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# Identification of Antiangiogenic and Antiinflammatory Proteins in Human Amniotic Membrane

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**Purpose.** To identify the potential antiangiogenic and antiinflammatory proteins expressed in human amniotic membrane tissue. **Methods.** Human amniotic epithelial and mesenchymal cells were isolated from human amniotic membranes by sequential trypsin and collagenase digestion. Total RNAs were harvested from freshly obtained human amniotic epithelial and mesenchymal cells. Antiangiogenic and antiinflammatory proteins were detected by the reverse transcriptase-polymerase chain reaction (RT-PCR) technique and further confirmed by DNA sequencing of PCR-amplified transcripts. The distribution of tissue inhibitors of metalloproteinase (TIMPs) were studied further by immunohistochemistry performed on paraffin-embedded amniotic membrane tissue. **Results.** RT-PCR results showed that both human amniotic epithelial and mesenchymal cells express interleukin-1 receptor antagonist, all four TIMPs, collagen XVIII, and interleukin-10. Thrombospondin-1 was expressed in all of the epithelial cell specimens and in one out of five mesenchymal cell specimens. Furthermore, immunohistochemistry studies performed on freshly prepared amniotic membrane confirmed that all members of the TIMP family were present in epithelial and mesenchymal cells as well as in the compact layer of the amniotic stroma. In cryopreserved amniotic membranes, positive staining was seen in residual amniotic cells and stroma. **Conclusions.** Human amniotic membrane epithelial and mesenchymal cells express various antiangiogenic and antiinflammatory proteins. Some of those proteins also were found in amniotic membrane stroma. These findings may explain in part the antiangiogenic and antiinflammatory effects of amniotic membrane transplantation.

**Key Words:** Amniotic membrane—Antiangiogenic proteins—Antiinflammatory proteins—RT-PCR—Immunohistochemistry.

The amniotic membrane is an avascular tissue that forms the innermost layer of the fetal membrane. It is composed of five layers: an epithelial monolayer, an acellular basement membrane layer, a compact layer, a mesenchymal cell layer, and a spongy layer.[1] Amniotic membrane was used clinically as a wound dressing for burn patients and in other surgical procedures as early as 1910[2–4] and was reported to promote epithelialization, reduce pain, and prevent infection.

In recent years, there has been resurgent interest in the use of amniotic membrane in the area of ophthalmic applications, especially in ocular surface reconstruction,[5–8] as a wound dressing,[9] and as a substrate for promoting wound healing.[10] Amniotic membrane transplantation has been noted to suppress corneal neovascularization and inflammation.[5,11] For example, in surgical reconstruction of the ocular surface in advanced ocular cicatricial pemphigoid and Stevens–Johnson syndrome, amniotic membrane-covered surfaces showed rapid epithelialization and reduced inflammation, vascularization, and scarring.[8,12]

Despite increasing interest in ophthalmic applications of amniotic membrane transplantation, the mechanisms underlying the observed antiangiogenic and antiinflammatory effects remain incompletely understood. We postulate that amniotic membrane tissue may possess antiangiogenic and antiinflammatory proteins that contribute, in part, to its ability to suppress corneal neovascularization and inflammation. The number of identified antiangiogenic and antiinflammatory proteins is large, and that number is constantly growing. In this study, we elected to examine the expression of a selected group of antiangiogenic and antiinflammatory proteins by the resident cells in human amniotic membrane using RT-PCR. The proteins include collagen a1(XVIII) (Col XVIII, precursor protein of endostatin); thrombospondin-1 (TSP-1); TIMP-1, -2, -3, -4; interleukin-1 receptor antagonist (IL-1ra); and interleukin-10 (IL-10). In addition, we studied the distribution of TIMPs by immunohistochemistry performed on both freshly harvested and cryopreserved human amniotic membranes.

## MATERIALS AND METHODS

### Human Amniotic Membrane

In accordance with a protocol approved by University of California, San Francisco (UCSF) Committee on Human Research, full term human placentas and fetal membranes were collected shortly after delivery at the UCSF Birth Center. The placenta with fetal membrane was first cleansed of blood clots and other contaminants with sterile phosphate buffered saline (PBS). The amnion was separated from the rest of chorion by blunt dissection. The separated amniotic membrane was thoroughly washed repeatedly with

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PBS supplemented with penicillin (100 U/mL) and streptomycin (100 μg/mL).

