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Differentiation and Mineralization of Murine Mesenchymal C3H10T1/2 Cells in Micromass Culture

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Abstract

The murine mesenchymal cell line, C3H10T1/2 in micromass culture undergoes chondrogenic differentiation with the addition of BMP-2. This study compares the use of BMP-2 vs. insulin, transferrin, and sodium selenite (ITS) to create a chondrogenic micromass cell culture system that models cartilage calcification in the presence of 4mM inorganic phosphate. BMP-2 treated cultures showed more intense alcian blue staining for proteoglycans than ITS treated cultures at early time points. Both ITS and BMP-2 treated cultures showed similar mineral deposition in cultures treated with 4mM phosphate via von Kossa staining, however FTIR spectroscopy of cultures showed different matrix properties. ITS treated cultures produced matrix that more closely resembled mouse calcified cartilage by FTIR analysis. ⁴⁵Ca uptake curves showed delayed onset of mineralization in cultures treated with BMP-2, however they had an increased rate of mineralization (initial slope of ⁴⁵Ca uptake curve) when compared to the cultures treated with ITS. Immunohistochemistry showed the presence of both collagens type I and type II in BMP-2 and ITS treated control (1mM inorganic phosphate) and mineralizing cultures. BMP-2 treated mineralizing cultures displayed more intense staining for collagen type II than all other cultures. Collagen type X staining was detected at Day 9 only in mineralizing cultures treated with ITS. Western blotting of Day 9 cultures confirmed the presence of collagen type X in the mineralizing ITS cultures, and also showed very small amounts of collagen type X in BMP-2 treated cultures and control ITS cultures. By Day 16 all cultures stained positive for collagen type X. These data suggest that BMP-2 induces a more chondrogenic phenotype, while ITS treatment favors maturation and hypertrophy of the chondrocytes in the murine micromass cultures.

Keywords

cartilage calcification; endochondral ossification model; C3H10T1/2; micromass; growth factors

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Introduction

During the process of endochondral bone formation, mesenchymal stem cells differentiate into chondrocytes, which can then go on to hypertrophy and become the cells that form calcified cartilage. Cartilage matrix calcification is a highly regulated and ordered process, which under abnormal conditions can lead to disease states such as osteoarthritis, chondrodysplasias, osteochondromas and other bone and cartilage diseases [1-7].

A number of different in vitro cell culture systems have been used to model cartilage calcification and mimic endochondral ossification. These studies have been used to identify significant proteins and mechanisms of mineralization. Our group, as well as others, have primarily used avian cultures of primary mesenchymal stem cells from chick limb buds in micromass cultures as an in vitro model of cartilage calcification [8-16]. Mouse limb bud micromass cultures have also been studied [17-20], but they require timed pregnancies, and, hence, are costly and time consuming. Although there is evidence that some of the events in bone formation are conserved between the species [21], there is a need for a convenient murine culture system that mimics the mechanisms of endochondral ossification in vitro, because more reagents are available for murine systems and the avian system does not necessarily recapitulate the mammalian systems [22-24]. It is possible to envision that information gathered about the mammalian mineralization process can be used to understand human disease states and further their treatment.

The factors that affect chondrogenic differentiation of mesenchymal stem cells during in vitro culture range from cell shape, to cell to cell contacts, to the presence of growth factors [15, 25-30]. Micromass (high density) cultures enhance chondrogenic differentiation due to considerable amounts of cell-cell interaction and preservation of three dimensional cell shape in a tissue-like culture. In a micromass system, C3H10T1/2 cells, a murine mesenchymal stem cell line, undergo chondrogenic differentiation with the addition of bone morphogenetic protein-2 (BMP-2) or transforming growth factor- β (TGF- β) [15,27-29]. These chondrogenic cultures produce proteoglycans and exhibit phenotypic chondrogenic markers early in their development.

While the calcification of C3H10T1/2 cells have not been examined in micromass culture, studies report that treatment with BMP2 and BMP7 can induce mineralization in these cells in monolayer cultures [31]. Similarly, chondrogenesis can be induced with the addition of insulin, transferrin, sodium selenite (ITS) [32-35] to another murine cell line (ATDC5), and the addition of inorganic phosphate to ATDC5 monolayer cultures has been shown to control the calcification of the extracellular matrices they produce [36-38]. The ATDC cell line, however, takes longer to undergo chondrogenic differentiation than the C3H10T1/2 cells [31] [36-38] which was confirmed by our own preliminary experiments.

