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Review

Progress in studies on the characteristics of human amnion mesenchymal cells

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Abstract

The amniotic membrane (AM) in humans is the innermost fetal membrane and is composed of amnion mesenchymal cells (hAMCs) and amnion epithelial cells (hAECs). The former are derived from the extra-embryonic mesoderm of the primitive streak, and the latter from the fetal ectoderm. Numerous studies have shown that both hAMCs and hAECs display stem cell characteristics. In this review, we examine the progress made in understanding the characteristics of hAMCs, including information on the structure and function of AM and hAMCs, their immunological features, and the pluripotency of hAMCs.

Keywords: Human amnion mesenchymal cells; Hypo-immunogenicity; Immunosuppression; Differentiation

1. Introduction

The amniotic membrane (AM) is a tissue of fetal origin. It is the innermost layer of the fetal membranes and comprises a single layer of epithelial cells on a thicker basement membrane and a spongy collagen layer containing mesenchymal cells. The amniotic cells have different embryological origins: human amnion mesenchymal cells (hAMCs) are derived from the extra-embryonic mesoderm of the primitive streak, while human amnion epithelial cells (hAECs) are derived from the fetal ectoderm at the eighth day of fertilization, prior to the start of organogenesis [1]. Many studies have demonstrated that cells derived from the AM are able to differentiate into many kinds of mature cells, including adipocytes, osteocytes, chondrocytes, myocytes, cardiomyocytes, hepatocytes, neurocytes and vascular endothelial cells. These observations suggest that the human AM contains stem cell-like cells and could therefore provide an alternative source of cells for regenerative medicine.

2. Structural features of AM and amniotic cells

There are no blood vessels, nerves, muscles, or lymphatics in the amnion. The amnion epithelium consists of a single layer of cells resting on a basal lamina, containing laminin heterotrimers composed of α-, αβ-, and γ-chains. The lamina, which contributes to regulation of cell differentiation, shape, movement and cellular function, is supported by a dense, acellular collagenous matrix [2].

Light microscopy shows that hAMCs are roundish in shape, with an average diameter of 15 μm, with abundant, multi-vacuolated and intensely basophilic cytoplasm. Transmission electron microscopy of hAMCs reveals a

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hybrid epithelial–mesenchymal ultrastructural phenotype: epithelial characteristics include non-intestinal-type surface microvilli, intracytoplasmic lumina lined with microvilli, and intercellular junctions; mesenchymal features include rough endoplasmic reticulum profiles, lipid droplets, and well-developed foci of contractile filaments with dense bodies [3]. These features are consistent with the view that hAMCs have pluripotent potential. Transmission electron microscopy shows that hAECs are cuboidal, with apical microvilli, and that their lateral cell borders are convoluted with frequent desmosomes and no obvious tight junctions. The basal epithelial cell surfaces are highly convoluted with frequent hemidesmosomes at the distal termini of cell processes, and wavy filament bundles are seen in the adjacent cytoplasm [4].

3. Functions of AM and amniotic cells

The AM not only protects the developing embryo from desiccation by enveloping it in the amniotic fluid, but also maintains the liquid balance between the mother and the fetus.

The amnion has the ability to produce human β-defensins (HBDs), elafin and the secretory leukocyte protease inhibitor (SLPI). HBDs are a major family of vertebrate natural antimicrobials, which are widely expressed at mucosal surfaces [5]. Elafin and SLPI are serine antiproteases that antagonize human neutrophil elastase, thereby preventing potential tissue injury caused by excessive release of proteolytic enzymes from inflammatory cells [6]. These factors suggest that the AM may play a role in the innate immunity of the amniotic cavity.

Katz et al. [7] found that amnion tissue explants synthesized and secreted six proteins of the complement system, indicating that the amnion may be a source of the complement proteins present in the amniotic fluid and may contribute to local host defense, together with the endometrial glandular epithelial cells that synthesize complement C3.

Prostaglandin E2 (PGE2) plays a pivotal role in the mechanisms involved in human parturition, both at term and at preterm. At the end of gestation, stretching of the AM and an increase in interleukin-1β (IL-1β) levels activate both nuclear factor kappa B and activator protein-1, leading to an increase in cyclooxygenase-2 (COX-2) mRNA expression, COX-2 protein synthesis, and PGE2 release [8,9].

