Published in final edited form as:

Connect Tissue Res. 2003 ; 44(Suppl 1): 196–201.

Perlecan-Stimulated Nodules Undergo Chondrogenic Maturation in Response to rhBMP-2 Treatment In Vitro

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Abstract

The heparan sulfate proteoglycan, perlecan, is localized to hypertrophic chondrocytes in the growth plates of long bones. Mice mutants for perlecan display severe cartilage and skeletal defects. Previously, we demonstrated that C3H10T1/2 fibroblasts cultured on perlecan stimulated extensive formation of dense nodules reminiscent of embryonic cartilaginous condensations. These nodules stain intensely with Alcian blue, and antibodies specific for collagen type II and aggrecan; however, nodules do not express collagen type X, a marker of chondrogenic maturation. In this investigation, we tested the hypothesis that addition of rhBMP-2 to perlecan-induced nodules would promote chondrogenic maturation in vitro. C3H10T1/2 fibroblasts were seeded in Lab-Tek® chambered “Permanox” slides uncoated or coated with perlecan (B&D, 5 µg/well), at a density of 2×10^5 cells/well. The cells were maintained in CMRL-1066 media supplemented with ascorbic acid, citrate, and pyruvate (50 ng/ml). C3H10T1/2 fibroblasts seeded on perlecan-coated wells began to condense and form cell aggregates within 15 min. On the third day postplating, the media was replaced and supplemented with or without rhBMP-2 (50 ng/ml, Genetics Institute®). On day 6 of culture, microscopy revealed that rhBMP-2–treated cultures had significantly proliferated; however, untreated cultures had not. By day 12 of culture, confocal microscopy revealed that perlecan-stimulated nodules treated with rhBMP-2 express a late stage marker of chondrogenesis (collagen type X). Morphologically, cells expressing collagen type X in rhBMP-2–treated nodules appear larger in diameter, relative to cells not expressing collagen type X. Cells cultured on plastic and treated with rhBMP-2 did not form nodules, but attached and spread, yielding a high-density monolayer. In response to rhBMP-2 treatment, these cells also express collagen type X. However, the appearance of collagen type X occurs at a later time point relative to the appearance of collagen type X in perlecan-stimulated nodules. Thus, perlecan-stimulated nodules do mature at a faster rate when treated with rhBMP-2 relative to monolayer cells.

Keywords

Cartilage; Chondrogenesis; Perlecan; Proteoglycan; rhBMP-2

INTRODUCTION

Chondrogenesis is a process of mesenchymal conversion leading to the formation of cartilaginous structures. Initially, prechondrogenic mesenchymal cells are recruited into a condensing core where they establish cell-cell contacts and gap junctions, and begin to differentiate into chondrocytes [1]. Precartilaginous condensation is characterized by the production of sulfated proteoglycans, including aggrecan, and the switch from type I to type II collagen synthesis [2]. Condensed chondrocytes proliferate and secrete increasing amounts

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of extracellular matrix (ECM) until each single cell is completely encased by matrix. Growth of the condensing core is supported by aggregation of surrounding mesenchymal cells. In the growth plate, maturing chondrocytes undergo hypertrophic growth during which they secrete and organize a unique ECM, characterized by expression of type X collagen [3–5]. Type X collagen is synthesized by growth plate chondrocytes after they have become hypertrophic, but prior to mineralization of the ECM.