The chondrocytic differentiation of the C3H10T1/2 cells has been studied extensively and is very well characterized in micromass cultures. In the present study, a micromass culture system was used to model cartilage calcification with C3H10T1/2 cells. We compared the use of exogenous BMP-2 vs. ITS to drive these cells to chondrogenesis and then to calcification upon addition of inorganic phosphate. These methods will provide a framework for future studies of the proteins that are involved in the mineralization process.

Methods

Cell Culture

C3H10T1/2 cells were obtained from ATCC (CCL-226) (Manassas, VA) and were expanded in T-75 flasks. They were maintained in DMEM (Invitrogen, Carlsbad, CA) supplemented

with 10% FBS (Invitrogen, Carlsbad, CA) and 100 units of penicillin, 100 µg of streptomycin, and 0.25 µg of amphotericin B/ml utilizing penicillin G (sodium salt), streptomycin sulfate, and amphotericin B as Fungizone® Antimycotic (1% antibiotics/antimycotics)(Invitrogen, Carlsbad, CA). Cells were passaged with 0.05% trypsin (Invitrogen, Carlsbad, CA) when they reached ~85% confluency. Cells were frozen in liquid nitrogen at passages 5 and 7 and used for the experiments below between passages 6 and 14.

Micromass cultures were plated at a density of 100,000 cells per 10µl spot in the center of 35mm tissue culture dishes (Corning, Corning, NY). The cells were allowed to attach for 1.5 hours at 37°C and 5%CO₂. The plates were then flooded with calcium free DMEM supplemented with 1% FBS [39], 6.25 µg/ml human recombinant insulin, 6.25 µg/ml human transferrin, 6.25 ng/ml selenious acid, and 5.35 µg/ml linoleic acid (1% ITS) (BD Biosciences, Bedford, MA), or calcium free DMEM with 100ng/mL BMP-2 with 10% FBS [15,26-28]. For some experiments cultures were treated with 100ng/mL of BMP-2 and 10% FBS for the first seven days of culture and then switched to 1% ITS and 1% FBS. BMP-2 was a generous gift of Genetics Institute (now Wyeth/Pfizer, Boston, MA). All cultures received 1% antibiotics/antimycotics and media was changed three times per week. On day 2 of cultures 1.3mM calcium, L-glutamine, and 25 µg/ml of ascorbic acid were added to the media and inorganic phosphate content was adjusted to 4mM (4P) for mineralizing cultures. Control cultures were left at 1mM (1P) inorganic phosphate. The calcium and phosphate content were confirmed for each batch of media by atomic absorption spectroscopy [40] and by colorimetry [41] respectively.

Determination of Cell Viability

Total number of cells per culture at day 35 was determined by the picoGreen assay (Invitrogen, Carlsbad, CA) as previously described [42]. Cultures were first digested with 12.5µg papain/mL to measure DNA content with picoGreen. The papain digested cultures were added to picoGreen dye solution in a 96-well plate, which was then excited at 485nm and fluorescence was read at 540nm on a Perkin Elmer Bioassay Reader HTS 7000. DNA content and cell number were calculated through linear interpolation using calf thymus DNA standards.

Flow cytometry was used to determine cell viability at day 35 with 1:4000 dilution of 4mM calcein (live) and 1:250 dilution of 2mM ethidium bromide (dead) stains (Invitrogen, Carlsbad, CA) as previously described [43]. In short, media from 12 samples were pooled with trypsin-released cultures (0.25%) (Invitrogen, Carlsbad, CA) and pelleted via centrifugation. The supernatant was discarded and cell pellets were then mixed with phosphate buffered saline (200 µL) (Invitrogen, Carlsbad, CA) and divided into four separate tubes prior to addition of stains. Unstained and single stained samples served as controls.

Histology

ECM Stains—To determine the presence of proteoglycans, cultures were rinsed with PBS and fixed with 90% ethanol in the dish. They were subsequently treated with acetic acid and then stained with alcian blue as previously described [43]. For demonstration/staining of mineral, micromass cultures were fixed in the dish with 10% formalin and then stained with the von Kossa technique as previously described [43].

Immunohistochemistry—The presence of type I, II and X collagen were localized by immunohistochemistry. Collagen type II and type X antibodies were from the Developmental Studies Hybridoma Bank (Iowa City, Iowa). Collagen type I antibodies were from Calbiochem (Madison, WI). Cultures were collected on days 16, 21, or 28 and either embedded in paraffin or fixed in the dish. For type I and type II collagen stains, micromass cultures were fixed with 10% neutral buffered formalin and the cultures were removed from the dishes after fixation.

