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The Immunomodulatory Properties of Amniotic Cells: The Two Sides of the Coin

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Abstract

Among the many cell types useful in developing therapeutic treatments, human amniotic cells from placenta have been proposed as valid candidates. Both human amniotic epithelial and mesenchymal stromal cells, and the conditioned medium generated from their culture, exert multiple immunosuppressive activities. Indeed, they inhibit T and B cell proliferation, suppress inflammatory properties of monocytes, macrophages, dendritic cells, neutrophils, and natural killer cells, while promoting induction of cells with regulatory functions such as regulatory T cells and anti-inflammatory M2 macrophages. These properties have laid the foundation for their use for the treatment of inflammatory-based diseases, and encouraging results have been obtained in different preclinical disease models where exacerbated inflammation is present. Moreover, an immune-privileged status of amniotic cells has been often highlighted. However, even if long-term engraftment of amniotic cells has been reported into immunocompetent animals, only few cells survive after infusion. Furthermore, amniotic cells have been shown to be able to induce immune responses in vivo and, under specific culture conditions, they can stimulate T cell proliferation in vitro. Although immunosuppressive properties are a widely recognized characteristic of amniotic cells, immunogenic and stimulatory activities appear to be less reported, sporadic events. In order to improve therapeutic outcome, the mechanisms responsible for the suppressive versus stimulatory activity need to be carefully addressed. In this review, both the immunosuppressive and immunostimulatory activity of amniotic cells will be discussed.

Keywords

amniotic membrane, amniotic mesenchymal stromal cells, amniotic epithelial cells, immunosuppression, immunostimulation

Introduction

Mesenchymal Stromal Cells

Mesenchymal stromal cells (MSCs), first identified in bone marrow (BM-MSCs) as adherent cells that form colonies[1], were subsequently isolated from virtually all adult and perinatal tissues. MSCs are defined as tissue-culture plastic adherent cells capable of differentiating into osteoblasts, adipocytes, and chondroblasts in vitro. MSCs express cluster of differentiation (CD)73, CD90, and CD105, and lack the expression of CD11b, CD14, CD34, CD45, CD79α, and human leukocyte antigen (HLA)-DR surface molecules[2]. An intriguing property of MSCs is their broad immunomodulatory activity both in vitro and in vivo. These immunomodulatory properties are usually referred as suppressive properties, and their ability to inhibit proliferation, inflammatory cytokine production, and functionality of different immune cell populations of the innate (monocytes, macrophages, dendritic cells, neutrophils, natural killer [NK] cells, mast cells), and adaptive (T and B cells) immunity, have been largely described[3–5]. Therefore, due to their trophic and immunomodulatory properties, MSCs have been successfully exploited in the preclinical (and clinical) treatment of inflammatory and immune-based disorders[6,7]. However, different studies indicate that the majority of MSCs do not persist following infusion, are able to induce in vivo immune responses, and are immune rejected[8–14]. Moreover, MSCs exposed to interferon γ (IFN-γ) in vitro can express significantly more major histocompatibility complex (MHC) class I and MHC class II than untreated MSCs and act as antigen-presenting cells[15–17]. In addition, MSCs in specific culture

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conditions can also stimulate an immune response inducing T cell proliferation[18–21] and respond to Toll-like receptor (TLR) ligands[22–24]. In sum, together with immunosuppressive properties, increasing evidence suggests that MSCs are not intrinsically immune privileged and can possess immunostimulatory properties[25,26].

Amniotic Membrane-Derived Cells

Among the many cell types useful in developing therapeutic treatments, human placenta-derived cells have been proposed as valid candidates[27,28]. Within placenta, human amniotic membrane (AM) is a fetal tissue that constitutes, together with the chorionic membrane, the amniotic sac that encloses the fetus during pregnancy. Human amniotic epithelial cells (hAECs) and human amniotic mesenchymal stromal cells (hAMSCs) are the 2 primary cell types that comprise the AM[29]. Isolation protocols and phenotype markers have been extensively described for both hAECs and hAMSCs. After isolation, hAECs express different markers, including CD324 (E-cadherin), CD326 (epithelial cell adhesion molecule), CD73, CD166 (activated leukocyte cell adhesion molecule), and stage-specific embryonic antigen (SSEA-4). hAECs do not express CD14 and CD45. On the other hand, hAMSCs express the classical MSCs markers CD90, CD44, CD73, and CD105 (endoglin)[29]. After isolation, hAMSCs also include a subpopulation of macrophages positive for CD14, CD11b, and HLA-DR, which has been shown to decrease markedly during culture passages[30,31]. In vitro, both hAECs and hAMSCs have been shown to differentiate toward mesodermal (osteogenic, chondrogenic, and adipogenic), ectodermal (neural), and endodermal (pancreatic) lineages[29].

