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REVIEW

Amniotic membrane: from structure and functions to clinical applications

A. C. Mamede · M. J. Carvalho · A. M. Abrantes · M. Laranjo · C. J. Maia · M. F. Botelho

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Abstract Amniotic membrane (AM) or amnion is a thin membrane on the inner side of the fetal placenta; it completely surrounds the embryo and delimits the amniotic cavity, which is filled by amniotic liquid. In recent years, the structure and function of the amnion have been investigated, particularly the pluripotent properties of AM cells, which are an attractive source for tissue transplantation. AM has anti-inflammatory, anti-bacterial, anti-viral and immunological characteristics, as well as anti-angiogenic and pro-apoptotic features. AM is a promoter of epithelialization and is a non-tumorigenic tissue and its use has no ethical problems. Because of its attractive properties, AM has been applied in several surgical procedures related to ocular surface reconstruction and the genito-urinary tract, skin, head and neck, among others. So far, the best known and most auspicious applications of AM are ocular surface reconstruction, skin applications and tissue engineering. However, AM can also be applied in oncology. In this area, AM can prevent the delivery of nutrients and oxygen to cancer cells and consequently interfere with tumour angiogenesis, growth and metastasis.

Keywords Amniotic membrane · Ocular surface reconstruction · Skin · Tissue engineering · Oncology

A. C. Mamede (✉) · M. J. Carvalho · A. M. Abrantes · M. Laranjo · M. F. Botelho
Biophysics Unit, IBILI, Faculty of Medicine, University of Coimbra, Coimbra, Portugal
e-mail: ana_mamede@hotmail.com

A. C. Mamede · C. J. Maia
CICS-UBI, Health Sciences Research Centre, University of Beira Interior, Covilhã, Portugal

A. C. Mamede · M. J. Carvalho · A. M. Abrantes · M. Laranjo · M. F. Botelho
Centre of Investigation on Environment, Genetics and Oncobiology, Faculty of Medicine, University of Coimbra, Coimbra, Portugal

M. J. Carvalho
Gynaecology and Obstetrics Service, University Hospitals of Coimbra, Coimbra, Portugal

Introduction

Fetal development is achieved by a complex system consisting in the umbilical cord, amniotic fluid and placenta. Fetal membranes are composed of two layers: an outer layer (chorion), which contacts maternal cells and an inner layer (amniotic membrane; AM). AM or amnion is a thin membrane on the inner side of the placenta; it completely surrounds the embryo/fetus and delimits the amniotic cavity, which is filled by amniotic fluid (Pollard et al. 1976; van Herendael et al. 1978). At present, following years of research on the structure, functions and properties of AM, it has numerous applications in the area of regenerative medicine. In 1910, Davis used AM for skin transplantation and, later, Stern and Sabella in 1913 used AM for management of skin burns and superficial wounds (Davis 1910; Stern 1913; Sabella 1913). Since then, this biological structure has begun to be considered an attractive treatment for problems associated with the genito-urinary tract, oral cavity, skin, stomach, larynx, head and neck (Amer and Abd-El-Maeboud 2006; Toda et al. 2007; Baradaran-Rafii et al. 2007; Fernandes et al. 2005; Dua et al. 2004). In ocular surface reconstruction, skin applications and tissue engineering, AM is an option in several treatments and has several clinical applications. The special properties and

availability of AM make it an ideal candidate for many clinical situations, such as oncology.

Amniotic membrane: structure and function

After implantation of the blastocyst in the endometrium, the walls of the blastocyst become the outer layer of membranes that surrounds the embryo during its development: the chorion. At the same time, the embrionic cells and the trophoblast separate, leading to the formation of the AM by the innermost trophoblastic cells. The amniotic sac fills with fluid, expands gradually and adheres to the inner surface of the chorion. The chorionic cavity disappears but the two layers do not fuse histologically and remain separable (Baradaran-Rafii et al. 2007; Sadler 2000). AM is a translucent biological structure that has no nerves, muscles or lymph vessels. Its source of nutrients and oxygen are the chorionic fluid, amniotic fluid and fetal surface vessels, being the nutrients supplied by diffusion (Toda et al. 2007; Sadler 2000; Benirschke 2000). Energy is primarily derived through an anaerobic glycolytic process because of the restricted oxygen supply (Benedetti at al. 1973). Glucose transporter proteins 1 and 3 have been found in the apical surface of the epithelial cells of AM (Wolf and Desoye 1993).

AM thickness varies from 0.02 mm to 0.5 mm and consists in three main histological layers: the epithelial layer, the thick basement membrane and the avascular mesenchymal tissue (Toda et al. 2007; Benirschke 2000; Bourne 1960; Danforth and Hull 1958). The inner layer, adjacent to the amniotic fluid, is constituted by a single homogeneous layer of cuboidal epithelial cells firmly fixed to the basement membrane, which is, in turn, attached to a condensed acellular layer composed of collagen type I, II and V (Sadler 2000). Amniotic epithelial cells have many microvilli at their apical surface and probably have an active secretory function and intra- and trans-cellular transport functions (Pollard et al. 1976). These cells have a large irregular nucleus with a large homogeneous nucleolus and many intracytoplasmic organelles and pinocytic vesicles (Sadler 2000). Amniotic epithelial cells express epidermal markers, such as glycoprotein CA125 and oxytocin receptors and are also positive for antigen CD44 and desmin (Toda et al. 2007; Benedetto et al. 1990). Erythropoietin and its receptors are expressed in human amniotic epithelial cells. Erythropoietin, whose functions are still unknown in the AM, stimulates the differentiation, proliferation and survival of erythroid precursors and its production is regulated by the concentration of oxygen in the blood. Ogawa et al. (2003) have reported that erythropoietin production in human amniotic epithelial cells is stimulated by progesterone but is not stimulated by hypoxia or 17β-estradiol.