Paraffin embedded samples were sectioned at 5-7 μ m, and then deparaffinized for immunohistochemistry. For all collagen staining an antibody dilution of 250ng of antibody in 100 μ L was used. Slides were then washed and linked to streptavidin via horseradish peroxidase and visualized with DAB.

Identification of Protein

For each condition, twelve micromass cultures were scraped off the dish and pooled with RIPA buffer (Pierce, Rockford, IL). Pooled samples were sonicated (Misonix 3000, Farmingdale, NY) for 10 seconds, three times on ice. Pre-cast 10% SDS-Page gels (Biorad, Hercules, CA) were loaded with 20 μ g of total protein per lane as determined by the Biorad Protein Assay (Biorad, Hercules, CA). Gels were blotted semi-dry onto nitrocellulose membranes (Biorad, Hercules, CA) for 1.5 hours and blots blocked in non-fat dry milk (Biorad, Hercules, CA) overnight at 4°C. Blots were then washed in tris-buffered saline with 0.1% TWEEN (TTBS) pH 7.4 and incubated with an antibody against collagen type X (Abcam, Cambridge, MA) at a dilution of 1:3000 for 1 hour at room temperature. Blots were incubated in goat anti-rabbit conjugated to horse radish peroxidase (HRP) secondary antibody (Santa Cruz Biotech, Santa Cruz, California) for one hour at room temperature after 15 minutes of washing with TTBS. Blots were visualized with enhanced chemiluminescent substrate (Thermo Scientific Pierce, Rockford, IL) for detection of HRP and film (Kodak, Rochester, NY) was exposed for 1 minute.

Mineralization Kinetics

To determine extent of mineralization, calcium uptake was measured with the addition of ^{45}Ca (Perkin Elmer, Waltham, MA) to the media. On day 7 of micromass cultures, 0.5 $\mu\text{Ci/mL}$ of ^{45}Ca was added to the media of mineralizing and control cultures; addition was at every media change thereafter. ^{45}Ca was measured on days 12, 16, 21, 26, 30 and 35 of culture. At these time points media was removed and discarded, dishes were washed with cold PBS, and then samples were transferred to scintillation vials. Dishes were washed with 200 μ L of 4N HCl to dissolve remaining mineral and this was also added to the scintillation vials. The sample-HCl mixtures were then heated to 60°C for 30 minutes and 5mL of Aquasol solution was added to each vial and vortexed until clear. ^{45}Ca uptake was measured by scintillation counting (Beckman LS 6500, Fullerton, CA). The values in control cultures were subtracted from mineralizing cultures and the differential values were normalized to Day 35 values. All results are expressed as a mean $\pm$ standard deviation. Data was fit to a sigmoidal curve in Slide Write 5.0 (Advanced Graphics Software, Encinitas, CA) to determine rate of ^{45}Ca uptake.

FTIR Spectroscopic Characterization of Mineral

Day 21 and day 35 cultures were analyzed by FTIR spectroscopy. Cultures were first washed with PBS and then with ethanol and allowed to air dry overnight. Freshly dried (120°C, 24 hours) KBr (200mg) was mixed with the air dried samples in the tissue culture plates and pellets were made for spectroscopic analysis. Spectra were recorded on a Thermo Nicolett 4700 FT-IR (Thermo Scientific Waltham, MA). Spectra were baselined in Biorad Win-IR Pro 3.1 and the mineral to matrix ratio was determined as the area under the phosphate peak (900-1200 cm^{-1}) divided by the area under the amide I peak (1585-1720 cm^{-1}). The collagen crosslinking ratio, a measure of collagen maturity, was estimated as the ratio of the intensities of the amide I sub-bands 1660 cm^{-1} and 1690 cm^{-1} [44]. The ratio of carbonate to phosphate was calculated as the area under the carbonate peak (840-892 cm^{-1}) divided by the area under the phosphate peak and reflects the amount of carbonate substitution in the mineral crystal. The hydroxyapatite crystallinity was calculated as the peak intensity ratios of the phosphate sub-bands 1030 cm^{-1} and 1020 cm^{-1} . This crystallinity is correlated to mineral crystal size and perfection as determined by x-ray diffraction [45]. At day 21, samples from 12 culture plates were combined to get sufficient material to generate one spectrum per group. For day

35 samples, only 3-6 plates were need to generate one spectrum, so calculated values are the average of three independent experiments.

