Journal of Cellular Biochemistry 93:454–462 (2004)

Chondrogenic Differentiation of Murine Embryonic Stem Cells: Effects of Culture Conditions and Dexamethasone

Hiroshi Tanaka,[1,2]* Christopher L. Murphy,[3] Chiho Murphy,[2] Mitsuhiro Kimura,[1,2] Shinya Kawai,[1] and Julia M. Polak[2]

[1]Department of Orthopedic Surgery, Yamaguchi University School of Medicine, 1-1-1 Minamikogushi, Ube, Yamaguchi 755-8505, Japan
[2]Tissue Engineering and Regenerative Medicine Center, Imperial College, London SW10, 9NH, United Kingdom
[3]Kennedy Institute of Rheumatology, Imperial College, 1 Aspenlea Road, London W6 8LH, United Kingdom

Abstract Pluripotent embryonic stem (ES) cells have the capability to differentiate to various cell types and may represent an alternative cell source for the treatment of cartilage defects. Here, we show that differentiation of ES cells toward the chondrogenic lineage can be enhanced by altering the culture conditions. Chondrogenesis was observed in intact embryoid body (EB) cultures, as detected by an increase in mRNA levels for aggrecan and Sox9 genes. Collagen IIB mRNA, the mature chondrocyte-specific splice variant, was absent at day 5, but appeared at later time points. Dexamethasone treatment of alginate-encapsulated EB cultures did not have a strong chondrogenic effect. Nor was chondrogenesis enhanced by alginate encapsulation compared to simple plating of EBs. However, disruption of day 5 EBs and culture as a micromass or pelleted mass, significantly enhanced the expression of the cartilage marker gene collagen type II and the transcription factor Sox9 compared to all other treatments. Histological and immunohistochemical analysis of pellet cultures revealed cartilage-like tissue characterized by metachromatically stained extracellular matrix and type II collagen immunoreactivity, indicative of chondrogenesis. These findings have potentially important implications for cartilage tissue engineering, since they may enable the increase in differentiated cell numbers needed for the in vitro development of functional cartilaginous tissue suitable for implantation. J. Cell. Biochem. 93: 454–462, 2004.

Key words: chondrogenic differentiation; embryonic stem cells; pellet culture; micromass; dexamethasone

Articular cartilage reconstruction is problematic due to its poor capacity for repair once damaged or diseased, and there is no well-established method for restoration available at present. Current strategies for articular cartilage repair have included the use of growth factors in biodegradable matrices, tissue transplantation, and cellular engraftment [Buckwalter, 2002]. Recent studies have suggested that cellular engraftment can be used to augment chondrocyte numbers in articular cartilage [Wakitani et al., 1989]. However, when proliferated in vitro, primary chondrocytes lose phenotype, and such cells may not be suitable in a repair process. Mesenchymal stem cells isolated from the bone marrow have the potential to differentiate to various cells of mesenchymal lineage, including adipocytes, chondrocytes, and osteoblasts [Caplan, 1991; Pittenger et al., 1999]. However, there are some limitations to the use of mesenchymal stem cells for tissue engineering and repair. These include the limited capacity for self-renewal [McCulloch et al., 1991] and, with increasing donor age, proliferative potential is impaired [Tanaka and Liang, 1995; Tanaka et al., 1999]. Identification of an alternative source of donor chondrocytes may thus be needed.

Embryonic stem (ES) cells are stem cell lines of embryonic origin, first isolated from the inner cell mass of mouse blastocysts [Martin, 1981;

Grant sponsor: Dunhill Medical Trust; Grant number: PC1358; Grant sponsor: Grant-in-aid-for Scientific Research (C)(2); Grant number: 14571381.

*Correspondence to: Hiroshi Tanaka, MD, PhD, Department of Orthopedic Surgery, Yamaguchi University School of Medicine, 1-1-1 Minamikogushi, Ube, Yamaguchi 755-8505, Japan. E-mail: htanaka@yamaguchi-u.ac.jp

Received 11 December 2003; Accepted 12 April 2004
DOI 10.1002/jcb.20171

Thomson et al., 1998]. These cells are characterized by their capacity to proliferate in an undifferentiated state for a prolonged period in culture [Martin, 1981; Thomson et al., 1998], and to differentiate to many different cell types by manipulating the culture conditions [Wiles and Keller, 1991; Bain et al., 1995]. In addition, chondrogenic differentiation of ES cells has been demonstrated using a culture system of embryoid body (EB) plating [Kramer et al., 2000; Hegert et al., 2002]. These attributes suggest that ES cells might represent a useful cell source for a much-needed alternative approach to the treatment of cartilage defects.

The aim of this study was to stimulate differentiation of ES cells toward the chondrogenic lineage. In addition to the previously used method of plating EBs [Kramer et al., 2000; Hegert et al., 2002], we also investigated EB encapsulation in alginate, since alginate culture is known to support the chondrocyte phenotype [Hauselmann et al., 1992; Petit et al., 1996]. High cell density micromass and pellet cultures of EB-derived cells were also investigated as both these methods enhance chondrogenesis in mesenchymal precursor cells [Denker et al., 1995]. Finally, we report the effects of dexamethasone, thought to stimulate chondrogenic differentiation [Zimmermann and Cristea, 1993], on EB cultures.