Perlecan (Pln) is a large multidomain heparan sulfate proteoglycan of 400–470 kDa, encoded by a 12 kb mRNA. Pln is found in a variety of species. As a component of nearly all basement membranes, it has wide tissue distribution and multiple potential functions [6]. The protein core of Pln is composed of multiple structural modules, arranged into five major domains. The amino terminal domain I of Pln is unique in sequence, whereas domains II–V share sequence similarities with other cell surface and ECM proteins. The cDNA cloning and expression of individual Pln domains in mammalian cell lines have provided valuable tools for the investigation of distinct functions of individual Pln domains [7–10]. In domain I three SGD consensus sequences, potential glycosaminoglycan attachment sites, occur near Pln's N-terminus. In the majority of cases, the glycosaminoglycans attached to Pln are of the heparan sulfate variety, although chondroitin sulfate may sometimes substitute for heparan sulfate at one or more of these sites [11]. The glycosaminoglycan chains located in domain I of the Pln core protein bind bFGF, promoting its mitogenic and angiogenic activities [12]. Additionally, since recombinant bone morphogenetic protein 2 (rhBMP-2) binds immobilized heparin and heparan-like polymers [13,14], its biological activity may also be modulated by the glycosaminoglycan chains located in domain I of the Pln.

Two reports describing the consequences of targeted disruption of the Pln gene in mice indicate that Pln plays important roles in multiple tissues [15,16]. While the majority of homozygous Pln null embryos die early in development, a small percentage survive until birth, presenting severe skeletal defects with striking abnormalities in growth plates of long bones. In Pln nulls, normal organization within the proliferative and hypertrophic zones of growth plate cartilage is lost. Thus, Pln appears to play a major organizational role during growth plate development. In vitro, Pln also may have important structural and/or signaling role(s) in cartilage development. Of particular interest is the observation made in our laboratory that Pln maintains the differentiated chondrogenic phenotype of both normal human and exostoses chondrocytes, and also promotes chondrogenic differentiation in multipotential mouse embryonic cells in vitro [17,18].

The multipotential mouse embryonic fibroblast cell line, C3H10T1/2, has been used by our laboratory and others to study chondrogenic conversion in vitro [17,19–21]. Collectively, these studies indicate that while C3H10T1/2 cells bind well to a variety of purified ECM components, Pln uniquely stimulates conversion to a chondrogenic phenotype. This phenotype is characterized by rapid cell condensation and aggregate formation, marked accumulation of Alcian blue staining material, as well as the appearance of type II collagen and aggrecan, markers of early chondrogenesis. However, even after 2 months in culture on Pln-coated substrates, C3H10T1/2 cell aggregates fail to express markers of cartilage maturation (i.e., type X collagen).

Recently, we completed studies to determine which Pln domain(s) promoted C3H10T1/2 cell aggregation and chondrogenic differentiation [22]. These “domain-mapping studies” employed individual recombinant Pln domains as coating matrices for C3H10T1/2 cells. In short, we found that C3H10T1/2 cells plated on surfaces coated with Pln domain I, but not with domains II–V, underwent aggregation and early chondrogenic differentiation. The response of C3H10T1/2 cells plated on Pln domain I-coated surfaces is identical, at least in early stages, to the response generated when plated on intact Pln. Our investigation also

demonstrated that the presence of heparan sulfate chains on intact Pln and recombinant domain I is required for this activity. Taken together, these studies demonstrated that Pln and domain I of Pln can promote the early stages of chondrogenic conversion, but alone they are not sufficient to drive subsequent stages of chondrogenic differentiation.

To pursue this further, we searched for soluble chondrogenic factors that could support maturation (expression of collagen type X) of Pln-stimulated nodules. Recombinant human bone morphogenic protein 2 (RhBMP-2), a commonly utilized growth factor for in vitro assay of chondrogenic differentiation, was chosen because of its expression in dorsal condensing mesenchyme, its ability to induce bone formation via an endochondral pathway [21,23], its known differentiation promoting effects on C3H10T1/2 cells, and its binding to heparin and heparan-like polymers [13,14]. Here we report our preliminary studies of Pln-stimulated nodules treated with rhBMP-2. We tested the hypothesis that rhBMP-2 addition to Pln-stimulated nodules would promote chondrogenic maturation in vitro.