Results

Both BMP-2 and ITS treated cultures showed glycosaminoglycan (GAG) deposition (alcian blue staining) by day 9 of culture (Figure 1A). The BMP-2 treated cultures produced more cells, measured by picoGreen assay (~420,000 cells in BMP condition, ~115,000 cells in ITS condition), more matrix, and also had darker alcian blue staining, however cell viability in both ITS and BMP-2 treated cultures were similar as measured by flow cytometry (70-77% cells alive with BMP treatment vs. 81-94% of the ITS treated cells).

All cultures showed increases in ^{45}Ca uptake (Figure 2), with an increase in mineralization in the 4P cultures with time. By day 28 in the ITS treated cultures ^{45}Ca uptake had plateaued, while the BMP-2 treated cultures took longer to begin to mineralize. However, the rate of mineralization (initial slope of ^{45}Ca uptake curve) was faster with BMP-2 treatment. Cultures treated with BMP-2 for the first seven days and then switched to ITS displayed ^{45}Ca curves that were a combination of the two treatments.

In both BMP-2 and ITS treated cultures there was significant von Kossa staining indicating mineral deposition by day 35 (Figure 1B). Based on the ^{45}Ca uptake data, different time points were selected for immunohistochemistry of BMP-2 vs. ITS treated cultures. Time points for staining were selected from the linear portion of the mineralization curve for these two groups. At Day 21 there was type I and type II collagen deposition in the micromass matrix of BMP-2 treated cultures (Figure 1C&D). BMP-2 treated cultures had more intense staining of type II collagen in the matrix of mineralizing cultures. ITS treated cultures had no noticeable differences in the amounts of type I or II collagen between the control and mineralizing cultures at Day 16 of staining, but did show positive staining in both control and mineralizing cultures (Figure 1E). Collagen type X was present at Day 9 based on immunohistochemistry in the mineralizing cultures treated with ITS but was not detectable in BMP-2 treated cultures or in the control ITS cultures. This was confirmed by western blotting (Figure 1F). At later time points (day 16 and 21) all cultures were positive for type X collagen by immunohistochemistry, with the most intense staining in the mineralizing ITS treated cultures (Figure 1G&H).

To confirm mineral deposition and characterize mineral properties FTIR spectroscopy was performed at both day 21 and day 35 of culture (Figure 3). At day 21 there was very little mineral in either treatment group and samples were pooled to get one spectrum for each treatment group. Mineral to matrix ratios were only about 0.7 for both groups at day 21. The mineral to matrix ratio was about 15 by day 35 in the BMP-2 treated cultures while in the ITS treatment the mineral to matrix ratio was about 9 (Table 1). We have previously reported the mineral to matrix ratios for calcified cartilage of 10 week old mice to be about 5 [46]. Collagen maturity was higher in the BMP-2 treated cultures indicating a higher degree of crosslinking in the collagen matrix, while cultures treated with ITS showed lower collagen maturity than what has been reported for the calcified cartilage in mouse bones. Crystallinity and the amount of $\text{CO}_3:\text{PO}_4$ were not different in BMP treatment versus ITS treatment.

Discussion

This study describes a model system for the study of cartilage calcification with a murine cell line in micromass culture. Previously, micromass cultures of this cell line were used to model the first stages of cartilage differentiation [15,26-28] and now it is shown that this system can be used to model endochondral ossification via the addition of inorganic phosphate. Similarly, exogenous inorganic phosphate can modulate calcification of primary chick limb bud

micromass spot cultures [8,11,13,16,43]. Furthermore, the present study shows that with certain alterations to this micromass model, the mineralization can be modified to mimic the process that occurs in vertebrate cartilage calcification.

In micromass culture, the increased cell-cell interactions help maintain the chondrocyte phenotype as well as create a more “tissue-like” environment. Previous work with chick primary cells in high density culture show promotion of chondrogenic differentiation and then the formation of matrix nodules, which then go on to mineralize with the addition of exogenous inorganic phosphate [13]. The cells produce calcified cartilage that has been shown to have similar properties to calcified cartilage of native tissues [13]. In the murine cell line the different culture conditions produced different types of matrix accumulation and variable extents of calcification.