MATERIALS AND METHODS

Culture of ES Cells

A mouse ES cell line denoted E14 was used, as described previously [Hooper et al., 1987]. Unless otherwise stated all reagents were from Life Technologies (Paisley, UK). The ES cell line was monolayer-cultured in Dulbecco's modified Eagle's medium [Martin, 1981] [Thomson et al., 1998]. Medium was supplemented with 10% (vol/vol) batch-tested fetal calf serum, 2 mM L-glutamine, 0.1 mM beta-mercaptoethanol (Sigma-Aldrich, Poole, UK), 50 U/ml penicillin, and 50 mg/ml streptomycin. Culture medium was also supplemented with leukemia inhibitory factor (LIF, 500 U/ml).

Stimulation of ES Cell Differentiation

To stimulate differentiation, ES cell monolayer cultures were lightly trypsinized for 2 min with trypsin-EDTA plus 2% (vol/vol) chicken serum (Sigma-Aldrich) at 37°C. Trypsinization was terminated by addition of DMEM containing 10% FCS. After brief centrifugation, cells were resuspended in medium and plated onto bacteriological grade petri dishes in the absence of fibroblast feeder layers or LIF. Cells were maintained for 5 days in culture, with medium being replenished on day 3. After 5 days in culture, ES cells formed free-floating aggregates or embryoid bodies (EBs) [Martin, 1981; Thomson et al., 1998].

Culture Conditions

EB cultures. Day 5 EBs were kept intact, and plated onto gelatin-coated culture dishes and allowed to attach, or were encapsulated in alginate hydrogel disks. For alginate cultures, a suspension of the EBs in 0.75 ml of 1.5% alginate in 150 mM NaCl was allowed to gel in 100 mM $CaCl_2$ [Hauselmann et al., 1994]. In parallel alginate cultures, medium was additionally supplemented with 1 mM dexamethasone.

EB-derived cultures. The 5-day-old EBs were collected by brief centrifugation and gently dissociated by 0.25% (vol/vol) trypsin-EDTA treatment for 3–5 min at 37°C. These cells were cultured as monolayer, high cell density micromass, or pelleted mass. For monolayer culture, cells were resuspended in culture media and allowed to adhere to six-well culture plates at a density of 5×10^3 cells/cm^2. For micromass culture, cells were resuspended at a density of 20×10^6/ml and 10 μl drops (2×10^5 cells) were spotted onto 24 well culture plates. After 1 h, wells were flooded with 0.5 ml of media. For pelleted mass culture, 2×10^5 cells were spun down in conical tubes in 0.5 ml of media. These cell aggregates were maintained in culture media for 14 days, which was changed every 2–3 days. In all cultures, media consisted of DMEM containing 15% FCS, non-essential amino acid solution (GIBCO BRL, Gaithersburg, MD), L-proline, 50 U/ml penicillin, and 50 mg/ml streptomycin.

Detection of Gene Expression by Semi-Quantitative RT–PCR

Alginate disks were dissolved in 55 mM sodium citrate, 150 mM NaCl, pH 6.8 (solubilization buffer), and gently centrifuged. The pellet were stored at −20°C. Samples of EBs, monolayers, micromasses, or pelleted masses at different time points were washed with phosphate-buffered saline (PBS) and stored at −20°C. Total RNA was isolated using the RNeasy Mini-Kit (Qiagen, Hilden, Germany).

The RNA concentrations were determined by measuring the absorbance at 260 nm. Semi-quantitative RT-PCR was conducted using Ready-To-Go RT-PCR beads (Amersham Pharmacia Biotech, Piscataway, New Jersey) according to the manufacturer's instructions. The number of cycles necessary to amplify cDNA but remain below saturation was determined for each primer set. PCR consisted of 34–45 cycles of 40 s denaturation at 95°C, 40 s annealing at the primer specific temperature (see below), and 50 s elongation at 72°C. Expression of the following genes was studied (oligonucleotide sequences are given in brackets in the order antisense-, sense-primer followed by the annealing temperature used for PCR and length of the amplified fragment): sox9 (5′-TCTTTCTTGTGCTGCACGCGC-3′, 5′-TGGCAGACCAGTACCCGCATCT-3′; 57°C; 135 bp) [Lefebvre et al., 1998], aggrecan (5′-TCCTCTCCGGTGGCAAAGAAGTTG-3′, 5′-CCAAGTTCCAGGGTCACTGTTACCG-3′; 60°C; 270 bp) [Walcz et al., 1994], collagen II (5′-AGGGGTACCAGGTTCTCCATC-3′, 5′-CTGCTCATCGCCGCGGTCCTA-3′; 60°C; 432 bp (splice variant A) and 225 bp (splice variant B) [Metsaranta et al., 1991], collagen I (5′-GAACGGTCCACGATTGCATG-3′, 5′-GGCATGTTGCTAGGCACGAAG-3′; 60°C). Finally, the house-keeping gene GAPDH (5′-CATCACCATCTTCCAGGAGC-3′, 5′-ATGCCAGTGAGCTTCCCGTC-3′; 59°C; 500 bp) was used as previous experiments had shown this to be the most appropriate internal control for differentiating ES cell cultures [Murphy and Polak, 2002]. Electrophoretic separation of PCR products was carried out on 2% agarose gels. Bands were scanned on an Epson flatbed scanner (Epson America, Inc., Long Beach, California), and OD was quantitated using NIH Image version 1.62f for Macintosh (Research Services Branch of the National Institute of Health, NIH).

Histology

Cell aggregates cultured as a pelleted mass at day 21 were fixed in 10% formalin solution, embedded in paraffin, and sections were prepared. The tissue sections were stained with hematoxylin and eosin (H&E), toluidine blue, and safranin O.

Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissues using the avidin–biotin–peroxidase complex method. Slides were incubated with a 1:1,000 dilution of the anti-collagen II antibody (LB1279; LSL/Cosmo Bio, Tokyo, Japan), and immunoperoxidase staining was performed using LSAB2 staining kit (DAKO Japan, Kyoto, Japan) according to the manufacturer's instruction. The antigen-antibody reaction was visualized using 3,3′-diaminobenzidine as chromogen. Slides were counterstained with Mayer's hematoxylin.

RESULTS

Temporal Gene Expression During EB Cultivation

We have analyzed the gene expression pattern of intact EBs either plated on culture dishes or encapsulated in alginate disks over a period of 3 weeks. After EB plating, a significant increase in gene expression for collagen type I, aggrecan, and Sox9 was observed during culture (Fig. 1B). Type II procollagen can be expressed in two forms by alternative splicing; the prechondrogenic splice variant (collagen IIA) and the adult or chondrocyte-specific splice variant (collagen IIB) [Sandell et al., 1991]. The expression level of collagen IIA was somewhat variable, whereas mRNA transcription of collagen IIB was detected at later time points—14 and 21 days after plating (Fig. 1B).

When EBs were encapsulated in alginate disks, gene expression of collagen type I, aggrecan, and Sox9 were significantly increased (Fig. 2B). Collagen type IIA mRNA expression was also enhanced, but the peak was observed at day 7. Collagen IIB transcripts appeared from 7 days onwards, although levels remained relatively low (Fig. 2A).

Effect of Dexamethasone on Gene Expression of EBs

Since dexamethasone was reported to modulate chondrogenic differentiation, we examined the effect of dexamethasone on gene expression of alginate-encapsulated EBs after 1 and 3 weeks culture (Fig. 3). When alginate encapsulated EBs were treated with dexamethasone, collagen type II mRNA expression was significantly enhanced after seven days ($P < 0.05$), while type I collagen gene expression was significantly decreased ($P < 0.01$) and expression of aggrecan and Sox9 was also somewhat reduced. Levels of type IIB collagen were unaffected by dexamethasone treatment.

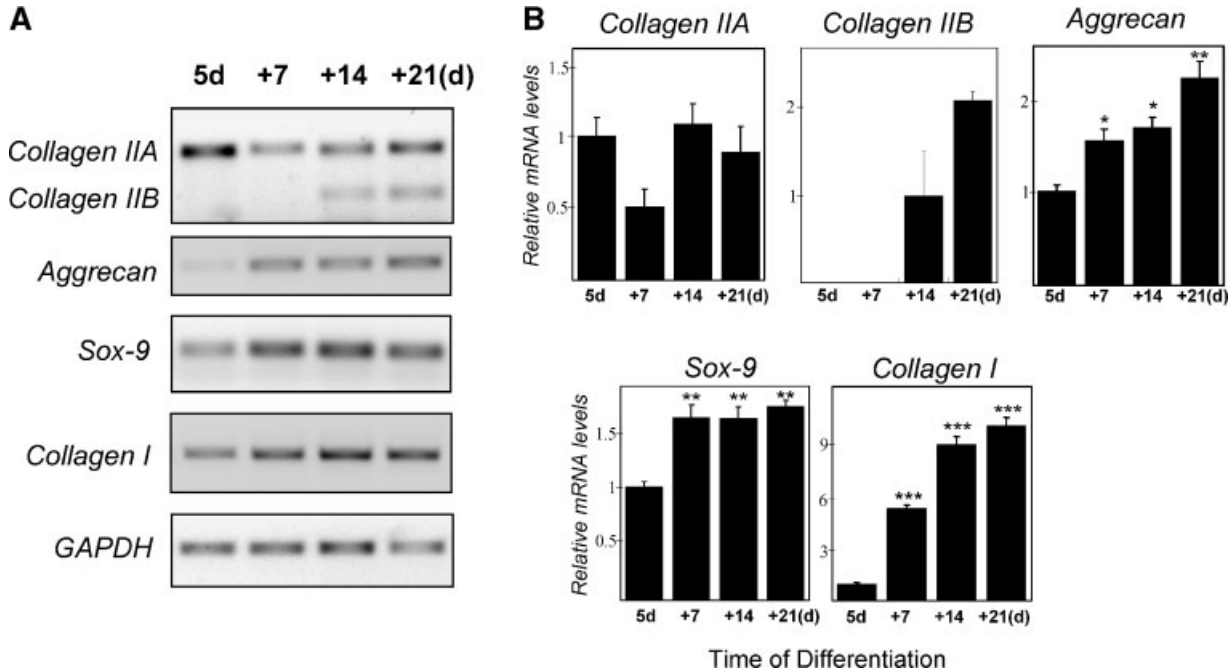

Fig. 1. Gene expression of plated embryoid bodies (EBs). Five-day-old EBs (5d) were plated and cultured for up to 21 days. Total RNA was isolated from the EBs and analyzed by RT-PCR using gene-specific primers. **A**: Representative RT-PCR amplification products are shown. **B**: Mean magnitude of mRNA levels normalized to GAPDH, and expressed relative to '5d' levels. Values are mean ± SE from four independent experiments. Note: for collagen IIB, expression is relative to day +14 levels. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to '5d' levels (unpaired *t*-test).

