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A new technique for seeding chondrocytes onto solvent-preserved human meniscus using the chemokinetic effect of recombinant human bone morphogenetic protein-2

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Abstract Many investigators are currently studying the use of decellularized tissue allografts from human cadavers as scaffolds onto which patients' cells could be seeded, or as carriers for genetically engineered cells to aid cell transplantation. However, it is difficult to seed cells onto very dense regular connective tissue which has few interstitial spaces. Here, we discuss the development of a chemotactic cell seeding technique using solvent-preserved human meniscus. A chemokinetic response to recombinant human bone morphogenetic protein-2 (rhBMP-2) was observed in a monolayer culture of primary chondrocytes derived from femoral epiphyseal cartilage of 2-day-old rats. The rhBMP-2 significantly increased their migration upto 10 ng/ml in a dose-dependent manner. When tested with solvent-preserved human meniscus as a scaffold, which has few interstitial spaces, rhBMP-2 was able to induce chondrocytes to migrate into the meniscus. After a 3-week incubation, newly-formed cartilaginous extracellular matrix was synthesized by migrated chondrocytes throughout the meniscus, down to a depth of 3 mm. These findings demonstrate that rhBMP-2 may be a natural chemokinetic factor in vivo, which induces migration of proliferative chondrocytes into the narrow interfibrous spaces. Our results suggest a potential application of rhBMP-2 for the designed distribution of chondrocytes into a scaffold to be used for tissue engineering.

Keywords BMP-2 · Chondrocytes · Cell migration · Chemokinesis

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Introduction

In most tissue engineering approaches, cells are typically isolated from a small biopsy sample and then expanded in culture. These expanded cells are then seeded onto a suitable three-dimensional scaffold and induced to produce a tissue-like structure in vitro. The composites of cells and scaffold are then implanted in vivo into an orthotopic site to repair damaged tissue. The phenotype of a cell-based tissue-engineered construct depends on a number of factors, such as the composition, size, porosity and biodegradability of the scaffold, as well as the cell type, cell density, and spatial distribution of the cells within the construct (Langer 2000). The cellularity of the engineered construct depends on the number of cells initially introduced into the construct, on the retention of these cells during the first few hours to days until cell-cell and cell-scaffold interactions have

been established, and on the mitotic activity of the cells incorporated into the construct (Solchaga et al. 2006). For any given scaffold, the initial seeding influences cell density, retention, and spatial distribution within the scaffold, all of which will eventually affect the function of the construct (Griffon et al. 2005; Holy et al. 2000; Mauck et al. 2002) .

Currently, there are a variety of methods used for seeding cells into a three-dimensional matrix with the aim of achieving uniform cell distribution (Griffon et al. 2005; Solchaga et al. 2006; Thevenot et al. 2008; Timmins et al. 2007; Wendt et al. 2003). In the passive methods of seeding, cells are laid on top of the scaffold and allowed to infiltrate into the scaffold over time (Wendt et al. 2003). The main disadvantage, particularly in the case of thick scaffolds with high degrees of tortuosity, is that the infiltration rates can be very low, resulting in poor loading and uneven distribution of cells within the scaffolds. In the active methods of seeding, an external force is applied to encourage the cells to infiltrate into the porous scaffold (Cimetta et al. 2007; Griffon et al. 2005; Mauck et al. 2002). The nature of the force varies from centrifugal to external pressure gradients, vacuum and compression force (Griffon et al. 2005; Griffon et al. 2005; Thevenot et al. 2008). However, it is difficult to seed cells into high density regular connective tissue which has few interstitial spaces. Here, we discuss the development of a chemotactic cell seeding technique using solvent-preserved human meniscus which has a highly dense collagen structure.

Bone morphogenetic protein (BMP)-2, a member of the transforming growth factor (TGF)-β superfamily (Chen et al. 1991a, b; Massague 1992; Sampath et al. 1990; Wozney et al. 1988) plays an important role in the differentiation of mesenchymal cells. Generally, BMP-2 stimulates cell differentiation when the concentration is higher than 100–500 ng/ml in vitro (Date et al. 2004; Kawasaki et al. 1998; Ryoo et al. 2006; Sekiya et al. 2005). On the other hand, BMP-2 also affects migration of mesenchymal cells, fibroblasts and osteoblasts (Bhardwaj et al. 2001; Fiedler et al. 2002; Lind et al. 1996; Uchida et al. 2007) but the concentration of BMP-2 required for cell migration is lower than that for differentiation. Migratory effects of BMP-2 have also been noted in endothelial cells or smooth muscle cells, but rarely in chondrocytes.

