Human amniotic epithelial cells induce apoptosis of cancer cells: a new anti-tumor therapeutic strategy

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Abstract

Background aims. Amniotic membrane (AM), the innermost layer of human placenta, is composed of a single layer of epithelial cells, a basement membrane and an avascular stroma. The AM has many functions and properties, among which angiogenic modulatory and immunoregulatory effects are applicable in cancer therapy. Because these functions belong to amniotic epithelial cells, in this study we compared the anti-cancer effect of amniotic epithelial cells and the whole AM.

Methods. The effect of the AM and the amniotic epithelial cells on cancer cell apoptosis was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay, terminal deoxynucleotidyl transferase dUTP nick end labeling assay and immunocytochemistry. The effect of the AM on angiogenesis in conditions both with and without epithelial cells was also evaluated using rat aortic ring assay. Results. There was a decrease in cancer cell viability after adding either AM or amniotic epithelial cell supernatant to cancer cells. A significant increase in caspase-3 and caspase-8 expression in cancer cells treated with amniotic epithelial cell supernatant was observed. The recorded media also demonstrated the possible induction of apoptosis in cancer cells treated with the amniotic epithelial cell supernatant. In the aorta ring assay, the AM showed an anti-angiogenic effect in the presence of its epithelial cells; however, this effect was altered to initiate angiogenesis when amniotic epithelial cells were removed from the AM.

Conclusions. These results suggest that amniotic epithelial cells, with their anti-angiogenic effect and induction of apoptosis, are candidates for cancer therapeutic agents in the near future.

Key Words: amniotic membrane, apoptosis, cancer, epithelial cells

Introduction

Cancer is one of the leading causes of death worldwide, and despite continuous progress in management, it still imposes high mortality and morbidity on patients (1). Routine treatments for cancer such as surgery, chemotherapy and radiotherapy are not always effective and may be followed by recurrence or metastatic spread (2,3). Many new ideas have emerged more recently in the field of cancer treatment, many of which focus on the role of stem cells in cancer, such as targeting cancer stem cells (1,3), differentiation therapy, stem cell transplantation (4) and cancer gene therapy using neural stem cells in malignant glioma (5).

Among new ideas that deal with the role of stem cells in cancer treatment, application of amniotic membrane (AM) is a promising idea for cancer therapy. The AM, the innermost layer of placenta, is a biologic barrier that supports the fetus by preparing an anatomically, physiologically and immunologically privileged space. The AM is composed of a thick basement membrane with a single layer of epithelial cells and an avascular stroma containing mesenchymal cells (6). Amniotic epithelial and mesenchymal cells have stem cell characteristics, which make them a great source of stem cell therapy.

A key element in tumor growth and metastasis is the development of a proper blood supply for the newly formed tumor cells and destroying the body’s immunologic barriers that prevent tumor invasion. The AM can prevent tumor metastasis because it has anti-angiogenic properties and immunoregulatory activity. Following the suggestion of these features for the AM, it was hypothesized that the AM can have anti-cancer effects (7,8). Other extra-embryonic cells, such as cord blood stem cells, also have been proven to have anti-cancer properties via a different mechanism, more likely apoptosis (9). The AM has also been shown to have an apoptotic effect on polymorphonuclear neutrophils (10) and...
macrophages (11) by secretion of certain substances. Apoptosis is a process of programmed cell death with significant roles in development and the homeostasis of multi-cellular organisms (12). Because the exact mechanism of the anti-tumor effect of the AM is unknown, the aim of this study was to evaluate whether the AM has anti-tumor properties through induction of apoptosis.

The origin of anti-cancer effect of the AM was the second question of this research. We hypothesized more recently that the amniotic epithelial cells are the source of anti-tumor properties of the AM (8). In this study, we evaluated the effect of the amniotic epithelial cells and the whole AM on proliferation of cancer cells.

