear translocation sequences of nuclear proteins. A mutant aFGF which lacked this sequence was prepared. The altered aFGF retained its ability to bind to heparin, but failed to induce DNA synthesis and cell proliferation. Re-attachment of the nuclear translocation sequence re-initiated the mitogenic activity in vitro and indicated that nuclear translocation was important for the biological response to aFGF.

The aFGF encoding open reading frame is flanked by translation stop codons and provides no signal peptide or internal hydrophobic domain for the secretion of aFGF (Jaye et al., 1986). The amino acid sequence of aFGF and interleukin-1 (Il-1) are homologous (Thomas et al., 1985). IL-1 also does not contain a stretch of hydrophobic amino acids (a signal that is common to most secreted proteins), either at the N-terminus or internally. This led March et al. (1985) to suggest that IL-1 is not actively secreted by macrophages, but is released by damaged cells. Stimulated monocytes or macrophages synthesize IL-1 actively in response to endotoxin stimulation but fail to release the protein efficiently. Auron et al. (1984) suggested it is entirely possible that the extracellular appearance of IL-1 from stimulated monocytes is predominantly the result of "cell leakage" rather than active transport.

Initially, this segment of the research project was fraught with difficulties. Several RNA isolation protocols were followed, but each method resulted in degraded RNA. The reason for this degradation of RNA was RNAase as a

contaminant. To overcome this problem, all glassware and surgical instruments were baked at 300^{0}F for 12 h to destroy this enzyme. In addition, the tissue homogenizor was soaked in DEPC-treated water to neutralize RNAase. To block the activity of any RNAase normally present in the tissues themselves, the fractured tibiae were repeatedly washed in DEPC treated water and tissues removed from the fracture site were snap frozen in isopropanol. Snap freezing the tissues inhibited RNAase and preserved the mRNA in the tissues until they could be homogenized in guanidium thiocyanate. Not until undegraded RNA could be obtained was it possible to perform PCR analysis. This was proven when degraded RNA was probed with primers to aFGF using PCR and no detectible signals were produced. As Figure 18 shows, following the RNA extraction procedure described by Chomczynski and Sacchi (1987) and neutralizing the activity of RNAase, produced total RNA of high quality (as indicated by the distinct 28 and 18 S banding).

Figure 18 does not initially convey to the reader the intense activity which is occurring during the stages of fracture repair. All that is depicted is an amplified signal stipulating the presence of mRNA to aFGF. When the histological data on the tissue types present during the four stages of fracture repair, the immunohistochemical distribution of four growth factors during the stages of fracture repair, and the distribution of mRNA to aFGF during the stages of fracture repair are considered together, some striking observations can be made. These are summarized in Table 7.

First, mRNA to aFGF is present at day 1 post-fracture while the aFGF protein itself is not detected immunohistochemically until day 3 post-fracture. Secondly, mRNA to aFGF is present in the tissues in the fracture site while aFGF protein is immunohistochemically detected from day 3 to day 5 post-fracture. Thirdly, mRNA to aFGF is present in tissues in the fracture site long after aFGF protein can be detected immunohistochemically. Finally, not only is mRNA to aFGF present beyond day 5 post-fracture, but its relative concentration increases up to day 10 post-fracture.

The discovery of mRNA to aFGF in tissues of the fracture site before aFGF protein can be detected immunohistochemically is not an unexpected observation. By necessity, mRNA must be present before a protein can be transcribed. Of interest though, is that mRNA is present two days before the aFGF protein and at the beginning, not the end of stage 1 of fracture repair (see Chapter 2). During stage 1, monocytes/macrophages are the main cell type responsible for cleaning up the fracture site and producing PDGF. Perhaps the release of phagocytizing chemicals and/or PDGF from these macrophages acts as an inducer stimulating the production of mRNA to aFGF. Gery et al. (1981) observed that macrophages produce IL-1 mRNA as a result of the stimulating influences of chemicals released by dead and damaged cells. It is possible that the lysis of dead and/or damaged cells as well as the phagocytic enzymes from macrophages act as chemical inducers to stimulate the production of mRNA to aFGF in the cells of the cambial layer of the periosteum.