Bhargava et al. (1999) have evaluated the migratory effects of several cytokines including BMP-2 on bovine meniscus cells. The meniscus-derived cells, which may contain chondrocytic cells, were not well characterized in the study. Isolated chondrocytes are easy to irreversibly dedifferentiate in monolayer culture (Mukaida et al. 2005) and therefore, it is unclear whether the cells used by Bhargava et al. (1999) still retained the biological characteristics of meniscus chondrocytes. In addition to the reported effect of BMP-2 on chondrocyte differentiation and cartilage matrix formation (Erickson et al. 1997; Stewart et al. 2004), we considered that investigation of the chemotactic and/or chemokinetic effect of BMP-2 on chondrocytes would be necessary. We also hypothesized that the migratory effect of BMP-2 on chondrocytes could be useful for chondrocyte seeding into solvent-preserved human meniscus.

In this study we used proliferating chondrocytes isolated from the distal epiphysis of 2 day old Wistar rats and evaluated the response of chondrocytes to BMP-2. We also developed a chondrocyte seeding technique into solvent-preserved human meniscus using the migratory response of chondrocytes to BMP-2.

Materials and methods

Preparation of proliferating chondrocytes

All the procedures involving animals were carried out in accordance with the guidelines of Kitasato University Animal Ethics Committee. Chondrocytes in the distal epiphysis were harvested from 2 day old Wistar rat femora. The distal femora were exposed and adherent soft tissues were stripped. The reserved zone of the epiphysis was removed by dissection. The cartilage fragments containing the reserved zone chondrocytes were washed three times in PBS (Wako) containing antibiotics (streptomycin, penicillin, fungizone (GIBCO-Invitrogen, Carlsbad, CA)) and digested for 4 h in a 0.3% solution of Collagenase P (GIBCO) in PBS in a shaking water bath at 37°C. The solution containing the isolated cells was filtered through 70-μ mesh and the cells recovered by centrifugation at 1,000 rpm at 4°C and resuspended in Ham's F-12 medium (Biochrom AG, Berlin, Germany) containing antibiotics and 10% FBS at a

final concentration of 2×10^5 cells/ml. The isolated cells were used for each experiment immediately after isolation.

Migratory response of chondrocytes to rhBMP-2

Disposable 96-well chemotaxis chambers (Neuro Probe, Gaithersburg, MD) were used (Albini et al. 1987; Uchida et al. 2007). Recombinant hBMP-2 (R & D systems, Minneapolis, MN) was added to a final concentration of 0.01, 0.1, 1, 10 or 100 ng/ml in Dulbecco's modified Eagle's medium (DMEM; Cambrex, Walkersville, MD) containing 1 % antibiotic mixture (GIBCO). Primary proliferating chondrocytes (1×10^4 cells/well) were seeded in Ham's F-12 medium onto the membrane of the chamber set in a 6-well plate. Cell number was standardized using a curve prepared from 0, 25, 50, 100, 200, 400, 800 and 1,600 cells/50 μl solution. The chemotaxis chamber was incubated in 5% CO_2 at 37°C for 48 h. Cells were then labelled with 2.5 μM Calcein acetoxymethyl ester (AM) after eliminating the non-migrant cells (Uchida et al. 2007) and the fluorescence of each sample was measured by a plate reader (Spectra Fluor Plus, Tecan, Finland).

The chemotactic responses of chondrocytes were tested by checkerboard analysis to determine whether cell migration was due to chemotaxis (i.e., cell migration in response to a concentration gradient) or chemokinesis (i.e., cell migration irrespective of a concentration gradient) (Fiedler et al. 2002). Four culture conditions were prepared, as follows. Equal concentration gradient: culture medium in both top and bottom wells of a Boyden chamber contained 100 ng/ml rhBMP-2; positive concentration gradient: only the medium in the bottom well contained 100 ng/ml rhBMP-2; negative concentration gradient: only the medium in the top well contained BMP-2. Absent concentration gradient was the condition in which neither the medium in the top nor the bottom contained 100 ng/ml rhBMP-2.