MATERIALS AND METHODS

Materials

Pln/HSPG2 was obtained from Becton-Dickinson Labware (Bedford, MA, USA). RhBMP-2 was a gift from Genetics Institute (Cambridge, MA, USA). The rabbit polyclonal antibody against the active form of caspase 3 was purchased from Cell Signaling Technology (Beverly, MA, USA). The mouse IgG antibody to link protein (9/30/8-A-4) was purchased from the Developmental Studies Hybridoma Bank (University of Iowa, Ames, USA). The rabbit polyclonal antibody against Pln was provided by Dr. J. Hassell (Shriner's Childrens Hospital, Tampa, FL, USA). The rabbit IgG antibody against mouse type II collagen was purchased from Biodesign International (Kennebunk, ME, USA). The rabbit antimouse antibody against type X collagen (PXNC1-88) was provided by Dr. G. Lunstrum (Shriner's Childrens Hospital, Portland, OR, USA). Species-specific, Texas-Red conjugated secondary antibodies were purchased from Amersham Corporation (Arlington Heights, IL, USA).

Culture of C3H10T1/2 Cells on Pln

Cell culture was performed as previously described [17] with some modification. For confocal microscopic analysis of cell aggregates, Nalge-Nunc "Permanox" chambered slides (Naperville, IL, USA) were used to culture cells. Briefly, 5 µg of Pln or recombinant protein plus Dulbecco's-phosphate buffered saline (D-PBS) without Ca^{2+} or Mg^{2+} was added to the well at a final volume of 200 µl, and incubated overnight at 37°C with lids askew. On the following day, wells were rinsed twice with D-PBS prior to addition of cells. C3H10T1/2 cells were added to wells at a density of 2×10^5 cells per well in CMRL-1066 media (Gibco Life Sciences, Rockville, MD, USA), supplemented with 15% (v/v) fetal calf serum, 100 U/ml penicillin, and 100 mg/ml streptomycin sulfate, ascorbic acid (50 µg/ml), citrate (50 µg/ml), and pyruvate (50 µg/ml), and every other day throughout the experiment. RhBMP-2 treatment (50 ng/ml) was initiated on experimental day 3, 2 days after aggregate formation.

Immunofluorescent Detection of ECM Components

After culture upon matrix for 6, 9, 12, or 15 days, cell aggregates and monolayers were rinsed twice with D-PBS without Ca^{2+} or Mg^{2+} , fixed, washed with D-PBS, and incubated with the primary antibody for 1 hr at 37°C in a humidified chamber. After 3 washes (5 min each at room temperature) in D-PBS, cell aggregates and monolayers were incubated with the secondary antibody for 45 min at 37°C in a humidified chamber, and finally washed 3 times (5 min each) with D-PBS before mounting.

Caspase 3 labeling was carried out following a 1 hr fixation at 4°C with 3% (v/v) paraformaldehyde. For Pln labeling, cell aggregates and monolayers were fixed in 100% methanol (10 min/at room temperature); however, for type II and X collagen labeling an ice cold solution composed of 95% (v/v) ethanol plus 5% (v/v) acetic acid (30 min on ice) was employed as fixative. To promote link protein, type II and X collagen antibody penetration, cell aggregates and monolayers were incubated with chondroitinase ABC (1U/ml, Sigma), 0.02% (w/v) Type IV-S testicular hyaluronidase (H3884, Sigma) for 30 min at 37°C (link protein and collagen type II and X, respectively).

Confocal Microscope Analysis of C3H10-T1/2 Cells Aggregates

All data were acquired on a Zeiss inverted 100M Axioskop equipped with a Zeiss 510 LSM confocal microscope and a Krypton Argon laser (488 nm and 568 nm excitation lines). Simultaneous acquisition of nuclei stained with Syto 13 (505-550 band-pass filter) and Texas Red-conjugated secondary antibodies (590 long-pass filter) utilized the fast-line switch feature for elimination of spectral bleedthrough. Samples were imaged using a Zeiss 63X C-Apochromat water immersion lens (N.A. 1.2).