The cryopreserved amniotic membrane was prepared by freezing freshly prepared amniotic membrane in Dulbecco's modified Eagle medium (DMEM) culture medium and glycerol at the ratio of 1:1 (vol/vol) at −80°C.[5]

### Isolation of Amniotic Epithelial Cells and Stromal Mesenchymal Cells

Isolation of amniotic epithelial and mesenchymal cells was accomplished by sequential trypsin and collagenase digestion, using a published method with minor modifications.[13] Briefly, amniotic membrane was minced and placed in 150 mL of DMEM culture medium containing trypsin (2 mg/mL of 1:250 trypsin, Sigma, St. Louis, MO, U.S.A.). The tissue and the medium were incubated and stirred at 100 rpm for 30 minutes at 37°C. The medium and tissue were then poured over sterile gauze to separate the dispersed amniotic epithelial cells from the tissue pieces. Tissue remnants were reprocessed through the same steps three more times. The first flow through was discarded to reduce potential tissue contamination. The epithelial cells in the combined last three flows through were then pelleted by centrifugation. The remaining tissue pieces were placed in DMEM to which collagenase (0.75 mg/mL; Sigma, St. Louis, MO, U.S.A.) and DNaseI (0.075 mg/mL; Sigma, St. Louis, MO, U.S.A.) had been added, and then incubated with stirring at 600 rpm for 60 minutes at 37°C. The dispersed mesenchymal cells were pelleted by centrifugation.

### RT-PCR

After human amniotic epithelial and mesenchymal cells were homogenized (QIAshredder, QIAGEN, Valencia, CA, U.S.A.), total RNA was extracted (RNeasy mini kit, QIAGEN, Valencia, CA, U.S.A.) and reverse transcribed into cDNA (Superscript Preamplification System, Gibco BRL, Grand Island, NY, U.S.A.). The PCR primers and expected PCR product sizes are listed in Table 1.

Reaction parameters for IL-1ra, IL-10, Col XVIII, and TIMPs were as follows: 94°C for three minutes; followed by 35 cycles of denaturation at 94°C for 20 seconds, annealing at 58°C for 20 seconds, and extension at 72°C for one minute. Reaction parameters for TSP-1 were as follows: 94°C for three minutes; followed by 35 cycles of denaturation at 94°C for 45 seconds, annealing at 58°C for 45 seconds, and extension at 72°C for four minutes. The products were electrophoresed in 1% agarose gel. A 732-base pair beta-actin sequence was simultaneously PCR amplified as an internal control. Identity of the PCR products was confirmed by sequencing or restriction enzyme digestion.

**TABLE 1.** *PCR reaction oligonucleotide pairs and resulting products*

| Gene | Oligonucleotide pairs | Product size (bp) |
|---|---|---|
| IL-1ra | 5′-CTG CAG TCA CAG AAT GGA AAT CTG C<br>3′-CCG TAC TAC TCG TCC TCC TGG AAG | 553 |
| IL-10 | 5′-ATG AAG GTC TCC GAG GCT GCC<br>3′-CTG GTC ATC ACT GGG AGT TGA | 371 |
| Col XVIIIa | 5′-ACA GCC ACC GCG ACT TCC AGC<br>3′-GCT ACT TGG AGG CAG TCA TGA A G | 551 |
| TSP-1 | 5′-CAC CAA CAG CTC CAC CAT GG<br>3′-TAT GAT CAG TCT TTC AAT CAA C | 3561 |
| TIMP-1 | 5′-CCA TGG CCC CCT TTG AGC C<br>3′-GCA GGA TTC AGG CTA TCT TGG ACC | 633 |
| TIMP-2 | 5′-GTC AGT GAG AAG GAA GTG GAC TCT G<br>3′-CGT TGG AGG CCT GCT TAT GGG TCC | 530 |
| TIMP-3 | 5′-CAG CAG CGG CAA TGA CCC CTT G<br>3′-TCT GGC GCT CAG GGG TCT GTG G | 656 |
| TIMP-4 | 5′-GCC AAA ATC TCC AGT GAG AAG GTA G<br>3′-CTA CTA GGG CTG AAC GAT GTC AAC | 530 |

bp, base pair.

