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Hyaluronic acid and autologous synovial fluid induce chondrogenic differentiation of equine mesenchymal stem cells: a preliminary study

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Abstract

Mesenchymal stem cells (MSC) have the potential to differentiate into distinct mesenchymal tissues including cartilage, which suggest these cells as an attractive cell source for cartilage tissue engineering approaches. Our objective was to study the effects of TGF-β1, hyaluronic acid and synovial fluid on chondrogenic differentiation of equine MSC. For that, bone marrow was aspirated from the tibia of one 18-month-old horse (Haflinger) and MSC were isolated using percoll-density centrifugation. To promote chondrogenesis, MSC were centrifuged to form a micromass and were cultured in a medium containing 10 ng/ml TGF-β1 or 0.1 mg/ml hyaluronic acid (Hylartil®, Ostenil®) or either 5%, 10% or 50% autologous synovial fluid as the chondrogenesis inducing factor. Differentiation along the chondrogenic lineage was documented by type II collagen and proteoglycan expression. MSC induced by TGF-β1 alone showed the highest proteoglycan expression. Combining TGF-β1 with hyaluronic acid could not increase the proteoglycan expression. Cultures stimulated by autologous synovial fluid (independent of concentration) and hyaluronic acid demonstrated a pronounced, but lower proteoglycan expression than cultures stimulated by TGF-β1. The expression of cartilage-specific type II collagen was high and about the same in all stimulated cultures. In summary, hyaluronic acid and autologous synovial fluid induces chondrogenesis of equine mesenchymal stem cells, which encourage tissue engineering applications of MSC in chondral defects, as the natural environment in the joint is favorable for chondrogenic differentiation.

Keywords: Equine bone marrow; Mesenchymal stem cells; Chondrogenesis stimulating factors; Cartilage; Tissue engineering

1. Introduction

The demand for effective treatment strategies of cartilage defects is continually increasing, especially considering the growing number of patients with degenerative diseases of the skeletal system. Osteoarthritis (OA) is a common cause of morbidity and loss of athletic performance not only in humans but also in horses (Jeffcott et al., 1982). Horses have naturally occurring OA, which is similar to that of humans. Therefore, new therapeutic strategies originally developed for horses may also be suitable for the treatment of human OA (Frisbie et al., 2002).

For cartilage repair at present, autologous chondrocytes are isolated, expanded ex vivo and re-implanted into the defect site, either as a cell suspension combined with a periosteal flap or seeded onto a biocompatible carrier material (Brittberg et al., 1994; Erggelet et al., 2003). The necessity to expand the cells in monolayer culture causes a dedifferentiation of the chondrocytes into fibroblastic cells, which has to be reversed by challenging and labor-intensive cell culture techniques (Benya and Shaffer, 1982). Because of that, and because of supply limitations of autologous chondrocytes

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for transplantation procedures, much attention was drawn to mesenchymal stem cells (MSC).

MSC can be found in bone marrow and give rise to different mesenchymal tissues, such as bone and cartilage (Owen and Friedenstein, 1988; Caplan, 1994). MSC of different species, such as human and equine, can be isolated and grown in vitro (Haynesworth et al., 1992; Fortier et al., 1998; Pittenger et al., 1999). Important characteristics of MSC are their ability to proliferate extensively in culture with an attached fibroblastic morphology and their in vitro multilineage potential (Pittenger et al., 1999). MSC have therefore the advantage that, in osteochondral defects, they are able to repair subchondral bone, as well as the covering articular cartilage layer (Wakitani et al., 1994). In a recent study, autologous MSC were used for the repair of cartilage defects in human osteoarthritic knees (Wakitani et al., 2002). Although the transplantation of human MSC resulted in an improved tissue development, the clinical benefit was not significantly enhanced.

The signals that induce the development of MSC into chondrocytes are not known in detail. Chondrogenic differentiation of equine MSC in monolayer cultures can be induced with transforming growth factor-β1 (TGF-β1) (Worster et al., 2000). In addition, chondrogenesis can be stimulated by forming a Pelleted micromass of MSC in a defined medium, supplemented with TGF-β1 or TGF-β3 (Johnstone et al., 1998; Mackay et al., 1998; Ringe et al., 2002). Factors like bone morphogenetic proteins (BMP)-2, -6, and -7 may enhance cartilage formation (Sekiya et al., 2001; Schmitt et al., 2003).