The presence of mRNA to aFGF while the aFGF protein is detected immunohistochemically is also intriguing. Again, research involving IL-1 offers some interesting insights into the molecular biology of aFGF. aFGF (Jaye et al., 1986) and IL-1 (March et al., 1985) both lack a stretch of hydrophobic amino acids which is a signal common to most secreted proteins. In order to explain the ability of macrophages to secrete IL-1, Auron et al. (1984) suggested that the extracellular appearance of IL-1 from stimulated monocytes was predominantly the result of "cell leakage" rather than active transport. Gery et al. (1981) demonstrated that injury to macrophages as well as chemicals induced macrophages to leak IL-1 extracellularly. Perhaps aFGF is leaked out of the cells of the cambial layer as long as there is chemical stimulation from macrophages as well as mechanical stimulation from movement of tissues by the fractured test animal. As the movement of the bone ends at the fracture site is reduced owing to the formation of a fibrous fracture callus, mechanical stimulation of movement in the fracture site ceases and so does leakage of aFGF. Macrophages have long since disappeared by day 5 post-fracture and any chemical stimulus they may have provided would also have been removed.

Cells might continue to produce mRNA to aFGF beyond day 7 post-fracture but the protein might not be secreted and might also be degraded beyond this point. As stated previously (Imamura et al., 1990), aFGF contains a signal sequence similar to nuclear proteins which allows nuclear proteins to pass through nuclear pores. Perhaps aFGF protein is produced beyond day 5 solely for

autostimulation of the cells.

Cessation in production of mRNA may result from a negative feedback mechanism. Once a certain level of mRNA transcription is reached, perhaps cells block the promotor sequence on the aFGF gene and mRNA production ceases. The aFGF protein has a signal sequence which allows it to pass through the nuclear pore (Imamura et al., 1990). Perhaps aFGF protein leaks out of cambial layer cells during the second stage of fracture repair. When leaking stops, the concentration of aFGF protein within the cell may reach a point at which the protein passes into the nucleus and interferes with its own promoter activity. A negative feedback mechanism is associated with proto-oncogenes. For example, c-fos promoter activity is repressed by fos protein (chap. 1.1.4) (Sassone-Cors et al., 1988).

Another reason for the lack of immunohistochemically detectable aFGF beyond day 5 post-fracture may be conformational changes in the protein. Baird et al. (1987) report that heparin and extracellular matrix have a protective effect on FGF stability. Flanders et al. (1989) using two antibodies to the amino-terminal 30 amino acids of TGF-β_1, were able to detect two types of staining patterns. One antibody stained TGF-β_1 bound to matrix, the other antibody stained TGF-β_1 intracellularly. Perhaps the aFGF protein secreted beyond day 5 is leaked out of cells but cemented into cell matrices in a configuration unrecognizable to the polyclonal antibody I used.

Regardless of when and how long mRNA to aFGF is transcribed by tissues in

the fracture site, the point is that tissues in the fracture site do indeed produce mRNA to aFGF. Therefore, the question asked in the introduction can be answered. Based on molecular biology studies, cells in the fracture site produce mRNA to aFGF and cells of the cambial layer of the periosteum stain positive for the presence of aFGF protein. It can be said, therefore, that aFGF performs an autocrine and/or paracrine function in stimulating fracture repair and does not have to be transported from outside the fracture site for this purpose.

Further studies are needed before actual quantifiable results can be obtained using PCR. A sample from nonfractured tibiae would establish the base line concentration of mRNA to aFGF in cells. A sample at day 20 would substantiate the trend toward decreased expression of aFGF mRNA after day 10 post-fracture. At least three sets of pooled samples would have to be analyzed for each day studied before accurate quantifiable measurements could be obtained.

A cautionary note should be added. Performing PCR over 40 cycles may lead to misleading results if DNA was present in the sample as a contaminant. A control would be treating the RNA sample with DNAase prior to PCR amplification.

Figure 17: Intact 28s and 18s ribosomal bands. Lanes 1-8 represent samples from days 1-8 post-fracture.