### Immunohistochemistry

Both live and cryopreserved amniotic membranes were fixed in 10% formalin in PBS solution (Sigma, St. Louis, MO, U.S.A.) overnight at 4°C, then embedded in paraffin. Sections were cut at a thickness of six micrometers laid onto Superfrost glass slides (Fischer Scientific, Pittsburgh, PA, U.S.A.), then incubated at 37°C overnight before being stained. For immunohistochemistry studies, the following antibodies were used: rabbit polyclonal antibodies against TIMP-1, TIMP-2, and TIMP-4 (1.0, 0.5, and 0.5 μg/mL, respectively, Chemicon International, Temecula, CA, U.S.A.); rabbit polyclonal antibodies against TIMP-3 (2.0 μg/mL, Triple Point Biologics, Portland, OR, U.S.A.). Rabbit polyclonal antibodies against mouse immunoglobulin G (IgG) (2.0 μg/mL, Jackson ImmunoResearch Laboratories, West Grove, PA, U.S.A.) were used as control primary antibodies. Following deparaffinization and rehydration, tissue sections were pretreated with 1:100 citric acid solution (Vector antigen unmasking solution, Vector Laboratories, Burlingame, CA, U.S.A.) at pH 3.0 for 30 minutes. Sections were then incubated with 5% normal goat serum for 30 minutes to block nonspecific binding. Primary antibodies were diluted with PBS with 5% goat serum. Sections were incubated with primary antibody solution at 4°C overnight, then incubated for 30 minutes with biotinylated secondary antibody solution (goat anti-rabbit IgG (H+L), Vector Laboratories) containing 2% normal goat serum. After washing in PBS, the sections were incubated with avidin-biotin-alkaline phosphatase complex (Vectastain ABC-AP kit, Vector Laboratories) for 30 minutes, then reacted with freshly prepared alkaline phosphate substrate (Vector Red, Vector Laboratories) for 20 to 30 minutes.

## RESULTS

### Transcription of Antiangiogenic Proteins

In the current study, the c-terminal coding region of Col XVIII mRNA was detected in both amniotic epithelial and mesenchymal cells by RT-PCR (Figs. 1 and 2). Col XVIII is a type of basement membrane heparan sulfate proteoglycan.[14] Its better known 20-kDa c-terminal fragment, endostatin, has been shown to be a potent antiangiogenic factor that can inhibit endothelial cell proliferation, angiogenesis, and tumor growth.[15,16] These results, therefore, suggest that amniotic membrane might express Col XVIII that, after proteolysis, could result in the production of endostatin.

TSP-1 is a multifunctional matrix protein secreted by many cell types and has been shown to have antiangiogenic activity.[17] The mRNA for TSP-1 was detected in all five specimens of amniotic epithelial cells, but only in one out of five specimens of mesenchymal cells (Figs. 1 and 2). This observation may reflect a differential tissue expression of TSP-1.

TIMPs are a family of multifunctional proteins present in many human tissues and play pivotal roles in regulation of extracellular

**FIG. 1.** RT-PCR analysis of antiangiogenic and antiinflammatory proteins in amniotic epithelial cells. Each amplified PCR product (arrows) was 553 bp for IL-1ra, 371 bp for IL-10, 633 bp for TIMP-1, 530 bp for TIMP-2, 656 bp for TIMP-3, 530 bp TIMP-4, 551 bp for Col XVIII and 3561 bp for TSP-1. Top of figure, 3.6 kilobase (kb); bottom of figure, 0.3 kb.

matrix (ECM) metabolism. It has been reported that TIMPs have diverse actions in inhibition of angiogenesis and tumor growth, invasion and metastasis.[18] TIMP-1 and TIMP-2 were identified previously in both amniotic epithelial and mesenchymal cells.[19,20] In our current study, the expression of mRNA for all four known members of the TIMP family was detected by RT-PCR in both amniotic cell types (Figs. 1 and 2). These results agree with and expand upon the earlier reports that found TIMP-1 and TIMP-2 expression in human amniotic membrane.[19,20]

**FIG. 2.** RT-PCR of antiangiogenic and antiinflammatory proteins in amniotic mesenchymal cells. Each amplified PCR products (arrows) was 553 bp for IL-1ra, 371 bp for IL-10, 633 bp for TIMP-1, 530 bp for TIMP-2, 656 bp for TIMP-3, 530 bp TIMP-4, 551 bp for Col XVIII. No expected PCR product for TSP-1 was observed on this gel. The band on the gel could be a nonspecific PCR artifact. Top of figure, 3.6 kilobase (kb); bottom of figure, 0.2 kb.

