BASIC SCIENCES

UPREGULATON OF NEUROTRANSMITTER RECEPTORS – A POSSIBLE MECHANISM FOR ACCELERATED FRACTURE HEALING.

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ABSTRACT

Excessive callus forms at a fracture site when there is an associated head injury. Underlying mechanism for this is unknown. We hypothesize that this exaggerated response is through an up regulation of neurotransmitter receptors in callus. Patients presenting with head injury and a long bone fracture are chosen in study group whereas those with only a long bone fracture taken as controls. Callus from fracture site is aspirated under local anesthesia starting from one week and repeated weekly till four weeks. Obtained cells are transported in a holding media (RPMI) to Dept of biotechnology CUSAT for real time PCR study.

Total RNA separated from callus is analyzed for various gene expressions. Here we used primers for GABA A, GABA B and 5HT receptors. Effect of head injury on callus cells was assessed using radiothymidine incorporation tests.

Patterns of neurotransmitter receptor expression in study and control subjects are analyzed and correlated with proliferative response. Our results showed that there is a demonstrable change in receptor expression for GABA-B in head injured group.

BACKGROUND

It has been noticed that excessive callus forms at a fracture site when the victim has also sustained a head injury. However the underlying biologic mechanism responsible for this excessive osteogenic response has remained obscure.

The callus formed at a fracture site is composed of an initial fibrin scaffold formed from fracture hematoma. This is then invaded by pleuripotent mesenchymal cells, which mark the beginning of repair phase. These mesenchymal cells are derived from cambium layer of periosteum, bone marrow , injured endothelium, surrounding muscles and other injured tissues. The callus also contains an array of specialized cells like osteoblasts, chondroblasts, fibroblasts etc —cells derived by differentiation of the aforementioned pleuripotent cells.

What triggers these normally dormant mesenchymal cells into rapid proliferation and subsequent differentiation is matter of debate. Current opinion says that local factors like local ischaemia, low oxygen tension and local inflammatory mediators which alter the micro environment play an important role here. These factors that augment cellular proliferation are said to exert their effects at the cellular level by regulating the cell cycle.

Humoral factors normally present at a healing fracture site include local hormones like arachidonates (*i.e.* prostaglandins, leukotrienes etc), derived from damaged tissues and extravasated platelets, coagulation pathway factors, fibrinolytic products etc. There is also a vast array of mediators derived from accumulated inflammatory cells and includes lymphokines and cytokines like interleukins, TNF-á etc.

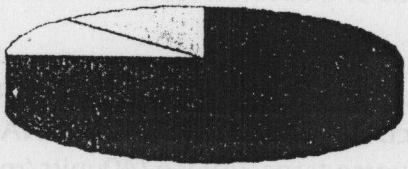
Most of these mediators exert their specific actions by binding with specific cell surface receptors and triggering an appropriate signal transduction cascade, which may terminally alter the transcriptional activity of these cells.

A study of those conditions producing exaggerated callus ,can provide insights into the activity of various factors that either accelerate or suppress the osteogenic activity. The scenario of a long bone fracture associated with a head injury is such a situation where exaggerated callus occurs. Increased callus formation here may involve either humoral or neural mechanism. Search is on for a single neurochemical factor acting as a link between activated CNS neurons and the proliferating peripheral cells. There is considerable amount of hypotheses in literature regarding candidate biomolecules. Circulating growth factors—bFGF, IGF, EGF, PDGF and TGFb family, hormones like prolactin,melatonin, endogenous opioids, cytokines, BMP,somatomedins etc have been considered. No study so far has produced the definitive evidence for a single factor being primarily involved.

In this study we are testing the possibility of brain derived neurotransmitters being the mediator of enhanced osseous formation after head injury and we intend to prove that their effect is exerted through expression of specific cell surface receptors on callus cells.

MATERIALS AND METHODS

Samples of callus were obtained from fourteen hospitalized patients. The patients were grouped into two groups. The first group comprised of 8 patients who had a long bone fracture and a documented head injury, whereas the second group of 6 had only a long bone fracture and no head injury. A head injury was defined as at least one instance of documented coma with GCS below 9 and a CT demonstrated intra cranial injury.