Hyaluronic acid (HA) based polymers alone or in combination with MSC were tested for their ability to enhance repair of osteochondral defects (Radice et al., 2000; Solchaga et al., 2000). HA plays an important role in morphogenesis. At that stage, cells divide and migrate within an extracellular matrix that is rich in HA. Modulations in both concentration and organization of HA accompany the cellular changes that occur as tissues and organs differentiate (Toole, 1991, 2001). HA is also a component of synovial fluid, in which it is secreted by synoviocytes, as are glycoproteins and protease inhibitors (Lynch et al., 1998). Synovial fluid nourishes articular cartilage, lubricates articular joint surfaces, functions as a shock absorber and contains growth factors like TGF-β1 (Okazaki et al., 2001). Both HA and synovial fluid were shown to have the potential to induce chondrogenic differentiation in chicken limb bud bioassays (Kujawa et al., 1986; Rodrigo et al., 1995; Maleski and Knudson, 1996a).

We hypothesized that hyaluronic acid, both alone and in combination with TGF-β, as well as autologous synovial fluid, have the potential to differentiate MSC along the chondrogenic lineage. Therefore, the purpose of the study reported here was to determine the chondrogenic effects of TGF-β1, the hyaluronic acids Ostenil® and Hylartil®, and autologous synovial fluid on high-density micromass cultures of equine mesenchymal stem cells. The results potentially will encourage tissue engineering applications of MSC in human and equine articular cartilage defects, as the natural environment is favorable for chondrogenic differentiation.

2. Materials and methods

2.1. Isolation and culture of equine MSC

Bone marrow aspirate was obtained at the Research Center of Medical Technology and Biotechnology in Bad Langensalza aseptically from the tibia of one 18-month-old horse (Haflinger) in this preliminary study. For collection of aspirates, horses were sedated with 0.08 mg/kg of body weight romifidine hydrochloride (Boehringer Ingelheim, Germany). The localization of the puncture was clean shaved, the harvest site was clipped, aseptically prepared, and infiltrated with 20 ml of 2% mepivacaine hydrochloride (Intervet, Germany). Bone marrow needles were used to aspirate marrow into 20 ml syringes containing 25,000 units of heparin-natrium (B. Braun Melsungen, Germany). Five milliliters of bone marrow was aspirated in one 20 ml syringe. Equine MSC were isolated according to modified protocols already established for human MSC (Haynesworth et al., 1992). Briefly, the bone marrow aspirate (10 ml) was washed with phosphate-buffered saline (PBS) (Biochrom, Berlin, Germany) and resuspended in DME-medium (Biochrom) supplemented with 10% fetal bovine serum of selected lots (Lennon et al., 1996) (Biochrom). Cells were purified by using a percoll-gradient of a density of 1.077 g/ml (Biochrom) and resuspended in DME-medium. 1×10^7 cells, mostly blood cells, were plated at a density of 200,000 cells/cm^2. The medium was changed after 72 h and then every 2–3 days thereafter. After reaching 90% confluence, cells were detached by the addition of a solution containing 0.5% trypsin–EDTA (Biochrom) and were replated at a density of 6000 cells/cm^2.

For cryopreservation, 1×10^6 equine MSC were suspended into a cryo medium consisting of 10% DMEM, 10% DMSO and 80% FBS, frozen at −80 °C for 24 h and then stored at −196 °C.

2.2. Cell differentiation studies

For chondrogenic differentiation, 250,000 cryopreserved equine MSC (passage 2) were centrifuged to form a pelleted micromass (Johnstone et al., 1998). Cells were cultured in a defined medium consisting of DME-medium, ITS + 1 (10 mg/l insulin, 5 mg/l transferrin, 5 μg/l selenium, 0.5 mg/ml bovine serum albumin and 4.7 μg/ml linoleic acid), 1 mM sodium-pyruvate, 100 nM dexamethasone, 0.35 mM proline and 0.17 mM L-ascorbic acid-2-phosphate (all purchased from Sigma, USA). TGF-β1 (R&D Systems, USA), hyaluronic acid and autologous synovial fluid were added to evaluate their influence on chondrogenic differentiation. Two kinds of hyaluronic acids were used: Hylartil® (Pharmacia & Upjohn, Germany) with a molecular weight (MW) of 3×10^6 Da and Ostenil® (Chemedica, Ger-

many) with a MW of 2×10^6 Da. Ten milliliters of autologous synovial fluid was obtained from the tarsometatarsal joint at the Research Center of Medical Technology and Biotechnology in Bad Langensalza. Arthrocentesis of this joint was performed from a lateral approach proximal of the fourth metatarsal bone. The synovial fluid was immediately centrifuged to clear it from cells, and was stored at 4 °C.