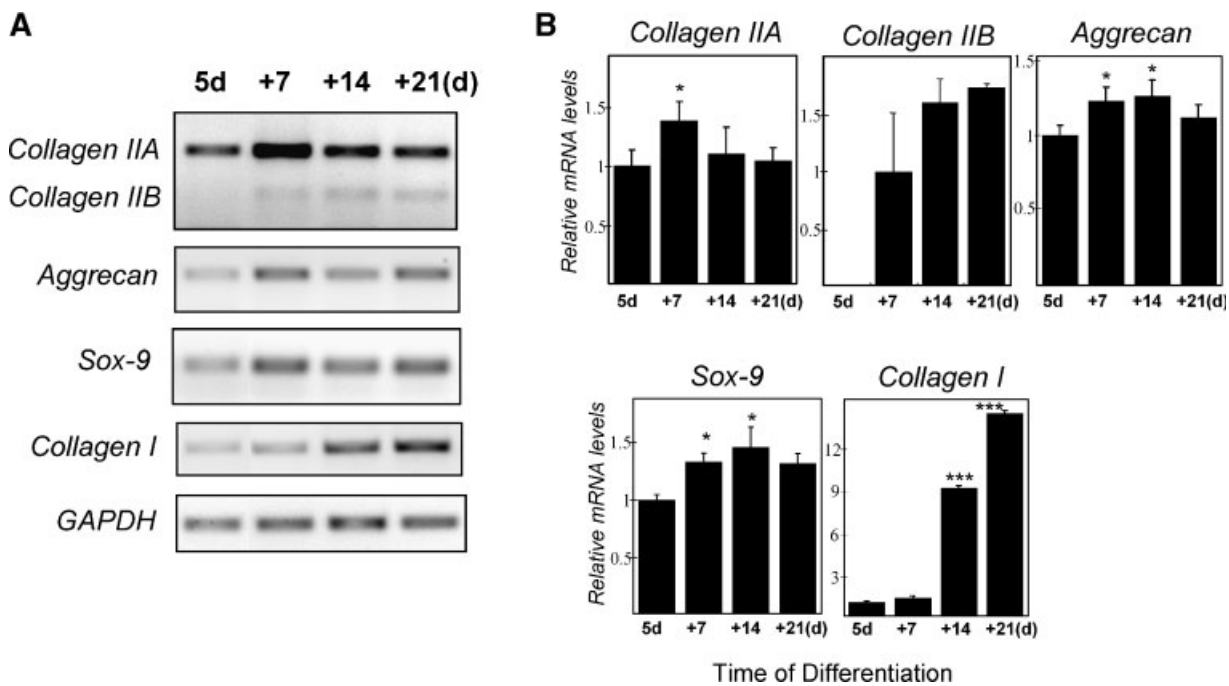

Fig. 2. Gene expression of alginate-encapsulated embryoid bodies (EBs). Five-day-old EBs (5d) were cultured in alginate disks for up to 21 days. Alginate disks were dissolved in solubilization buffer for separation of EBs. Total RNA was isolated and analyzed by RT-PCR using gene-specific primers. **A**: Representative RT-PCR amplification products are shown. **B**: Mean magnitude of mRNA levels normalized to GAPDH, and expressed relative to '5d' levels. Values are mean ± SE from four independent experiments. Note: for collagen IIB, expression is relative to day +7 levels. $^{*}P < 0.05$, $^{***}P < 0.001$ compared to '5d' levels (unpaired *t*-test).