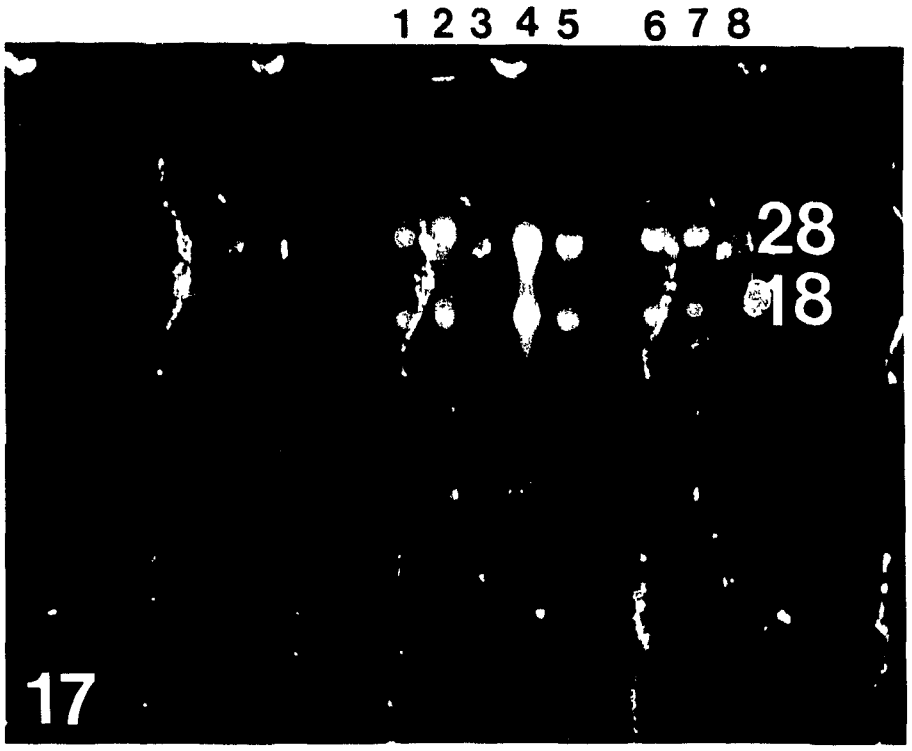

1 2 3 4 5 6 7 8
28
18
17

Figure 18: PCR amplified aFGF products.


603
310
aFGF
actin
18

Table 6: Densitometry readings for aFGF pro ' :cts which have been normalized with respect to actin concentration in samples.

Selection Set:				
No.	afgf	actin		corr
1	122584	222391		0.55120936
2	109626	188999		0.58003481
3	125601	161279		0.77878087
4	146289	218329		0.67003925
5	166630	194086		0.85853694
6	210228	187834		1.11922229
7	35184	73074		0.48148452
8	180717	182743		0.98891339
9	198816	181066		1.09803055
10	235489	163779		1.43784612
14	148596	153595		0.96745337
Normalization Factor:				

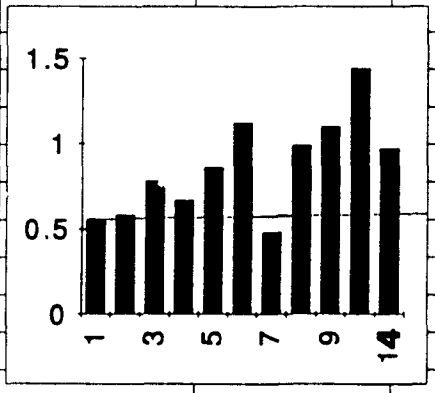


Table 7 Relative distribution of mRNA and protein to aFGF.

Time	mRNA	Protein
Stage 1 Day 0-2 post-fracture	+ ve	- ve
Stage 2 Day 2-5 post-fracture	+ ve	+ ve
Stage 3 Day 5-9 post-fracture	+ ve	- ve
Stage 4 Day 9-21 post-fracture	+ ve	- ve

Chapter 6 Summary and Conclusions

This chapter will summarize the experimental results obtained in this research project. I will also offer my conclusions based on these results.

The literature revealed that several animal models had existed prior to my study. The earlier animal models concentrated on producing a fracture, but little emphasis was placed on the importance of the reproducibility of the fracture model. These earlier models were unable to document easily recognizable histological stages of the repair of the fracture.

A major problem was that animals used were very large and the repair process took months to complete. e.g. rabbits, dogs (Ketenjian and Arsenis, 1975; Schenk and Willenegger, 1967). With my reproducible mouse model, the entire healing process was completed within 21 days. In this time frame, histological examination of the fracture repair process demonstrated the existence of four distinct stages of repair based on tissue types and tissue processes occuring during each stage. The previous animal models took too long to heal, and the distinction between different stages of fracture repair became blurred. With my model I was able to resolve both these problems. To use an analogy, studying the histology of fracture repair using previous animal models was like watching a very long movie on a screen. Using my model, I was able to reduce the length of the movie (healing process) so that tissue interactions were more distinct and recognizable.