In addition to their differentiation potential, amniotic cells downregulate inflammation, and both hAECs and hAMSCs have emerged as valid candidates for the potential use in inflammatory and immune-based disorders[32–35]. As with BM-MSCs, amniotic cells also seem to exert their biological function through trophic mechanisms, including the secretion of cytokines and growth factors with antiapoptotic, proangiogenic, and immune-regulatory properties[36]. However, as for BM-MSCs, some immunogenic and stimulatory activity has also been raised.

In this review, we will focus on the immunomodulatory properties of amniotic cells, discussing both their main immunosuppressive potential and their sporadically described immunostimulatory activity. Moreover, we will discuss some controversial results that remain to be clarified.

Immunosuppressive Properties of Amniotic Cells

In Vitro Immunosuppression

Multiple reports have provided evidence of the immunosuppressive properties of amniotic cells that could derive from their role in maintaining fetomaternal tolerance during pregnancy. Different in vitro studies have shown that both hAECs[37–39] and hAMSCs[30,40–43], or a mix of the 2 obtained from the total AM digestion[44,45], strongly suppress T lymphocyte proliferation in a dose-dependent manner. Inhibition was observed when T cell proliferation was induced by allogeneic stimuli in vitro (in mix lymphocyte cultures [MLCs])[30,37,38,40,41,44–46], T cell receptor cross-linking (anti-CD3/anti-CD28)[30,41], mitogens such as Concanavalin A[37,39,43] and phytohemagglutinin[38,40–42], or by recall antigen[37]. Interestingly, amniotic cells can also suppress the proliferation of peripheral blood mononuclear cells (PBMCs) isolated from patients with rheumatoid arthritis[47]. Some groups have reported that hAECs and hAMSCs contact with PBMCs is a prerequisite for immunosuppressive effects[37,38], whereas other groups have shown that inhibition occurs regardless of cell–cell contact[30,40,41]. Moreover, the conditioned medium (CM) generated from the culture of amniotic cells has been shown to possess antiproliferative effects on lymphocytes[30,43,48,49], thus providing evidence of a paracrine-mediated immunosuppressive activity. Amniotic cells and their CM suppress the proliferation of both activated CD4 and CD8 T cells[50,51] and reduce different T cell subsets and related cytokines, such as T helper (Th)1 (IFN-γ, tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β), IL-12p70), Th2 (IL-5, IL-6, IL-13), Th9 (IL-9), and Th17 (IL-17A, IL-22)[43,47,50–53]. Moreover, different inflammatory cytokines are shown to be suppressed by hAMSCs in PBMCs activated in MLCs, including IL-21, IL-12/IL-23p40, regulated on activation, normal T cell expressed and secreted (RANTES), interferon gamma-induced protein 10 (IP-10), monokine induced by gamma interferon (MIG), macrophage inflammatory protein (MIP)-1α, MIP-1β, monocyte chemoattractant protein-1 (MCP-1), and the soluble Fas-ligand (FAS-L) and soluble CD40-ligand (sCD40-L)[41]. On the other hand, amniotic cells and their CM possess the ability to promote the induction of regulatory T cells in MLCs[47,50–52], which could in turn significantly contribute to the suppressive activities exerted by amniotic-derived cells.

Besides T cells, amniotic cells were found to influence the activity of several other immune cells. Indeed, CM from hAECs culture was shown to induce murine B cell apoptosis and inhibit B cell proliferative responses to lipopolysaccharide (LPS)[48].

Moreover, hAECs and hAMSCs have been shown to inhibit the cytotoxicity of NK cells against K562 cells in a dose-dependent manner[54]. Inhibition of NK cytotoxic activity was correlated with downmodulation of NK-activated receptors (NKp30, NKp44, NKp46, NKG2D, CD69) and was reversible since the reduced NK cytotoxicity was recovered by continuous culturing without amniotic cells. Together with cytotoxic activity, the release of pro-inflammatory IFN-γ by NK cells significantly decreased after amniotic cell coculture[54]. Interestingly, amniotic cell immortalization does not alter the suppressive properties toward NK cells[54], as also observed toward T cells[42].

Neutrophils have also been reported to be a target of amniotic cells. Indeed, CM from hAECs has been shown to inhibit the migration of murine neutrophils in vitro[48], while CM from AM accelerated apoptosis of neutrophils[55].