### Transcription of Antiinflammatory Proteins

IL-1ra and IL-10 have been well studied as potent antiinflammatory cytokines.[21,22] IL-1ra is structurally similar to IL-1 beta but lacks agonist activity. Il-1ra competes with IL-1 for IL-1 receptor binding that blocks the inflammatory responses initiated by IL-1.[23,24] IL-10 has been reported to function as a broad spectrum antiinflammatory cytokine by inhibiting production of IL-1, TNF-$\alpha$ and other proinflammatory factors.[25,26] It has also been reported that IL-10 promotes TIMPs production and suppresses matrix metalloproteinase expression.[27–29]

The mRNAs for IL-1ra and IL-10 were detected by RT-PCR in both epithelial and mesenchymal cells (Figs. 1 and 2). The IL-10 band was only weakly detected after 35 cycles of amplification. This observation could be explained by suboptimal conditions for the PCR reaction or a relatively low abundance of IL-10 mRNA. Support for the latter possibility is provided by reports indicating that while high concentrations of constitutive IL-10 are found in the maternal decidua and placental chorionic villi, amnion itself has limited expression of IL-10 protein.[30,31] The existence of IL-1ra (and to a lesser extent, IL-10) could be one of the factors that contributes to the observed antiinflammatory properties of amniotic membrane.

### Immunolocalization of TIMPs

In order to corroborate our RT-PCR findings, we further examined the expression and distribution of TIMPs by immunohistochemistry performed on both freshly prepared and cryopreserved amniotic membrane (the predominant form used in ophthalmic clinical applications). Immunohistochemical studies in fresh tissue demonstrated the presence of all four TIMPs in both amniotic epithelial and mesenchymal cells (Fig. 3), which is consistent with the results of RT-PCR experiments. Furthermore, abundant deposition of all four TIMPs proteins was observed within the compact layer of the stroma.

In cryopreserved tissue, positive staining was detected in residual epithelial cells and in the stromal layer. However, post-preservation tissue changes prevented detailed differentiation of the different layers of the stroma and identification of mesenchymal cells (Fig. 4).

## DISCUSSION

In this study, we have shown that amniotic membrane can express many antiangiogenic and antiinflammatory proteins. However, in light of the fact that amniotic membrane is one of the few avascular tissues that maintains its avascularity even though it is next to a highly vascular tissue (the chorion), it would not be surprising that the amniotic membrane produces a variety of anti-angiogenic proteins. Furthermore, many of those antiangiogenic and antiinflammatory proteins may play important roles in the normal function of the amniotic membrane.

One of the main functions of the amniotic membrane is to maintain the integrity of the fetal membrane until term.[29–31] Premature proteolysis of the amniotic membrane sac through activation of endogenous tissue proteases or secretion of proteases from infiltrating leukocytes because of inflammation can result in pre-

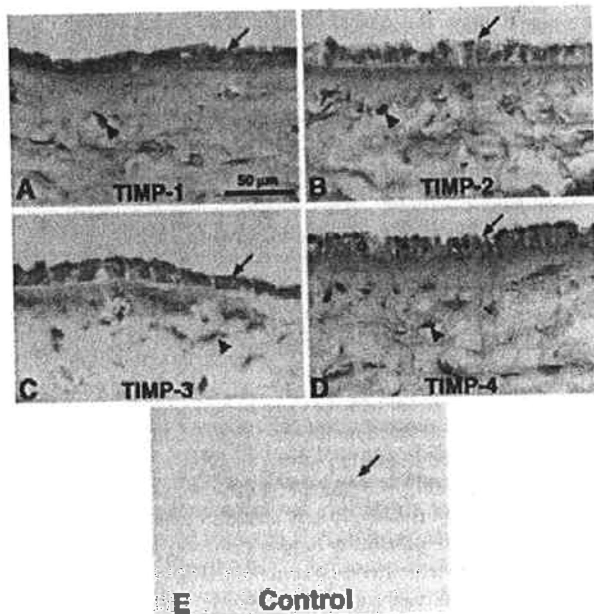


**FIG. 3.** **(A)**, TIMP-1; **(B)**, TIMP-2; **(C)**, timp-3; and **(D)**, TIMP-4 staining in freshly isolated amniotic membrane. Positive staining was observed in both amniotic epithelial (arrows) and mesenchymal cells (arrow heads). There were significant deposits of TIMPs in the amniotic stroma, especially in the compact layer of amniotic membrane. The control rabbit antibody **(E)** did not yield any staining in the amniotic membrane.

mature rupture of the fetal membrane.[32] Therefore, we speculate that protease inhibitors and antiinflammatory proteins may play important roles in maintaining the integrity of the amniotic membrane during pregnancy. Besides TIMPs, other protease inhibitors, such as plasminogen activator inhibitors-1 and -2, can be detected in human amniotic membrane by RT-PCR (data not shown).