The basement membrane contains large amounts of proteoglycans that are rich in heparan sulphate and that serve as a permeable barrier to amniotic macromolecules and several molecules with a structural function enabling the maintenance of membrane integrity. These molecules are actin, α-actinin, spectrin, ezrin, several cytokeratins, vimentin, desmoplakin and laminin (King 1985; Wolf et al. 1991; Akashi et al. 1999). The expression of laminin has been widely investigated, because this molecule contributes to cell survival, differentiation, shape and movement and is involved in the maintenance of tissue phenotypes (Akashi et al. 1999; Takashima et al. 2008).

The outer layer of AM is composed of mesenchymal fibroblast-like cells that are probably derived from the mesodermal embryonic plate and that are scattered in a full-term membrane. The content of collagen-rich mesenchymal layer increases its tensile strength. Some authors call the outermost layer of the amnion the zona spongiosa, because its abundant content of proteoglycans and glycoproteins produces a spongy appearance in histological preparations. This layer lying adjacent to the chorion laeve is an almost acellular structure and contains a nonfibrillar meshwork mostly of type III collagen (Toda et al. 2007; Parry and Strauss 1998). The spongy layer, closely connected to the chorionic membrane, consists in wavy bundles of reticulum bathed in mucin; hence, AM is easily separated from the chorion by means of blunt dissection (Niknejad et al. 2008). As expected, amnion varies in histological appearance from conception to maturity and several different patterns are often noted, even at term (Pollard et al. 1976).

AM is not just a simple avascular structure; it has multiple metabolic functions such as the transport of water and soluble materials and the production of bioactive factors, including vasoactive peptides, growth factors and cytokines (Cunningham 2001). One of the basic functions of the AM is to provide the developing embryo with protection against desiccation and an environment of suspension, in which the embryo can grow free from pressures of the structures that surround its body. Indeed, tractional resistance of AM is mainly related to the condensed layer of interstitial collagen type I, II and elastin. On the other hand, the elasticity of the amnion is mainly attributable to collagen type III (Sadler 2000). Because of the presence of interstitial collagens, one of the important properties of AM is its resistance to proteolytic factors (Fukuda et al. 1999). AM also has an important role during birth, because the substances produced by the epithelium of AM allow the initiation and maintenance of uterine contractility. Prostaglandins, especially prostaglandin E2 (Okazaki et al. 1981) and enzymes integrated into the synthesis of prostaglandins, such as phospholipases and prostaglandin synthase, are some of the molecules that are produced in the amniotic epithelium and that have a role in the physiology of contraction (Toda et al. 2007; Bryant-Greenwood et al. 1987). Human chorionic gonadotrophin, corticotrophin-releasing hormone and glucocorticoids regulate

prostaglandin production (Gibb and Lavoie 1990; Jones and Challis 1989; Toth 1992a; 1992b; Toth et al. 1996). The presence of prostaglandin dehydrogenase, a prostaglandin-inactivating enzyme, has also been demonstrated by Cheung et al. (1990). Interleukin (IL) 4 has also been shown to suppress the activity of prostaglandin-H synthase-2 in amnion epithelial cells (Keelan et al. 1999). During pregnancy, amniotic epithelium is highly metabolically active and has an important role in maintaining the pH of the amniotic fluid, keeping it at a constant value of 7.10 (Shumway et al. 1999). Carbonic anhydrase isoenzymes CA-1 and CA-2 are found in amniotic epithelial cells (Crescimanno 1993; Muhlhauser et al. 1994). This enzyme, which is involved in bicarbonate/carbon dioxide metabolism, is thought to have a regulatory role in maintaining constant the pH of amniotic fluid.

Relevant properties of the amniotic membrane

Pluripotency of amnion-derived cells

Cells of AM have pluripotent properties and, for this reason, are an attractive source for transplantation (Toda et al. 2007). During the development of the fetus, the inner layer of the blastocyst provides the hypoblast and epiblast, the latter producing the amniotic epithelial layer (amniotic ectoderm; Enders and King 1988). Since the epiblast is also the source of all of the germ layers of the embryo, the layer of amniotic epithelial cells also possesses a wide differentiation capacity (Miki et al. 2005). Indeed, several in vitro and in vivo studies suggest that amnion-derived cells have the potential to differentiate into all three germ layers: endoderm, mesoderm and ectoderm. Several studies have documented that amniotic cells express various surface markers associated with embryonic stem cells, e.g. stage-specific embryonic antigen 3 and 4 (SSEA-3 and −4), TRA-1-60 and TRA-1-81 (Niknejad et al. 2008). Epithelial and mesenchymal amniotic cells also express various stem cell markers, such as octamer-binding transcription factor 4 (OCT-4), hepatocyte nuclear factor 3β (HNF-3β), nanog and nestin. These data suggest that both epithelial and mesenchymal amniotic cells have a pluripotent potential to differentiate (Miki et al. 2005; Wei et al. 2003; Takashima et al. 2004).