Finally, immunomodulation and paracrine effects have been observed toward antigen-presenting cells (APCs). Indeed, it was demonstrated that hAMSCs and hAECs act directly on monocytes decreasing the production of TNF-α and IL-6 cytokines induced by LPS stimulation[54]. Moreover, amniotic cells and their CM have been shown to block differentiation and maturation of monocytes into dendritic cells (DCs)[31,50,56–58] or into inflammatory M1 macrophages[50,59], switching monocyte differentiation toward macrophages with anti-inflammatory M2-like features. Indeed, macrophages generated in the presence of amniotic cells and their CM usually show reduced expression of costimulatory molecules CD40, CD80, CD86, and HLA-DR, and reduced secretion of different pro-inflammatory factors such as IL-12p70, TNF-α, CCL5/RANTES, CXCL10/IP-10, CXCL9/MIG, MIP-1α. Moreover, these cells show increased production of the anti-inflammatory cytokine IL-10[58,59], and the increase expression of the immunosuppressive molecules HLA-G was also reported in monocytes differentiated toward DCs in the presence of hAECs[56]. As a consequence, these cells were shown to be poor inducers of allogeneic T cell proliferation and inflammatory Th1 cell generation, favoring the emergence of regulatory T cells[31,59]. Additionally, phenotype, migration, and cytokine expression of murine macrophages have been affected by hAMSCs and CM from hAECs[48,60–62]. Interestingly, activation of human microglia (the resident macrophages in the brain and spinal cord) has been described to be modulated by amniotic cells and their CM. In fact, the proliferation and TNF-α inflammatory cytokine production was suppressed in microglia cocultured with hAMSCs[63]. In addition, hAMSCs or their CM promoted M2 microglial polarization in organotypic cortical brain slices exposed to ischemic injury by oxygen–glucose deprivation[64].

Overall, the ability of amniotic cells (both hAECs and hAMSCs), and their CM to dampen in vitro inflammatory conditions by suppressing the proliferation, inflammatory cytokine production, stimulatory, and cytotoxic activity of different immune cell subpopulations, and by inducing T cells and monocytes to acquire anti-inflammatory and regulatory functions, has been widely demonstrated.

In Vivo Immunosuppression

The ability of amniotic cells, and their CM, to downregulate inflammation offers significant therapeutic potential for treating inflammatory diseases. Indeed, amniotic cells and their CM have been successfully applied in different preclinical disease models where exacerbated inflammation occurs[28], such as lung fibrosis[60,65–69], liver fibrosis[70–72], wound healing[73–76], collagen-induced arthritis[47,77], inflammatory bowel disease[47], sepsis[47], colitis[47,61], experimental autoimmune encephalomyelitis (EAE, an animal model for multiple sclerosis)[47,78], and traumatic brain injury (TBI)[64]. In these models, the modulation of inflammation is thought to be a key element used by amniotic cells and their CM to trigger the restoration of tissue integrity, by dampening pro-inflammatory signals (cytokines and cells), and enhancing anti-inflammatory immune components (Tregs and M2-macrophages)[79]. Indeed, beneficial effects were associated with reduced infiltration of inflammatory cells such as neutrophils, such as neutrophils, monocytes/macrophages, and/or T cells in the injured site[47,60,61,65,70,77,78]. A reduction of inflammatory in of inflammatory microglia/macrophages has also been observed after hAECs infusion in fetal sheep brains after LPS-induced injury[80].

In addition to the reduced cell inflammatory infiltration, amniotic cell treatment was shown to be associated with decreased levels of different cytokines/factors that are linked to inflammation, such as MCP-1, TNF-α, IL-1, INF-γ, IL-6, TGF-β, platelet-derived growth factor (PDGF)-α, and PDGF-β[47,61,67,70,81–84]. Moreover, splenocytes from hAECs-treated EAE mice produced less inflammatory Th1- (IFN-γ) and Th17- (IL-17) related cytokines and increased the number of Th2 (IL-5) cells, naive CD4+ T cells, and peripheral T regulatory cells[78,85]. Similarly, amniotic cells significantly reduced the incidence and severity of collagen-induced arthritis by decreasing the development of autoreactive Th17 and Th1 cells in the lymph nodes[47]. Moreover, these draining lymph node cells were reported to produce high levels of IL-10. In addition, treated mice induced peripheral generation of antigen-specific regulatory T cells with suppressive functions, able to prevent arthritis progression when transferred to mice with collagen-induced arthritis[47]. Not only regulatory T cells but also anti-inflammatory/wound healing M2 macrophages, able to promote the switch from the inflammatory phase to the tissue-repair phase, were the predominant macrophages found in the lungs[60], in the liver[70], in the skin[59], and in tendon lesions[86], of the different animal models treated with amniotic cells or their CM.

