Combined effects of melatonin and FGF-2 on mouse osteoblast (MC3T3-E1) behavior within IP-CHA constructs – *in vitro* analysis

多孔性ハイドロキシアパタイト内で培養したマウス骨芽細胞におけるメラトニンと FGF-2 の併用作用に関する基礎的研究

Introduction

Bone grafting plays an essential role in craniofacial surgery performed for both reconstructive and aesthetic purposes, which has led to discovery of different biomaterials, including interconnected porous hydroxyapatite (IP-CHA). It is not only biocompatible and osteoconductive but it also has the distinct advantage of being molded in desirable shape and size. The interconnection of pores is considered to be an essential factor for osteoconduction inside the IP-CHA for cell viability and function.

Fibroblast growth factor 2 (FGF-2) is a versatile member of the 23-polypeptide growth factor family. It has been found to participate in a various of biological processes from angiogenesis, embryonic development to bone remodelling. Furthermore, FGF-2 has been reported to stimulate osteoblast proliferation in immature cells rather than differentiation.

Melatonin (MEL) on the other hand is a pineal hormone that normally regulates the sleep cycle in humans. The effects of MEL on differentiation of mouse pre-osteoblast cells have been reported. Further reports suggest that MEL inhibits RANKL-induced bone resorption and thereby promote bone formation. These observations imply that MEL positively influences bone formation.

Even though both MEL and FGF-2 play significant roles in bone formation, their combined effects on osteoblast activity and bone formation have not been reported. Also, their combination within useful biomaterials, such as hydroxyapatite, remains undocumented. Here, we investigated the effects of FGF-2 and MEL in combination on mouse osteoblast cells when cultured within an IP-CHA construct.

Experimental design

Experiment 1: Determining the most efficient method for cell-seeding

To evaluate the most efficient way to seed cells within IP-CHA, we compared, four different cell seeding methods - static, agitation, centrifugation and vacuum-assisted. Mouse pre-osteoblast cells, MC3T3-E1 cells were cultured under normal culture medium until they were confluent. They were pipetted onto IP-CHA block of 4 mm diameter, 7 mm in height, and 75% porosity by concentrated cell suspensions.

For static seeding, IP-CHA blocks were immersed in cell medium containing $1X10^5$ cells and incubated for upto 3 days. For centrifugation method, IP-CHA block were placed in plastic tube along with $1X10^5$ cells and centrifuged at 2000 rpm with re-suspension at 1 min interval for 3 mins. For agitation method, IP-CHA block with $1X10^5$ cells was continuously agitated at 200 rpm at 37^oC for 1 hour. For vacuum-assisted method, IP-CHA block with $1X10^5$ cells were subjected to vacuum condition (100mmHg) for 100s followed by incubation for 3 days. All the resultant IP-CHA/MC3T3 composites were incubated for 3 days and then evaluated for cell viability using the MTT assay.

Experiment 2: Determining the optimum concentration of FGF-2 and MEL

Next we determined the optimum concentration of FGF-2 and MEL that would illicit a positive osteobastic response. This was done by treating the IP-CHA/MC3T3 composites with varying concentration of FGF-2 (2, 20 and 100 µg/ml infused cell culture medium) and MEL (50, 200, 1000 nM infused cell culture medium) independently. Following treatment, the composites were incubated for upto 3 days after which they were evaluated was based on real time PCR and RT-PCR mRNA expression of ALP and COL1 for FGF-2; OCN and OPN for MEL.

Experiment 3: Evaluating the combined effect of FGF-2 and MEL on osteoblastic activity.

Once the optimum concentration was determined, we treated IP-CHA/MC3T3 composites with or without FGF-2 and/or MEL and evaluated the proliferation assay (MTT assay), the mRNA expression on bone markers (Osteopontin and Osteocalcin), the Alkaline Phosphatase enzyme activity for 1, 3, 5 and 7 days.

Experiment 4: Evaluating the combined effect of FGF-2 and MEL on osteoblastic mineralization.

For illustrating mineralization, Alizarin Red staining was used. Primarily, monolayer cultures of MC3T3-E1 cells were prepared and in sub-confluent stage treated with or without FGF-2 and/or MEL infused cell culture medium. Secondarily, to evaluate the degree of mineralization of MC3T3 cells within the IP-CHA construct, quantification of Alizarin Red stained cells were carried out. For this, IP-CHA/MC3T3 composites were treated with/without FGF-2 and/or MEL for 2 weeks and then stained with Alizarin Red solution according to the manufacture's protocol except they were subjected to vacuum to allow the Alizarin Red solution to reach the

cells situated at the core of the IP-CHA. After substantial vortexing, readings were obtained using a spectrophotometer at 450 nm of absorbance.

Results

Experiment 1: Compared to all the methods of cell seeding in our study, we found that vacuum-assisted cell seeding method was most efficient in terms of cell viability.

Experiment 2: 20µg/ml of FGF-2 was the most optimum concentration. The mRNA expressions of COL-1 and ALP was significantly lower in 2µg/ml whereas not significantly higher at 100µg/ml. For the MEL, 200 nM was the most optimum concentration. The mRNA expression of both OCN and OPN was significantly lower in 20 nM whereas it was not significantly higher in 1000 nM.

Experiment 3: For the proliferation assay of combined effect – mainly FGF-2 influenced cell proliferation compared to control and MEL treatment. Interestingly, the MEL did not interfere with the proliferative potential of FGF-2. For the ALP enzyme activity, mainly MEL induced ALP enzyme activity compared to the FGF-2 treatment and control. Furthermore, the combination of MEL and FGF-2 showed an even greater effect on the ALP enzyme activity. The mRNA expression of OCN and OPN was significantly higher for MEL treated composites compared to FGF-2 treatment and controls. However, the combined effect of MEL and FGF-2 on mRNA expression was significantly greater.

Experiment 4: In monolayer cultures, both MEL and FGF-2 shows signs of mineralization however, the combination of MEL and FGF-2 showed more intense stains of mineralization. For the IP-CHA/MC3T3 composites, the absorbance showed mineralization in both FGF-2 and MEL treatment. As anticipated, the combination of MEL and FGF-2 illustrated greater mineralization.

Discussion and Conclusion

Based on our findings, we would like to suggest that FGF-2 mainly influences proliferation whereas MEL mainly influences osteoblastic differentiation. Moreover, the combination of MEL and FGF-2 shows increased

mineralization. Therefore we conclude that the combination of MEL and FGF-2 is effective for increased mineralization of osteoblast cells within IP-CHA constructs.

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