Hepatocytes

Takashima et al. (2004) have found that several hepatic genes are expressed in amniotic epithelial cells, namely: albumin, α1-antitrypsin, cytokeratin-18, glutamine synthetase, carbamoyl phosphate synthetase I, phosphoenolpyruvate carboxykinase, cytochrome P450 2D6 and 3A4 (genes involved in drug metabolism). Hepatocyte nuclear factor 3γ (HNF-3γ), one of the isoforms of transcription factor HNF-3 and CCAAT-enhancer-binding protein-α (C/EBP-α) are genes involved in the regulation of the process of transcription in hepatocytes and have been identified in amniotic cells. The HNF-3γ gene induces the expression of albumin, α-fetoprotein, α1-antitrypsin and transthyretin. On the other hand, the C/EBP-α gene controls glycogen storage and the gene expression of albumin, α-fetoprotein, transthyretin and tyrosine aminotransferase (TAT; Takashima et al. 2004). Immunohistochemical studies have identified albumin and glycogen in cultured amniotic epithelial cells. Albumin production and amnion survival have been found in vivo after the implantation of amniotic tissue in the peritoneal cavity of mice (Takashima et al. 2004; Nakajima et al. 2001). Amniotic epithelial cells do not express ornithine transcarbamylase (ammonia metabolism-related genes), glucose-6-phosphatase or tryptophan 2,3-dioxygenase (amino acid metabolism-related genes; Toda et al. 2007).

Cardiomyocytes

The differentiation potential of amniotic cells into cardiomyocytes has been evaluated in order to establish a possible cellular source for transplantation. The amniotic cells exhibit some specific cardiac transcription factors, namely cardiac-specific transcription factor GATA4, cardiac-specific genes such as myosin light chain (MLC)-2a, MLC-2v, cTnI and cTnT, α-subunits of the cardiac-specific L-type calcium channel (α1c) and the transient outward potassium channel (Kv4.3; Toda et al. 2007). After stimulation with specific growth factors, such as fibroblast growth factor (FGF) or activin A, amniotic cells express a specific cardiomyocyte transcription factor (Nkx2.5) and a cardiac-specific marker (atrial natriuretic peptide; Tanaka et al. 1999). Co-culture experiments have confirmed that amniotic cells have the ability to integrate into cardiac tissue and to differentiate into heart cells. In vivo studies have also demonstrated cardiomyocyte differentiation after the injection of amniotic cells into scar tissue post-myocardial infarction (Kasahara et al. 2001). Despite the encouraging results, the contractile capacity of these cells is not yet established (Toda et al. 2007).

Chondrocytes

The amniotic cells express genes related to chondrocytes, such as the SOX (SRY-related HMG box) family, bone morphogenic proteins (BMP) and their receptors (Toda et al. 2007). Toda et al. (2007) have shown that induction in vitro with BMP-2 leads to expression of collagen II and aggrecan. In a study reported by the same authors, amniotic cells were implanted into non-cartilage tissue in an animal model with BMP-2 or were implanted with a collagen scaffold into defects generated in rat bone. As a result, morphological changes with the deposition of collagen type

II were observed. These results suggest that amniotic cells have the potential to differentiate into chondrocytes in vivo and in vitro, suggesting a potential role of amniotic cells in the treatment of damaged cartilage tissue (Toda et al. 2007).

Pancreatic β cells

The possibility of inducing insulin production by amniotic epithelial cells was investigated by Wei et al. (2003) by in vitro stimulation with nocitinamide. The authors verified the expression of insulin mRNA in cultivated amniotic epithelial cells and the normalization of blood glucose values for several weeks after the implantation of amniotic cells into streptozotocin-induced diabetic mice (Wei et al. 2003). Despite the results obtained so far, we need to obtain mature β cells through the differentiation of amniotic cells in order to understand several regulatory pathways, particularly those related to the Maf , HB9 and snail family (Toda et al. 2007; Nishimura et al. 2006; Wang 2004; Rukstalis et al. 2006).

Neuronal cells

The expression of acetylcholine, catecholamines, dopamine, neurotrophic factors, activin and noggin has been found in epithelial and amniotic basement membrane cells (Sakuragawaa et al. 1997; Koyano et al. 2002). Musashi-1, a marker of central nervous system stem cells has previously been detected in undifferentiated cells of the amniotic basement membrane (Kaneko et al. 2000). Neural grafts with amniotic epithelial cells in animal models of Parkinson's disease result in the synthesis and release of catecholamine and neurotrophic factors, such as nerve growth factor, neurotrophin-3 and brain-derived neurotrophic factor (Toda et al. 2007; Kakishita et al. 2000; Uchida et al. 2000).