Methods

The ethical committee of Shahid Beheshti University of Medical Sciences approved all experimental procedures. The placenta was obtained from mothers with normal pregnancy following elective cesarean section at gestational ages of about 36–38 weeks. Serologic tests for human immunodeficiency virus, hepatitis B and C and syphilis were negative for all cases, as checked before delivery. Although placenta is a waste material after delivery, adequate information about the study process and the usage of placenta was given to all parents who signed the informed consent and agreed to participate in this study.

Placenta tissues were transferred immediately to our laboratory, under sterile condition, at 4°C. The AM was separated from the chorionic membrane by a peeling method. After being washed thoroughly with phosphate-buffered saline (PBS) solution, the anti-cancer property of the AM was evaluated in different conditions.

The epithelial cells were harvested from the AM using enzymatic digestion by trypsin-ethylendiaminetetraacetic acid (EDTA) (TrypNik; Yavandan Andisheh, Tehran, Iran) 0.15%, as described previously (13). Briefly, to release amniotic epithelial cells, the AM was incubated at 37°C with 0.15% trypsin-EDTA. Cells from the first 10 min of digestion were discarded to exclude debris. The solutions from the second and third 40-min digests were pooled. Trypsin was inactivated with fetal bovine serum (FBS), and the solution was centrifuged at 2500 rpm for 12 min. Cells were washed with PBS and suspended in -Dulbecco’s modified Eagle’s medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) containing 100 U/mL penicillin-streptomycin solution and 10% FBS. Viability of amniotic epithelial cells was determined by exclusion of trypan blue dye. The purity of the isolated cells was determined by immunostaining with antibody against the epithelial marker pan-cytokeratin conjugated with fluorescein isothiocyanate (1:100; Sigma-Aldrich).

Effects of AM supernatant on cancer cell viability

To evaluate the effects of the AM on viability and proliferation of cancer cells, the AM was used on two different cancer cell lines including HeLa cancer cells and breast cancer cells (MDA-MB-231) and in two different protocols, as follows: (i) The AM was cut into 12 small pieces that were each 2 × 2 cm, and the pieces were individually cultured epithelial side up in a 12-well plate. To each well, 1 mL of prepared DMEM containing FBS 10% and penicillin-streptomycin 1% was added. The plates were incubated in 37°C with 5% CO2 for 24 h, after which the supernatant was collected from each well. (ii) The obtained epithelial cells were seeded on tissue culture collagen I (CollaNik I, Yavandan Andisheh, Tehran, Iran) coated plates at a density of 2 × 10^4 cells per cm² in DMEM containing FBS 10% and penicillin-streptomycin 1%, incubated at 37°C with 5% CO2. After 24 h, the supernatant was separated from cells. In the amniotic epithelial cells group, the collected supernatant was filtered with 0.22-µm filters to remove all the possible epithelial cells from the medium.

The supernatant from both procedures was added to cancer cells, which were previously cultured in 24-well plates in prepared RPMI medium (Chemicon International, Temecula, CA, USA) (20% FBS and 1% penicillin-streptomycin) and incubated at 37°C with 5% CO2, about 24 h before adding the supernatant. The amount of supernatant added to the cancer cells in each well ranged from 200–800 µL, added in increments of 200 µL. The cancer cells without treatment served as the control group. The cancer cells were incubated for another 24 h at 37°C. After 24 h, the vitality of the cancer cells, which had been cultured with the supernatant of the previously cultured amniotic epithelial cells or AM, was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay.

MTT assay

MTT solution (5 mg of MTT/mL of distilled water) was filter sterilized. Solution was added to growing culture of cancer cells (40 µL in each well). The mixture was incubated for 4 h at 37°C. The cells were checked afterward, and the presence of crystal formation was evaluated. When the amounts of formed crystals among cells were acceptable, the formazan crystals were dissolved adding dimethyl sulfoxide (Sigma-Aldrich), 900 µL in each well. Formazan has maximum absorption in 570 nm.
Using a spectrophotometer (CE7500; Cecil, Cambridge, UK), we evaluated the absorption rate of the formazan made by viable cancer cells in different concentrations of supernatant. All experiments were carried out as four independent experiments with triplicates.

