

The Effect of Changes in Oxygen Tension During Fracture Repair on Mesenchymal Stem Cell and Bone Activities

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Abstract: Fracture healing is a complex physiological process that results in regenerative of bone. This study looks in greater detail into the effect of oxygen tension, one of the multiple parameters that successful fracture healing depends on. A summary of the biology of bone and bone repair is described before delving into the effect of oxygen tension on fracture healing. The preponderance of evidence appears to suggest that hypoxia taken in isolation promotes the up regulation of several signalling molecules involved in the fracture repair cascade in *In vitro* studies by many cells especially osteoblasts, however there is some suggestion to the contrary. The influence of oxygen tension on fracture healing will continue to be investigated especially how HIF-1 affects the various cells responsible for this regenerative process.

Key words: Hypoxia, fracture, healing, bone, cell signalling, mesenchymal stem cells

INTRODUCTION

Fracture healing is a complex physiological process. It involves several processes acting in a coordinated manner including the recruitment of cells from the bone marrow, surrounding tissues and circulation. These cells include many haematopoietic cells and mesenchymal stem cells. This is basically a recapitulation of the molecular mechanisms that regulate skeletal tissue formation during embryological development and makes bone healing and fracture repair unique as a regenerative process than repair.

Successful regeneration and healing of fractures depend on multiple parameters present locally at the injured tissue and systemically including growth factors, hormones, nutrients, pH, oxygen tension, the electrical environment and the mechanical stability obtained at the site. This study will look in greater detail into the effect of one of these parameters namely oxygen tension on bone healing however, as stated earlier, fracture healing is a well orchestrated process and each factor is vital in optimal skeletal repair resulting in restoration of skeletal function.

Biology of Bone and Bone Healing: The skeleton is made up of over 200 individual bones which contribute to providing support and locomotion for the body and has metabolic functions mainly in calcium metabolism. Each bone is made of the cells and the matrix with its neurovascular supply. The matrix accounts for over 90%

of the volume of the tissue with the remainder made up of cells and blood vessels (Buckwalter *et al.*, 1995). The cells include osteocytes which are the most numerous and are surrounded by the matrix, and osteoblasts that line the surface of the bones laying down the osteoid both of which are derived from mesenchymal stem cells; and the large multinucleated osteoclasts derived from haematopoietic stem cell precursors needed for the breakdown of bone.

The bone receives a significant amount of the cardiac output which is not surprising considering its huge metabolic functions for the body. However different parts of the bone and indeed different bones receive varying amounts of this cardiac output ranging from 5 mL/min/100g in cortical bone and periosteum to 20 mL/min/100g in cancellous bone (McCarthy, 2006). This circulation is affected in fractures which leads to vascular damage the degree of which will depend on the degree of injury to that bone. Various attempts at repairing the fracture such as internal and external fixation also leads to vascular damage and circulatory compromise.

Bone fracture healing is a remarkable process which leads to regeneration of the anatomy and complete return to function unlike soft tissue healing which leads to scar tissue formation. The various bone cells mentioned above are responsible for this regenerative process. Bone fractures heal by either primary (direct) or by secondary (indirect or spontaneous) methods. Primary healing occurs by gap or contact healing and would usually not occur in an anaerobic environment. Secondary healing occurs in

three distinct phases, namely inflammatory, reparative and remodelling phases. In all these vascular invasion is essential for the formation of bone.

As in many repair or regenerative processes in the body, fracture healing starts with the induction of an immune response. A haematoma is formed leading to an inflammatory process being set in motion. This is brought about by cytokines, platelets and bone morphogenetic proteins (BMP). These have a chemotactic effect on other inflammatory cells leading to the recruitment of mesenchymal stem cells. The MSCs proliferate and differentiate under the influence of several other factors into a chondrogenic and osteogenic lineage, which in the presence of ongoing angiogenesis leads to development and consolidation of the callus needed for bone regeneration. Without angiogenesis, osteogenesis would not occur.

Molecular Aspects of Fracture Healing: The signalling molecules fall into three categories namely the pro-inflammatory cytokines, the growth factors and the angiogenic factors. Interleukins 1 and 6 (IL-1, IL-6) and tumour necrosis factor-alpha (TNF-alpha) secreted by inflammatory cells have a chemotactic effect on other inflammatory cells and on the recruitment of MSCs, usually in the first three days after the fracture (Dimitriou *et al.*, 2005). At about the same time platelets activated by thrombin and subendothelial collagen release platelet derived growth factors (PDGF) and transforming growth factor-beta (TGF-beta). These induce mesenchymal cell migration, activation and proliferation, angiogenesis and further aggregation of platelets.