Auditory system

Amniotic epithelial cells express Cx26 on their cell membranes and nuclei and also express sodium-potassium pumps on their cell membranes and in their cytoplasm, characteristic of cochlear fibrocytes, indicating that human amniotic epithelial cells can act as functional fibrocyte-like cells (Spicer and Schulte 1998). The transplantation of amniotic cells into an animal model has also revealed the same expression of Cx26 and sodium-potassium pumps but only for a period of 3 weeks (Yuge et al. 2004). These results encourage further studies in the context of the pathology of the inner ear (Toda et al. 2007).

Anti-inflammatory, anti-bacterial, anti-viral and immunological characteristics

Cells of AM are ideal candidates for allotransplantation because of their anti-inflammatory, anti-bacterial, anti-viral effects and their low immunogenicity. The mechanisms by which AM exerts its anti-inflammatory properties are not yet clear but some theories that might explain this effect have however been proposed. AM can serve as a physical barrier, confining the inflammatory cells to the affected area and reducing the inflammatory mediators. Shimmura et al. (2001) have found that, when the membrane is applied as a patch in vivo, AM entraps T lymphocytes. This result corroborates experimental data obtained by Park and Tseng (2000) in a study of rabbit eyes; the authors state that the stromal matrix of AM attracts inflammatory cells. Li et al. (2005) have reported that amniotic epithelial cells secrete soluble factors that inhibit both innate and adaptive immune system cells. Hyaluronic acid also plays an important role at the level of adhesion of inflammatory cells to the stroma of AM, since it is a ligand for CD44, an antigen expressed by inflammatory cells (Higa et al. 2005). Furthermore, amniotic cells produce a variety of anti-inflammatory factors, such as IL-1 and IL-2 receptor antagonists and IL-10, plus endostatin, all of which inhibit endothelial cell proliferation, angiogenesis and tumour growth (Hao et al. 2000). IL-1β is produced by amniotic epithelial cells in vitro and is also thought to be involved in the regulation of prostaglandin production (Rote 1993). IL-6 and IL-8 have been found in high concentrations in the amniotic fluid at term and the expression of these cytokines is increased in the presence of IL-1β, tumour necrosis factor alpha (TNF-α) and bacterial lipopolysaccharide, being the expression of both cytokines down-regulated by steroids (dexamethasone; Keelan et al. 1997). AM also expresses some inhibitors of matrix metalloproteinase (MMPs), enzymes that are expressed by infiltrating polymorphonuclear cells and macrophages (Hao et al. 2000; Kim et al. 2000). Curiously, the supernatant of cultured human amniotic epithelial cells inhibits the chemotactic activity of neutrophils and macrophages, reduces the proliferation of T and B cells and induces their apoptosis (Li et al. 2005).

AM might decrease the risk of infection because of its anti-microbial and anti-viral properties, as a result of not only its function as a biological barrier but also the expression of several molecules that determine these properties. Cystatin E, present in AM, is an analogue of the cysteine proteinase inhibitor and has several anti-viral properties (Ni et al. 1997). AM might function as a barrier against bacterial infiltration by adhesion to the wound surface, thereby reducing bacterial load. In clean surgical wounds, the haemostatic property of collagen fibres in the amniotic basement membrane prevents haematoma formation, reducing microbial accumulation and the risk of infection (Baradaran-Rafii et al. 2007). Adhesion to the wound surface prevents dead space formation and serous discharge accumulation, which is another mechanism of action against infection. Furthermore, the formation of fibrin

filaments during wound healing results in adhesion of the wound to AM collagens, leading to bacterial entrapment and stimulation of phagocyte migration (Baradaran-Rafii et al. 2007). Evidence is available that AM decreases bacterial proliferation, even in contaminated wounds (Baradaran-Rafii et al. 2007; Bari et al. 2002). Cells of AM have the ability to produce β-defensins, especially β3-defensin, a group of anti-microbial peptides that are expressed in mucosal surfaces by epithelial cells and leucocytes and that belong to the innate immune system (Krisanaprakornkit et al. 1998; Harder et al. 2000; King et al. 2007; Buhimschi et al. 2004). Secretory leucocyte proteinase inhibitor (SLPI) and elafin are two elastase inhibitors that are expressed by AM and that possess anti-inflammatory and anti-microbial properties (King et al. 2003, 2007; Buhimschi et al. 2004). Treatment of AM with IL-1 receptor antagonists or lactoferrin results in anti-inflammatory and anti-microbial effects (Niknejad et al. 2008; Kanyshkova et al. 2001; Gomes et al. 2005).