There was one exception, however. Joyce et al. (1990b) quantified the fracture repair process into four stages (Chapt. 2.4.). The model devised for my experimental purposes defined the fracture repair process in four distinct stages. The difference between my model and that of Joyce et al. (1990b) is that their model did not include the pivotal importance of the role of the periosteum in fracture repair. Periosteal activity formed a very distinct stage in my model of fracture repair. A correlation was made in my animal model between the different stages, and the duration of each stage. These latter facts have not been documented previously in an animal fracture repair model. Reproducibility was an essential component of my experimental model in order to carefully document the repair process from day 1 post-fracture to day 21. This enabled me to examine tissue types and record the presence and/or absence of certain cell types and to discover the meaning of these changes.

In order to explore the role of growth factors during these stages of fracture repair an appropriate immunohistochemical method had to be devised. Previous existing methods of decalcifying bone and cartilage were detrimental to the preservation of antigenic sites (chap. 4.1.). To solve this problem, I developed a decalcification, tissue processing, embedding procedure, which preserved antigenic sites in calcified tissue. As a result, I was able to document the presence of four growth factors during the stages of fracture repair (PDGF, aFGF, IGF-I and TGF-$ß_1$).

Growth factors are known to be involved in the physiology of bone and

cartilage (chap. 1.2.-1.4.). They are also known to be initiators of cell differentiation and/ or cell replication (chap.1.2.-1.4.). My interest was to locate the growth factors during the repair process. Based on the review of the literature regarding growth factors in bone and cartilage, and the histological review of the fracture repair process, I was able to hypothesize that certain growth factors may be present during certain tissue events post-fracture. PDGF was discovered to be present in macrophages during stage 1 of fracture repair. aFGF tested positively in cambial layer cells during stage 2. During the 3rd stage of fracture repair, IGF-I was detected in chondroblasts, and TGF-$ß_1$ was detected in calcified chondrocytes during stage four of the fracture repair process.

My theory of the activity of these four growth factors in the repair process is as follows. Fracture repair can be visualized as a series of steps or stages similar to a staircase. A growth factor is present at each stage of the repair, and is associated with the major cellular activity occurring at that stage. I believe PDGF is responsible for initiation of cell division and may also act as a chemoattractant for other cells during stage 1. aFGF continues the replication process during stage 2, until the fibroblast-like mesenchymal cells fill the gap and form the first bridge across the fracture gap. IGF-I is present as a stimulator of cartilage matrix production during stage 3. Since cartilage is a temporary tissue in the fracture site, TGF-$ß_1$ is present during the transformation of the tissue from cartilage to bone during stage 4. The stages of fracture repair can be thought of as steps in a stair case. A slinky stepping down the stairs is analagous to the transition of

cellular activity from stage to stage. Each growth factor acts as the force which pushes the slinky off a step and fuels its decent to the next step (much like PDGF initiating cell division and aFGF fueling cell division) A diagram of this "Slinky down a stair case model" is presented as Figure 19. I believe that the growth factors work in succession to ensure the completion of the fracture repair process.

A question remained, "were these growth factors brought in from outside the fracture site, or were they produced in situ?" Using primers to mouse aFGF, I was able to detect the presence of mRNA to aFGF. This in fact, signified that the growth factor aFGF was produced at or in the fracture site. Not only was mRNA to aFGF present during the same time frame as the aFGF protein; it was also present before, during, and after, the protein was detected immunohistochemically. I believe that all four growth factors are produced in the fracture site, but was unable to establish this fact due to a lack of information available on the mouse mRNA sequence to PDGF. IGF-I, and TGF-$ß_1$.

The existence of mRNA to aFGF beyond the presence of aFGF protein, may offer new insights into the understanding of how growth factors control their own expression or self-modulate themselves. I propose that a negative feedback mechanism may explain this observation. The aFGF protein can pass into the nucleus. Since the protein is not secreted from cells but leaks out, high levels of the protein may accumulate within the cell to the point that it accumulates within the nucleus and interferes with the promoter site for its mRNA. This is similar to the negative feedback mechanism used by proto-oncogenes and discussed in

chapter 1.1.4.