The recruited MSCs proliferate and differentiate into chondroblasts. Proliferation of these into new chondrocytes occurs from day 7 to 21 resulting in the formation of soft callus. Simultaneously BMPs released from the bone matrix and also expressed by MSCs push for the differentiation down chondrogenic and osteogenic lineages. Vascular ingrowth into the developing callus is regulated by fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF) and angiopoietin 1 and 2. The calcifying hypertrophic chondrocytes are removed by chondroclasts as the vasculature begins to invade leading to its replacement by woven bone which undergoes significant remodelling to become weight bearing bone.

MATERIALS AND METHODS

Past study was performed looking at studies that have investigated the effect of oxygen tension on fracture healing and the regulation of signalling molecules involved in the fracture repair cascade including HIF-1.

RESULTS

The Role of Oxygen Tension: Numerous studies have shown the significant role of oxygen tension in fracture repair. Oxygen tension in arterial blood is about 95

mmHg and 40 mmHg in venous and capillary blood. In normal tissues the median interstitial oxygen tension values range from 24 to 66 mmHg. Thus, the cellular oxygen tension at which most physiologic activity is conducted is a narrow range which is tightly controlled as any slight shift in either direction may be disastrous. All cells functioning in this environment are therefore influenced by changes in oxygen tension. Hypoxia can cause a failure to generate the required amounts of ATP to power cellular functions while hyperoxia results in the generation of dangerous reactive oxygen intermediates causing suppression of cellular proliferation (Tuncay *et al.*, 1994).

Since, the regulation of oxygen tension is of such importance to the body it is therefore not surprising that most oxygen breathing species express the highly conserved transcriptional complex hypoxia inducible transcription factor (HIF). This heterodimer composed of alpha and beta subunits controls oxygen-sensitive gene expression. HIF signalling cascade mediates the effects of hypoxia on the cell (Jiang *et al.*, 1996). In turn the degradation of HIF is performed by a family of HIF prolyl-hydroxylases in an oxygen-dependent reaction requiring 2-oxoglutarate and iron as a cofactor.

In normoxic or hyperoxic conditions HIF is quickly degraded. However, in hypoxic conditions HIF alpha is stabilised as dimerises with HIF beta and binds hypoxia response elements (HRE) in target gene promoter sequences leading to the up regulation of several genes including VEGF, insulin-like growth factor-2 (IGF-2), TGF-beta 1 among many and possibly the down regulation of tissue inhibitors of matrix metalloproteinase-1 (Wan *et al.*, 2008; Wang *et al.*, 2004).

Vascular disruption secondary to fracture creates a hypoxic zone of injury where the oxygen tension at the center of the wound is very low (Heppenstall *et al.*, 1976; Kofoed *et al.*, 1985). This hypoxia would normally keep cells from differentiating. For the fracture to heal as described above there needs to be significant cell signalling to regulate the complex process of fracture healing. HIF pathway has been shown to be the central regulator of adaptive responses to the low oxygen availability and is activated during bone repair.

Hypoxia as a Promoter of Fracture Healing: Insulin-like growth factor is released from the bone matrix and produced by endothelial cells, osteoblasts and chondrocytes. IGF-I (or somatomedin-C) promotes bone matrix formation by production of type I collagen and non-collagenous matrix proteins while IGF-II (skeletal growth factor) acts at a later stage to stimulate production of type I collagen and cellular proliferation during endochondral bone formation. Steinbrech *et al.* (2000) have shown that in hypoxic conditions there was a 60% increase in IGF-II messenger RNA expression which continues to increase for over 48 h by the osteoblast. However, IGF-I showed no increase suggesting that the differential expression of these growth factors may underscore important differences in behaviour of osteoblasts in the hypoxic fracture environment.

The transforming growth factor superfamily is a large family of growth and differentiation factors including BMP, TGF-beta, growth differentiation factor (GDF) and many others. Many members of this family promote the various stages on intramembranous and endochondral bone ossification during fracture healing. TGF-beta in particular is produced by degranulating platelets, inflammatory cells, endothelium and osteoblasts, and is a potent mitogenic and chemotactic factor for bone forming cells. It is expressed from very early stages of fracture and on throughout healing. Hypoxia has again been shown to cause a marked elevation of TGF-beta 1 gene expression (Saadeh *et al.*, 1999; Warren *et al.*, 2001). Warren *et al.* (2001) also demonstrated a striking decrease in the expression of TIMP-1 in response to hypoxia suggesting that extracellular matrix turnover which helps in fracture repair and release of stored growth factors is enhanced. They therefore suggest that hypoxia can affect osseous healing by altering the expression of cytokines, bone specific extracellular matrix molecules and their regulators.