Interestingly, not only AM but also the amniotic fluid has antimicrobial properties in wound healing. Thadepalli et al. (1978) have studied the antimicrobial effect of amniotic fluid obtained during the first (AF1) and second (AF2) trimesters and compared it with that of the third (AF3) trimester against anaerobic bacteria; the authors conclude that AF1 is the least inhibitory, AF3 the most inhibitory and AF2 intermediate for the organisms tested. Miller and colleagues (1976) have also studied the antimicrobial properties of amniotic fluid. To this end, sixty-one amniotic fluid samples from women in their second and third trimesters of pregnancy were examined for antimicrobial activity. The authors reported that 70% of the fluids were found to be active (Miller et al. 1976). The immunological response to the transplantation of AM is negligible, as demonstrated in several studies that have found that no human volunteers show clinical signs of acute rejection (Kamiya et al. 2005; Akle et al. 1981). Initially, amniotic epithelial cells were believed not to express human leucocyte antigen system (HLA) -A, -B or -DR antigens (Adinolfi et al. 1982). However, several studies suggest that in-vitro-cultured epithelial and mesenchymal amniotic cells produce small quantities of all HLA class I molecules, including class Ia (HLA-A, -B, -C, -DR) and class Ib (HLA-G, -E; Adinolfi et al. 1982; Kubo et al. 2001). Houlihan et al. (1995) have found the expression of class 1b HLA antigen on full-term amnion but extremely limited expression of class 1a antigens. However, HLA class II antigens are not expressed by amnion epithelial cells (Adinolfi et al. 1982; Hammer et al. 1997). The expression of Fas ligand by epithelial and mesenchymal amniotic cells in apoptosis might exert an immunosuppressive effect (Shimmura et al. 2001; Kubo et al. 2001; Hammer et al. 1997; Runić et al. 1998). Clinical applications of AM depend on the risk of immunogenicity, being the locality of transplantation and rejection dominant factors (Toda et al. 2007). Moreover, several studies have indicated that the immunogenicity of cryopreserved AM is lower than that of fresh AM, with cryopreserved cells being expected to be nonviable (Kubo et al. 2001).

Anti-angiogenic and pro-apoptotic characteristics

Both AM and the amniotic cell culture supernatant have anti-angiogenic properties. The study of Shao (2004) has revealed that human AM inhibits endothelial cell growth and suppresses corneal neovascularization. Other studies have shown that transplantation of human AM decreases the vascularization of the ocular surface, being this anti-angiogenic effect one of main reasons for therapeutic application of the membrane (Shao 2004; Kim and Tseng 1995a). These properties can be explained, in part, by the inhibition of cell growth and migration of vascular endothelial cells because of the action of the membrane as a physical barrier, thereby preventing the diffusion of promoters of vascularization (Kobayashi et al. 2002). AM has also been established to contain large amounts of extracellular matrix proteins such as collagen α2(IV), laminin-1, laminin-5, fibronectin and collagen type VII, proteins that are involved in the suppression of corneal neovascularization (Fukuda et al. 1999). Amniotic cells additionally produce various kinds of cytokines, including several anti-angiogenic factors, endostatin and thrombospondin-1 (Hao et al. 2000). Thrombospondin-1 is a potent anti-angiogenic chemical produced by only 20% of mesenchymal cells, whereas endostatin is a powerful anti-angiogenic and endothelial cell growth inhibitor. MMP-1, MMP-2, MMP-3, MMP-4, IL-1 receptor antagonist, collagen XVIII and IL-10 are some of the proteins found in AM and that might have anti-angiogenic activity (Hao et al. 2000). Pigment epithelium-derived factor (PEDF) expressed in the AM plays a major role in eliciting the anti-angiogenic activity of AM (Shao 2004). Obviously, if the anti-angiogenic effects are attributable to chemical inhibitors, we can expect that fresh AM will be more effective than cryopreserved membrane.

AM secretes some substances that act primarily as pro-apoptotic agents and can accelerate apoptosis of polymorphonuclear neutrophils (Zhou et al. 2003). Li et al. (2006) have reported that the apoptosis of interferon-γ-activated macrophages promoted by AM is not mediated directly by nitric oxide (NO) and TNF-α. However, this effect is commonly caused by the interruption of survival pathways mediated by both nuclear factor κB (NF-κB) and Akt-FKHR (Li et al. 2006).

Amniotic membrane as a promoter of epithelialization

AM act as a basement membrane that facilitates epithelial cell migration (Tseng et al. 1997), reinforces adhesion of basal epithelial cells (Shimazaki et al. 1998), promotes epithelial differentiation (Guo and Grinnell 1989) and prevents epithelial apoptosis (Boudreau et al. 1995). It produces various growth

factors that can stimulate epithelialization (Koizumi et al. 2000) and might also reduce the pain experienced by patients. In a prospective study, the safety, reliability and healing effect of AM in venous leg ulcers was evaluated. All patients showed a significant reduction in pain after the transplantation, with no adverse effects. Epithelialization was promoted and excessive fibrosis was reduced (Mermet et al. 2007). These effects can be explained because the fibroblasts are naturally responsible for scar formation during wound healing and are activated by transforming growth factor β (TGF-β). Once the AM inhibits the expression of TGF-β receptors in fibroblasts, less fibrosis occurs (Hao et al. 2000; Choi and Tseng 2001; Khouw et al. 1999). Therefore, the membrane can modulate the healing of the wound by promoting tissue reconstruction, rather than promoting scar tissue formation (Tseng et al. 1999; Lee et al. 2000). Koizumi et al. (2000) have found TGF-β1, -β2 and -β3 mRNA by reverse transcription with the polymerase chain reaction (RT-PCR) and the respective proteins by enzyme-linked immunosorbent assay in the amniotic epithelium and stroma. They have also found high levels of TGF-α, epidermal growth factor (EGF), keratocyte growth factor (KGF), hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF) and two growth factor receptors (KGFR and HGFR), all these growth factors being expressed by the amnion, in both epithelium and stromal regions and proposed that these growth factors play several important roles in ocular surface wound healing. AM also prevents wound surfaces from drying out, which accelerates wound healing. Notably, the presence of proteinase inhibitors might facilitate wound healing (Kim et al. 2000). AM relieves the pain in burn patients because of its adhesion to the wound surface and coverage of dermal nerve endings (Hajiiski 1990). Lee and Tseng (1997) have reported the successful re-epithelialization in 10 out of 11 cases of persistent epithelial defect following AM transplantation.