I did not examine immunohistochemically for the presence of TGF-$ß_1$ during Stage 1 of fracture repair. Based on the literature reviewed in Chapter 1.2.1-1.2.4, TGF-$ß_1$ is present as an initiator of bone and cartilage formation in skeletal tissue. The activity in and around the fracture site was the frame of reference for studying the stages of fracture repair. Cartilage and bone do not appear in the fracture site until stages 3 and 4. In support of this hypothesis, Andrew et al. (1992) detected mRNA to human TGF-$ß_1$ only during endochondral ossification. PDGF was looked for immunohistochemically during stages 1 and 2 but not later. This was because the literature stated PDGF was a powerful initiator of cell division, primarily in fibroblast-like cells. mRNA to human PDGF has been detected only in macrophages during the early response to fracture repair (J.G.Andrew, pers. comm.)

Clinically, growth factors have enormous potential as enhancers of wound repair. They could be delivered to a wound site by injection or implanted as a diffusible component from a biodegradable matrix. However, I have only characterized the activity of four growth factors in fracture repair. Other bone inducing proteins, e.g. bone morphogenetic protein, are involved in bone physiology. I believe we should understand as many components of bone physiology as possible before clinically intervening with growth factors in cases such as non-unions. Failure to understand the many components of bone physiology may lead to disappointing and poorly understood clinical results.

Summary

1. A fracture repair animal model has been developed which is reproducible and quantifiable. The fracture produced is closed and transverse, with initial minimal displacement between bone ends.

2. The fracture repair process can be divided into four stages. The duration of these stages is consistently reproducible.

3. Stage 1 was termed the "inflammatory response stage". This stage occurred immediately post-fracture, and lasted until day 2 post-fracture. Histologically, this stage was marked by the formation of a haematoma, influx of polymorphonuclear leukocytes and monocytes into the fracture site, and the transformation of many monocytes into macrophages.

4. Stage 2 was termed the " periosteal-fibrous callus stage". This stage was present from days 2-5 post-fracture. In the beginning, cellular activity was observed in the cambial layer of the periosteum. At the end of this stage the cambial layer had increased in size and cell number, and at the same time, a large number of fibroblast-like mesenchymal cells appeared and bridged the fracture gap.

5. Stage 3, the "chondrogenic stage" was present from days 5 to 9 post-fracture. During this stage, the fibrous callus, composed of fibroblast-like mesenchymal cells was transformed into cartilage.

6. Stage 4, the "endochondral stage" was present from days 9 to 21 post-fracture. The cartilage formed during the chondrogenic stage was replaced by new bone trabeculae through the process of endochondral ossification.

7. A new tissue processing method was devised which preserves tissue architecture and antigenic sites on growth factors during decalcification of skeletal tissues (bone, cartilage).

8. Using this new tissue processing technique, the growth factors, PDGF, aFGF, IGF-I and TGF-β_1, were immunohistochemically detected in tissues during fracture repair.

9. PDGF was visualized in macrophages toward the end of stage 1 of fracture repair. These PDGF-positive macrophages occur at the periphery of the fracture site and in close proximity to the periosteum.

10. aFGF was visualized in cells of the expanding cambial layer during stage 2 of fracture repair. The increase in the number of aFGF positive cambial layer cells coinicided with the accumulation and rapid increase in the population of fibroblast-like mesenchymal cells.

11. IGF-I was visualized in matrix forming chondroblasts during stage 3 of the fracture repair process. These young chondroblasts occur at the periphery of the cartilage mass.

12. TGF-$ß_1$ was visualized in calcified matrix producing chondrocytes during endochondral ossification. These TGF-$ß_1$ positive chondrocytes are present at the edge of the ossification front where the transformation from cartilage matrix to bone matrix was occurring.