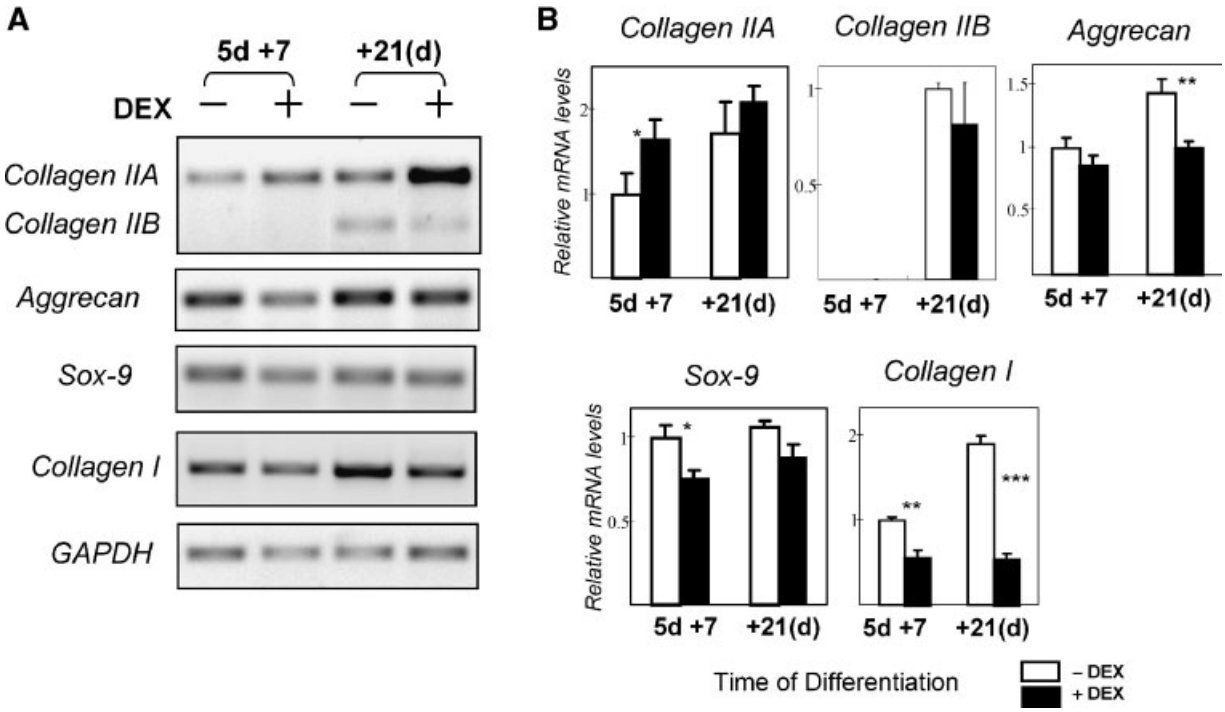

Fig. 3. Effect of dexamethasone on gene expression of embryoid bodies (EBs). Five-day-old EBs (5d) were cultured in alginate disks for 7 or 21 days with or without dexamethasone. Total RNA was isolated from the EBs and analyzed by RT-PCR using gene-specific primers. **A**: Representative RT-PCR amplification products are shown. **B**: Mean magnitude of mRNA levels normalized to GAPDH, and expressed relative to '5d+7' (−DEX) levels. Values are mean ± SE from four independent experiments. Note: for collagen IIB, expression is relative to '+21(d)' (−DEX) levels. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared to control (−DEX) levels (unpaired *t*-test).

Effects of the in Vitro Culture Conditions on the Expression of Cartilage Marker Genes

Next, we investigated the effect of disrupting the EBs and culturing as monolayer, high cell density micromass, or cell pellets (Fig. 4). Intact EB cultures were run in parallel. All marker genes were detected in the various culture conditions. Comparing to monolayer levels, expression of type II collagen was enhanced

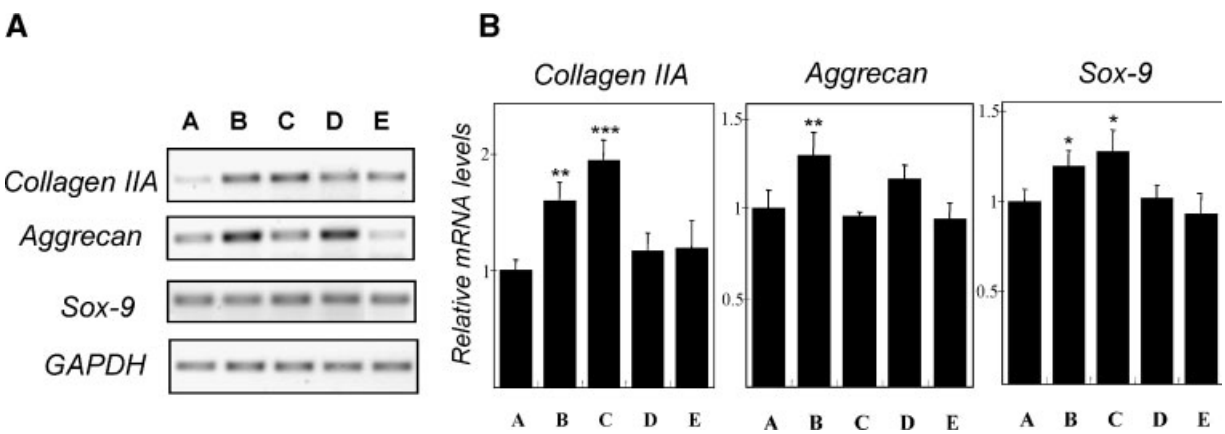

Fig. 4. Effect of the in vitro culture conditions on the expression of cartilage marker genes. Five-day-old embryoid bodies (EBs) were either gently dissociated and cultured as monolayer (**A**), micromass (**B**), or pelleted mass (**C**); or intact EBs were plated (**D**) or encapsulated in alginate disks (**E**). After 14 days culture, total RNA was isolated and analyzed by RT-PCR using gene-specific primers. A: Representative RT-PCR amplification products are shown. B: Mean magnitude of mRNA levels normalized to GAPDH, and expressed relative to monolayer culture levels (A). Values are mean ± SE from four independent experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared to monolayer (A) levels (unpaired *t*-test).

for both micromass ($P < 0.01$), and pellet cultures ($P < 0.001$). (Fig. 4B). In addition, Sox9 expression was significantly upregulated under both of these conditions ($P < 0.05$). The expression of aggrecan mRNA was also enhanced in micromass cultures ($P < 0.01$). Monolayers (A) and intact EB cultures (D and E) expressed lest chondrogenic phenotype in these experiments, and were not significantly different ($P > 0.05$) (Fig. 4B).

Histology and Immunohistochemistry of EB-Derived Pellet Cultures

Collagen type II, the major protein component of cartilage, was expressed most highly in pellet mass culture. Thus, histology and immunohistochemistry was performed on these pellet cultures. Photomicrographs of pelleted mass sections showed that cells were round and resembled hyaline chondrocytes, forming cartilaginous lacunae (Fig. 5A,B). Cells were separated by extensive regions of diffuse, metachromatically stained extracellular matrix, indicative of a chondrogenic stage of differentiation (Fig. 5C,D). Immunohistochemical analysis of these tissues demonstrated a type II collagen-containing matrix. Type II collagen immunoreactivity was found extracellularly, predominantly within the pericellular matrix of chondrocytes (Fig. 5E). Control sections that were stained only with the secondary antibody showed no staining indicating the specificity of the response (data not shown).