Non-tumorigenicity of amniotic membrane

No significant tumorigenicity has been reported in humans receiving transplants of amniotic cells or patients in which an attempt has been made to correct congenital lysosomal storage diseases (Kamiya et al. 2005; Akle et al. 1981; Sakuragawa et al. 1992; Scaggiante et al. 1987). Robinson et al. (2002) confirmed, by molecular analysis of AM, the presence of trisomy mosaicism in 16 (48%) of 33 cases studied. However, the trisomy is absent from most fetal tissues (Robinson et al. 2002). So far, no known effects have resulted from the clinical application of these amnions.

Few ethical issues

Immediately after birth, the ovular membranes and placenta are usually eliminated. Use of AM thus does not involve ethical issues and research into the clinical application of AM is therefore acceptable. As a research interest at the moment, groups undertaking investigations with AM should seek the approval of the ethical committees of their research institutions and women should be asked for their consent for the utilization of AM. To diminish the possibility of contamination, AM should be obtained by caesarean section with AM integrity being assured (mainly as an elective procedure). As a routine procedure, all women should be screened by means of infectious serological studies. The most relevant properties of amniotic membrane are summarized in Fig. 1.

Amniotic membrane: most common clinical applications

AM can be applied in clinical practice in various fields of medicine, particularly in the treatment of skin burns and prevention of tissue adhesion in surgery of the head, neck, abdomen, genito-urinary tract and larynx (Kim and Tseng 1995b). We will next focus on the largest, best known and most auspicious applications of AM: ocular surface reconstruction, wound healing, gynaecology and tissue engineering.

Ocular surface reconstruction

The transplantation of human AM was introduced in ophthalmology over seventy years ago. This type of transplantation was first described in 1940 by De Rotth in the treatment of conjunctival defects and symblepharon (Rotth 1940).

Six years later, good results were reported in the treatment of acute chemical burns by Sorsby et al. (1947). Thereafter, for no evident reason, the use of AM was abandoned or went unreported until recently, when renewed interest has emerged in its potential role in the treatment of ocular surface disorders. Three types of applications for human AM transplantation in ocular surface disorders are available, as we can see in Table 1. Since 1995, AM transplantation has been successfully applied to ocular surface reconstruction in patients with a variety of ocular surface diseases, including conjunctival surface reconstruction (pterygium surgery, conjunctival tumour excision, symblepharon release, repair of leaking blebs, scleral melt, fornix formation, socket reconstruction, chemical burns, cicatrizing keratoconjunctivitis), corneal surface reconstruction (persistent epithelial defects, non-healing stromal ulcers, partial and total limbal stem cell deficiency, painful bullous keratopathy, band keratopathy) and as a substrate for the *ex-vivo* expansion of limbal and conjunctival stem cells (Burman et al. 2004). AM is used not only as a substitute but also as a scaffold upon which cells can migrate and regenerate, forming new and healthy tissue.