Collection of tissue sample was done as a minor surgical procedure under local anaesthesia. Callus from fracture site is aspirated at the end of second week following the fracture.

After taking proper aseptic precautions, patient is draped and given local anaesthesia. Once the desired effect of local anaesthesia is confirmed, aspiration from the local site is done using a wide bore needle, to obtain cells for neurotransmitter receptor assay. The collected tissue transferred into a holding media (RPMI media). The sample in the holding media is then immediately transported to centre for neurosciences, Dept of biotechnology, CUSAT, in ice cooled flasks.

At CUSAT the further steps of this study were conducted as soon as the sample was reached. The steps included an intial radiothymidine incorporation study to assess the proliferative potential of the sample. The steps involved in receptor assay included homogenization of the callus tissue into cellular components & centrifugation to isolate the mRNA component and finally real time PCR analysis of this mRNA for any over expression of the target genes(receptor genes).

ISOLATION OF m RNA

2.5-50 mg of callus tissue/serum is homogenized in 0.5 ml Tri reagent. The homogenate is centrifuged at 12000Xg for 10 minutes at 4 C .The clear supernatant is transferred to a fresh tube and it is allowed to stand at room temperature for 5 minutes.100 ml of chloroform is added to it, shaken vigorously for 15 seconds and allowed to stand at room temperature for 15 minutes. The tube is then centrifuged at 12,000xg for 15 minutes at 4 C.

Three distinct phases appear after centrifugation. The bottom red organic phase contains protein, interphase contains DNA and upper aqueous phase contain RNA. The upper phase is transferred to a fresh tube and 250 ml of isopropranalol is added and the tubes allowed to stand at room temperature for 10 minutes. The tubes are then centrifuged at 12,000xg for 10 min at 4 °C.

RNA precipitate forms a pellet on the sides and bottom of the tube. The supernatant is removed and the RNA pellet washed with 500 ml of 75% ethanol, vortexed and centrifuged at 12,000xg for 5 minutes at 4 °C. The pellet is semi dried and dissolved in minimum volume of DEPC treated water. 2 ml of RNA is made up to 1 ml and absorbance is measured at 260nm and 280nm. For pure RNA preparation the ratio of absorbance at 260/280 is ³1.7. The concentration of RNA is calculated as one absorbance 260 = 42 mg.

RT-PCR of GABA B receptor_subunits

RT-PCR is carried out in a total reaction volume of 20ml in 0.2ml tubes. RT-PCR is performed on an Eppendorf Personal thermo cycler. cDNA synthesis of 2 mg RNA is performed in a reaction mixture containing MuMLV reverse transcriptase (40units/reaction), 2mM dithiothreitol, 4 units of human placental RNAse inhibitor, 0.5 mg of random hexamer and 0.25mM dNTPs (d ATP, dCTP, dGTP and dTTP). The tubes are then incubated at 42 °C for one hour. After incubation heating at a temperature of 95 °C inactivate the reverse transcriptase enzyme, MuMLV.