The following combinations were evaluated to induce chondrogenesis: (1) 10 ng/ml TGF-β1, (2) 0.1 mg/ml Hylartil®, (3) 0.1 mg/ml Ostenil®, (4) 10 ng/ml TGF-β1 and 0.1 mg/ml Hylartil®, (5) 10 ng/ml TGF-β1 and 0.1 mg/ml Ostenil®, (6) 5%, 10% and 50% autologous synovial fluid and (7) the defined medium as described above as a control. The medium was changed every 2 days and the high-density micromass cultures were cultured for 26 days. For histological staining, pellets were cryopreserved in OCT (Sakura, The Netherlands) and 6 μm sections were used for further analysis.

2.3. Histological methods

Proteoglycans, typically found in cartilage matrix, were visualized with Alcian blue (Roth, Germany) at pH 2.5. Quantitative analysis of the staining was performed with Adobe Photoshop 4.0 software as described previously (Sittinger et al., 1994). In brief, for analysis of histological staining, a distinct standard was defined, which represents the particular color for the specific staining. The tool "color range" was used to select areas of that particular color. The selected area is given as a number of pixels shown in "image histogram." Subsequently, the percentage of the stained pixels in relation to the total number of pixels per specimen area was determined. Image analysis was performed with three to six stained micrographs for each individual experiment. Subsequently, mean values and standard deviation (S.D.) were determined.

2.4. Immunohistochemistry

Cryosections (6 μm) of chondrogenic cultures were mounted on aminoalkylsilane-coated slides, fixed for 5 min in acetone, and stored at −20 °C. For immunohistochemistry, sections were incubated for 5 min in 1% H_2O_2 and blocked with 10% goat serum (DAKO, Germany) in phosphate-buffered saline for 30 min. Slides were incubated for 1 h with primary antibody at room temperature (rabbit anti-human type II collagen antibody) (DPC Biermann, Germany). Slides were then incubated with biotinylated anti-rabbit antibody (DAKO) for 30 min. After subsequent washes, slides were covered with peroxidase-conjugated streptavidine (DAKO). The color reaction was developed by 3-amino-9-ethylcarbazol (AEC) (DAKO) substrate, followed by counterstaining using hematoxylin (Sigma).

3. Results

3.1. Mesenchymal stem cell harvest and culture

Equine mesenchymal stem cells were harvested from bone marrow aspirates using a percoll-gradient of a density of 1.077 g/ml. 1×10^7 bone marrow-derived cells were plated, consisting mostly of round shaped erythrocytes and nonadherent hematopoietic cells (data not shown). By day 8, nonadherent hematopoietic cells were removed due to the exchange of the culture medium. At this time, equine MSC appeared as single stretched cells and, as they multiplied, they led to large clusters of stellate cells (Fig. 1a). Further cultivation revealed proliferation of MSC growing outward in a swirling fibroblast-like pattern. Equine MSC, which were cultivated and expanded, still presented a stable fibroblast-like phenotype and demonstrated no obvious reduction in mitogenic properties (Fig. 1b).

3.2. Chondrogenic differentiation

To study the influence of TGF-β1, the hyaluronic acids Hylartil® and Ostenil®, and autologous synovial fluid on chondrogenic differentiation, equine MSC (passage 2) were cultured up to 26 days in high-density micromass cultures ("pellets"). The potential of MSC to differentiate along the chondrogenic lineage was characterized by the staining of

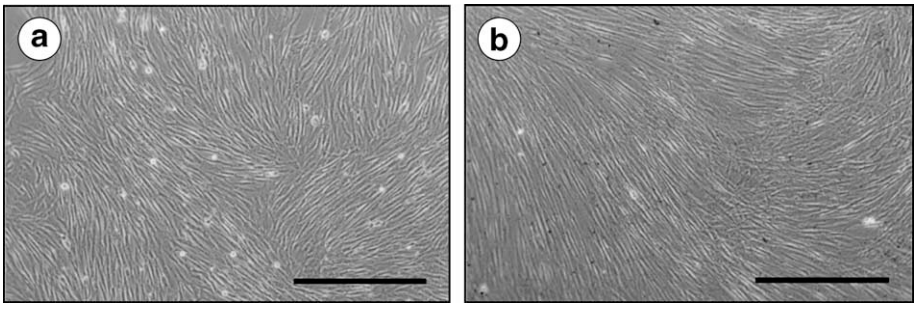

Fig. 1. Phase-contrast photomicrographs of equine mesenchymal stem cells (MSC) grown in culture. Equine bone marrow-derived cells were purified according to protocols described for human MSC. Cells were plated and consisted mostly of round shaped erythrocytes and nonadherent hematopoietic cells. (a) By day 8, equine MSC exhibited a stretched fibroblast-like phenotype and large swirls of cells had formed. (b) Sub-cultivated equine MSC expanded up to passage 2 presented a stable fibroblast-like morphology and were used for chondrogenic differentiation. Bar = 250 μm for (a) and (b).