**Immunocytochemistry**

Immunocytochemistry was performed to evaluate the possible expression of apoptotic markers caspase-3 and caspase-8 in cancer cells cultured with amniotic epithelial cell supernatant. Cells were fixed at room temperature with 4% paraformaldehyde for 10 min, washed with PBS and incubated with 10% goat serum and 0.1% Triton X-100 (Sigma-Aldrich) for 1 h. The cells were allowed to incubate in the primary antibodies anti-caspase 3 (1:100; Sigma-Aldrich) and anti-caspase 8 (1:100; Chemicon) overnight at 4°C, in a humidified chamber. Anti-rabbit and antimouse IgG secondary antibodies conjugated with rhodamine and fluorescein (1:100; Chemicon) were applied for 1 h after washing with PBS. Isotype-matched rabbit or mouse IgG at concentrations similar to specific antibodies were added as negative control in each experiment. The total number of cells was counted by staining nuclei with 4,6-diamino-2-phenylindole (Sigma-Aldrich).

**Terminal deoxynucleotidyl transferase dUTP nick end labeling assay**

The cells were fixed for 30 min with 4% paraformaldehyde, and permeabilization was achieved with Triton X-100 and sodium citrate 0.1%. The cells were assessed using the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (In Situ Cell Death Detection Kit; Roche, Mannheim, Germany) according to the manufacturer’s protocol. Briefly, the cells were incubated with a reaction mixture containing biotin-dUTP and terminal deoxynucleotidyl transferase for 60 min. Fluorescein-conjugated avidin was applied to the samples, which were then incubated in the dark for 30 min. Positively stained fluorescein-labeled cells were visualized and photographed using fluorescence microscopy. To provide a negative control for the TUNEL, we omitted terminal deoxynucleotidyl transferase from the labeling mixture.

**Multi-media**

The whole process was repeated, and the data were recorded using multi-media technique. In this technique, HeLa cancer cells and breast cancer cells (MDA-MB-231) were co-cultured with AM-derived medium and amniotic epithelial cell supernatant. Images of the cancer cells were taken every 10 min for 24 h, and cancer cell growth, proliferation and possible apoptosis were evaluated with the video made by the captured photos using Windows Movie Maker software, version 6.0 (Microsoft, Redmond, WA, USA).

**Rat aortic ring assay**

We evaluated the anti-angiogenesis property of the AM to indicate if the anti-angiogenic effect depended on specific components of the AM. For this purpose, rat aortic ring assay was performed using the aorta ring of 12-week-old rats (14). Before dissection of the aorta, the AM was prepared in different conditions including non-scraped and scraped AM. Non-scraped AM was the intact AM, whereas scraped AM was prepared when the amniotic epithelial cells were removed by extensive scraping of the epithelial side of the AM after it was placed in a sterile enzymatic solution (trypsin-E EDTA 0.03%) for 10 min. Both non-scraped and scraped AM were cut into 2×2 cm pieces and expanded in the bottom of 12-well plates. To each well, 1 mL of prepared DMEM (with FBS 20%, basic fibroblast growth factor 10 ng/mL and penicillin-streptomycin 1%) was added, and the plates were incubated at 37°C with 5% CO₂. The pieces of AM were placed in two different positions: the epithelial side up and the mesenchymal side up.

To prepare the aorta ring, the left descending thoracic aorta of 12-week-old rats was dissected and washed with an antibiotic solution (penicillin-streptomycin). Being placed in a sterile culture dish, the aorta was cut into 2-mm ring-shaped sections after its surrounding fibroadipose tissues were removed. These aortic rings were immediately transferred to cultured wells that contained pieces of the AM. The culture medium was changed every other day, and the results were evaluated after 7 days.