Scratch assay

We assessed the chemokinetic effect of rhBMP-2 using the scratch assay. Rat primary proliferating chondrocytes (2×10^4 cells/well) were seeded into a 6-well plate when the cells reached sub-confluence.

After culturing in DMEM containing 10% foetal bovine serum (FBS; Sigma, Japan) and antibiotics for 8 h, medium was replaced with the same medium without FBS, then a scratch was gently made with a sterile 200 μl pipette tip across the centre of the plate. After two washes with serum free DMEM, the cells were incubated in the same medium in the presence or absence of 10 ng/ml rhBMP-2. Cell migration into the scratched area was photographed at 0, 12, 24, 36 and 48 h and the cell number was counted using NIH image software (NIH, Bethesda, MD) (Katsumoto et al. 2005; Nishio et al. 2005; Zhang et al. 2004).

Preparation of preserved menisci

Preserved menisci were provided by Tutogen Medical Inc. (Neunkirchen, Germany). Menisci were retrieved from cadaveric donors who gave informed consent for cadaveric donations. Donors did not receive any monetary compensation. Donor suitability was established by the medical director in accordance with American Association of Tissue Banks (AATB) standards and Food and Drug Administration (FDA) regulations. Prior to retrieval, a physical examination, medical, social and sexual history inquiry, medical history and medical records review were performed to screen for infections, adverse conditions, risk factors and malignancies. The autopsy report was also checked. Serological tests for infectious diseases were performed.

The retrieved menisci were processed through delipidation, osmotic treatment, oxidation, dehydration and irradiation. Tissue underwent an acetone bath to remove the fat tissues, and the graft was washed in alternating baths of distilled water and saline for osmotic contrast treatment to remove cellular materials. The graft was bathed in hydrogen peroxide washes for oxidative treatment to destroy proteins and immunogenic structures. The graft was then passed through a series of acetone baths for solvent dehydration and viral inactivation to prohibit the growth of microbes and inactivate viruses. Following product packing, the graft was subjected to low-dose gamma irradiation (17–20 kGy). The process required only validated minimal doses of irradiation, thereby preserving the biomechanical integrity of the graft while ensuring post-packaging sterility.

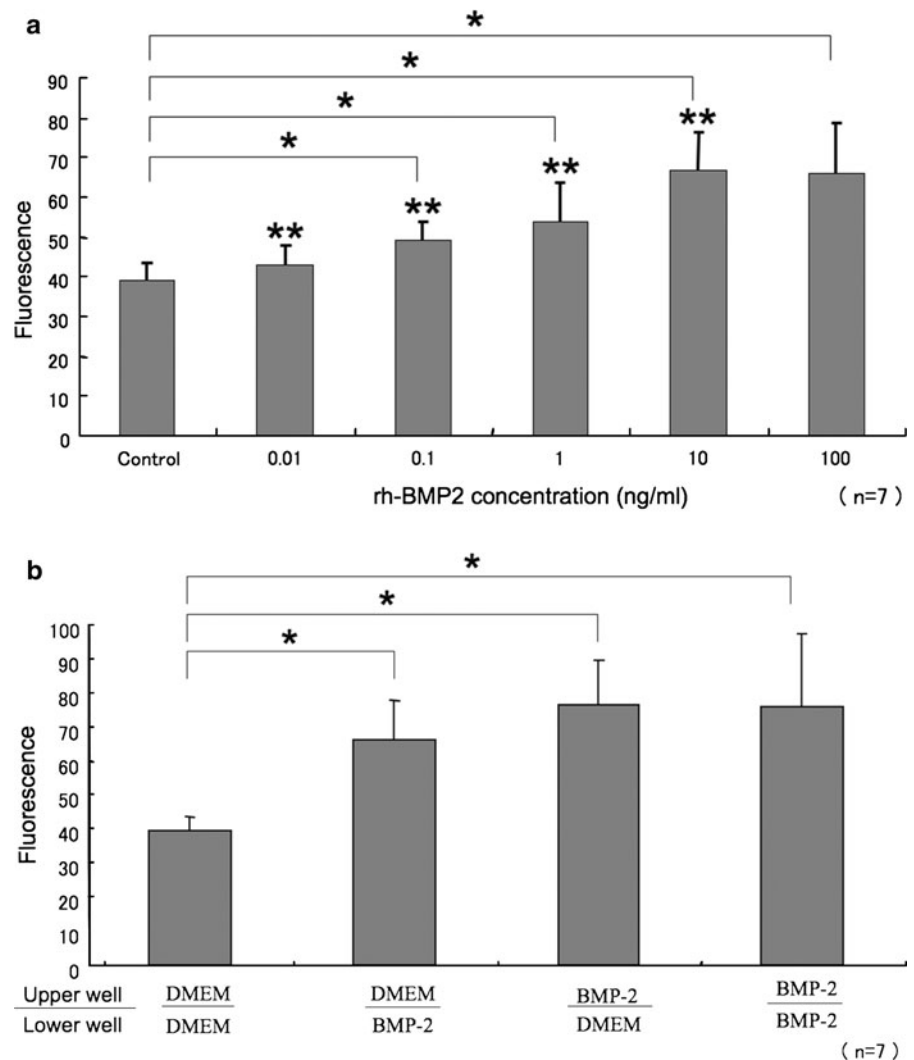Evaluation of migrated chondrocytes
within the dense tissues