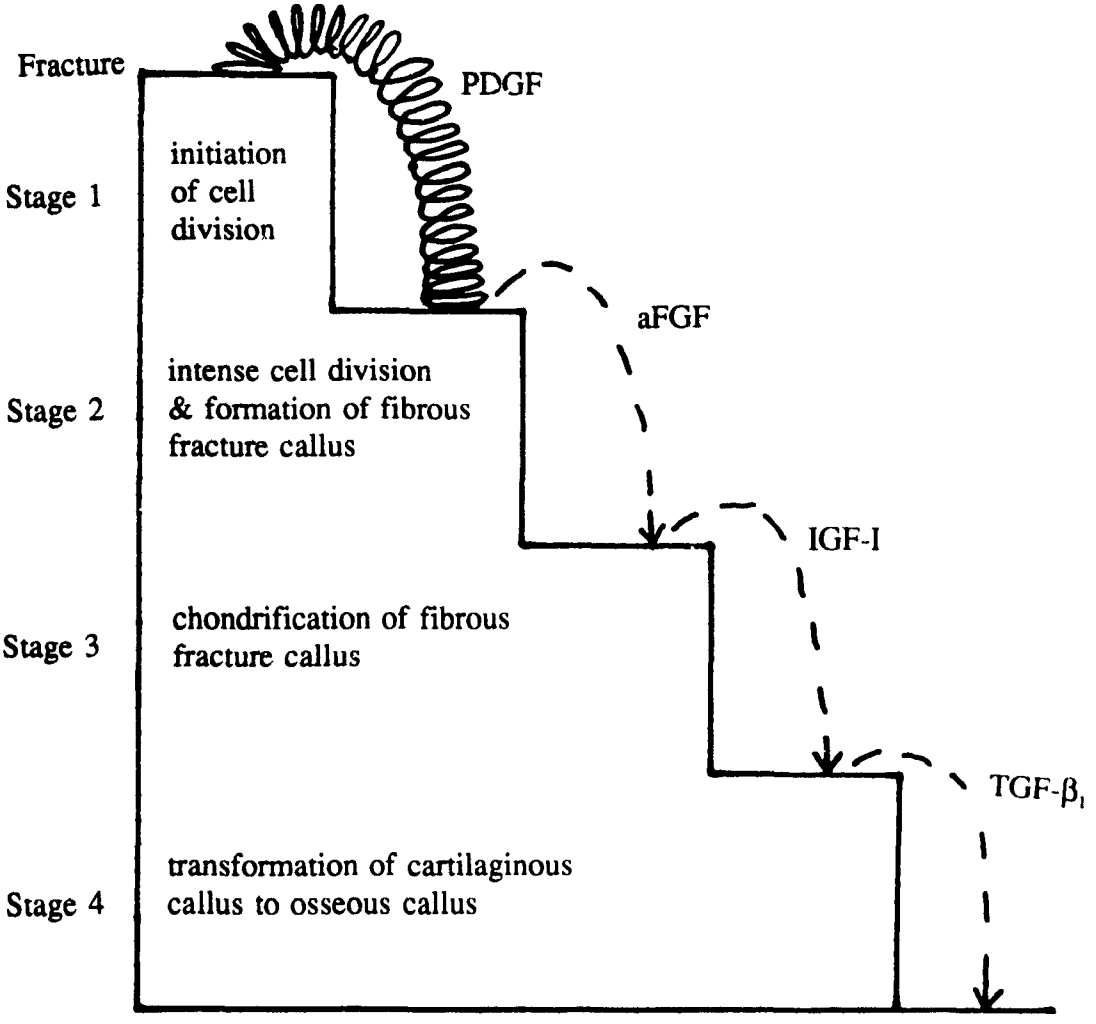
13. The presence of mRNA to aFGF was successfully demonstrated in tissues harvested from the fracture site during the stages of fracture repair. This proved the hypothesis that aFGF is produced by cells <u>in situ</u> during the repair process.

14. mRNA to aFGF was detected from day 1 to day 14 post-fracture. The aFGF protein was detected only from day 3 to day 5 post-fracture. A theory involving the negative-feedback control of mRNA to aFGF expression was proposed. aFGF protein may interfere with the promoter site for aFGF mRNA.

15. I propose a model termed the "Slinky down a stair case" to explain the relationship between the cellular activities occurring during each stage of fracture repair, and the presence of a growth factor during each stage.

Figure 19: Slinky down a stair case model.

19


Fracture
PDGF
initiation of cell division
Stage 1
aFGF
intense cell division & formation of fibrous fracture callus
Stage 2
IGF-I
chondrification of fibrous fracture callus
Stage 3
TGF-β1
transformation of cartilaginous callus to osseous callus
Stage 4

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