Thermo cycling *profile for Real Time-PCR*

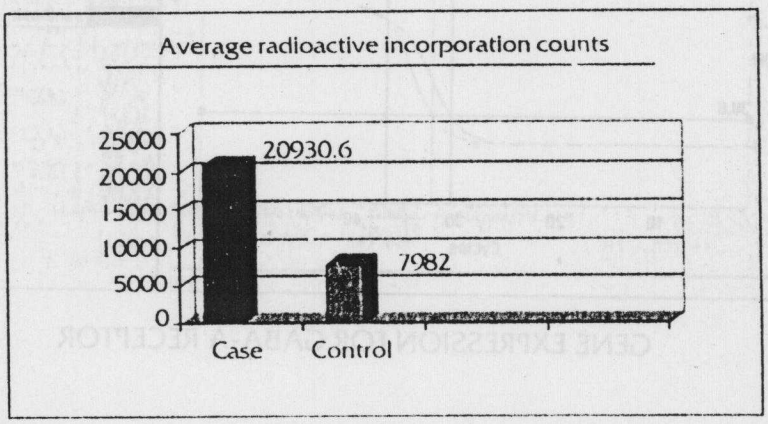
For obtaining higher stringency conditions RT-PCR profile is adopted. PCR is carried out in a 25 ml volume reaction mixture in the specially designed Real Time PCR tubes provided by Takara, Japan, containing 2 ml c DNA, 12.5 ml reaction mixture and 1ml of primer and 9.5 ml DEPC water. The reaction mixture is SYBR Premix EX Taq of which the unit definition is —one unit is the amount of the enzyme that will incorporate 10nmol of dNTP into acid insoluble products in 30 minutes at 74 °C with activated salmon sperm DNA as the template-primer. The ingredients of the reaction mixture are TAPS (pH 9.3 at 25 °C, KCl, Mg Cl2, 2-mercaptoethanol, d ATP, dGTP, dTTP, [a-32]-dCTP and activated salmon sperm DNA). Following is the thermo cycling profile used.

95°C — 30 seconds	Initial denaturation	
95°C — 10 seconds	Denaturation	
5°C — 30 seconds	Annealing	45 cycles
72°C — 30 seconds	Extension	

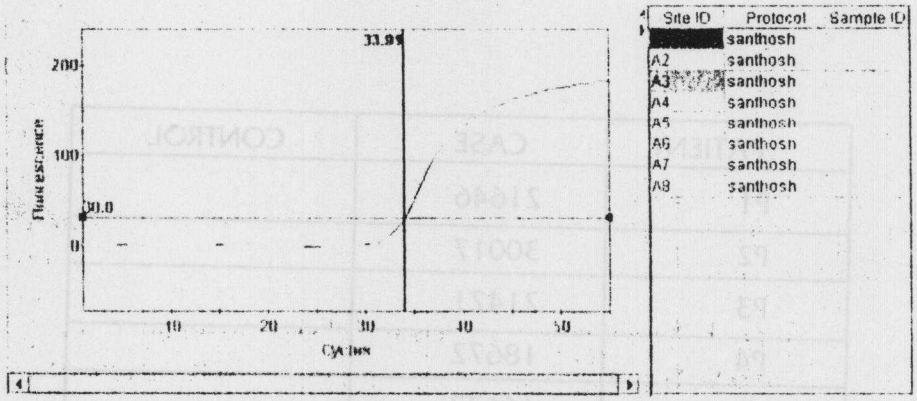
RESULTS.

The radiothymidine test was used as an intial screening test and it demonstrated an increased proliferative tendency in the study group.

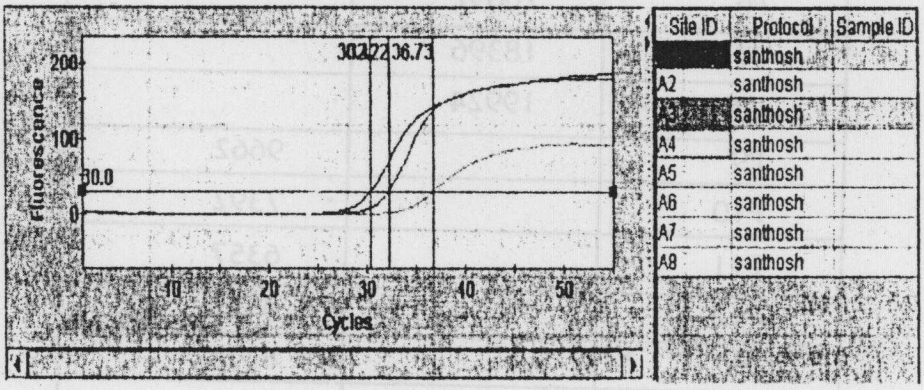
PATIENT	CASE	CONTROL
P1	21646	
P2	30017	
P3	21421	
P4	18672	
P5	16677	
P6	20692	
P7	18396	
P8	19924	
P9		9662
P10		7392
P11		6357
P12		8532
P13		8378
P14		7578
mean	20930.6	7983



