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Expression of Bone Morphogenic Proteins in Rats with and without Brain Injury and a Tibia Fracture

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Abstract: Patients sustaining severe head injury and fractures of long bones or large joints often show enhanced osteogenesis, with hypertrophic callus formation and/or heterotopic ossification. Early consolidation of fractures in these patients may be a result of the enhanced bone formation. However, extensive periarticular calcification may result in complete ankylosis of the affected joint. In spite of numerous efforts aimed at clarifying the way in which severe head injury can influence osteogenesis at a distant site, this phenomenon is still not understood. Prophylactic indomethacin and low dose radiation are two independent agents that help inhibit heterotopic ossification. The major prerequisite for testing therapeutic intervention is to create an animal model of upregulated bone formation and to define parameters of an early diagnosis of enhanced osteogenesis. One possible link between brain injury and bone formation is through the bone morphogenic proteins (BMP's). Thus, the aim of this study was to test this phenomenon at the histologic and molecular level by using a well-established head injury model in rats. The lateral fluid percussion model is the most widely used and characterized method of inducing brain injury in rats. Rats were subjected to severe experimental lateral fluid percussion (FP) injury (3.0–3.6 atm) as described by McIntosh et al. [18]. We utilized a reliable tibia fracture model described by Bourque et al. [2] and Otto et al. [23] for rats. One group (n = 5) of rats TBI, another group (n = 5) of rats was exposed to TBI and an experimental fracture, the third group was only exposed to experimental fracture (n = 5) and sham animals (n = 5) were subjected to anesthesia without injury. Muscle specimens from around the hip as well as the entire area of the fractured tibia were taken and processed. Rt-PCR showed an upregulation of BMP 2 and 4 (2/4) in muscle around the hip of all brain injured animals and also in the soft tissue around the tibia. BMP 2/4 was upregulated in the fractured tibia and the surrounding soft tissue in all animals with a fractured tibia. BMP 2/4 was not upregulated in muscle tissue around the hip in these animals (fractured tibia group). Our study revealed differences at the tissue level in animals with head injury with or without tibia fracture when compared to their controls. There was differential expression of several genes including BMP 2/4 that was activated by head injury, perhaps by a circulating factor or direct nerve signaling pathway. This might be a possible mechanism by which head injury induces ectopic ossification.

Introduction

There are several clinical phenomena that present with an enhancement of bone formation. Clinical presentation of upregulated bone formation, like heterotopic ossification (HO), is either acquired, which occur with incidents such as brain injury, spinal cord injury, blunt trauma, burns, infection, neurologic disease and postsurgical complications, or inherited, such as fibrodysplasia ossificans progressiva (FOP) and progressive osseous heteroplasia. There is a well-established relationship between brain injury and HO. Patients with brain injury are known to have an increased tendency to form ectopic bone and are given corticosteroids, nonsteroidal anti-inflammatory drugs, disphosphonates, and radiotherapy as a preventative measure. Depending on the method used for diagnosis and length of follow-up, there is a reported 10–86% incidence of HO associated with brain injury [6]. These patients also present with an accelerated rate of fracture healing. In clinical studies of patients with combined fractures and brain injury, Spencer (1987) and Perkins and Skirving (1987) both found a significant increase in the volume of bone growth at the callus site, as well as a significant decrease in the time to union of the fractured bone [25,28]. This phenomenon has also been observed in brain injured patients with soft tissue trauma: they have an increased rate of ectopic bone growth at the injured sites.

Urist made the key discovery that demineralized bone fragments implanted either subcutaneously or intramuscularly in animals induce bone formation [31]. The search for the factors responsible for this effect has resulted in the identification of a family of bone morphogenetic proteins (BMP's). Bone morphogenic protein 4 (BMP-4) has been proven to induce ectopic cartilage or bone formation when implanted into extraskeletal sites (subcutaneous or intramuscularly) [9,10,29,31,32,34]. Bone morphogenic proteins are members of the transforming growth factor beta (TGF β) superfamily, as classified on the basis of similarities in their amino acid sequence, and they regulate the growth and differentiation of a variety of cell types in diverse tissues [4,27]. BMPs have been shown to be important in the development of the skin, heart, gonads, kidneys, eye, brain, and liver, although their ability to induce in vivo chondro-osteogenesis has been the focus of the majority of BMP clinical research efforts [4,12,27,38]. However, in the CNS, BMP ligands and their receptor subunits are expressed throughout development and are upregulated in the brain after traumatic brain injury (TBI). Among other functions they help regulate cellular proliferation, survival, differentiation, apoptosis, and lineage commitment [5,37]

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Creation of an animal model that mimics human clinical scenarios is necessary for investigators to understand the biochemical mechanism(s) underlying these phenomena. Our goal was to create an animal model of unregulated bone formation and investigate the role of potential chemical mediators known to be involved in the regulation of bone formation.