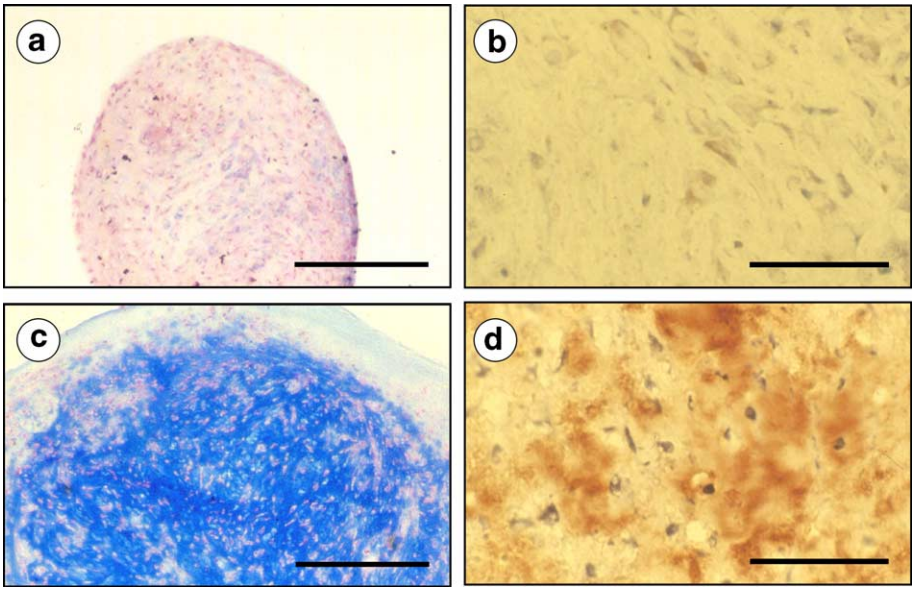

Fig. 2. Photomicrographs of histochemical analysis of equine MSC (passage 2) undergoing TGF-β1-induced chondrogenesis. (a) High-density micromass control cultures, treated with dexametasone alone, showed a weak expression of cartilage extracellular matrix proteoglycan at day 26, as demonstrated by Alcian blue staining, and (b) no type II collagen expression, as indicated by negative monoclonal antibody staining. (c) By day 26, 10 ng/ml TGF-β1 chondrogenic-induced pellets were larger and showed strong expression of proteoglycan, and (d) of type II collagen. Bar = 250 μm for (a) and (c) and 65 μm for (b) and (d).

extracellular cartilage matrix proteoglycans with Alcian blue at pH 2.5. Type II collagen, as the crucial component of extracellular cartilage matrix, was identified by immunohistochemistry. In all pellets, including the dexamethasone-treated control pellets, proteoglycans were found (Figs. 2–4). Type II collagen could be detected in all induced pellets, but not in the control pellets (Figs. 2–4). Proteoglycans and type II collagen were focused more in the central part of the high-density micromass cultures (Figs. 2–4), whereas type I collagen was found more in the border zones (data not shown).

3.3. TGF-β1 and hyaluronic acid as chondrogenesis inducing factors

In comparison to the control pellets (Fig. 2a and b), 10 ng/ml TGF-β1 induced high-density cultures showed a high secretion of extracellular matrix proteoglycans (Fig. 2c), type II collagen (Fig. 2d), and a pronounced increase in pellet size (Fig. 2a and c). Compared to TGF-β1-treated cells, equine MSC, stimulated with 0.1 mg/ml of the hyaluronic acids Hylartil® or Ostenil®, demonstrated – independent of the tested hyaluronic acid – a clearly lower Alcian blue staining and pellet size (Fig. 3a and c), but a comparable type II collagen expression (Fig. 3b and d). Chondrogenic induction with a combination of 10 ng/ml TGF-β1 and 0.1 mg/ml hyaluronic acids (Hylartil® or Ostenil®) leads to a proteoglycan and type II collagen secretion and a pellet size similar to 10 ng/ml TGF-β1 induced pellets (Fig. 3e–h).