DISCUSSION

Recent studies have documented chondrogenic differentiation of ES cells using a culture system of EB plating [Kramer et al., 2000; Hegert et al., 2002]. Our initial experiments therefore focused on intact EB cultures. In addition to plating EBs, we also investigated EB encapsulation in alginate, since the alginate culture system is known to support the chondrocyte phenotype [Hauselmann et al., 1992; Petit et al., 1996]. Levels of mRNA for collagen type I upregulated dramatically from 5 to 21 days of EB cultivation (Figs. 1, 2), suggesting

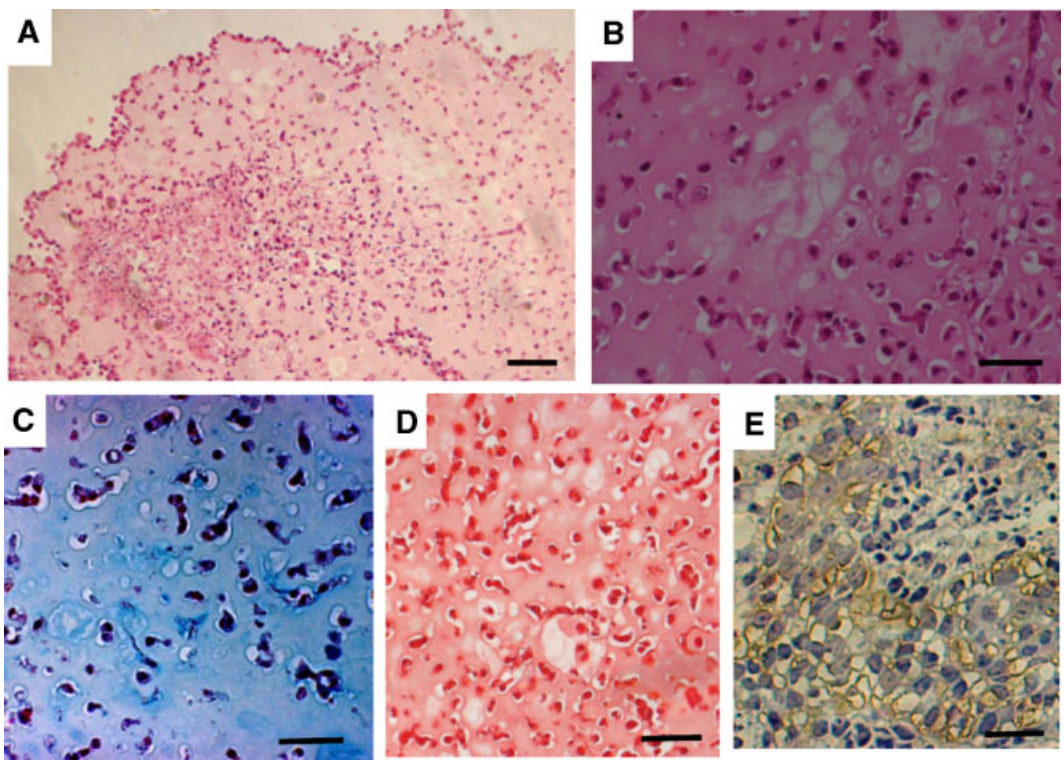

Fig. 5. Histological analysis of pellet cultures. Day 5 EBs were disrupted and cultured as pellets for a further 21 days. **A**: Paraffin sections of cell pellets were stained with hematoxylin and eosin—shown in higher power in (**B**). Cartilagenous tissue formation in the cell pellets was assessed with Toluidine blue staining (**C**), safranin-O staining (**D**), and type II collagen immunoreactivity (**E**). For the latter, antigen was detected using an avidin–biotin–peroxidase system visualized with DABA. Note: Bar = 40 μm for B–E and 120 μm for A. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

undifferentiated ES cells, mesenchymal intermediate cells, or osteoblastic cells may also be proliferating in this phase—all these cells might express high levels of collagen type I. However, another possible contributing factor is outgrowth of monolayer-forming cells from the EBs, which occurred in both plated and encapsulated cultures. Collagen IIB mRNA (the mature chondrocyte-specific splice variant) was absent at day 5, but appeared at later time-points. Similar observations have been documented previously, showing that the collagen type IIA gene was already expressed at the day of plating, while collagen IIB gene was detected later at day 7 after plating [Hegert et al., 2002]. Aggrecan, Sox9, and collagen IIB, all cartilage-associated marker genes, were increased during EB cultivation for both plated and encapsulated cultures, indicating chondrogenic differentiation of ES cells. However, alginate encapsulation of EBs did not increase chondrogenic differentiation over EB plating.

In pellet culture [Kato et al., 1988; Ballock and Reddi, 1994] and high cell density micromass culture [Ahrens et al., 1977; Denker et al., 1995], cells form three-dimensional aggregates that allow cell–cell interactions similar to those observed in precartilage condensations found during embryonic development. Therefore, in an attempt to enhance the level of chondrogenic differentiation from ES cells, we disrupted 5-day-old EBs and cultured the released cells as high cell density micromasses, or pelleted masses. In both micromass and pelleted mass cultures, the expression of cartilage marker genes was significantly enhanced. Histology of cultured pellet masses revealed cartilage-like tissues stained with an anti-type II collagen antibody. This suggests that a high-density microenvironment is favorable for chondrogenic differentiation of ES cells—similar to the case of mesenchymal stem cells. Interestingly, at day 14, the aggrecan gene expression pattern did not closely reflect that of collagen II and Sox9 for the various treatments (Fig. 4). This may be due, in part, to the fact that we are looking at a snap-shot of mRNA levels, since we have previously shown that for redifferentiating chondrocyte cultures, the temporal expression of aggrecan and collagen II did not shadow that of Sox9 [Murphy and Polak, 2004]. It appears that other factors are also involved in the regulation of these cartilage matrix proteins. In addition, it may be that the pelleted cultures downregulated aggrecan expression at an earlier time-point due to the enhanced cell density in these cultures. Further time course experiments would shed more light on these issues.