In Vivo Cell Survival and Immune Tolerance

Long-term engraftment has been observed after xenogeneic and allogeneic amniotic cell transplantation into different immune-competent animals without the use of immune suppressants, including rabbits[87], mice[71,88], rats[89–92], guinea pigs[93], and bonnet monkeys[94]. Additionally, human DNA was detected in several organs of newborn swine and rats after xenogenic amniotic cell transplantation[45]. Similarly, human DNA was observed in the mouse liver 6 months after hAECs transplantation[95]. Moreover, the human[95] or rat[96] metabolic activity observed in the recipient liver, and the correction of the hepatic metabolic defect in a maple syrup urine disease model[97] observed after AEC transplantation, have suggested a long-term engraftment of viable cells with functional activity. Further, several clinical studies have proven that allogeneic transplantation of the AM, or cells derived thereof, does not induce acute immune rejection in

the absence of immunosuppressive treatment[28,34]. hAMSCs and hAECs are usually described as poorly immunogenic. This feature is associated with the low or limited expression on their surface of HLA class II (HLA-DR) and costimulatory molecules responsible for T cell activation, such as B7-1 (CD80), B7-2 (CD86), B7-H2 (CD275 or inducible costimulator molecule ligand), and glucocorticoid-induced tumour necrosis factor receptor ligand[32,35]. This low immunogenicity is thought to contribute to the survival of amniotic cells in the immune-competent animals. However, different studies have highlighted how amniotic cells may not actually be considered immune privileged but, on the contrary, can stimulate both an innate and adaptive immune response (see following sections). Thus, such immune tolerance seems to be mediated more by active amniotic immune-suppressive properties rather than by their true lack of immunogenicity, but this aspect remains to be clarified. Within immunosuppressive molecules, nonclassical HLA class Ib molecule HLA-G, B7-H3, programmed death ligands 1 (PD-L1) and PD-L2 have been largely supposed to be involved in amniotic cell tolerance. Indeed, hAMSCs and hAECs express HLA-G, and its expression and secretion increase after amniotic cell treatment with IFN-γ[37,41,52,98,99]. Further, immunohistochemical analyses have shown that hAECs express B7-H3 (CD276)[100]. In addition, hAMSCs express PD-L1 and PD-L2[41,63,101], and IFN-γ treatment has been shown to increase their expression in hAMSCs[41], and to induce them in hAECs, which do not constitutively express these molecules[37,100]. These molecules appear to play a role in maintaining immunologic tolerance during pregnancy[102–104], consistently downregulate human T cell cytokine production and proliferation[105,106], and direct CD4-T cells toward an immunosuppressive phenotype[104,107]. Moreover, HLA-G inhibits NK cell toxicity[108] and can lead to the generation of suppressive phagocytes[109]. Several studies have associated the presence of HLA-G with induction of tolerance after allogeneic organ transplantation[110–112]. Therefore, amniotic cell long-term engraftment observed into immunocompetent animals was often easily correlated with the expression of these tolerogenic molecules[99]. However, there is no clear demonstration of the involvement of these molecules in the in vitro and in vivo immunosuppressive activities and in vivo survival of amniotic cells. Interestingly, hAMSCs have been found to be tolerated long term in the hearts of immunocompetent rats[92]. In this study, the authors observed that pretreatment of hAMSCs with IL-10 or progesterone markedly increased hAMSCs survival in vivo, and pretreatment with IL-10 increased the level of HLA-G expressed by hAMSCs. However, after transplantation, no membrane-binding isoform of HLA-G was detected in the surviving hAMSC-derived cardiomyocytes, and there was no correlation between continuous secretion of the soluble HLA-G in the sera and survival of hAMSC-derived cardiomyocytes. Thus, the authors speculated that HLA-G might play a role in the initial process of tolerance, while it might not play a major role in the maintenance of tolerance[92]. Not only tolerogenic molecules, but the induction of regulatory T cells is also thought to be involved in tolerance. In line with this, forkhead box P3 (FOXP3)-positive regulatory T cells were reported to be constantly detected adjacent to the surviving hAMSC-derived cardiomyocytes and they were able to survive more than 4 wks in the infarcted rat hearts, suggesting that they could be involved in maintenance of tolerance[92]. Moreover, long-term graft tolerance in a mouse skin transplantation model induced by coinfusion of hAECs with limited numbers of donor unfractionated bone marrow cells was associated with deletion of donor-reactive T cells and expansion of regulatory T cells[52].