Amniotic cells release physiologic levels of cytokines relevant to wound healing, including platelet-derived growth factor, vascular endothelial growth factor (VEGF), angiogenin, transforming growth factor-β2 (TGF-β2), tissue inhibitor of metalloproteinase-1 (TIMP-1), and TIMP-2. These cytokines promote cell proliferation, reduce inflammation, and regulate many of the processes that are crucial for wound healing [10–12].

4. Surface marker expression profile of hAMCs

Immunocytochemistry, flow cytometry analysis and reverse transcription polymerase chain reaction (RT-PCR) have revealed that hAMCs express surface markers including CD13, CD29, CD44, CD49d, CD54, CD59, CD73, CD90, CD105, CD166, fetal-liver kinase-1, very late antigen (VLA)-5, intercellular adhesion molecules, and integrins, such as L-selectin, α-M β-2 integrin, and p-selectin ligand-1 [3,13–18]. They do not express CD14, CD31, CD34, CD45, CD106, or CD117 [3,13–15,17,18].

5. Immunosuppression and hypo-immunogenic characteristics of hAMCs

Many studies have concluded that hAMCs express HLA-A, -B, and -C antigens, but not HLA-DR antigens [3,13,14,16–18], and are weakly positive for HLA-G [13]. In their study, Wolbank et al. [14] found that amniotic cells inhibited the proliferation of activated peripheral blood mononuclear cells (PBMCs) in a dose-dependent manner. The hAMCs and hAECs significantly reduced PBMC proliferation in mixed lymphocyte reaction experiments by 34% and 23%, respectively. When PBMCs were activated using phytohemagglutinin, similar levels of inhibition were observed: 33% for hAMCs and 28% for hAECs. Subcultivation did not alter the immunoinhibitory properties of the AM cells, whereas cryopreservation significantly reduced their immunomodulatory potential. Krampera et al. [19] and Di Nicola et al. [20] found that bone marrow mesenchymal stem cells (BM-MSCs)-induced inhibition of T-cell proliferation was a reversible and transient phenomenon, but this aspect has not been investigated in hAMCs.

In addition, cells isolated from the amnion did not induce allogeneic or xenogeneic lymphocyte proliferation responses and were able to actively suppress lymphocyte responsiveness [14,15]. In limbal transplantation, although some CD4 and CD8 T cells surrounded the amniotic graft, the response was mild. In intracorneal transplantation, all grafted AM were accepted and remained clear, without host cell infiltration, while skin grafts were rejected [21].

In some cases, class II antigens can be expressed by amniotic fibroblasts [21], and the presence of hAMCs can result in an enforced PBMC reaction [14]. Intact amniotic epithelium (AE) from enhanced green fluorescent protein transgenic mice (C57BL/6 background) and wild-type C57BL/6 mice was transplanted to the cornea, conjunctiva, or anterior chamber of normal BALB/c mice, C57BL/6 mice, or BALB/c mice presensitized to donor antigens. Graft survival was markedly shorter in presensitized recipients and recipients that underwent repeated AE implantation (in which AE was grafted in the other eye 7 days after the first grafting) compared with normal recipients. Delayed hypersensitivity was induced at 2 weeks in these animals, but failed to be induced in the normal recipients

[22]. This suggests that allogeneic AE is vulnerable to immune rejection in specifically sensitized recipients.

In conclusion, although the AM and the cells derived from it have been recognized as an immuno-privileged material, their immunogenicity should not be ignored in further research.

6. Immunosuppression and anti-inflammatory mechanisms of hAMCs

In order to examine the possible immunosuppressive mechanisms of hAMCs, Wolbank et al. [14] cultured hAMCs and PBMCs in transwell systems and found no significant inhibition. They therefore concluded that hAMC inhibited PBMC proliferation via cell contact, rather than by soluble factors.