Previous in vitro studies have demonstrated that growth factors as well as hormones can influence anabolic and catabolic processes in chondrocytes. BMPs [Duprez et al., 1996], TGF-b [Seyedin et al., 1988], bFGF [Sachs et al., 1982; Froger-Gaillard et al., 1989], and IGF-I [Hunziker et al., 1994] each can stimulate mitotic activity, proteoglycan synthesis, and chondrogenic differentiation. Several in vitro studies demonstrated that dexamethasone has the potency to induce multiple end-phenotypes [Grigoriadis et al., 1990; Shalhoub et al., 1992]. Dexamethasone is not a specific chondrogenic differentiation factor, however, several publications have shown that dexamethasone facilitated chondrogenic differentiation of undifferentiated mesenchymal cells. Dexamethasone induced chondrogenesis in organoid cultures of murine embryonic cells [Zimmermann and Cristea, 1993] and mesodermal progenitor cells [Poliard et al., 1995]. In the current study, dexamethasone did not appear to strongly stimulate chondrogenic differentiation of EBs encapsulated in alginate. Although collagen type IIA mRNA expression was significantly enhanced, type IIB was not. Type I collagen gene expression was significantly decreased by dexamethasone. However, this may be partly due to a reduction of outgrowth of monolayer-forming cells in the presence dexamethasone. Further work will be needed to investigate the effect of dexamethasone under different culture conditions, for example, pellet or micromass cultures.

In this study, we have shown that ES cells can differentiate into chondrocytes in vitro, and that this process is enhanced in high cell density cultures. These findings have important implications for cartilage tissue engineering since they enable the increase in differentiated cell numbers needed for the in vitro development of functional cartilaginous tissue suitable for implantation. Furthermore, this system may be useful for studying early chondrogenesis with respect to its initiation and specific factors that can influence cartilage repair. However, successful use of ES-derived donor cells would require the generation of essentially pure chondrocyte cultures, as engraftment of ES cells would likely result in teratoma formation

[Wakitani et al., 2003]. For this purpose, it will be worthwhile to further develop strategies to enrich for ES cell-derived chondrocytes, which include in vitro conditions, growth factors, immunological selection, and genetic selection. In addition, if chondrocyte engraftment proves to be of therapeutic value, the generation of human chondrogenic ES cell lines would be needed.

REFERENCES

Ahrens PB, Solursh M, Reiter RS. 1977. Stage-related capacity for limb chondrogenesis in cell culture. Dev Biol 60:69–82.

Bain G, Kitchens D, Yao M, Huettner JE, Gottlieb DI. 1995. Embryonic stem cells express neuronal properties in vitro. Dev Biol 168:342–357.

Ballock RT, Reddi AH. 1994. Thyroxine is the serum factor that regulates morphogenesis of columnar cartilage from isolated chondrocytes in chemically defined medium. J Cell Biol 126:1311–1318.

Buckwalter JA. 2002. Articular cartilage injuries. Clin Orthop 21–37.

Caplan AI. 1991. Mesenchymal stem cells. J Orthop Res 9: 641–650.

Denker AE, Nicoll SB, Tuan RS. 1995. Formation of cartilage-like spheroids by micromass cultures of murine C3H10T1/2 cells upon treatment with transforming growth factor-beta 1. Differentiation 59:25–34.

Duprez D, Bell EJ, Richardson MK, Archer CW, Wolpert L, Brickell PM, Francis-West PH. 1996. Overexpression of BMP-2 and BMP-4 alters the size and shape of developing skeletal elements in the chick limb. Mech Dev 57: 145–157.

Froger-Gaillard B, Charrier AM, Thenet S, Ronot X, Adolphe M. 1989. Growth-promoting effects of acidic and basic fibroblast growth factor on rabbit articular chondrocytes aging in culture. Exp Cell Res 183:388–398.

Grigoriadis AE, Heersche JN, Aubin JE. 1990. Continuously growing bipotential and monopotential myogenic, adipogenic, and chondrogenic subclones isolated from the multipotential RCJ 3.1 clonal cell line. Dev Biol 142:313–318.

Hauselmann HJ, Aydelotte MB, Schumacher BL, Kuettner KE, Gitelis SH, Thonar EJ. 1992. Synthesis and turnover of proteoglycans by human and bovine adult articular chondrocytes cultured in alginate beads. Matrix 12:116–129.

Hauselmann HJ, Fernandes RJ, Mok SS, Schmid TM, Block JA, Aydelotte MB, Kuettner KE, Thonar EJ. 1994. Phenotypic stability of bovine articular chondrocytes after long-term culture in alginate beads. J Cell Sci 107: 17–27.

Hegert C, Kramer J, Hargus G, Muller J, Guan K, Wobus AM, Muller PK, Rohwedel J. 2002. Differentiation plasticity of chondrocytes derived from mouse embryonic stem cells. J Cell Sci 115:4617–4628.