Immunostimulatory Properties of Amniotic Cells

Expression of HLA- and Costimulatory Molecules

The immunostimulatory activity of a cell, that is the ability to induce a humoral and/or cell-mediated immune response, is usually referred to as its immunogenicity. Expression of human leukocyte antigen (HLA) and costimulatory molecules on the surface of APCs are the principal elements that govern T cell proliferation, differentiation, and fate[113,114]. hAMSCs and hAECs constitutively express HLA-ABC[31,37,41,89], and the expression of HLA-DQ in hAECs, shown to increase during cell expansion, has also been reported[115]. Culture of hAECs in serum-free media has been shown to induce the expression of CD58[115], the ligand of CD2, and the primary costimulatory molecules of CD28(-) CD8(+) T cells[116]. Moreover, INF-γ stimulation augments the expression of HLA-ABC and CD40 in both hAECs and hAMSCs and induces the expression of HLA-DR in hAMSCs[41]. In addition, the presence HLA-DR and CD86 was described in freshly isolated hAMSCs preparations[30], and in the stromal layer of cryopreserved AM[89]. The expression of these immunogenic markers could confer antigen-presenting properties to hAMSCs and hAECs, and thus could be responsible for their stimulatory activities.

Expression of TLR Molecules

TLRs belong to pattern-recognition receptors and are crucial regulators of the innate immune system. TLR recognize a wide variety of pathogens (bacterial and viral products), as well as endogenous danger signals released after cell damage[117]. The effects of TLR ligands on MSCs immune-regulatory functions have been investigated, and different pro-inflammatory (MSC1) or anti-inflammatory (MSC2) MSCs phenotypes have been reported, depending on the TLR-ligand concentration, timing, and kinetics of activation[24,118–121]. In the case of amniotic cells, transcripts for all TLR (TLR1-10) were detected in both hAECs[122] and hAMSCs[123]. hAECs also expressed functional TLR5, TLR2/6, and TLR4. Indeed, activation by TLR5 and TLR2/6

agonists induced the production of inflammatory cytokines such as IL-6 and IL-8. In contrast, TLR4 activation reduced hAECs viability and induced cell apoptosis[122]. Similarly, protein expression of TLR2, 4, and 6 was detected in cultured hAMSCs, and TLR2/6 ligand led to secretion of IL-4, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-6, and IL-8[123]. The expression of TLR supports the idea that amniotic cells are sensitive to foreign pathogens and could be activated by microbial compounds contributing to inflammatory responses. However, how TLR ligands influence immunomodulatory properties of amniotic cells, generating a pro-inflammatory or anti-inflammatory phenotype (as described for MSCs from other sources) needs to be further investigated.

In Vitro Immunostimulation

Amniotic cells have been shown to be unable to induce lymphocyte proliferation when cocultured with unstimulated allogenic PBMCs at high concentrations (PBMCs: amniotic cells ratio of 1:1)[30,37,38]. Instead, low concentrations of hAECs and hAMSCs have been shown to stimulate PBMCs proliferation[38,53]. Maximum lymphocyte response was observed at amniotic cell concentrations between 3.1% and 12.5%, whereas values at lower and higher cell concentrations approximated the unstimulated state of naive PBMCs[38]. Amniotic cell concentration determined also the fate of T cells stimulated through anti-CD3/anti-CD28. Indeed, at high amniotic cell concentrations (T cell: amniotic cell ratios of 1:1 or 1:1.3), T cell proliferation was suppressed, but lower concentrations not only failed to inhibit T cell proliferation but strongly induced it[30]. Moreover, hAMSCs were shown to induce the proliferation of purified T cells cultured with anti-CD3[30]. Since stimulation with anti-CD3 is unable to induce proliferation of T cells unless APCs are also present, this reinforces the notion that hAMSCs could provide costimulatory signals and could act as APCs and activate immune responses.

In Vivo Immunostimulation

Different in vivo studies have pointed out the immunogenicity of amniotic cells. For example, in the clinical setting, repeated transplantation of AMs was shown to result in a localized immunologic reaction, such as hypopyon (a leukocytic exudate) that developed after the second and the third AM transplantation onto the ocular surface, suggesting that immunologic responses of the recipient to donor tissue may have been involved[124]. Also, macrophage infiltration into the grafts have been reported when hAECs have been grafted into healthy human volunteers and patients with lysosomal storage diseases[125,126]. Similar macrophage infiltration was observed after allogeneic AM transplantation in the cornea of healthy mice, confirming the induction of an innate immune reaction[127]. In addition, in preclinical studies, a mild T cell infiltration was present in the limbal area 1 wk after transplantation of cryopreserved AM[89]. Furthermore, hAECs transplanted in healthy mice were reported to elicit a B cell immune response. Indeed, murine anti-hAECs antibodies were detected in the mice sera collected 2 wks after hAECs injection[70]. Thus, these studies highlight how amniotic cells may not actually be immune privileged but how sometimes they can stimulate both innate and adaptive immune response.