Supplied menisci with an average pore size of 10 μm
were punched out perpendicularly to the surface (see
Fig. 3 for a representative meniscus) to form a small
column. The same culture medium as that used in the
scratch test was prepared. Fifty microliters of
medium with or without 10 ng/ml rhBMP-2 was
added to the micro-tube. Each piece of meniscus was
plugged tightly into a micro-tube so that the bottom
of the meniscus was soaked in the solution. Chon-
drocytes (2×10^4) were then placed on top of the
column and cultured for 24 h in 5% CO_2 at 37°C.

Following this 24-h incubation, the meniscus col-
umns were washed 3 times with medium to remove
rhBMP-2 and non-adherent cells. The cells which
had penetrated into the columns were then cultured
in DMEM with 10% FBS and antibiotics for 3 weeks.
Paraffin sections were prepared for Hematoxy-
lin-Eosin and Safranin-O staining for histological
observation.

Statistical analysis

One-way ANOVA with Tukey's multiple comparison
test was used. All tests were two-tailed, with
differences considered significant * at $P < 0.05$.

Fig. 1 Migratory effect of recombinant human BMP-2 (rhBMP2) on chondrocytes. **a** Chemotaxis chamber analysis. Increasing concentrations of rhBMP-2 from 0.01 to 10 ng/ml increased the intensity of fluorescence. However the levels of intensity between 10 and 100 ng/ml were similar. **b** Checkerboard analysis. This analysis showed that the migratory effect of rhBMP-2 on chondrocytes was due to chemokinesis
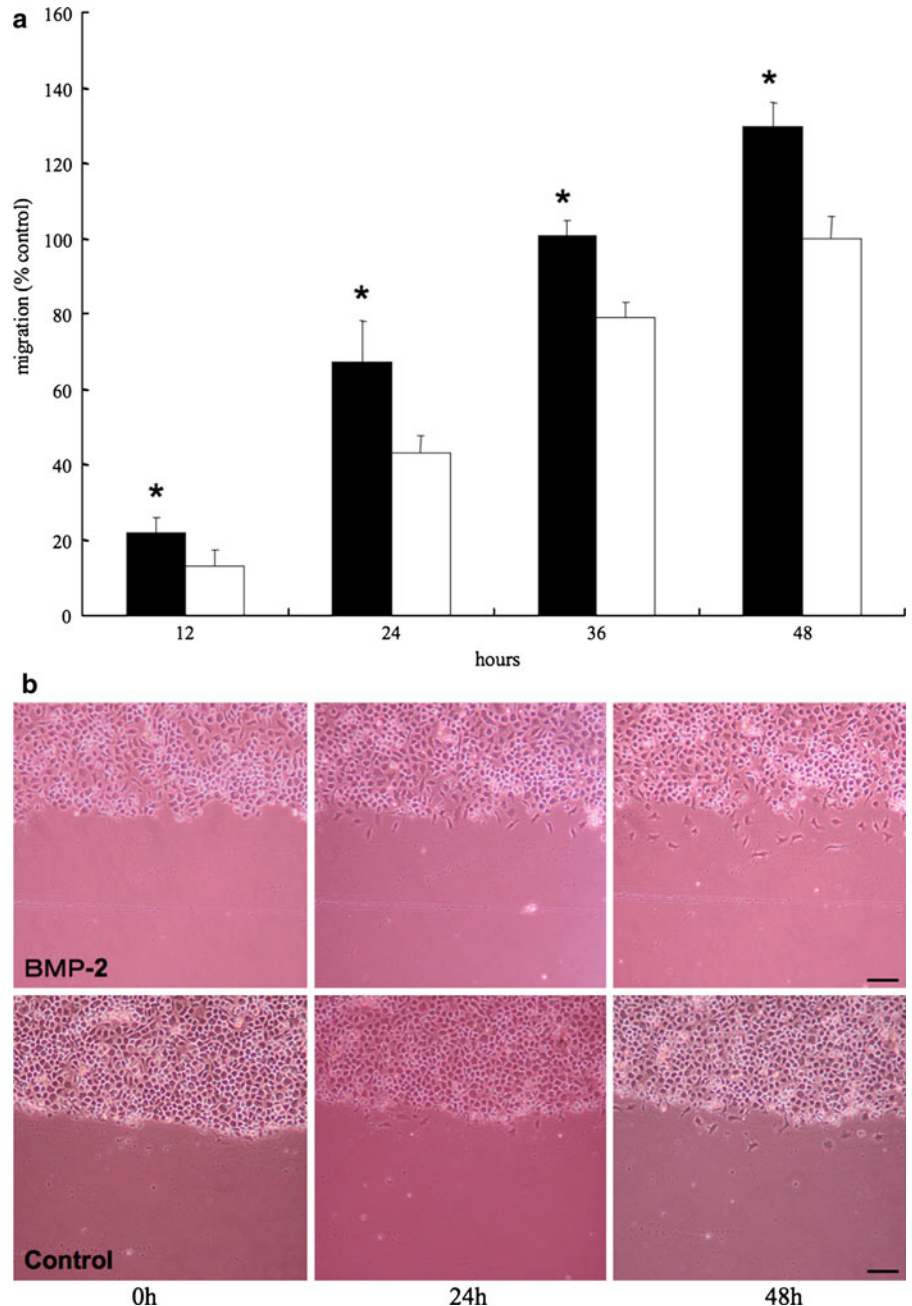
Results

Migratory response of epiphyseal proliferating chondrocytes to rhBMP-2