3.4. Synovial fluid as chondrogenesis inducing factor

All tested concentrations (5%, 10%, and 50%) of autologous synovial fluid lead to an induction of chondrogenesis as indicated by an extensive secretion of cartilage specific proteoglycans (Fig. 4a, c, and e) and by a high expression of type II collagen concordant to the sites stained with Alcian blue (Fig. 4b, d, and f). The pellet size apparently increases with higher concentration of autologous synovial fluid and reached the size of TGF-β1-induced cells (Fig. 4a, c, and e).

3.5. Quantitative expression of Alcian blue in chondrogenic-induced MSC

Pronounced differences were found in the amount of secreted proteoglycans. This was evaluated by analyzing digitalized pictures of Alcian blue stainings. In 0.1 mg/ml HA stimulated micromass cultures, proteoglycans in ratio to the whole pellets were much higher (35–38%) than in the dexamethasone (<1%) stimulated control group, but clearly lower than in pellets induced with 10 ng/ml TGF-β1 (60%) (Fig. 5). 10 ng/ml TGF-β1 in combination with 0.1 mg/ml Hylartil® or Ostenil® leads to micromass cultures with almost the same amount of proteoglycans in ratio to the whole pellet as with TGF-β1 alone. Comparing the effects of the two types of HA, Hylartil® and Ostenil®, Hylartil® alone and in combination with TGF-β1 had the stronger effect on proteoglycan secretion (Fig. 5). For proteoglycans in ratio to the whole pellet, 5% autologous synovial fluid seems to be the most favorable concentration (Fig. 5). Both HA and synovial fluid stimulated