Lack of In Vivo Cell Survival

Although the aforementioned studies describe long-term engraftment of amniotic cells in immune-competent hosts, only small number of cells engraft and are usually detected after allo- or xeno- transplantation[91,127]. On one hand amniotic cells might not persist in vivo due to adverse conditions encountered during transplantation (eg, lack of attachment, nutrient deprivation, unfavorable level of oxygen, or pH), on the other hand, an active immunological process could be responsible for their loss after transplantation. Several groups reported that they were not able to detect amniotic cells injected into different immune-competent animals. For example, Murphy and colleagues[82] did not detect hAECs transplanted in a mouse model of bleomycin-induced lung fibrosis, in any of the host tissues investigated, including lungs, brain, heart, spleen, liver, and kidneys, 7 and 14 d after cell administration. In addition, hAMSCs locally injected in the brain of a mouse model of TBI were not detected 5 wks after infusion, neither in the brain nor in the liver, lungs, or spleen[64]. Similarly, in a rat model of penetrating ballistic-like brain injury, no surviving amniotic cells were identified anywhere in the brain, at any time point (1, 2, 3, and 4 wks) after injection into the sublingual vein or directly into the injury site. Of note, cells were detected after intracerebral ventrically administration[128], suggesting that the injection route, and thus the tissue microenvironments, provides favorable or inauspicious sites for the survival of transplanted cells. Among the mechanisms that could underline the rejection of transplanted cells, the complement system has been recently proposed as central component implicated in the rapid clearance of systemically circulating MSCs after infusion[129]. Indeed, it was shown that MSCs activated complement in contact with the sera and were injured by the complement activation product membrane attack complex, both in vitro and in vivo[129]. On the other hand, MSCs express the complement-regulatory proteins CD46 (membrane cofactor protein), CD55 (decay accelerating factor), and CD59 (protectin), and that upregulating CD55 levels in MSCs were demonstrated to help in reducing their cytotoxicity after infusion[129]. Similar to BM-MSCs, hAMSCs and hAECs secrete the complement inhibitor factor H[130] and express the complement inhibitory proteins CD46, CD55, and CD59[115,131–133], and CD59 and CD55 were shown to protect the amniotic cells from lysis by human complement[133]. Thus, the balance between these mechanisms of defense and the complement-activated environment could determine the survival or the complement-mediated lysis of transplanted cells.

Other Critical Aspects and Open Questions of Amniotic Cells

Heterogeneity of Amniotic Cell Preparations

The fetal membrane has areas with different structural characteristics, including a "zone of altered morphology"[134]. Not only morphology, but also functional activity, such as mitochondrial activity, was reported to differ through the anatomical region (placental amnion and reflected amnion)[135]. Moreover, the anatomical region and the type of delivery (labor vs. no labor) have a substantial impact on the transcriptional program. For example, HLA-G, TGF-β signaling proteins, and IL-1β mRNA expression in reflected amnion was different than that in placental amnion[136]. Thus, the area sampled to isolate amniotic cells should be relevant to identify and define for consistency and comparison with other studies and could explain some controversial results that have been reported. For example, in hAECs, the expression of HLA-ABC was described to be low or moderate for some authors[88,137], or at high level for others[31,37,39,115,138], indicating the phenotypic and functional heterogeneity of amniotic cell preparations[101,139]. Moreover, the expression of CD40 is reported to be constitutively for some authors[39], or induced after INF-γ stimulation for others[37]. Also, passage culture (and the expansion culture media) influence immunologic phenotype of hAECs and hAMSCs[31,115], reinforcing the notion of heterogeneity of amniotic cell preparations and how culture conditions (passage number, culture media, INF-γ activation) influence their immunologic phenotype.