RESULTS

Within hours of plating on a Pln-coated surface, C3H10T1/2 cells aggregate, forming tiny clusters of cells. In contrast, cells plated in uncoated wells do not aggregate, but attach and spread, forming dense monolayers. By 24 hr, larger cell aggregates have formed as smaller clusters merge. By day 6 of culture, cell aggregates stain intensely for Alcian Blue and aggrecan, early markers of chondrogenesis, as well as collagen type II and link protein (Table 1). Even after long-term culture (>20 days), Pln-stimulated aggregates do not express markers of cartilage maturation, i.e., collagen type X, a phenomenon that we attributed to a lack of requisite growth factors or hormones. We tested the hypothesis that treatment of Pln-stimulated cartilage nodules with rhBMP-2 would facilitate their maturation. Wells were coated with Pln for each time point while control wells were left uncoated. Then, 2 days after cell plating and aggregate formation on perlecan coated wells, rhBMP-2 (50 ng/ml) was added to culture media. Throughout the experiment, culture media was replaced with supplements every other day. Samples were collected on days 6, 9, 12, and 15 of culture on matrix, which correspond to days 4, 7, 10, and 13 of rhBMP-2 treatment.

During the first 3 to 4 days of rhBMP-2 treatment (day 6 of culture), a proliferative response was observed in all wells. Light microscopic observations suggested that a marked increase in cell density had occurred and that monolayer cultures became multilayered. In Pln-coated wells, nodules formed leaving some parts of the well bare; however, in response rhBMP-2 treatment, the vacant plastic rapidly filled with cells around the nodule; however, a significant increase in nodule size resulting from rhBMP-2 treatment was not observed. Between days 4 and 7 of rhBMP-2 treatment, multilayered cultures also underwent obvious morphological alterations. In these cultures, densely packed spindle-shaped fibroblasts became rounded, with a clear and defined matrix deposition between them. Marked proliferation and the morphological changes suggestive of a chondrocyte phenotype were not observed in untreated cultures.

On day 6 of culture, samples were collected and prepared for immunocytochemical analysis. Immunofluorescent signal intensity for both collagen type II and Pln was greatly enhanced in cultures treated with rhBMP-2, compared with untreated cultures (Table 1). The signal intensity of both collagen type II and Pln in rhBMP-2-treated aggregates was especially remarkable at 4–5-fold above untreated aggregates. For these two markers, a gradient of signal intensity can be clearly established (rhBMP-2-treated aggregate>>> untreated aggregate>> rhBMP-2-treated monolayer> untreated monolayer). Interestingly, immunofluorescent signals for

collagen type II and Pln in rhBMP-2–treated and–untreated aggregates are localized to the outer/top 2–4 cell layers.

The detection of immunofluorescent signals specific for collagen type X is first observed, at low levels in rhBMP-2–treated aggregates from day 9 cultures (Table 1). At this time point, collagen type X labeling is localized to the lower half of the aggregate, and only rhBMP-2–treated aggregates label with collagen type X antibodies. By day 12, collagen type X expression is prominent within rhBMP-2–treated aggregates (Figure 1) and detectable within some cells from rhBMP-2–treated multilayered cultures. The collagen type X signal is localized to the ECM surrounding individual cells in the middle of the aggregate. Morphologically, cells surrounded by the most intense signal appeared larger in diameter than cells with little or no collagen type X. In contrast, collagen type X expression was not detectable in untreated aggregates (Figure 1) or monolayer cultures. At this time point, expression of both collagen type II and perlecan remained detectable and localized to the top of the aggregate, but at reduced signal intensity relative to initial observations (Table 1).