Hao et al. [23], however, found that both hAMCs and hAECs expressed the interleukin-1 receptor antagonist (IL-1ra), TIMPs, collagen XVIII, IL-10 and thrombospondin-1 (TSP-1). IL-1ra is structurally similar to IL-1β but lacks agonist activity. IL-1ra competes with IL-1 for IL-1 receptor binding, thereby blocking the inflammatory responses initiated by IL-1. TIMPs are a family of multifunctional proteins present in many human tissues and play pivotal roles in regulation of extracellular matrix (ECM) metabolism. TIMPs have demonstrated diverse actions in the inhibition of angiogenesis and tumor growth, invasion and metastasis. Collagen XVIII is a potent antiangiogenic factor that can inhibit endothelial cell proliferation, angiogenesis, and tumor growth. IL-10 functions as a broad-spectrum anti-inflammatory cytokine by inhibiting the production of IL-1, TNF-β and other pro-inflammatory factors. IL-10 has also been reported to promote TIMP production and suppress matrix metalloproteinase expression. TSP-1 is a multifunctional matrix protein secreted by many cell types and has been shown to have anti-angiogenic activity. These findings may partly explain the antiangiogenic and anti-inflammatory effects of AM and the cells derived from it.

7. Pluripotency of hAMCs

Many studies have demonstrated that hAMCs express stem cells markers, including the transcription factors Oct-4, GATA-2, GATA-4, Pax-6, TRA-1-60, SSEA-3, SSEA-4, STAT-3, Rex-1, stem cell factor, neural cell adhesion molecule, nestin, bone morphogenetic protein-4, hepatocyte nuclear factor-4α, vimentin, CK-18, Sox-2, homing cell adhesion molecule-1, Brachyury and Notch-1 receptor [13,15,24]. A few studies, however, have failed to detect expression of the genes for Brachyury, fibroblast growth factor-5, Pax-6, TRA-1-60, SSEA-3, SSEA-4, vascular cell adhesion molecule-1, platelet/endothelial cell adhesion molecule-1 and TRA-1-81 [16,25]. Telomerase is a ribonucleoprotein that synthesizes and directs the telomeric repeats to the 3′ ends of existing telomeres. It is specifically expressed in immortal cells, and its expression has been confirmed in hAMCs and mouse amnion-derived stem cells (ADSCs) [16,26], consistent with the proliferative capacity of these cells.

The hAMCs simultaneously express markers characteristic of three distinct embryonic germ layers. Bailo et al. [15] demonstrated that two pneumocyte markers, surfactant protein-A (a type II pneumocyte marker), and aquaporin-5 (a type I pneumocyte marker) were both expressed by amnion cells. The hAMCs express nestin, CD133, neurofilament-medium (NF-M), β-tubulin III (Tuj-1), MAP-2, and Musashi-1 [1,17], demonstrating that hAMCs have the phenotypes of both neural stem cell and mature neurons. The hAMCs express some markers specific for cardiomyocytes, such as MLC-2a, MLC-2v, cTnT, and cTnI. Cardiac-specific transcription factor GATA-4, and the cardiac-specific ion channel genes, α1c and kv4.3, were also expressed [27]. Tamagawa et al. [28] analyzed the expression of hepatocyte-specific genes in hAMCs prior to the induction of differentiation and found mRNAs encoding albumin, α-fetoprotein, cytokeratin-18, and α1-antitrypsin. Mouse ADSCs expressed markers specific for bone (osterix and alkaline phosphatase), fat (adipsin), and smooth muscle (SM22-a) [26].

These results demonstrate that hAMCs not only express markers characteristic of stem cells, but also possess the phenotypes of cells derived from ectodermal, mesodermal and endodermal origins.

8. Differentiation of hAMCs

Under appropriate culture conditions, hAMCs retain the ability to differentiate into adipocytes, osteocytes, chondrocytes, myocytes, cardiomyocytes, hepatocytes, neurocytes, and vascular endothelial cells.

8.1. Adipocytes

After culture under suitable adipogenic conditions, hAMCs differentiated into single adipocytic multivacuolar cells, in small and large colonies, the size increasing with the time of induction. Large aggregates displayed intense secretion of large neutral lipid drops [29]. Oil Red-O stain showed numerous neutral lipid droplets that were present in the cytoplasm of hAMCs, indicating the accumulation of lipid vacuoles [14,15,19,28], and mRNA for lipoprotein lipase was detected by RT-PCR [13]. Mouse ADSCs were also capable of differentiating into fat cells [26].