The Inflammatory Microenvironment

Several studies indicate that BM-MSCs need to be "licensed" by inflammatory signaling to become fully immunosuppressive[140–144]. For example, Ren et al. reported that BM-MSCs do not suppress IL-2-driven T cell proliferation. Such T cell blasts do not produce cytokines, thus highlighting the necessary of inflammatory cytokines to suppress T cell proliferation. IFN-γ along with other inflammatory cytokines (TNFα, IL-1α, or IL-1β) were found to boost BM-MSCs suppressive functions[140]. In line with these data, MSCs cultured in transwell, or their CM, did not exert suppressive effects if they were not exposed to IFN-γ or to additional immune cells (monocytes)[143,145,146]. In the case of amniotic cells, priming by inflammatory cytokines does not seem to be a prerequisite for their suppressive effects[30,49]. However, Banas et al. observed that hAECs are unable to inhibit IL-2-preactivated T cell blast proliferation. The authors hypothesized that preactivated T cells, in contrast to naive or memory T cells, may be less prone to inhibitory effects of amniotic cells[37]. In a different setting, preincubation of amniotic cells with inflammatory INF-γ was reported to enhance the antiproliferative properties of hAMSCs toward stimulated PBMCs[41] and even amplify inhibitory effects of hAECs toward maturation of monocyte-derived DCs[37]. Not only INF-γ, but also IL-1β, another inflammatory cytokine, was described to induce the production of the immunosuppressive molecule prostaglandin E2 (PGE2) in amniotic cells[147]. Moreover, the degree of inhibition induced by amniotic cells toward proliferating T cells has been reported to depend on the type of responder cells; in fact, hAMSCs showed a significantly enhanced capacity to suppress stimulated PBMCs rather than purified T cells[41]. Further, the type of stimulation (allogeneic stimulus, mitogens, or recall antigen) can influence the degree of T cell inhibition induced by hAECs[37]. Since each stimulation method induces dissimilar activation status of T cells, and of the other immune cells present within PBMCs, it is likely that the diverse inflammatory microenvironment uniquely influences the suppressive capabilities of amniotic cells.

Cryopreservation

Cryopreservation of cells enables their long-term storage and, in prospect of their availability for a cell-treatment, MSCs and cell products are usually cryobanked. Preserved AM has been widely used in various clinical fields, including ophthalmology and wound care[34]. In cryopreserved AM, variable amounts of amniotic cells have been shown to remain viable, to grow in culture, and to maintain some immune molecule expression[89,148,149]. For example, they retained the expression of HLA-ABC, HLA-DR, CD45, although the degree of HLA-ABC signal intensity and the number of HLA-DR-positive cells were significantly reduced in cryopreserved compared to fresh AM[148]. Thus, cryopreserved AM still induces a certain degree of immune reaction[89,127]. Compared to nonpreserved AM, cryopreserved AM was shown to secrete low levels of different immune inflammatory factors, including IL-6, IL-8, IFN-γ, leptin, MCP-1, tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2, and thrombopoietin[150]. Thus, immunogenicity of cryopreserved AM seems to be inferior than that of fresh tissues, and this was associated with the low presence of viable cells in cryopreserved AM[127]. However, when looking at the immunosuppressive potential of amniotic cells, both hAMSCs and hAECs have shown a significant reduction in the ability to inhibit T cell proliferation after cryopreservation[38]. This effect was independent of HLA-class I/II levels, which were found unaltered by the freezing process[38]. In sum, cryopreserved and nonpreserved AM and derived cells display different immunogenic and immunosuppressive properties that should be extensively addressed and considered for clinical application.

Expression of Hematopoietic Markers

hAECs and hAMSCs are usually described to be negative for CD45, CD34, or CD14, a trait that distinguishes them from hematopoietic cells[40–43,137,138,151–156]. However, in freshly isolated hAMSCs preparations, there is subpopulation of cells (5%-15%) which have been shown to express the monocyte/macrophage markers CD45, CD14, and

CD11b[30,31]. Moreover, a CD34-positive subpopulation, able to ameliorate liver fibrosis in mice with drug-induced liver injury, was identified, enriched, and characterized in AM[72]. In addition, the culture of hAECs in serum-free media induce the expression of different hematopoietic markers, including CD34 (the hematopoietic stem cell marker), CD77 (the germinal centre B cell marker, usually expressed on Epstein-Barr virus infected B cells), or CD108 (the glycosylphosphatidylinositol [GPI]-linked protein, expressed on erythrocytes, lymphocytes, lymphoblasts, and lymphoblastic cell lines)[115]. Expression of hematopoietic markers CD45, CD34, CD14 has been described also in amniotic fluid stem cells[157]. Of note, amniotic fluid is heterogeneous in composition and cells contained in it, mostly of epithelial nature, could derived also from AM[158]. In addition, Wharton's jelly MSCs may express monocyte–macrophage antigens CD68 and CD14[159,160]. Whether the expression of these hematopoietic markers could represent a distinct cell group (of fetal origin) with hematopoietic potential has yet to be determined, as well as if this subpopulation is present only in perinatal cells (amniotic cells, amniotic fluid, and Wharton's jelly MSCs) or also in adult MSCs.