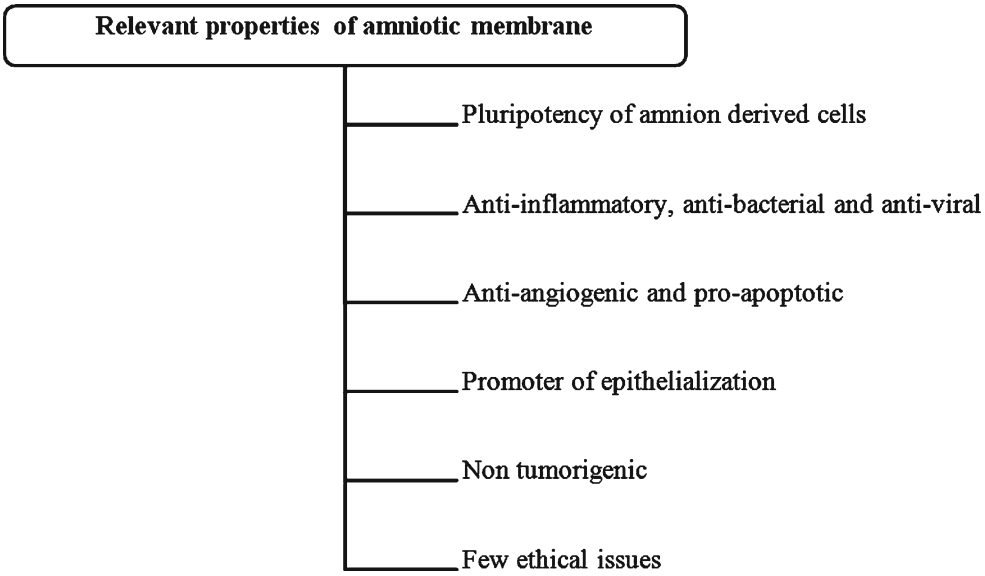

Fig. 1 Relevant properties of the amniotic membrane

A variety of characteristics, such as the promotion of re-epithelialization, the decrease of inflammation and fibrosis and the inhibition of angiogenesis, make AM useful in clinical applications. Another advantage of AM for use in ocular surface is that it is an avascular tissue. Inhibition of neovascularization during corneal surface reconstruction is important. With regard to anti-angiogenic activity of human AM, PEDF (expressed by the basement membrane of AM) is thought to play a major role in inhibiting endothelial cell growth in the cornea (Shao 2004). PEDF promotes the proliferation of bovine cornea epithelial cells but inhibits the proliferation of human umbilical vein endothelial cells and bovine retinal capillary endothelial cells (Toda et al. 2007).

Skin applications

AM has unique characteristics, including anti-adhesive effects, bacteriostatic action, wound protection, pain reduction and the capacity for initiating epithelialization. In a prospective study, the safety, reliability and healing effect of AM in venous leg ulcers has been evaluated. All patients showed significant pain reduction after transplantation of membrane without adverse effects, being re-epithelialization promoted and excessive fibrosis reduced (Toda et al. 2007).

Gynaecology

Asherman's syndrome is defined as the presence of intrauterine adhesions that are formed during the healing of defects of the endometrial surface and that partially or completely obliterate the uterine cavity. Patients present hypomenorrhea or amenorrhea, infertility and obstetric complications (Pabuçcu et al. 1997). Surgical treatment has been the standard method for resolving this complication. Since the 1970s, hysteroscopic treatment has been the preferred method, followed by hormonal treatment. In a study that involved the assessment of the safety and efficacy of an AM graft following hysteroscopic lysis of uterine adhesions, the authors described the reduction of the recurrence of adhesions and improved endometrial regeneration (Amer and Abd-El-Maeboud 2006).

Regenerative medicine

AM is an option for tissue engineering because it is easy to obtain, is readily available, can be used with specific applications and has a small risk of infection or immune reaction. Thus, AM can be used as a support matrix in regeneration, promotes re-epithelialization, reduces inflammation and

Table 1 Types of application for amniotic membrane (*AM*) transplantation in ocular surface disorders

Type of AM tranplantation	Application
Graft	Transplantation of AM on corneal or scleral stroma induces proliferation and differentiation of the ocular surface epithelium
Patch	AM can be used to cover the inflamed ocular surface, especially when associated with epithelial defects. It is also useful in the acute phase of chemical/thermal burns or Stevens Johnson syndrome
Stuff	Deep corneal/scleral ulcerations or small perforations refractory to conventional medical therapy can be treated by stuffing small pieces of AM into the stromal defects, followed by AM grafting and patching over the area

suppresses fibrosis (Burman et al. 2004; Portmann-Lanz et al. 2007; Yeh et al. 2005). Because of various structural components of AM, such as collagen, laminin, fibronectin and vitronectin, adhesion and cell migration can be expected. These adhesion ligands activate signal pathways after interactions at cell surface receptors (Niknejad et al. 2008). A recent study of tissue engineering applied to the healing of iatrogenic defects of fetal membranes compared amniotic native supports and a synthetic support, namely polyesterurethane (DegraPol), in an animal model (rabbit; Ochsenbein-Kölble et al. 2007). Native supports were better preserved than polyesterurethane at the end of 7 days but the integrity of the membrane was comparable in both groups. Native supports suffered abundant re-epithelialization with minimal signs of inflammation. The synthetic support crumbled in some cases, showing moderate local re-epithelialization (Ochsenbein-Kölble et al. 2007).

AM can be used either with the amniotic epithelium (intact AM) or without it (denuded AM). Several experimental studies have been performed with AM as a scaffold. The human amnion is reported to be an effective conduit for peripheral nerve regeneration, being AM also a biodegradable scaffold with unique biochemical and mechanical characteristics for nerve regeneration (Mohammad et al. 2000; Mligiliche et al. 2002). Denuded AM has been investigated as a carrier of chondrocytes and AM has been suggested as serving as a carrier matrix for cartilage regeneration (Jin et al. 2007). The culturing and seeding of epithelial cells on an amnion scaffold is a frequently used method for ocular surface and skin reconstruction (Fatima et al. 2006; Yang et al. 2006; Capeáns et al. 2003). Culture of endothelial cells on an AM scaffold has also been reported as a potential approach for vascular tissue engineering (Ishino et al. 2004; Tsai et al. 2007).

Amniotic membrane as a therapy against cancer: utopia or reality?

The onset of cancer is attributable to a complex interaction between genetic and environmental factors. Only 5% of all cancers are estimated to result from genetic inheritance, with environmental factors being determinant in the onset of cancer. Exposure to chemicals carcinogens, to ionizing radiation (e.g. X-rays, gamma rays, charged particles, neutrons) and to non-ionizing radiation (e.g. infrared, ultraviolet, microwave) and chronic inflammatory states, together with diet, tobacco and lifestyle, are factors that need to be taken into account in the aetiology of cancer. The ethnicity, gender, age and general state of health also influence the rates of incidence and mortality of this disease (Perera and Weinstein 2000). As a result of the interaction between genetic and environmental factors, proto-oncogenes, tumour suppressor genes and genes that regulate cell growth can mutate and carcinogenic alterations can occur in cells (Knudson 1993; Weinberg 1991). These mutations in cells are associated with the acquisition of autonomy, growth and insensitivity to inhibitory signals and the cells acquire an unlimited replicative potential. Furthermore, cells acquire the ability to form new vessels and to undertake invasion and metastasis, thus escaping to the natural mechanisms of control that, under normal conditions, would lead to cell death (Hanahan and Weinberg 2000; Yu and Zhang 2004; Cooper and Hausman 2003).