Some effects seen in the BMP-2 treated cultures may be from the higher percentage of serum used in these cultures. BMP-2, however, is important in bone and cartilage development in vivo and in vitro applications [47]. ITS is often used as a supplement for low serum or serum free media due to its mitogenic capabilities. Previous work with the ATDC5 cell line indicates that ITS is not only a supplement for cell adhesion, but can also induce chondrocyte differentiation, maturation, and hypertrophy and in combination with exogenous phosphate and ascorbic acid can lead to the formation of a calcified matrix [34,37]. It is likely that the differentiation and further calcification of the two model systems in the present study are based on the two different growth factors added to the media and not due to the serum concentration. It is possible, however, that the increased proliferation with BMP-2 is due to the high serum concentrations. In the present study, the data show that the use of ITS both promotes chondrogenesis and aids in calcification of the C3H10T1/2 murine mesenchymal cells in micromass culture.

BMP-2 has been shown to promote chondrogenic differentiation of this murine mesenchymal cell line when used in micromass culture [27,28], and has been shown to induce mineralization in monolayer cultures of this cell line [31]. Our data shows results similar to theirs at early time points with GAG deposition and collagen type II production in micromass cultures. However, at later time points, BMP-2 treated cultures had delayed onset of mineralization compared to ITS treated cultures but once mineralization was initiated its rate was faster with BMP-2 than with ITS. The cultures treated with BMP-2 for only 7 days and then switched to ITS also showed the delay in the commencement of mineralization, but not the increase in the rate of mineralization.

FTIR spectroscopy of the murine micromass cultures revealed that the calcified matrix of cultures given ITS more closely resembled that of the growth plate of mouse bone with respect to the collagen crosslinking ratio and mineral to matrix ratio. The higher mineral to matrix ratio in the BMP treated cultures may represent a deficiency of matrix rather than increased mineral accumulation. Both BMP-2 and insulin are important in endochondral ossification and bone growth and remodeling [48-50], and in these micromass cultures they did give different responses. BMP-2 when given to the cultures also promoted cell proliferation, however cell viability did not differ significantly between groups.

While the mechanisms of BMP-2 driven chondrogenesis is still not completely clear, it has been suggested that cartilage specific transcription factors such as SOX9 and scleraxis, as well as proteoglycan expression is increased over the course of differentiation in BMP-2 driven chondrogenesis [27,28,51] and requires N-cadherin [27] modulation. In other studies it has been suggested that BMP-2 chondrogenesis in these cells induces SOX6 and that SOX9 can act synergistically in this system to enhance BMP-2 driven chondrocyte formation [52]. In embryonic stem cells, continuous treatment with BMP-2 drives osteogenesis, and may lead to

a more osteoblast like phenotype, while additions of ascorbic acid and insulin can prevent dedifferentiation of chondrocytes into adipocytes [51]. Continuous treatment with BMP-2 can induce hypertrophy of chondrocytes and collagen type X production [51]. In this study, the BMP-2 treated micromass cultures produced collagen type X later than the ITS treated cultures. By day 16 all cultures showed the presence of collagen type X, indicating chondrocyte maturation and hypertrophy. The BMP-2 treatment may have induced a more proliferative state in the chondrocytes so that maturation and ECM production was delayed. It appears in the present study and by others that BMP-2 is a strong inducer of cartilage expression, as shown by the high amounts of collagen type II and the intense alcian blue stains, but continuous treatment with BMP-2 may inhibit calcification [53]. This model may be suited to the study of chondrocyte gene expression and cartilage versus endochondral ossification or bone growth.

Insulin in a number of chick micromass studies triggers maturation, but may impair hypertrophy [54,55]. In contrast to these studies, in our micromass cultures it is likely that the ITS treated cultures are driven towards hypertrophy as evidenced by the type X collagen production, which is consistent with results from another murine cell line [32,34,37]. Furthermore, both hypo- and hyperinsulinemia have been shown to have deleterious effects on bone and bone mineralization [49,56]. It is unclear as to whether or not insulin acts through its own receptor (IR) or through insulin like growth factor receptors (IGF-IR), as there is affinity for insulin to these receptors, albeit lower than to its own. Insulin receptors, IGF-I receptors, and hybrid receptors can all bind insulin and stimulate chondrocyte differentiation and cartilage growth [56]. Because the downstream events following binding to the IGF-IR and the insulin receptor can follow the same pathways and events, it is not easy to determine which receptor is binding insulin to promote chondrogenic potential or effects [57-61], although it has been reported that in a murine fibroblast cell line there seems to be different genes up and downregulated with insulin vs. IGF [62]. From recent literature it appears that low concentrations of insulin binding to the IR can promote differentiation of ATDC5 cells into chondrocytes [63]. It also has been shown that compared to insulin, IGF-I has strong mitogenic effects and that insulin may act more as a differentiating agent. In mouse bone explant cultures insulin's primary effect was on the hypertrophic zone, indicating that insulin is a differentiating agent rather than a proliferating agent. In the present study we observe similar effects of insulin and believe that it stimulates onset of chondrocyte hypertrophy and calcification.