Labeling, using an antibody specific for the active form of caspase 3, a marker of apoptosis, suggested a low level of apoptosis is occurring in aggregates from day 12 and 15 rhBMP-2–treated cultures (Table 1). Caspase 3 signal appeared throughout untreated aggregates, with the exception of the first 2–3 cell layers. However, in rhBMP-2–treated aggregates, caspase 3 labeling was localized to the aggregate middle, an area where collagen type X signal also was localized. Caspase 3 immunofluorescence also was detectable in rhBMP-2–treated multilayer and untreated monolayer cultures; however, only a few cells in each well were positive.

DISCUSSION

Mesenchymal condensation is the pivotal stage and primary mechanism by which the vertebrate skeleton develops [24–26]. Cells within these condensations or nodules express elevated levels of cell surface adhesion molecules and ECM components, especially heparan sulfate proteoglycans. Together, the sequential and joint functions of these molecules facilitate the required cell-cell and cell-matrix interactions that initiate condensation. The progression from condensation and growth to chondrocyte differentiation also involves members of the fibroblast growth factor and bone morphogenetic protein families [27,28]. Thus, the temporal and spatial patterning of heparan sulfate proteoglycans may alter the presentation of heparin-sulfate-binding growth factors to their signaling receptors to regulate nodule growth and differentiation.

In vitro, C3H10T1/2 cells plated on Pln condense, differentiate, and express specific markers of developing chondrocytes [17,22]. However, the cell-cell and/or cell-matrix interactions provided by Pln alone are not sufficient to promote the maturation and terminal differentiation (expression of collagen type X) of the aggregates. Thus, we set out to determine if addition of a heparin-binding growth factor, rhBMP-2, to our in vitro model system would facilitate maturation of Pln-stimulated nodules (Figure 2). In support of our hypothesis, addition of rhBMP-2 to Pln-stimulated aggregates elicits proliferation, differentiation, maturation (collagen type X expression), and some subsequent terminal differentiation (caspase 3 expression). Moreover, treatment of aggregates with rhBMP-2 promoted a structural organization and localized protein expression that most resembles the growth plate. However, it has yet to be determined if perlecan plays a direct role in the rhBMP-2 response.

The growth plate is structurally organized into columns, and functionally into zones, defined by cellular activity and marker expression (Figure 2). Collagen type II expression is high in the proliferative zone of the growth plate [2,29]. In contrast, Pln accumulates in the prehypertrophic and hypertrophic zones, and collagen type X is expressed only in the hypertrophic zone [30–32]. Interestingly, confocal microscopic analysis suggests that

rhBMP-2-treated aggregates organize into columns and express chondrogenic markers in a zone/region specific manner. Cells at the top of these aggregates appear densely packed and flat, contrasting the rounded phenotype, increased spacing, and linear stacking observed in the aggregate middle. Indeed, a more rigorous structural analysis needs to be completed; however, the appearance and localized expression of collagen type II and X, as well as caspase 3 in treated aggregates, suggest the presence of region specific cues and functional organization.

In contrast, expression of Pln in treated aggregates suggests that these aggregates are not organized like growth plates. In growth plates of developing long bones, Pln first appears in the prehypertrophic zone, accumulates, and finally reaches its highest concentration in the hypertrophic zone [17]. Thus, if treated aggregates are organized like growth plates, then Pln and collagen type X expression should appear in similar regions. However, Pln is localized to the outer 3–4 cell layers of treated aggregate and is not found in areas where collagen type X is localized. Perhaps, the absence of internal Pln labeling is due to the inability of antibodies to penetrate the aggregate. This may be likely since the measures employed to enhance collagen X labeling are not routinely employed for Pln. An alternative explanation may be that the nutrient content, pH, oxygen tension, etc., within the first 3–4 cell layers provide an environment optimal for collagen type II and Pln expression. It is also possible that proteases rapidly degrade Pln expressed in the aggregate middle to facilitate terminal differentiation. At the hypertrophic cartilage/mineralization interface of developing long bones, Pln expression is abruptly lost as mineralization occurs. Perhaps, future investigations will provide data that will simplify our interpretation.