Hooper M, Hardy K, Handyside A, Hunter S, Monk M. 1987. HPRT-deficient (Lesch–Nyhan) mouse embryos derived from germline colonization by cultured cells. Nature 326:292–295.

Hunziker EB, Wagner J, Zapf J. 1994. Differential effects of insulin-like growth factor I and growth hormone on developmental stages of rat growth plate chondrocytes in vivo. J Clin Invest 93:1078–1086.

Kato Y, Iwamoto M, Koike T, Suzuki F, Takano Y. 1988. Terminal differentiation and calcification in rabbit chondrocyte cultures grown in centrifuge tubes: Regulation by transforming growth factor beta and serum factors. Proc Natl Acad Sci USA 85:9552–9556.

Kramer J, Hegert C, Guan K, Wobus AM, Muller PK, Rohwedel J. 2000. Embryonic stem cell-derived chondrogenic differentiation in vitro: Activation by BMP-2 and BMP-4. Mech Dev 92:193–205.

Lefebvre V, Li P, de Crombrugghe B. 1998. A new long form of Sox5 (L-Sox5), Sox6 and Sox9 are coexpressed in chondrogenesis and cooperatively activate the type II collagen gene. Embo J 17:5718–5733.

Martin GR. 1981. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci USA 78: 7634–7638.

McCulloch CA, Strugurescu M, Hughes F, Melcher AH, Aubin JE. 1991. Osteogenic progenitor cells in rat bone marrow stromal populations exhibit self-renewal in culture. Blood 77:1906–1911.

Metsaranta M, Toman D, de Crombrugghe B, Vuorio E. 1991. Mouse type II collagen gene. Complete nucleotide sequence, exon structure, and alternative splicing. J Biol Chem 266:16862–16869.

Murphy CL, Polak JM. 2002. Differentiating embryonic stem cells: GAPDH, but neither HPRT nor beta-tubulin is suitable as an internal standard for measuring RNA levels. Tissue Eng 8:551–559.

Murphy CL, Polak JM. 2004. Control of human articular chondrocyte differentiation by reduced oxygen tension. J Cell Physiol 199:451–459.

Petit B, Masuda K, D'Souza AL, Otten L, Pietryla D, Hartmann DJ, Morris NP, Uebelhart D, Schmid TM, Thonar EJ. 1996. Characterization of crosslinked collagens synthesized by mature articular chondrocytes cultured in alginate beads: Comparison of two distinct matrix compartments. Exp Cell Res 225:151–161.

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. 1999. Multilineage potential of adult human mesenchymal stem cells. Science 284:143–147.

Poliard A, Nifuji A, Lamblin D, Plee E, Forest C, Kellermann O. 1995. Controlled conversion of an immortalized mesodermal progenitor cell towards osteogenic, chondrogenic, or adipogenic pathways. J Cell Biol 130: 1461–1472.

Sachs BL, Goldberg VM, Moskowitz RW, Malemud CJ. 1982. Response of articular chondrocytes to pituitary fibroblast growth factor (FGF). J Cell Physiol 112:51–59.

Sandell LJ, Morris N, Robbins JR, Goldring MB. 1991. Alternatively spliced type II procollagen mRNAs define distinct populations of cells during vertebral development: Differential expression of the amino-propeptide. J Cell Biol 114:1307–1319.

Seyedin SM, Rosen DM, Segarini PR. 1988. Modulation of chondroblast phenotype by transforming growth factor-beta. Pathol Immunopathol Res 7:38–42.

Shalhoub V, Conlon D, Tassinari M, Quinn C, Partridge N, Stein GS, Lian JB. 1992. Glucocorticoids promote development of the osteoblast phenotype by selectively modulating expression of cell growth and differentiation associated genes. J Cell Biochem 50:425–440.

Tanaka H, Liang CT. 1995. Effect of platelet-derived growth factor on DNA synthesis and gene expression in bone marrow stromal cells derived from adult and old rats. J Cell Physiol 164:367–375.

Tanaka H, Ogasa H, Barnes J, Liang CT. 1999. Actions of bFGF on mitogenic activity and lineage expression in rat osteoprogenitor cells: Effect of age. Mol Cell Endocrinol 150:1–10.

Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. 1998. Embryonic stem cell lines derived from human blastocysts. Science 282:1145–1147.

Wakitani S, Kimura T, Hirooka A, Ochi T, Yoneda M, Yasui N, Owaki H, Ono K. 1989. Repair of rabbit articular surfaces with allograft chondrocytes embedded in collagen gel. J Bone Joint Surg Br 71:74–80.

Wakitani S, Takaoka K, Hattori T, Miyazawa N, Iwanaga T, Takeda S, Watanabe TK, Tanigami A. 2003. Embryonic stem cells injected into the mouse knee joint form teratomas and subsequently destroy the joint. Rheumatology (Oxford) 42:162–165.

Walcz E, Deak F, Erhardt P, Coulter SN, Fulop C, Horvath P, Doege KJ, Glant TT. 1994. Complete coding sequence, deduced primary structure, chromosomal localization, and structural analysis of murine aggrecan. Genomics 22:364–371.

Wiles MV, Keller G. 1991. Multiple hematopoietic lineages develop from embryonic stem (ES) cells in culture. Development 111:259–267.

Zimmermann B, Cristea R. 1993. Dexamethasone induces chondrogenesis in organoid culture of cell mixtures from mouse embryos. Anat Embryol (Berl) 187:67–73.