The basal numbers of migrated cells in the chemotaxis chambers were in proportion to the numbers of seeded cells (data not shown). The number of migrated cells increased with the addition of rhBMP-2 in a dose dependent manner between 0.01 and 10 ng/ml (Fig. 1a), and then levelled off above 10 ng/ml. The increments showed a significant difference compared to the control value as depicted in Fig. 1. The numbers of migrated cells in positive, negative and equal concentration gradients were significantly higher than that in the absent concentration gradient, and there

Fig. 2 Scratch assay. **a** Percentage of cell migration. Each value was calculated as the percentage of the area covered at each time point divided by the area covered at 48 h in control. *Black* and *white* bars symbolize the percentages of the recombinant human BMP-2 (rhBMP-2) group and control, respectively. A significantly higher migration rate in proportion to time was observed in the rhBMP-2 group when compared to control. **b** The appearance of the scratched area. The number of migrated cells in the scratched area of a 6-well plate was increased in the rhBMPf-2 group when compared to control. *Scale bars* represent 100 μm

were no differences between positive, negative and equal concentration gradients (Fig. 1b). The scratch assay showed a significantly higher rate of migration in the rhBMP-2 group at all time points measured (Fig. 2a, b). These results indicate that the effect of rhBMP-2 on chondrocyte migration is not chemotactic but chemokinetic.