Efforts are ongoing to complement these experiments with studies employing Pln domain I stimulated aggregates and rhBMP-2 treatment. In addition, RT-PCR analysis of Pln-stimulated aggregates also is underway to establish the temporal expression of chondrogenic markers in response to rhBMP-2 treatment. Last, disruption of Pln expression in C3H10T1/2 cells also is underway to more specifically test the requirement of Pln in aggregate formation, differentiation, maturation, and response to rhBMP-2.

ACKNOWLEDGMENTS

The authors wish to acknowledge Dr. Kirk Czymmek for his expert assistance with the confocal microscopy; Genetics Institute for the generous gift of rhBMP-2; Dr. Frank Manuzzo at Becton Dickinson Labware for assistance with obtaining Pln. The authors are also indebted to the members of the Farach-Carson and Carson laboratories for helpful discussion.

This work is supported in part by fellowships from the Delaware Biotechnology Institute and a National Institutes of Health, National Research Service Award (NRSA-1F32-AG20078) to Dr. Gomes, as well as a grant from the National Institutes of Health (RO1-DE13542) to Dr. Carson and Dr. Farach Carson.

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Figure 1.

Detection of collagen type X in perlecan-induced nodules treated with rhBMP-2 by indirect immunofluorescence. As antibody specificity controls, frozen longitudinal serial sections of d18 mouse hindlimb were labeled with either a rabbit-antimouse antibody against collagen type X (A) or normal rabbit serum (B) and detected with species-specific Texas-red secondary antibodies (red). The green signal is the nucleic acid stain, Syto 13. The collagen type X antibody specifically labels to hypertrophic chondrocytes of the growth plate (GP). RZ-resting zone; PZ-proliferative zone; Pre HZ-prehypertrophic zone; HZ-hypertrophic zone; M-metaphysis. Panels C and D are optical sections captured with a confocal microscope of perlecan-induced nodules treated (C) and untreated (D) with rhBMP-2, and labeled with collagen type X specific antibodies. Intense pericellular as well as intracellular, potentially golgi labeling, of collagen type X can be observed in nodules treated with rhBMP-2 (C). Collagen type X is not detected in untreated nodules (D).

Figure 2.

Comparison of the role of perlecan (Pln) during in vivo and in vitro chondrogenesis. In vivo, Pln-stimulated nodules, treated with rhBMP-2, express markers of mature chondrocytes (collagen type X) and organize into column-like structures. In the growth plate of developing long bones, Pln accumulates in the prehypertrophic and hypertrophic zones. Since BMP receptors are also sequestered in these regions, Pln, via the GAGs at its N-terminus, may modulate the biological activity of BMPs in the growth plate.

TABLE 1

Relative comparison of chondrogenic marker intensity in perlecan-stimulated aggregates, untreated or treated with rhBMP-2 (50 ng/ml).

	Day 6	Day 6	Day 9	Day 9	Day 12	Day 12	Day 15	Day 15
	rhBMP-2	+rhBMP-2	rhBMP-2	+rhBMP-2	rhBMP-2	+rhBMP-2	rhBMP-2	+rhBMP-2
Chondrogenic marker	–	+	–	+	–	+	–	+
Link protein	+	+++	++	++++	++	++++	+	+++
Collagen II	++	++++	++	+++	+	++	+	++
Perlecan	++	++++	++	+++	+	++	+	++
Collagen X	–	–	–	+	–	++++	–	+++
Caspase 3	N/A	N/A	N/A	N/A	+++	++	++++	+++

The 6-, 9-, 12-, and 15-day tissue analysis time points refer to time on coated matrix and correspond to 4, 7, 10, and 13 days of rhBMP-2 treatment.

(–) = not detected; (+) barely detectable, (++) = moderate intensity levels, (+++) = high intensity levels, (+++++) = highest levels of detection, N/A = not done.