Conclusions and Future Perspectives

Amniotic cells and their CM possess broad immunosuppressive properties and have been proposed for the treatment of chronic inflammation and immune alterations. However, increasing experimental data indicate that amniotic cells, as BM-MSCs, also possess stimulatory ability, both in vitro and in vivo. It has been questioned whether MSCs innately perform immunoregulatory activities, but this is now unlikely, since their primary "mission" was very likely to generate bone, cartilage, and fat[161]. In the case of amniotic cells, due to the unique role of placental tissue in inducing fetal-maternal tolerance avoiding the immunological attack of the semiallogeneic fetus by the maternal immune system, immunomodulation is likely an intrinsic property. However, if on one hand, placental cells play the critical role in fetal-maternal tolerance, on the other hand they must be ready to respond and to induce immune activation against foreign pathogens (such as bacteria or virus). Therefore, a balance between immunosuppression and immunostimulation could exist in cells isolated from the AM of placenta (hAECs and hAMSCs), and this needs to be carefully addressed before their clinical use. Recognizing the existence of both suppressive and stimulatory properties and understanding the mechanisms that underline the duality of the immune reaction may help in the design of successful immunotherapeutic approaches that reach therapeutic benefit through the manipulation of the immune system. In multiple diseases, there is an exacerbation of inflammatory conditions that need to be dampened, but in other diseases, such as cancer, the stimulation of immune system has been proposed as an efficient therapeutic strategy[162].

Immunogenicity of amniotic cells, like BM-MSCs, should not be ignored. In the case of AM transplantation, abstaining from repeated transplantation of AM from the same donor has been suggested to limit antidonor response[127]. Within host immune reaction after AM or amniotic cell transplantation, the generation of antidonor antibodies has also been observed[70]. Of note, a second infusion of amniotic cells did not lead to further increases in circulating antihuman donor antibodies[70]. It still needs to be reported whether transplantation of amniotic cells induces the generation of the classical memory B and plasma cells or rather a different (eg, regulatory) B cell subpopulation[163].

Usually, the number of engrafted cells (amniotic cells as well as MSCs) is low. Increasing amniotic cell and MSCs survival and persistence could prolong their effect and avoid repeated administrations. In the case of MSCs, different strategies have been proposed to prolong their in vivo persistence, such as their encapsulation in alginate matrix[164], or genetic engineering to overexpress IL-13[8], or other immunosuppressive factors (eg, PGE2, IDO, HLA-G, IL-10)[165,166]. Moreover, increased expression of complement inhibiting molecules, or of HLA-ABC (after INF-g treatment), was proposed as mechanism to avoid complement- or NK-mediated cytotoxicity[3,167]. However, beneficial effects were observed despite the absence of transplanted cells in injured tissue, thus the persistence of cells seems to be not required for a therapeutic effect. In the field of neurological injuries, a new interesting vision focusing on the response of the host niche to the cell graft was recently speculated[168]. In this perspective, stromal cell grafting induces an inflammatory process that leads to hypoxia-mediated apoptotic death of grafted cells, neutrophil invasion, microglia and macrophage recruitment, astrocyte activation, and neo-angiogenesis within the stromal cell graft site. These immune remodeling processes, and not only the soluble factors secreted by grafted stromal cells, are of substantial importance to the regenerative processes[168].

In order to improve the successful application of MSCs in regenerative medicine, the necessity of the development of potency assays has been underlined[169,170]. These assays consist of in vitro tests to predict the in vivo immunosuppressive activity of MSCs, and thus their therapeutic efficacy[171,172]. Among these assays, it is fundamental to consider the immunogenicity of the cells to ensure that transplanted cells possess characteristics which will minimize, if not eliminate, any possibility of rejection. Moreover, donor variability and cell heterogeneity due to culture conditions, passage number, and cell treatment (eg, INF-γ activation) represent critical aspects that could influence immunologic phenotype of cells[101101] and therefore their therapeutic outcome.

A further understanding of amniotic cell and MSCs mechanisms of action, and specifically how they interact with the microenvironment, and balance immunosuppressive and immunostimulatory activities, will be crucial in improving and developing new clinical protocols for MSC-based cell therapy.

Declaration of Conflicting Interests

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