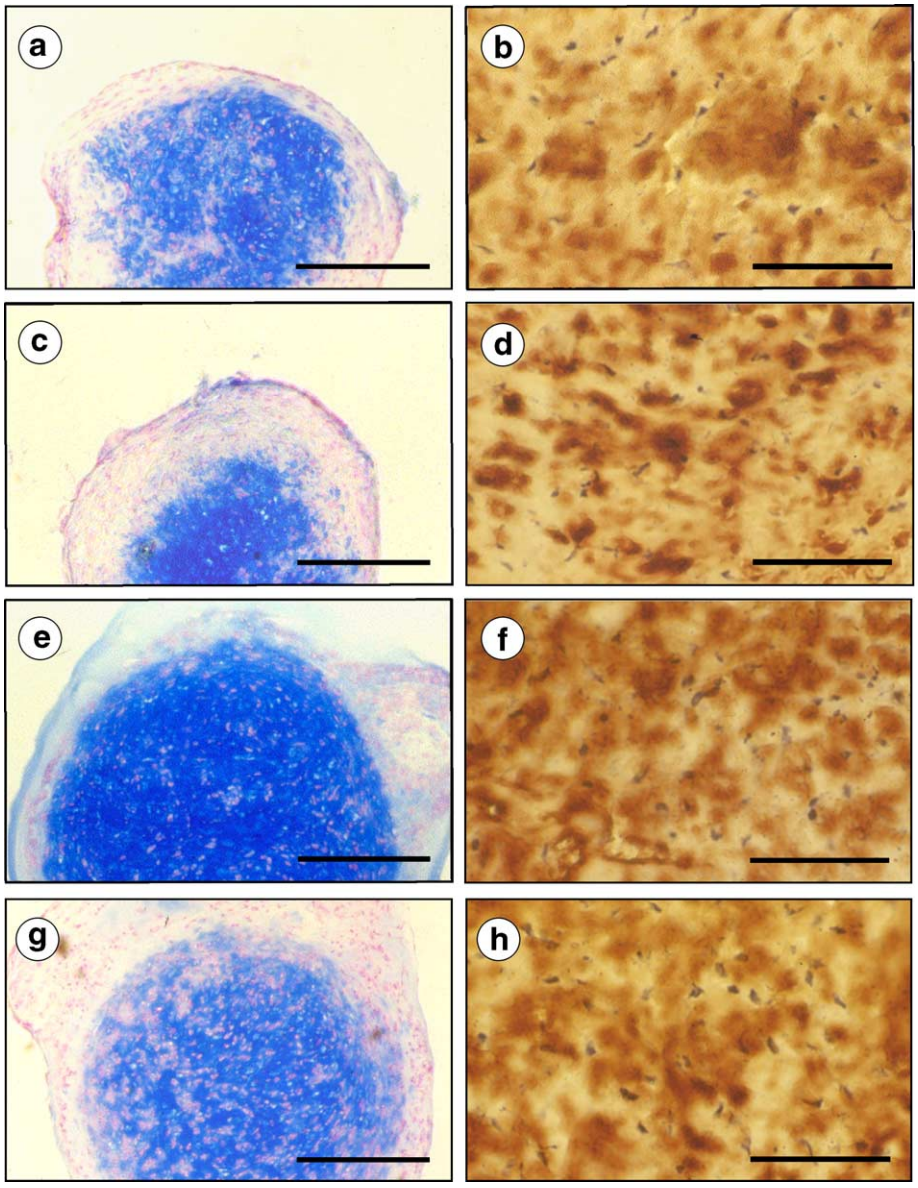

Fig. 3. Photomicrographs of histochemical analysis of equine MSC (passage 2) undergoing hyaluronic acid-induced chondrogenesis. (a) After prolonged stimulation with 0.1 mg/ml of hyaluronic acid Hylartil®, at day 26, equine MSC showed an Alcian blue staining of cartilage proteoglycan, and (b) monoclonal type II collagen antibody staining demonstrated a pronounced expression of type II collagen. (c and d) 0.1 mg/ml Ostenil® stimulated micromass cultures showed similar behavior. (e and g) By day 26, pellets induced with a combination of 10 ng/ml TGF-β1 and 0.1 mg/ml Hylartil® or Ostenil®, were larger and showed a 'more pronounced Alcian blue staining' of extracellular matrix proteoglycan as compared with pellets stimulated by hyaluronic acid alone. (f and h) On the contrary, at day 26, type II collagen expression was similar to the Hylartil® and Ostenil®-induced cultures. Bar = 250 μm for (a), (c), (e), and (g) and 65 μm for (b), (d), (f), and (h).

equine MSC demonstrated a lower amount of proteoglycans in ratio to the whole pellet (35–42%) compared to TGF-β1 (60%).

4. Discussion

In recent studies, the chondrogenic differentiation potential of equine bone marrow-derived mesenchymal stem cells, isolated without performing standard percoll-density centrifugation, was evaluated using either monolayer cultures and TGF-β1 or three-dimensional fibrin matrices and insulin-like growth factor-1 (Worster et al., 2000, 2001). In our study, we have demonstrated that equine MSC, isolated by percoll-density centrifugation and culture expanded, undergo chondrogenic differentiation when using high-density micromass cultures ("pellets") and appropriate differentiation stimulants, such as TGF-β1, hyaluronic acid (Hylartil®, Ostenil®) or autologous synovial fluid.

Pellet culture systems allow cellular interactions analogous to those that occur in precartilage condensation during embryonic development (Solursh, 1991). Under these con-