8.2. Osteocytes and chondrocytes

Osteogenic differentiation was revealed by morphological changes into flattened cells and by expression of alkaline phosphatase, followed by the expression of osteocalcin and collagen I in the ECM [13,14,16,17,29], and at the end of the induction period, by the formation of mineralized matrix and calcium deposits. The mRNAs for core-binding factor alpha-1 (CBFA-1) were detected

by RT-PCR in hAMCs after osteogenic differentiation [13]. CBFA-1 is a prerequisite transcription factor for osteoblastic differentiation, and is crucial for maintaining an equilibrium between bone formation and resorption [30]. Marcus et al. [26] found that mouse ADSCs exposed to osteocyte-inducing medium attained cuboidal morphologies and exhibited extensive mineralized matrix deposition.

In our study, we used an animal model and transplanted hAMCs-bovine demineralized bone matrix into a femoral head defect. New bone tissue was formed in the graft. Light microscopy showed a typical trabecular structure, osteoblasts, chondrocytes, and neovascularization in the new bone tissue. Immunohistochemical analysis revealed anti-human mitochondrial-positive cells, indicating that hAMCs survived in the graft, while osteopontin and osteocalcin expression demonstrated the construction and remodeling of new osseous tissue. Anti-factor VIII-positive cells demonstrated neovascularization of the new bone tissue (unpublished data). These phenomena indicate that under suitable circumstances, hAMCs are able to differentiate into osteocytes and endothelial cells and to form capillary-like structures.

Chondrogenic differentiation was inferred after induction by the appearance of abundant ECM and by detection of type II collagen [13,16,17,29]. Decorin mRNA was detected after chondrogenic differentiation, and its levels were higher in differentiated hAMCs than in undifferentiated cells [13].

8.3. Myocytes and cardiomyocytes

Myogenic differentiation of hAMCs was shown morphologically by the formation of long, multinucleated cells representing the precursors of myotubes. This process was accompanied by the expression of transcription factor MyoD1 and the skeletal muscle myosin heavy chain [17]. RT-PCR analysis demonstrated the expression of the myogenic-specific transcription factors, MyoD and myogenin, after induction. The skeletal muscle protein desmin was also detected after induction, in line with the fact that lineage-specific cytoskeletal filaments are synthesized during late myogenesis [29].

After stimulation with basic fibroblast growth factor (b-FGF) or activin-A, hAMCs expressed Nkx2.5 (a cardiac-specific transcription factor and the earliest marker of heart precursor cells in all vertebrates) and atrial natriuretic peptide (a cardiomyocyte-specific gene expressed in ventricular myocytes in vivo and thought to be an in vivo target gene of Nkx2.5 or α-myosin heavy chain (α-MHC)). Activin-A-stimulated hAMCs expressed α-MHC, which is a contractile protein gene specific for cardiomyocytes and is observed at the onset of spontaneous contraction in embryoids. These results indicate that hAMCs can express the genetic characteristics of cardiomyocytes after stimulation with b-FGF or activin-A. After transplantation into infarcted rat myocardia, hAMCs survived in the scar tissue for at least 2 months and differentiated into cardiomyocyte-like cells [27].

8.4. Hepatocytes

Immunocytochemistry revealed that albumin and α-fetoprotein were abundantly produced after hAMCs were cultured on type I collagen-coated dishes to induce differentiation into hepatocytes. After induction of hepatocyte differentiation, periodic acid-schiff staining showed that hAMCs had the ability to store glycogen, which is a feature of hepatocytes [28]. After exposure to hepatocyte-inducing medium, mouse ADSCs assumed a cobblestone appearance, similar to that of mature hepatocytes, and differentiated cells had the ability to take up low-density lipoprotein and to accumulate glycogen, which are functions indicative of hepatic differentiation [26].

8.5. Neurocytes

After treatment to induce neural cell differentiation, hAMCs initially assumed elongated fibroblastic morphologies, then, several days later, some of the cells converted from flat, amoeboid figures to cells displaying compact, light-refractile cell bodies, with elaborate, long processes emanating from the cell body. In many cases, the cellular processes formed networks with neighboring cells, similar to those found in primary neural cultures [1,17,24]. After neural differentiation, expression of NeuN, Gal C, MAP-2, ChAT, tyrosine hydroxylase (TH), nestin, neuron-specific enolase, NF-M, Tuj-1, and glial fibrillary acidic protein (GFAP) was significantly increased [1,15,16,24]. Similar results were obtained in mouse ADSCs [26]. These studies demonstrate that hAMCs can display the phenotypes of neural progenitor cells and can differentiate into neural phenotypes under optimal differentiation protocols.

