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Operative Techniques in

Sports Medicine

Amniotic Fluid, Cells, and Membrane Application

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Orthobiologics is a rapidly growing field, as the potential of stem cells becomes better understood. Amniotic tissue has a long history of clinical use and its anti-inflammatory and paracrine function makes it an attractive source for cells. The use of amniotic membrane for cartilage damage has been evaluated primarily in preclinical settings. Multiple in vitro studies have shown that amniotic membrane and amniotic mesenchymal stem cells can produce a chondrocyte phenotype with accumulation of glycosaminoglycans, collagen, and chondrogenic markers. Both autologous and allogeneic sources are available, with the latter having the benefit of decreased morbidity to the patient. A wide array of placental-derived allograft tissue forms are currently available in both tissue and injectable formats. Sheets generally consist of one or more intact layers of placental membrane, namely amnion, amnion and chorion, double layer amnion, or umbilical cord. Liquid products consist of morselized tissues such as amnion or chorion or both, suspensions containing cells such as amniotic fluid stem cells, purified variants of amniotic fluid, or some combination. Clinical and basic science investigations are underway to define the cellular mechanisms of action and appropriate clinical indications for the use of these placental-derived products.

KEYWORDS Amniotic fluid stem cells, amniotic membrane stem cells, mesenchymal stem cells, cartilage, osteoarthritis

Introduction

The field of orthobiologics is undergoing rapid expansion, as treatment failures migrate toward biologic deficiencies with improved implants. The term orthobiologics, as it is in use today, can refer to specific molecule preparations (eg, bone morphogenic proteins), composites (demineralized bone matrix and platelet-rich plasma), mesenchymal stem cells (MSCs), and placental components that offer a unique source of extracellular matrix, biologically active proteins and proteoglycans, and hyaluranons.

First identified in bone marrow, MSCs have been more recently isolated from almost all adult tissues,[1] including adipose tissue[2] and muscle.[3] In addition to these adult connective tissues, MSCs can also be isolated from fetal annexes, such as cord blood, placenta, and amniotic fluid.[4] These sources of MSCs do not share some of the ethical concerns inherent to embryonal tissue, as they are routinely discarded after birth, thus making them both readily available and affordable. Placental-derived cell sources are an attractive cell source for allogeneic applications, because these tissues are thought to be immunoprivileged as these MSCs express low levels of major histocompatibility complex (MHC) class I, no MHC class II, and do not induce activation of allogeneic lymphocytes.[5,6] Moreover, placental MSCs are able to inhibit

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proliferation of both autologous and allogeneic T cells, suggesting that their immunosuppressive properties do not depend on MHC expression by MSCs and lymphocytes.[7]

An important benefit of allogeneic sources of stem cells is the lack of donor site morbidity. This allogeneic source more importantly obviates the need for additional procedures, thus making this approach the least invasive and most efficient.

Independent of their allogeneic or autologous origin and in addition to their ability to participate in the regenerative process through their direct differentiation into tissue-specific cell types, MSCs have been demonstrated to possess even more important therapeutic functions, such as immunomodulation and trophic activities. In response to injury, MSCs are able to release a variety of different molecules, including cytokines and growth factors, with immunomodulatory and trophic effects. For this reason, they now are viewed as "drugstores,"[8] as they provide the microenvironment with specific molecules through paracrine signaling.[9] Caplan originally coined the term mesenchymal stem cells (MSCs), however, more recently has proposed changing the term MSC to stand for medicinal signaling cells to better represent their paracrine function.

Further, recent evidence seems to demonstrate that the interaction of MSCs with other cells of their native niche is crucial for the maintenance of an active stem cell pool and for their differentiation pathway.[10,11] In this view, the protection of the stem cell niche in producing cell-based treatment is important, whereas the number and purity of cells is less crucial.

Among fetal annexes, placenta and its membranes have undergone the most investigation. In particular, undifferentiated cells from amniotic fluid and membrane have shown very promising results, and clinical trials as well as early clinical experience with these cells are increasing.