Several studies have demonstrated the critical role of angiogenesis for successful osteogenesis during endochondral ossification and fracture healing. It has been suggested that without angiogenesis there can be no osteogenesis. Angiogenesis is believed to be regulated by one of two pathways. The VEGF dependent and Angiopoietin-dependent pathways are thought to be functional during fracture repair. Many studies have shown that VEGF is up-regulated during membranous fracture healing, in many other tissues in response to hypoxia and in osteoblasts in response to hypoxia (Mayer *et al.*, 2005; Richard *et al.*, 1999). Steinbrech *et al.* (1999) showed that hypoxia regulates the expression of VEGF in osteoblast like cells and increased alkaline phosphatase suggesting that osteoblasts through the expression of VEGF may be responsible for angiogenesis in fracture healing. Wan *et al.* (2008) have suggested that this is achieved through the HIF-1 pathway. Hypoxia has been shown to not only influence VEGF but both in turn up regulate BMP thereby directly influencing osteogenesis (Bouletreau *et al.*, 2002).

Hypoxia as a Deterrent to Fracture Repair: Utting *et al.* (2006) have demonstrated that osteoblast proliferation was significantly decreased when studied in a hypoxic environment compared to a normoxic one. They also showed that there was decreased alkaline phosphatase activity in hypoxic osteoblasts during the mineralising phase of culture. Most importantly they suggest that all these and other findings lead to prevention of production of mineralised matrix by disrupting collagen formation and alkaline phosphatase activity.

In another study they had demonstrated that hypoxia actually had a powerful stimulatory effect on osteoclast formation hence on bone resorption (Arnett *et al.*, 2003). This study was interesting because it showed that though hypoxia caused reduction in the number of osteoclasts the

osteolytic effect was equivalent to the maximum stimulation observed in bones. So the reduction in osteoclasts numbers which might have promoted fracture healing in the way that bisphosphonates act is countered by increased activity. They also suggest that hypoxia consistently reduced the pH and that this effect also promotes osteoclast activity.

DISCUSSION

The expression of HIF and the hypoxic induction of signalling molecules which promote fracture repair would suggest that low oxygen tension promoted fracture healing. However the work of Arnett *et al.* is contradictory and would at first glance be more logical thereby raising doubts about the hypoxia theory. They however do not explain sufficiently the influence and need for the HIF. The recent development of therapeutic agents which act as selective HIF prolyl-hydroxylase inhibitors to treat some forms of anaemia with a possible influence on fracture healing may add to the evidence in support of the hypoxia theory (Bruegge *et al.*, 2007; Yen *et al.*, 2005). Two probable reasons are suggested below to explain these apparent differences.

Firstly, this highlights one of the challenges of *In vitro* studies which try to mimic in-vivo environment but are not quite the same. It is quite plausible that the same gene and signalling molecule may act differently in different environments hence what is observed in the hypoxic model *Ex vivo* may be totally at odds to what happens *In vivo*.

Secondly and slightly different from the case above is the *In vitro* practice of testing for effects of genes and signalling molecules in isolation without consideration the effects of other genes and cytokines acting simultaneously in the *In vivo* environment. Taking oxygen tension for instance, how does the apparent hypoxic increase in IGF influence the regulation of TGF's and VEGF and vice versa? This strengthens the arguments of Arnett *et al.* (2003), Utting *et al.* (2006) whose testing appears to investigate several molecules at the same time. As stated in the introduction several factors influence fracture repair and not necessarily oxygen tension alone. It would be interesting to determine what influence pH has on oxygen tension, or other factors such as hormones, electrical and mechanical activity for that matter *In vivo*.

Oxygen tension is known to have an effect in fracture healing especially as there is evidence that there is a hypoxic gradient at the fracture site. The preponderance of evidence appears to suggest that hypoxia taken in isolation promotes the up regulation of several signalling molecules involved in the fracture repair cascade in in-vitro studies by many cells especially osteoblasts, however there is some suggestion to the contrary. The influence of oxygen tension on fracture healing will continue to be investigated especially how HIF-1 affects the various cells responsible for this regenerative process.

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