Angiogenesis is a characteristic associated with malignancies and inflammatory, ischaemic, infectious or immunological diseases. Vascular support brings nutrients and oxygen to the tumour. At the same time, it provides a means for tumour cells to migrate from the primary tumour location and for metastization to other organs. Thus, high vascularization increases local tumour aggression and metastatic potential of the tumours (Bussink et al. 2003).

AM is a biological structure with peculiar characteristics that make it extremely useful in the area of reconstructive medicine. However, its anti-angiogenic, immunoreactive and pro-apoptotic characteristics are likely to determine its application in oncology (Seo et al. 2008). On the other hand, AM has a low immunogenicity and thus is not rejected by the human body. Human amniotic epithelial cells express small quantities of the (HLA) -A, -B, -C, or -DR antigens and are presumably less likely to be rejected by the host's immune system. Li et al. (2005) have reported that amniotic epithelial cells secrete soluble factors that inhibit both innate and adaptive immune system cells. On the other hand, as AM secretes various anti-angiogenic substances and is a non-vascular structure, the membrane can be expected to act as a barrier and to hinder the delivery of nutrients and oxygen to tumour cells, thereby preventing angiogenesis, tumour growth and metastasis. AM contains large amounts of extracellular matrix proteins that are involved in suppression of corneal neovascularization: collagen IV, laminin-1 and −5, fibronectin and collagen type VII (Fukuda et al. 1999). Amniotic cells also produce various kinds of cytokines, including several anti-angiogenic factors, endostatin and thrombospondin-1 (Hao et al. 2000). Tissue inhibitor of metalloproteinase (TIMP-1, -2, -3, and −4), interleukin-1 receptor antagonist, collagen XVIII and interleukin-10 are some of the chemicals that are found in the structure of AM and that might have anti-angiogenic activity (Hao et al. 2000). Shao (2004) has revealed that human AM specifically inhibits endothelial cell growth and suppresses corneal neovascularization, as mentioned above. Other studies have shown that the transplantation of human AM decreases vascularization of the ocular surface due to the activity of PEDF (Shao 2004; Kim and Tseng 1995a). As a physical barrier, AM can inhibit cell growth and migration of vascular endothelial cells, preventing diffusion of promoters of vascularization (Kobayashi et al. 2002). Kobayashi et al. (2002) have

demonstrated that the supernatant of amniotic cell cultures contains potent inhibitors of neovascularization and suggest that this supernatant can be used in diseases involving neovascularization. In another study by Mahgoub et al. (2004) whose objective was to use AM as a biocompatible natural immune barrier and assess their revascularization, AM were obtained from full-term pregnant female dogs. AM were formed into macrocapsules that were implanted into the peritoneal cavity. The capsules were removed after 3, 10, 15 and 30 days and examined histopathologically. The authors concluded that implanted amniotic sac capsules were vascularized within the omental tissue from day 10 onwards, with significant blood vessel formation starting on day 3 (Mahgoub et al. 2004).

Several studies have shown that AM secretes several molecules with pro-apoptotic activity, an important feature in cancer treatment. Li et al. (2006) have reported that the apoptosis of macrophages activated by interferon-gamma promoted by AM is not mediated directly by NO and TNF-α. However, this effect is commonly caused by the interruption of survival pathways mediated by both NF-κB and Akt-FKHR.

In a curious and recent study performed by Mencucci et al. (2011), the authors examined the possible inhibition of viral replication in vitro by the use of AM treated with antivirals. Preliminary results published by these authors suggest that AM treated with anti-viral drugs can indeed inhibit viral replication, demonstrating that AM has the ability to absorb antivirals and sustain drug release (Mencucci et al. 2011). This study might open new frontiers in the concept of controlled drug release and AM impregnated with drugs could play an important role in the treatment of various diseases, including cancer.

Concluding remarks

AM is an extremely useful tool in the development of therapeutic strategies in ocular surface reconstruction, skin applications, gynaecology and tissue engineering. This broad clinical application is attributable to the various and particular characteristics of AM, which has anti-inflammatory, antibacterial, anti-viral and immunological characteristics, together with anti-angiogenic and pro-apoptotic features. AM cells have pluripotent properties, are promoters of epithelialization and are non-tumorigenic, their use raises no ethical problems. Despite knowing a great deal about the structure, function and characteristics of AM, we need to continue studying this amazing fetal tissue. Further studies about the structure, function and properties of AM will certainly bring to light new clinical applications, such as in oncology. In this area, AM can probably prevent the delivery of nutrients and oxygen to cancer cells and consequently interfere with tumour angiogenesis, growth and metastasis.

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