Amniotic Membrane

A basic understanding of the amniotic and chorionic membranes helps us understand their applications. The amniotic and chorionic membranes make up the fetal portion of the placental membranes; the amniotic membrane (AM) is adjacent to the fetus, whereas the chorionic membrane is adjacent to the endometrium, which is maternal tissue. The AM is made up of 3 basic components, namely the epithelium, the basement membrane, and the stroma. The epithelium is a single layer of cells that have secretory and transport functions. The underlying basement membrane is thick and contains type IV collagen. The stromal layer is composed of a compact layer, fibroblastic layer, and then intermediate or spongy layer that lies closest to the chorionic membrane. The stromal layer contains collagens, hyaluronic acid (HA), and proteoglycans. Connected to the amnion is the chorion, which is made up of an outer trophoblast layer that interfaces with the uterine decidua, and an inner layer formed by the somatic mesoderm, which comes in direct contact with the amniotic stroma. The inner layer is further divided into 2 additional layers, namely the reticular layer, which is composed of collagen types I, III, IV, V, and VI in addition to various proteoglycans, and the underlying basement membrane, which is primarily composed of collagen type IV, fibronectin, and laminin.[12]

The AM has multiple roles contributing to its uniqueness. One of the key functions of the AM is to suppress inflammation. Although the exact mechanism is unknown, the membrane releases multiple anti-inflammatory components that suppress proinflammatory markers. Transforming growth factor (TGF)-beta is a soluble factor that is known to induce fibrotic responses through activation of fibroblasts. The AM is able to inhibit the expression of TGF-beta, reducing inflammation and scar formation. Additionally, unique to amniotic tissues is a special iteration of HA known as heavy-chain HA.[13] This form of HA is formed through the covalent linkage of HA to the heavy chain of inter-alpha-trypsin inhibitor. This specific iteration of HA has been demonstrated to exert immunomodulatory properties using mechanisms such as the inhibition of the complement system cascade,[14] and the polarization of inflammatory M1 macrophages to their anti-inflammatory M2 phenotype in the context of activation by foreign antigens.[15]

The AM also promotes epithelialization while inhibiting fibrosis. Amniotic mesenchymal and epithelial cells produce and release growth factors involved in epithelialization and wound healing such as epidermal growth factor, keratinocyte growth factor, keratinocyte growth factor receptor, hepatocyte growth factor, and hepatocyte growth factor receptor. Not only does the AM facilitate the migration of epithelial cells, but also reinforces adhesion to the basement membrane. Various growth factors released by the AM stimulate epithelialization. Amniotic membrane reduces the risk of fibrosis by down-regulation of TGF-beta and its receptor expression by fibroblasts.

Further, amniotic tissues have been reported to have antimicrobial properties that may contribute to a decreased risk of infection. The membrane itself acts as a mechanical barrier against microbial inoculation, but the cells of the AM also produce antimicrobial molecules such as bactricidin, betalysin, transferrin, and 7S immunoglobulin.

The AM is also important as a mechanical structure. The extracellular matrix has an abundance of various forms of collagen, laminin, HA, and glycosaminoglycans, leading to its function as a scaffold. The success of the AM in various clinical applications is also related to its minimal immune response. The immune privileged cells of the AM lack the polymorphic antigens human leukocyte antigen-A (HLA-A), HLA-B, and HLA-DR on their surfaces, which is directly related to a reduced risk of rejection or immune reaction when the allograft is applied. This property is unique to the membranes of the placenta.

Cells of the Amniotic Membrane

Amniotic tissues contain population groups of undifferentiated cells associated with the AM. Contained within the membrane are 2 specific subsets known as AM epithelial cells (AECs), and cells derived from the embryonic mesoderm, referred to as AM stromal cells. The amniotic epithelial cells, displaying multiple

cytokeratins, form the first layer of the AM, sitting on the basement membrane (the thickest basement membrane in human tissues). AECs have the ability to differentiate into all 3 germ layers. Amniotic fluid is a heterogeneous mixture predominated by 3 main cell types.[16-18] Two cell types, known as AF-type (amniotic fluid specific) cells and F-type (fibroblast type) cells, exhibit spindle-shaped morphologies, whereas E-type (epithelioid type) cells exhibit a rounded morphology.

Comparison of Amnion-Derived Cells vs Other Cell Sources

Several metrics have been used to compare amniotic fluid and membrane-derived cell populations to other cell types such as MSCs. For example, previous studies have compared the expansion rate of second trimester amniotic fluid-derived stem cells (AFSCs) with bone marrow–derived MSCs (BM-MSCs).[19] In all studies, the cellular growth rate of AFSCs was greater compared with BM-MSCs. Further, studies were performed assessing the osteogenic and chondrogenic capabilities of AFSCs in comparison with MSCs. For example, a study performed by Peister et al[20] using spindle-shaped second trimester AFSCs demonstrated lower levels of initial alkaline phosphatase activity than BM-MSCs, yet greatly increased levels of mineral deposition over time. In another study, adipose, bone marrow, and amniotic fluid–derived cell populations were compared in terms of their chondrogenic potential.[21] This study found that AFSCs had similar chondrogenic capability, but expressed collagen I to a lesser extent than either adipose or bone-derived cell populations, indicating a higher likelihood for the production of hyaline cartilage. However, recent studies have shown that direct tissue deposition and engraftment of implanted stem cell populations is an unlikely mechanism of action and that these cells are more likely to coordinate the local healing environment through signaling and immunomodulation.