Materials and Methods

Brain injury and fracture

Adult male Sprague-Dawley rats (n = 16) weighting 350–450 g were initially anesthetized with sodium pentobarbital (nembutal, 65 mg/kg, i.p.) prior to surgical preparation. At all times, we strictly adhered to the principles outlined in the guide for the care and use of laboratory animals, prepared by the Committee on Care and Use of Laboratory Animals from the Institute of Laboratory Resources, National Research Council (1995).

Once anesthetized, all animals were placed in a sterotaxic frame. Ninety minutes following administration of anesthesia, animals were subjected to fluid-percussion brain injury, while still fully anesthetized. The scalp and left temporal muscle were reflected. A 5.0 mm hollow female Luer-Lok fitting was rigidly fixed with dental cement to the skull of all animals, fitting exactly into a craniotomy site centered over the left parietal cortex, midway between lambda and bregma and 4 mm lateral to the sagittal suture. The dura was left intact at its opening. A well characterized experimental model of parasagital (lateral) fluid percussion (FP) brain injury was utilized in the anesthezied rat. This model yields a predictable degree of brain injury and simulates many aspects of brain injury in humans, including increases in intracranial pressure (ICP), changes in cerebrovascular reactivity, regional hypoperfusion and cognitive dysfunction. Experimental brain injury was induced using an FP injury device consisting of a plexiglas, cylindrical, saline-filled reservoir bound at one end by a plexiglas plunger mounted on O-rings. The opposite end of the cylinder was capped with a male Luer stub, which was connected to the animals via the female Luer-Lok fitting. A pendulum was dropped, striking the plunger and producing a pulse of increased intracranial pressure (2–2.5 atm ICP) of 21–23 milliseconds (ms) duration via the rapid injection of saline into the closed cranial cavity. The FP device uses hydraulically-induced pressure changes to produce quantifiable mechanical deformation of the brain. This results in brief displacement and deformation of neural tissue. This pressure pulse is measured extracranially by a transducer (Gould, Inc.) housed in the injury device and is recorded on a computer monitor.

After brain injury, the Luer-Lok fitting was removed from the skull and the skin was sutured closed. A reproducible tibia fracture in rats is produced using a well established apparatus (Bonnarens et al., 1995). This three-bending-point apparatus allows the examination the effects of fracture repair, influenced by brain injury. The leg of the rat was placed across the fulcrum of the apparatus and a lever was brought down to produce the fracture. A rat will normally use the injured leg within 24 hours and show no signs of pain when using the injured leg.

Messenger Ribonucleic Acid (mRNA) extraction and reverse transcriptase polymerase chain reaction (RT-PCR)

Messenger ribonucleic acid (mRNA) was extracted from the bone and the surrounding soft tissue as well as from hip muscles using the Micro-FastTrack mRNA isolation kit (Invitrogen Corp., San Diego, CA) according to the manufacturer's specifications. As a positive control, mRNA was also extracted from a rat osteosarcoma (Dunn osteosarcoma) in the same way. The mRNA from each sample (10 nanograms) was reversed transcribed in a 20 microliter (μl) reaction mixture containing 400 units (U) Molony murine-leukemia virus reverse transcriptase and 50 pmol antisense primer for mBMP 2/4. Thereafter, 1 μl of each reaction product was amplified in 25 μl polymerase chain reaction (PCR) mixture containing 0.125 U Taq DNA polymerase and 12.5 pmol each of primers (sense and antisense) to detect mBMP 4 specific RNA. The PCR protocol of 30 cycles at 94°C for 30 s, at the annealing temperature for 30 s, and at 72°C for 1 min followed by extension at 72°C for 5 min. The PCR products were subjected to 1.5% agarose gel electrophoresis.