This study demonstrates that ITS and BMP-2 can separately promote differentiation of the C3H10T1/2 cells into chondrocytes and can aid in the production of a calcified matrix. The mechanisms or pathways through which calcification occur in this in vitro system are still unclear. This micromass system, however, does mimic endochondral ossification and will be useful to elucidate the factors that are important in the mineralization process.

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Figure 1.

A. Proteoglycan Distribution on Whole mount Cultures. Alcian blue staining of BMP-2 treated cultures (Day 9) indicates proteoglycans were present mostly at the outer edges of the micromass. Alcian blue staining of ITS treated cultures shows more disperse and less intense alcian blue staining in the micromass culture. Original magnification - 4x

B. Von Kossa Staining of Control and Mineralizing Cultures. At Day 35, von Kossa stains of control cultures showed no mineral deposition. Both BMP2 and ITS treated cultures given 4mM inorganic phosphate stained similarly indicating mineral deposition with both treatment groups at Day 35 of culture. Original magnification – 20x

C. Collagen Type I Deposition in Control and Mineralizing Cultures. All cultures were positive for collagen type I, and were not visibly different with ITS (Day 16) vs. BMP (Day 21) treatment. Original magnification – 20x

D. Collagen Type II Immunohistochemistry. Mineralizing BMP-2 (Day 21) treated cultures showed more collagen type II deposition in the matrix than control and ITS (Day 16) treated cultures. Original magnification – 20x

E. Collagen Type X Immunohistochemistry at Day 9. Only ITS treated cultures given 4mM exogenous inorganic phosphate stained positive for collagen type X via immunohistochemistry at Day 9. Original magnification – 20x

F. Collagen Type X Western Blot. Western blot confirmed the presence of collagen type X in ITS treated cultures give 4mM phosphate. All other cultures showed very faint bands for collagen type X.

G. Collagen Type X Immunohistochemistry at Day 16. By Day 16 all cultures exhibited staining for collagen type X. Original magnification – 20x

H. Collagen Type X Immunohistochemistry at Day 21. At Day 21 cells in all conditions still stained positive for collagen type X. Original magnification - 20x

□ BMP ● ITS + BMP 7 Days /ITS

Figure 2.

⁴⁵Ca Uptake of Mineralizing Cultures. ⁴⁵Ca uptake of mineralizing cultures was first corrected by subtracting the control values and then normalizing to Day 35. Mineralization was delayed in BMP-2 treated cultures, but once mineralization commenced there was a faster rate of mineral accretion than ITS treated cultures. Cultures treated with BMP-2 for 7 days and then switched to ITS showed a combination of the features of the BMP-2 and ITS alone curves. ⁴⁵Ca was added to cultures on Day 9 and monitored starting on Day 12 by which time calcium uptake by the mineralizing cultures was already higher than control cultures. Data was fit to a sigmoidal curve to determine rate of ⁴⁵Ca uptake.

FTIR Spectroscopy

Figure 3.

FTIR Spectra of Mineralizing Cultures. Both BMP-2 treated cultures and ITS treated cultures showed peaks in the mineral region. The amide I and amide II peaks, mainly due to collagen, in the BMP-2 treated samples seemed to suggest deficiencies in matrix.

Table 1

FTIR Analyses of Micromass Cultures at Day 35

Day 35	CO ₃ : PO ₄	Crystallinity	Collagen Maturity	Mineral:Matrix
Micromass Spot with BMP-2	.007 ± 5.8 E-5	1.13 ± 0.01	11.9 ± 4.03	14.61 ± 4.21
Micromass Spot with ITS	0.0065 ± .00037	1.05 ± 4.73 E-5	1.9 ± 0.0009	8.8 ± .276
Calcified Cartilage from 10 week old mouse bones ⁴⁶	0.004±0.0009	1.18±0.003	5.4 ± 0.4	5.2 ± 0.8