Currently, only few studies have been performed in which BM-MSCs are compared with either AFSCs or amniotic membrane-derived stem cells (AMSCs) in terms of the immunomodulatory capabilities. However, these studies hint at possible differences among BM-MSCs, AMSCs, and AFSCs. In a study, MSCs were collected from decidual tissue (maternal-derived MSCs) and amniotic fluid (fetal-derived MSCs).[22] The cells were then compared with each other in their ability to suppress lymphocyte proliferation and produce anti-inflammatory cytokines. Initial flow cytometric analysis of both maternal- and fetal-derived MSCs found them to be negative for HLA-G, CD133, and HLA-DR. However, this is to be expected, as AFSCs downregulate HLA-G during in vitro culture and re-express HLA-G on stimulation with inflammatory cytokines.[23] In mixed lymphocyte reaction, fetal-derived MSCs (both AFSCs and AMSCs) were found to have a greater inhibitory response than their maternal-derived counterpart. The main mechanism behind this inhibition was determined to be enhanced production of IL-10 in fetal-derived MSCs relative to the maternally derived MSC counterparts. Additionally, gene expression analysis demonstrated enhanced gene expression of TGF-beta for fetal-derived MSCs relative to the maternally derived MSCs.

In another study,[24] AFSCs were compared with BM-MSCs by assessing the expression of immunogenic and immunomodulatory cytokines such as HLA-ABC, HLA-DR, and PD-L1 among others. In relation to BM-MSCs, AFSCs express PD-L1 (also known as CD274) to a greater extent in their inactivated state. This is in addition to the fact that AFSCs have lower expression of HLA-ABC and HLA-DR. Additionally, AFSCs were found to have a much greater immune inhibition to T, natural killer (NK), and B cells as well as a much lower susceptibility to NK-mediated cell lysis than BM-MSCs.

Although AMSCs have not been compared with BM-MSCs in terms of their immunomodulatory capability, they have been compared with adipose-derived MSCs (AD-MSCs).[25] In this study, AMSCs were compared with AD-MSCs in terms of their capability to suppress immune effector cells proliferation in mixed lymphocyte reaction and by quantifying their production of multiple immunomodulatory cytokines. The study found that AMSCs had a lower expression of HLA-DR antigen, greater expression of PD-L1 antigen, and had greater production of the immunomodulatory cytokines IL-10 and IL-6, among others. Additionally, AMSCs inhibited peripheral blood mononuclear cells proliferation to a greater degree compared with AD-MSCs.

Available Amniotic Preparations in the United States

Several preparations of placental or amniotic tissues are available in the United States today under the Food and Drug Administration minimally manipulated tissue guidelines (HCT/P 361). Owing to the rapidly changing marketplace, we decided against the mention of specific products and elected to discuss broad categories instead.

A wide array of placental-derived allograft tissue forms are currently available. These include both sheet and injectable formats. Sheets generally consist of one or more intact layers of placental membrane: amnion, amnion and chorion, double layer amnion, or umbilical cord. Liquid products consist of morselized tissues such as amnion or chorion or both, suspensions containing cells such as AFSCs, purified variants of amniotic fluid, or some combination. Whether delivered as sheets or in liquid form, there are various processing methodologies used to preserve placental-derived allograft tissues including dehydration, cryopreservation, and hypothermic or fresh storage. Dehydration is a broad term that encompasses several techniques for removing water content using lyophilization, dessication, or dry heating. Grafts processed in this way are typically stored at ambient temperatures and can be stored for extended periods (5 years). These methods do not preserve cell content, but are intended to deliver extracellular matrix proteins and growth factors. Cryopreservation and hypothermic or fresh processing techniques also aim to preserve the extracellular matrix and growth factors and may or may not preserve viability of the native cell population, depending on the details of the processing methodology used. These

methods typically result in grafts with a shorter shelf life. The various graft forms may be subject to terminal sterilization, or may be aseptically processed instead. Aseptic grafts are processed in a fashion designed to minimize the possibility of contamination with harmful bacteria, viruses, or other micro-organisms. They are thoroughly tested preprocessing and postprocessing according to the standards and guidelines established by the American Association of Tissue Banks (AATB) and the Food and Drug Administration. In comparison, sterilized grafts undergo a final step to destroy any harmful living contaminants. This may be accomplished by gamma or electron-beam irradiation at various doses. Other techniques include ethylene oxide and autoclaving, although they are less commonly used.