The following primers specific for BMP2 and 4 were used:

BMP 4 (3′): GCTGAAGTCCACATAGAGCGAGTG
BMP 4 (5′): AACTGGTCCACCACAATGTGACACG
(product size 346 bp, restriction site Alu I, restriction products 153+193)
BMP 2 (3′): GCTGTACTAGCGACACCCAC
BMP 2 (5′): TCATAAAACCTGCAACAGCCAACTCG
(product size 671 bp. Restriction side TaqI, restriction products 24+55+89)

Northern blot was performed from one sample of each lesion in which sufficient RNA for analysis (at least 15 μg) was available. Equal amounts (15 μg) of electrophoresed, total, RNA was transferred to a nytran membrane. Hybridization was carried out using end labeled digoxigenin oligonucleotide probes, antisense to BMP-4. The membranes were washed after hybridization and probes were detected using a commercially available chemiluminescent kit (Boehringer-Mannheim) and autoradiography. The membranes were the stripped and hybridized to guanine-adenine phosphate dehydrogenase (GAPDH) as a control.

Eight rats were head-injured using the FP. Four had head injury and a mid-tibial fracture. Eight animals underwent fracture but no head injury. Four animals were controls (neither head-injury nor fracture). The animals were sacrificed at 2 days. Bone and soft-tissue from the injured tibia region and specimens taken from hip muscles were snap-frozen.

Results

There was no BMP 2/4 expression detectable in all naïve animals. After fracture analysis by RT-PCR, BMP 2/4 ex-

pression was detected in animals with and without brain injury in the broken tibia and surrounding soft tissue at fracture side (Fig. 1). After the RT-PCR products were electrophoresed in an agarose gel and stained with ethidium bromide, there was a strong band for BMP 2/4 in all brain injured rats (with and without a tibia fracture) not only in the tibia and the surrounding soft tissue, but also in the muscles around the hip.

Northern blot showed that BMP-4 was expressed at a higher level in animals with brain injury and a tibia fracture than in animals with only a tibia fracture without brain injury. There was no upregulation of BMP 2/4 in the muscles about the hip in the sham animal group or in animals with only a fracture (Fig. 2).

In summary, all brain injured rats showed an upregulation of BMP 2/4 in the tibia and the soft tissue around the hip, whether or not ther was a fracture present. However, the animals which have only been exposed to an experimental fracture showed only an upregulation of BMP 2/4 in the broken tibia and the surrounding soft tissue at fracture site but not in muscles around the hip.

Discussion

In the United States an estimated 1.5 to 2 million people incur traumatic brain injury (TBI) each year. There is a well-established clinical relationship between brain injury and heterotopic ossification (HO). Depending on the method used for diagnosis and length of follow-up, there is a reported 10–86% incidence of HO associated with brain injury [6,25,28]. These patients also demonstrate accelerated rate of fracture healing [1,25,28]. BMP-2 and BMP-4 have been shown to have potent ectopic and orthotopic bone-induction activities [9,10,29,31,32,34], while individual BMPs may have differences in dose response for exhibiting bone inducing activities. During development, members of the BMP family of proteins have been shown to induce mesenchymal migration, proliferation and differentiation, leading to cartilage and bone formation [22]. The bone-inducing ability of the mBMP-4 in systems in vivo was confirmed by implanting the gene product with pure collagen carrier into the muscle of rats and mice [3]. Their actions at the cellular level include inducing chemotaxis in monocytes and differentiation of mesenchymal cells into chondroblasts or osteoblasts. More specifically BMPs commit mesenchymal progenitor cells to differentiate into cells of chondroblast and osteoblast lineage, promote the expression of markers that are characteristic of the chondroblast and osteoblast phenotype, and enhance the synthesis of the extracellular matrix. The precise role of individual BMPs during cartilage and bone formation still remains unclear.

BMP ligands and receptor subunits are present throughout neural development within discrete regions of the embryonic brain and within neural crest-derived pre- and postmigratory zones. BMPs initially inhibit the formation of neuroectoderm during gastrulation while, within the neural tube, they act as a gradient to promote the differentiation of dorsal and intermediate cell types throughout co-operative signaling [19]. In the peripheral nervous system, BMPs act as instructive signals for neuronal lineage commitment and promote graded states of neuronal differentiation. These observations suggest that the BMPs exhibit a broad range of cellular and context-specific effects during multiple stages of neural development.

BMPs and their receptors have also been proven to be upregulated in the brain after traumatic brain injury [14]. Investigators hypothesized that the BMPs and their receptors are involved in neuronal plasticity that occurs after TBI.

In the present study, expression of the genes encoding BMP 2/4 was studied in animals with brain injury, animals with brain injury *and* a tibia fracture, animals with a tibia fracture alone and naïve animals.

The present results provide us with two new findings. BMP 2/4 is upregulated in all brain injured animals in the bone and soft tissue (muscle). As seen in our study, 48 hours postinjury BMP 2/4 was expressed in the bone and surrounding soft tissue in all animals with brain injury and those with a tibia fracture alone. Furthermore, the brain injured animals showed an upregulation of BMP 2/4 in muscle specimen around the hip.