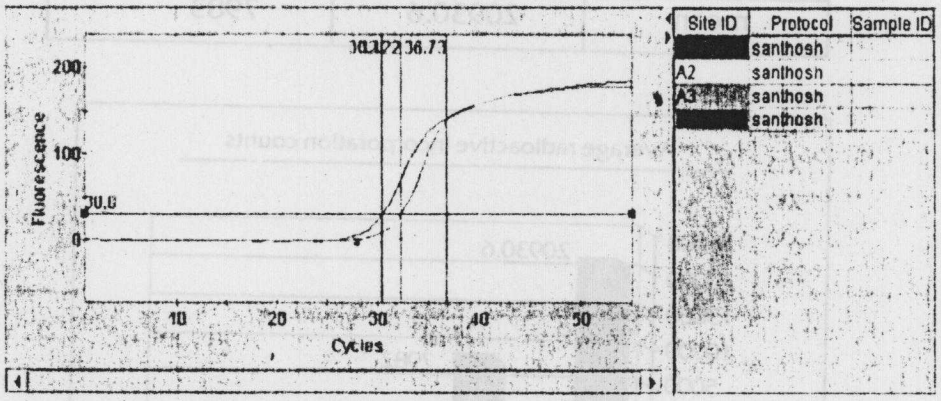
The real time PCR study for assessing the levels of gene expression of neurotransmitter receptors, showed the following results.



GENE EXPRESSION OF 5HT RECEPTOR



GENE EXPRESSION OF GABA-B RECEPTOR



GENE EXPRESSION FOR GABA-A RECEPTOR

The measure of PCR data is expressed as Crossing threshold (Ct) value, which is inversely proportional to the gene expression. So Ct value low means more gene expression compared to control .Here while running the melt curve for accuracy of PCR results, we found that the primer we used for GABA-A lacked homology with human genome. Hence we excluded it from the results. The Ct values for 5HT and GABA-B are as follows.

Receptor	Sample	Ct Value*
GABA B	Control	35.97
	Head injury	31.80
5 HT 2C	Control	31.22
	Head injury	31.21

*Values are mean of 6-8 samples

Here the Ct values of test and control groups are identical for 5HT receptor expression indicating only a marginal or no up regulation, whereas there is a significant difference in Ct values in case of GABA-B receptor.

DISCUSSION

It is an old orthopaedic teaching that fractures of long bones, when associated with head injuries, heal with excessive callus and at a faster rate than normal.

This enhanced osteogenic response may involve either humoral or neural mechanisms, or both.

Most of the studies in the literature have postulated a humoral mechanism. There is considerable amount of hypotheses in literature regarding candidate

biomolecules like circulating growth factors.bFGF,

IGF, EGF, PDGF and TGFb family, hormones like

prolactin,melatonin endogenous opioids, cytokines,

BMP,somatomedins etc . But no study has conclusively proved the role of a single substance.

Neural mechanisms could involve direct stimulation through small nerve endings or through release of trophic factors from nerve endings.

In this study, we tried to prove the role of neurotransmitters in accelerated fracture healing in head injury by directly looking for upregulated receptors on the callus cells. Our real time pcr results show that the receptors are indeed unregulated at the fracture site. This result opens a new concept,that accelerated fracture healing in head injury is mediated through an interplay of neurotransmitters released either from the damaged CNS directly or from the peripheral nervous system in response to CNS injury. Our results also suggest that selective upregulation of their receptors could be the possible mechanism.

According to our study, neurotransmitters show preferential mitogenic activity for the osseous cells. Although co-mitogenic effect of neurotransmitters like GABA has been reported previously in literature, it has not been reported in osseous cells.

CONCLUSION

We conclude that, neurotransmitters like GABA play an important role in accelerated

fracture healing and that this is mediated by selective upregulation of cell surface receptors for these molecules. How the binding of these neurotransmitters to their receptors brings about cell proliferation needs further evaluation at cellular level

ACKNOWLEDGEMENTS

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