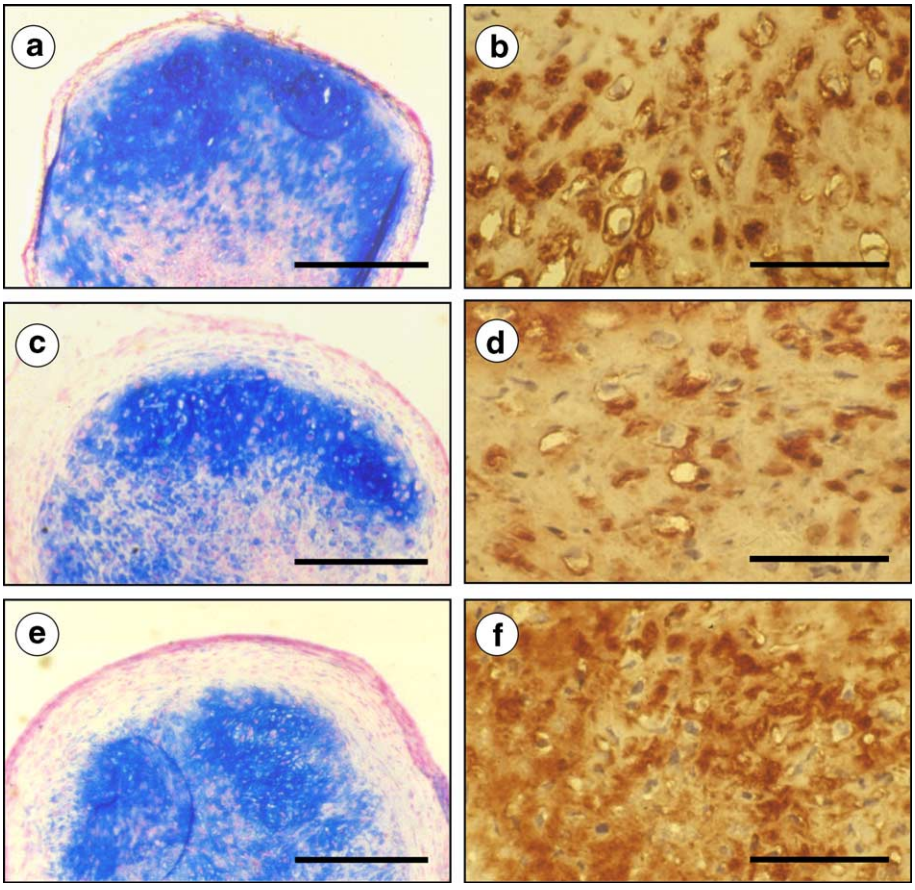

Fig. 4. Photomicrographs of histochemical analysis of equine MSC (passage 2) undergoing autologous synovial fluid-induced chondrogenesis. Autologous synovial fluid was obtained from an 18-month-old horse (Haflinger) using a standard aspiration technique. (a, c, and e) Independent of the synovial fluid concentration (5%, 10%, 50% in medium), at day 26, all pellets showed a strong cartilage proteoglycan expression and they were as large as TGF-β1 stimulated pellets. (b, d, and f) At day 26, type II collagen expression demonstrated a chondrogenic development of equine MSC and was similar in all autologous synovial fluid-treated pellets. Bar = 250 μm for (a), (c), and (e) and 65 μm for (b), (d), and (f).

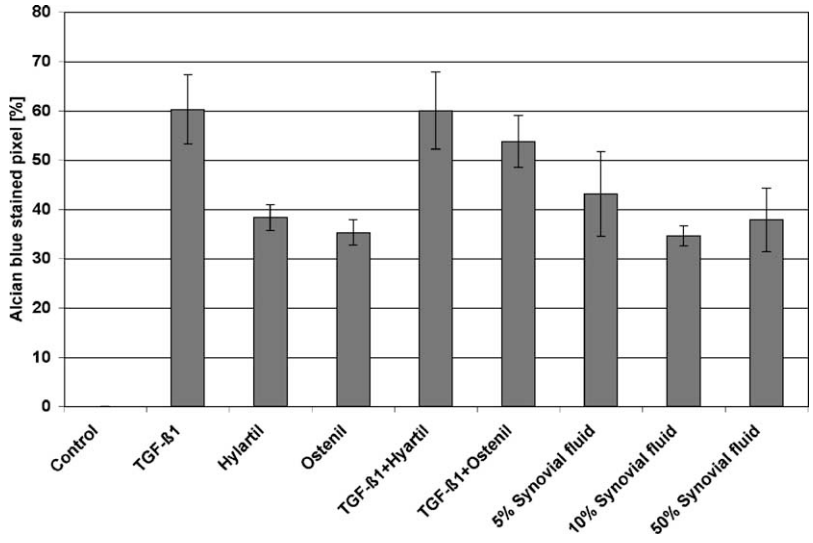

Fig. 5. Quantitative expression of Alcian blue stained proteoglycan in chondrogenic-induced equine MSC cultures (passage 2). Quantitative analysis of the staining was performed at day 26 with Adobe Photoshop 4.0 software by counting the percentage of the stained pixel in relation to the total number of pixels per specimen area. Results clearly indicated that TGF-β1 has induced the highest proteoglycan expression in ratio to the whole pellet, whereas hyaluronic acids and synovial fluid has caused less expression. The proteoglycan expression in ratio to the pellet size was very low (<1%) in the control pellets.

ditions, at day 26, the control group did not express type II collagen, but slightly expressed extracellular cartilage matrix proteoglycan, which could also be due to dexamethasone that was part of the defined medium used in all cultures (Johnstone et al., 1998; Ringe et al., 2002).

Members of the TGF-β superfamily of growth and differentiation factors have been shown to induce chondrogenesis in MSC from various species (Johnstone et al., 1998; Mackay et al., 1998; Worster et al., 2000, 2001; Sekiya et al., 2001; Ringe et al., 2002). In our investigation, TGF-β1 was the most effective differentiation factor. The pronounced chondrogenic capacity of TGF-β1 is not surprising, since in embryonic cartilage TGF-β1 is expressed abundantly and may be involved in the chondrogenic transformation of primitive mesenchymal condensations (Cancedda et al., 1995).