Chondrocyte migration into the dense meniscus tissue

Scanning electron microscopy showed that the diameter of the interstitial spaces within the preserved human menisci used for this study was approximately 10–20 μm with the fibrous tissue structure maintained (data not shown). H.E. stained sections of preserved meniscal tissue suggested the homogeneous orientation of collagen fibres and interstitial spaces (Fig. 3b). Both cells and extracellular matrix could be recognized in the meniscus columns cultured for 3 weeks after 24-h rhBMP-2 treatment, but not in the columns cultured without BMP treatment (Fig. 4). In the H.E.

stained sections, newly synthesized extracellular matrix was observed between the existing fibrous components (Fig. 4a). This area stained red with Safranin-O (Fig. 4c). The cells and matrix were distributed homogeneously from the tissue surface to the bottom of the column (data not shown). The fibrous structure of the meniscus was either distorted or apparently degraded by the newly formed extracellular matrix (Fig. 4a, c). No cells or newly synthesized extracellular matrix were seen in the control cultures not treated with rhBMP-2 (Fig. 4b, d).

Discussion

In dense regular connective tissues such as tendons and ligaments, parallel arrays of fibrils and fascicles are uniaxially aligned into cable-like fibrillar structures that maintain the mechanical integrity and strength necessary for their load-bearing function. Many investigators are currently studying the use of decellularized tissue allografts from human cadavers
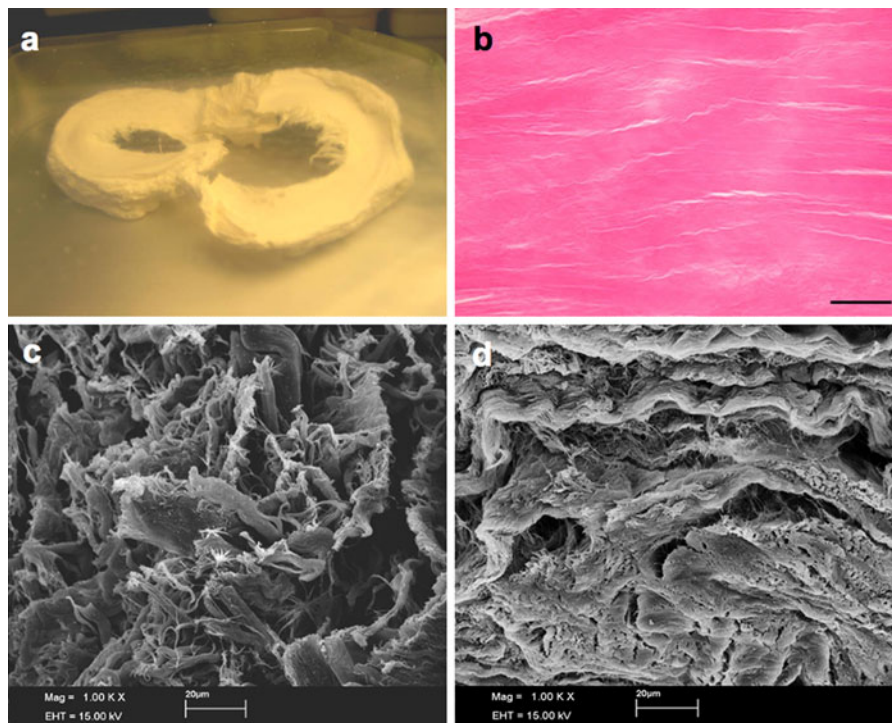
Fig. 3 Evaluation of solvent preserved meniscus from human cadavers. **a** Gross appearance of solvent preserved meniscus. The meniscus was punched out perpendicularly to the surface to form a small column. **b** H.E. stained section of preserved meniscus. No cells were evident and collagen fibers were homogeneously stained with eosin. *Scale bars* represent 100 μm
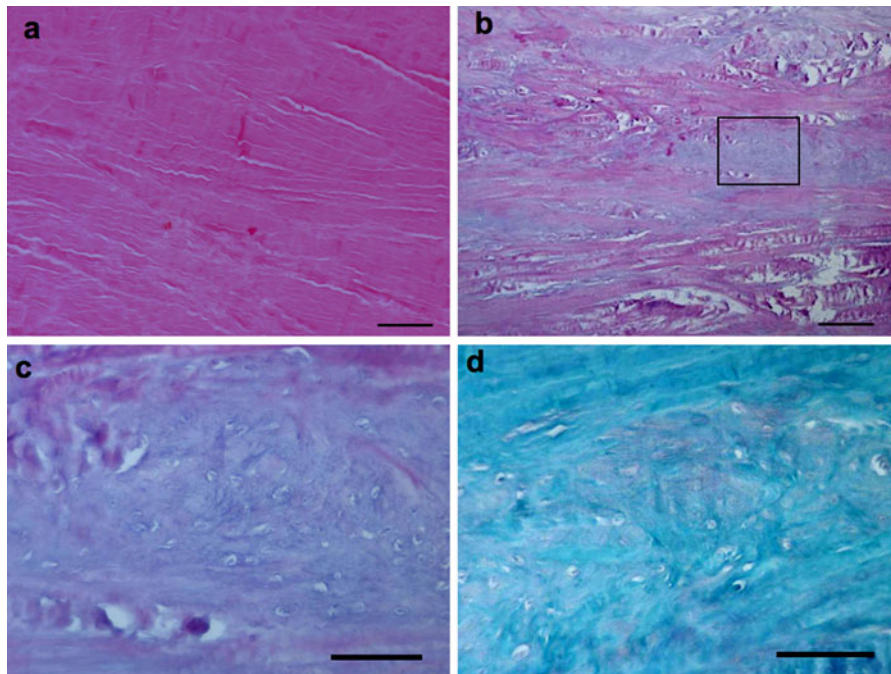
Fig. 4 Histology of migrated chondrocytes within the dense tissue. **a** H.E. stained section of solvent preserved meniscus with recombinant human BMP-2 (rhBMP-2). Migrated chondrocytes and newly-formed extracellular matrix were observed in H.E stained sections. High (×400) magnification is shown below right. **b** H.E. stained section of solvent preserved meniscus without rhBMP-2 as a control. **c** Safranin O stained section of solvent preserved meniscus with rhBMP-2. Migrated chondrocytes and newly-formed extracellular matrix were observed in Safranin O stained sections. High (×400) magnification is shown below right. **d** Safranin O stained section of solvent preserved meniscus without rhBMP-2 as a control