Clinical Applications

Amniotic tissues have been used clinically for more than 100 years. The first applications were for the treatment of burns and skin ulcerations. Amniotic membrane is now widely used in ophthalmology, for example, for the treatment of corneal ulcers. The use of amniotic tissues in orthopaedics is correspondingly expanding. In 1938, Shimberg et al[26] described a clinical study in which amniotic fluid concentrate was injected into joints for a number of purposes including arthrotomies, closed manipulation of joints, articular and periarticular fractures, and joint effusions.

More recently, several orthopaedic applications have been studied such as treatment of plantar fasciitis, tendon disorders, and cartilage defects. Hanselman et al[27] performed a randomized, controlled, double-blind, single-center pilot study comparing cryopreserved human AM (chAM) and corticosteroid injections for the treatment of plantar fasciitis. Although most outcomes measures showed no statistical difference between the 2 treatments, the authors concluded that chAM injection may be safe and comparable with corticosteroid injection for the treatment of plantar fasciitis. Similarly, Zelen et al[28] examined the efficacy of micronized dehydrated human amniotic and chorionic membrane (dhACM) injection as a treatment for chronic refractory plantar fasciitis. Compared with saline injections, patients receiving dhACM had improvement in symptoms. Anderson et al[29] used human amniotic allograft (HAA) containing AFSCs and AM as an adjunct to surgical treatment of talar osteochondritis dissecans lesions. The addition of HAA to arthroscopic microfracture repair of talar dome lesions measuring less than 2 cm^2 significantly improved postoperative visual analogue scale scores, when compared with preoperative scores. Werber et al[30] performed injections of cryopreserved human amniotic membrane and amniotic fluid product into the tissues of patients who experienced severe heel pain unresponsive to existing therapies. Significant improvements in pain were observed 4 weeks after treatment in all patients, though longer time points are necessary.

The use of AM for cartilage damage has been primarily evaluated in a preclinical setting. Multiple in vitro studies have shown that AM and amniotic MSCs can produce a chondrocyte phenotype with accumulation of glycosaminoglycans, collagen, and chondrogenic markers. Amniotic membrane–derived products have also been applied in animal studies. Willett et al[31] conducted a study evaluating AM's effect on cartilage degradation. Rats underwent transection of the medial meniscus to induce arthritis, and 24-hours postsurgery they were injected with micronized dhACM or saline. The authors noted that treatment with dhACM prevented the development of cartilage lesions at 21 days and the number of partial erosions was significantly reduced. In this study, the AM showed a potential protective effect against the development of osteoarthritis. A recent pilot study of 6 patients with end-stage osteoarthritis treated with HAA demonstrated safety and a positive trend of efficacy for up to 1 year (Vines et al).[32] Garcia et al[33] created full-thickness cartilage defects in the knees of sheep and treated them with 1 of 4 options: no treatment of the defect, filling with fresh AM, with chAM previously cultivated with BM-MSCs, or with chAM alone. All 3 treatment groups demonstrated improved appearance of the cartilage as compared with the defect without treatment. Tabet et al[34] showed evidence of diffuse chondrocyte-like cell proliferation of a stromal matrix similar to hyaline cartilage in a sheep model in which cartilage defects were filled with human AM mixed with demineralized bone matrix.

Conclusions and Future Perspectives

In summary, the field of orthobiologics is expanding rapidly, specifically regarding the use of stem cells. Both autologous and allogeneic sources are available, with the latter having the benefit of decreased morbidity to the patient. Amniotic membrane and fluid cells have a long history of clinical use, with an initial focus on wound care and ophthalmology. Their anti-inflammatory and paracrine functions make them attractive cell sources for the treatment of orthopaedic diseases, such as osteoarthritis and tendinosis. Clinical and basic science investigations are underway to define the cellular mechanisms of action and appropriate clinical indications for the use of these placental-derived products. Although cautiously optimistic, the orthopaedic community should remain focused on patient safety until the peer review process validates these studies.

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