Previously, hyaluronic acid (HA) has been shown to induce chondrogenesis in stage 24 limb mesenchyme cell cultures (Kujawa et al., 1986; Maleski and Knudson, 1996a). It was found that the differentiation response of these cells is related to the molecular weight (MW) and the concentration of the HA. A MW between 2×10^5 Da and 4×10^5 Da turned out to be the most active size (Kujawa et al., 1986). Hyaluronic acid higher in MW than 1×10^6 Da was observed to inhibit chondrogenesis because it blocked the aggregation of cells, regarded as necessary for differentiation. In our study, we evaluated the chondrogenic effects of Ostenil® with an MW of 3×10^6 Da and Hylartil® with an MW of 2×10^6 Da—two distinct hyaluronic acids—since they are approved for in vivo applications: specifically Ostenil® in humans and Hylartil® in horses. Despite the high MW of both HA, they clearly demonstrated the potential to induce chondrogenesis of equine MSC. As we used high-density micromass cultures that promote cell aggregation, the MW of HA most likely becomes less important. The chondrogenic effect of Hylartil® and Ostenil® may be explained by the interaction between HA and its receptor CD44, which is expressed by mesenchymal stem cells (Pittenger et al., 1999). Previous studies have shown the importance of those interactions for regulation of chondrogenesis in embryonic development, as well as for regulation of proliferation and matrix synthesis in chondrogenesis (Maleski and Knudson, 1996b; Ishida et al., 1997). Also of special interest for our experimental approach was the report that adhesion of chondrocytes to HA via CD44 augmented TGF-β mRNA expression (Ishida et al., 1997). After 26 days in culture, the size of HA-treated pellets was smaller compared to TGF-β1 stimulated pellets. This is due to a lower expression of extracellular matrix molecules, such as proteoglycans in HA cultures. As reported previously, when human MSC were cultured as pellets, TGF-β leads to an increase in pellet weight and size (Sekiya et al., 2001). A pronounced synergic effect of hyaluronic acid and TGF-β1, however, could not be detected.

Autologous synovial fluid was shown in previous studies to induce chondrogenesis in perichondrium cells in vivo, as well as in vitro (Skoog et al., 1990; Rodrigo et al., 1995). Moreover, chondrogenesis could be verified using a chicken limb bud bioassay. Here, we demonstrated that autologous synovial fluid of a healthy horse has a noteworthy potential to induce chondrogenic differentiation. The concentration of hyaluronic acid in synovial fluid is approximately 2–4 mg/ml. Thus, we calculated that 5% autologous synovial fluid would have about 0.1–0.3 mg/ml hyaluronic acid in our synovial fluid assay and therefore be similar in concentration to the hyaluronic acid assays. Concentrations of 10% and even 50% synovial fluid were also tested to reflect a more physiological situation. Pellets induced with synovial fluid appear to be considerably larger in size than those induced with hyaluronic acid (Figs. 3a and c and 4a, c, and e). Although the secreted proteoglycan in ratio to the whole pellet that is found in pellets induced with synovial fluid is almost the same as in the ones induced with hyaluronic acid, the larger size implies that besides hyaluronic acid, synovial fluid contains further factors that also promote and enhance the development of cartilage from mesenchymal stem and progenitor cells. The hormone prolactin is one such factor of human synovial fluid that modulates the growth and chondrogenic differentiation of human MSC (Ogueta et al., 2002). Synovial fluid also contains TGF-β1, which may have caused a pellet size similar to the TGF-β1-treated micromass cultures (Okazaki et al., 2001).

Although the quality of the cartilage obtained has not yet reached the high standards of native articular hyaline cartilage and the effects of pathological synovial fluid on MSC still needs to be explored, the results are promising. Synovial fluid can act as a model for finding effective combinations of differentiation factors and deserves further research on its components and their potentials to induce chondrogenesis. The results encourage further steps toward tissue engineering applications of MSC in chondral defects of the joint, as the natural environment in the joint would be favorable for chondrogenic differentiation of the MSC. The results also suggest using hyaluronic acid based biomaterials as the carrier for cartilage tissue engineering transplants (Radice et al., 2000; Solchaga et al., 2000). HA would probably act as a differentiation factor and be a source for building up extracellular matrix, where HA is one of the main components.

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