We propose that one useful function for transplanted amniotic membrane is as a drug delivery vehicle from which various antiinflammatory and antiangiogenic factors can diffuse and permeate the underlying ocular surface. We hypothesize that in the early phase after amniotic membrane transplantation soluble antiangiogenic and antiinflammatory factors present within the membrane, such as TIMP-1, -2, -4 and IL-1ra and IL-10, would diffuse into the surrounding ocular tissue. In the late phase after transplantation, proteolysis of the amniotic membrane could release less soluble factors, such as TIMP-3, TSP-1 and endostatin.

Corneal inflammation and neovascularization are closely related processes. Corneal neovascularization is frequently the consequence of an extended inflammatory reaction due to various insults, such as chemical burns, microbial infection, and autoimmune diseases.[33] Proteins, such as IL-1ra, could suppress corneal neovascularization by exerting an antiinflammatory effect. It has been reported that the topical application of IL-1ra suppresses corneal neovascularization.[34,35] In addition, IL-10 has been shown to play a more direct role in blocking the angiogenic process by suppressing secretion of matrix metalloproteinase-2 and -9 (MMP-2 and -9) and promoting TIMP-1 secretion.[29]

In present clinical use, the most commonly transplanted form of amniotic membrane is cryopreserved tissue. Many of the soluble antiinflammatory and antiangiogenic factors that reside in the amniotic membrane would be expected to be depleted in a relatively short time after transplantation. Therefore, it would possibly be advantageous to replace the amniotic membrane at regular intervals in order to maintain its ability to release factors that can counter ocular surface inflammation and angiogenesis. In addition, because most of the antiinflammatory and antiangiogenic proteins are produced and stored in amniotic epithelial cells, it would seem, on a theoretical basis, to be more effective to apply amniotic membrane with the epithelial cell side toward ocular surface to maximize the availability of therapeutic proteins.

Not all antiinflammatory and antiangiogenic effects can be attributed to the diffusible therapeutic proteins expressed in the amniotic membrane. The antiinflammatory and antiangiogenic effects appear to persist well beyond the critical period of engraftment. This suggests that other mechanisms (e.g., physical covering of the ocular surface, speeding of reepithelialization) are important con-

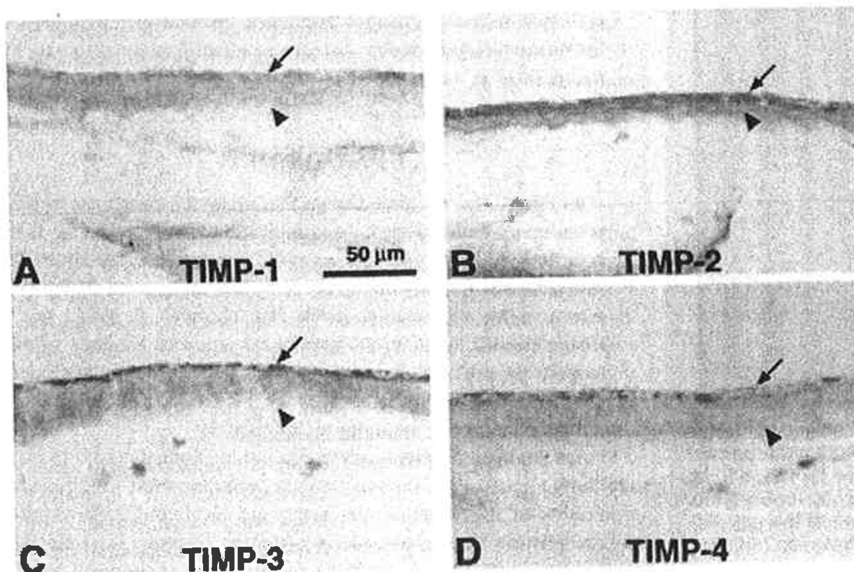


**FIG. 4.** **(A)**, TIMP-1; **(B)**, TIMP-2; **(C)**, TIMP-3; and **(D)**, TIMP-4 staining in cryopreserved amniotic membrane. Positive staining was observed in residual amniotic epithelial (arrows) and mesenchymal cells. There were significant deposits of TIMPs in the amniotic stroma (arrow heads).

stituents to the beneficial effects of amniotic membrane transplantation.

In summary, our study shows that amniotic membrane epithelial and mesenchymal cells can produce various antiangiogenic and antiinflammatory proteins. Some of these proteins appear to be abundantly deposited within the stroma of the amniotic membrane. These findings may explain, in part, the clinical observation that amniotic membrane can mitigate ocular surface neovascularization and inflammation.

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