as scaffolds onto which patients' cells could be seeded, or as carriers for transplantation of genetically engineered cells. However, it is difficult to seed cells into high density regular connective tissue which has few interstitial spaces.

We turned our attention to cancer cells to solve the difficulty of cell induction into these connective tissues. Cancer cell migration is critical in the invasive growth of cancer into the connective tissues (Albini et al. 1987). Various growth factors and cytokines are involved in cellular infiltration into the narrow interstitial spaces in a relatively short time (Zigmond and Hirsch 1972). The purpose of this study was to investigate the potential of chondrocytes to infiltrate into dense meniscus tissue in relation to the chemotactic/chemokinetic effect of BMP-2.

BMP-2 has been reported to induce migration of bone marrow derived undifferentiated mesenchymal stem cells, osteoblasts, fibroblasts and cells derived from menisci. Although chondrocyte differentiation by BMP-2 has been well studied (Erickson et al.

1997), the migration response of chondrocytes to BMP-2 has not been investigated. Our results indicate that rhBMP-2 has a chemokinetic effect on chondrocytes. The optimal concentration, 10 ng/ml, for chondrocyte migration is one fifth of that required for induction of cellular differentiation (Erickson et al. 1997). Within the tissue, the local concentration of BMP-2 is predicted to gradually decrease in relation to the distance from the site where the cytokine is synthesized. Thus cell migration may be induced at sites distant from BMP-2 synthesizing cells, while cell differentiation may be induced at sites close to these cells.

We have shown that chondrocytes migrate deeply into the preserved meniscal tissues if induced by BMP-2. Characteristics of the scaffold such as pore size, shape, interconnectivity, and material stiffness or surface chemistry and degradability influence tissue regeneration. Of note, the scaffold pore size is highly related to the proliferation and differentiation of chondrocytes in culture (Bhardwaj et al. 2001; Malda

et al. 2005; Nehrer et al. 1997; Yamane et al. 2007). In our study, we show that BMP-2 induces chondrocyte migration and proteoglycan production throughout the meniscus tissue in vitro. By combining appropriate scaffolds with application of a low concentration of BMP-2, effective application of chemokinesis to cartilage tissue engineering is expected even with seeding of a small number of recipient cells.

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