In terms of the fracture group without brain injury our data are correspondent to the results of Nakase and Yaoita [21,36]. Both found mRNA of BMP 2/4 upregulated at the fracture site 48 hours postinjury after rats and mice had been exposed to an experimental femur fracture. They did not look at remote sites such as the hip muscles as we did in our study.

It remains unclear if the response of BMP 2/4 to trau-

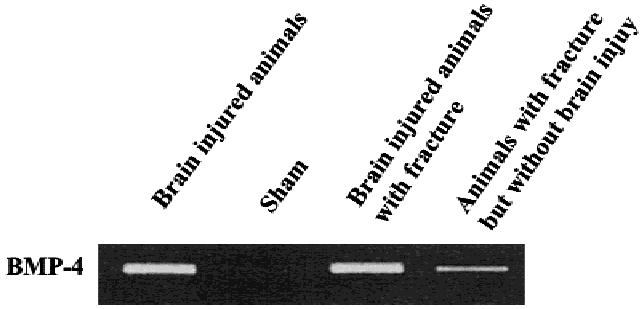

Fig. 1. BMP-4 is upregulated in bone and the surrounding soft tissue in all brain injured animals and all animals with a fracture (same results for BMP-2). There is no BMP2/4 overexpression in shams.

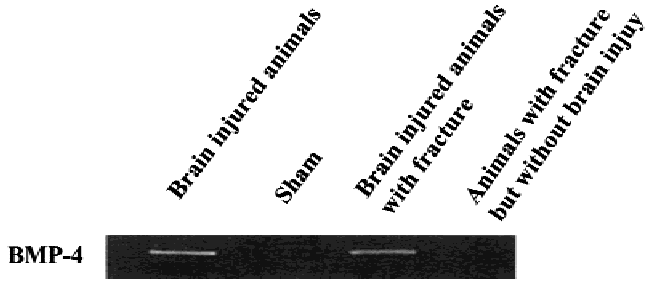

Fig. 2. BMP-4 is also overexpressed in the muscles around the hip in all brain injured animals and those with brain injury and fracture but not in shams or animals with a fracture alone (same results for BMP-2).

matic brain injury is mediated by a circulating factor or direct nerve signaling pathway. Histopathological studies of pre-osseous lesions in FOP patients reveal a pattern of lymphocytic infiltration and muscle-cell degeneration followed by the appearance of highly vascular, fibroproliferative tissue and then endochondral ossification with mature lamellar bone and marrow elements [13]. These same patients were found to overexpress BMP-4 in their lymphocytes. The working hypothesis resulting from these studies is that FOP patients sustain a muscular injury, creating a muscular vascular bed, which attracts and accumulates inflammatory cells, which in turn transport and deposit their load of BMP-4. This is proposed to lead to the differentiation of mesenchymal cells into osteoblasts, forming ectopic bone. BMP-4 has more recently been shown to be excluded from the developing central nervous system by specific binding proteins [19]. If these proteins are unable to bind BMP-4, then ectopic bone is formed in that region of the developing brain. Therefore TBI may lead to an upregulation of bone growth via BMP or similar chemical mediators, directly or indirectly, released from the brain. In support of this, Groot et al. (1994) recently demonstrated the differentiation of fetal mouse chondrocytes into functional osteoid producing osteoblasts when co-cultured with brain tissue [7]. The central nervous system is endowed with cytokines, in addition to BMPs, which have a functional and embryological role [19]. Nervous tissue, for example, produces a great number of neuropeptides and neurotransmitters, which are known to affect osteoblast metabolism, possibly through receptors on osteoblastic cell lines [17]. These mediators would also likely play a critical role in the internal homeostatic switch relating to bone formation. Several studies have addressed the issue of released mediators, in addition to BMP, in brain-injured patients in relation to increased bone growth [1,26]. Trauma to the CNS may increase the release of, or decrease uptake of, bone formation mediators that can enter the systemic circulation. Alternatively, other chemicals may be released from the brain which act to stimulate local production of BMP or other mediators. Both scenarios would result in altered bone formation.

To our knowledge this is the first study showing an upregulation of genes in bone and soft tissue which are closely related to enhanced bone formation in brain injured animals.

Further studies have to be conducted to characterize the interaction of TBI and bone formation; the results of such studies will aid understanding of the functional roles of growth factors as part of a systemic response to TBI in various morphogenic processes during bone formation.

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