In our study, we found that cultured human amnion cells (hACs) were able to express markers characteristic of neural stem cells (nestin, Musashi-1, and 3-polysialylated neural adhesion molecule), neural cells (TH, AchT, NeuN, MAP-2 and myelin basic protein), astrocytes (GFAP), and oligodendrocytes (2′, 3′-cyclic nucleotide 3′-phosphodiesterase) [31–33]. The hACs survived after transplantation into the brain of mice with Parkinson's disease, and the majority of the grafted mice showed a significant increase in both the duration of rotation and the number of spontaneous movements. This suggests that the transplanted hACs were able to promote endogenous neurogenesis in the subventricular zone and enhance preservation of the nigrostriatal system. Our results also showed that transplantation of hACs could increase brain-derived neurotrophic factor and glial-derived neurotrophic factor levels in the striatum, which could help to increase the survival of dopaminergic neurons [31].

Based on the previous studies, we are now focusing on the neurobiology and stem cell characteristics of hAMCs. In our recent research, we detected the expression of the

mesenchymal stem cell marker (vimentin) and the neural stem cell marker (nestin) in the stroma of the AM. The mesenchymal stem cell markers (vimentin and STRO-1), the neural stem cell marker (nestin), the dopaminergic neuron marker (TH), and the neuron marker (Tuj-1) were also expressed in cultured hAMCs (data to be published).

8.6. Vascular endothelial cells

The hAMCs can spontaneously differentiate into endothelial cells and form capillary-like structures, and these behaviors can be enhanced by VEGF. Both VEGF receptor-1 and receptor-2 (FLT-1 and KDR) were found in hAMCs, and the expression of endothelial-specific markers such as FLT-1, KDR, and intercellular adhesion molecule-1 were increased after exposure to VEGF, together with the occurrence of CD34- and von Willebrand factor-positive cells [29]. We also detected VEGF receptor-2 expression in hAMCs in vitro, and found that capillary-like structures were formed after transplantation of hAMCs into the femoral head of dogs (data not shown).

9. Advantages of hAMCs

There is growing evidence to suggest that hAMCs have many advantages, which make them a potentially important source of material for regenerative medicine:

High productivity: The number of cells in hAMC cultures was significantly higher than that in BM-MSC cultures [18,29]. The number of hAMCs yielded by primary culture (based on $4\,cm^2$ of AM) ranged between 1.3×10^6 and 1.5×10^6. Thus, considering the whole area of the AM (about $1300\,cm^2$), about 4×10^8 cells could ideally be generated, which is a suitable amount for cell therapy in a human clinical setting [29].

Powerful reproductivity: Alviano et al. [29] found that passage three hAMCs expanded approximately 300-fold in 21 days and yielded 2.9 million cells. The hAMCs expressed higher levels than BM-MSCs of the Oct-4 transcript, which codes for a regulatory protein involved in the maintenance of stem cell renewal capacity and the undifferentiated state in embryonic stem cells.

No ethical problems: Ethical concerns, and in some cases governmental policies, restrict the isolation and cultivation of human embryonic stem cells. Since the AM is usually discarded, the use of hAMCs is not associated with any trauma to the mothers or babies, and should therefore not present any ethical problems.

Hypo-immunogenicity: Many studies have found that hAMCs do not express HLA-II antigens. Cells isolated from the amnion did not induce allogeneic or xenogeneic lymphocyte proliferation responses and were able to actively suppress lymphocyte responsiveness and proliferation of PBMCs [14,15].

Other advantages: The AM is the only source of mesenchymal stem cells free from contamination with non-fibroblastoid cells [29]. In addition, no tumorigenicity of the AM has been reported after transplantation into animals or human beings.

10. Conclusion

The hAMCs have been shown to not only exhibit stem cell characteristics but also to display immunosuppressive and hypo-immunogenic features, providing a potentially useful, new therapeutic approach for treating various forms of diseases. AM has been used for treating chemical or thermal burns and ocular disorders, such as corneal ulcers and pterygium, and is an easily accessible, high yield, and ethically acceptable source of cells for regenerative medicine, with potential applications in bone remodeling, hepatic regeneration, cardiac repair, and neurological reconstruction.

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