**Statistical analysis**

All data were expressed as mean ± standard deviation. The difference between data was assessed using one-way analysis of variance with Tukey post-test. *P* values < 0.05 were considered statistically significant.

**Results**

**Effects of amniotic epithelial cell supernatant on cancer cells**

Pan-cytokeratin marker was expressed in approximately all cells isolated from fresh human AM, confirming their epithelial nature and the lack of contamination with mesenchymal fibroblasts (Figure 1A). Cancer cell viability was significantly
decreased after being treated with the amniotic epithelial cell supernatant, and the decrease was dose dependent.

HeLa cells treated with 600 µL and 800 µL of amniotic epithelial cell supernatant showed significant differences in cell viability compared with the control group. There was also a significant difference between 200-µL and 800-µL groups. (C) MDA-MB-231 breast cancer cells treated with 800 µL, 600 µL, and 400 µL of epithelial cell supernatant showed a significant decrease in cell viability compared with the control group. The group that received 200 µL of epithelial cell supernatant still had a statistically significant difference compared with the control group. (D) HeLa cells treated with different concentrations of condition medium of the AM showed different cell viabilities; 800 µL and 600 µL of the AM supernatant significantly decreased cancer cell viability compared with the control group. HeLa cell viability was also significantly different between groups that were treated with 200 µL versus 800 µL of the AM supernatant. (E) MDA-MB-231 breast cancer cells treated with the AM tissue culture supernatant had a statistically significant decrease in cell viability in all doses except for 200 µL. The difference in cell viability in the 200 µL group versus the 800 µL group was also statistically significant. *P < 0.05. **P < 0.01.

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HeLa cells treated with 600 µL and 800 µL of amniotic epithelial cell supernatant showed significant differences in cancer cell viability compared with the control group (600 µL versus control, P < 0.05, and 800 µL versus control, P < 0.01); the decrease in HeLa cell viability was not significant when treated with 200 µL of amniotic epithelial cell supernatant. Higher doses of amniotic epithelial cell supernatant had a significantly more potent effect on cancer cell viability (Figure 1B).

In MDA-MB-231 breast cancer cells, there was a significant difference between control group and each of the intervention groups. Even 200 µL of epithelial cell supernatant had a statistically significant effect on breast cancer cell viability (200 µL versus control group, P < 0.05) (Figure 1C).
Effects of whole AM supernatant on cancer cells

The effect of the AM supernatant on cancer cell viability was also dose dependent. With 800 µL of the AM supernatant, a decrease in cancer cell viability was detected in both HeLa and MDA-MB-231 cancer cells. Similar to the amniotic epithelial cell supernatant, higher doses of the AM supernatant resulted in a more significant decrease in cancer cell viability because HeLa cell viability was significantly different when treated with 800 µL versus 200 µL of the AM supernatant (Figure 1D). The viability of the MDA-MB-231 breast cancer cells was also significantly decreased after being treated with 800 µL of the supernatant from the AM tissue culture, and this decrease was significantly different with the group that received 200 µL of the AM supernatant. Treatment with 200 µL supernatant from the cultured AM showed no statistically significant decrease in breast cancer cell viability, in contrast to when MDA-MB-231 cancer cells were treated with the epithelial cell supernatant. In the rest of the groups, the decrease in cell viability was statistically significant compared with control group (600 µL versus control group, $P < 0.01$, and 400 µL versus control group, $P < 0.05$) (Figure 1E).

Immunocytochemistry

To evaluate the mechanism by which amniotic epithelial cell supernatant decreases cell viability of cancer cells, immunocytochemistry was carried out on cancer cells treated with supernatant for 24 h with apoptotic markers caspase 3 and caspase 8. In each visual field (eight fields/well, 12 wells/group), the number of caspase-3-positive and caspase-8-positive cells was calculated and compared between groups treated with low dose (200 µL) and high dose (800 µL) of supernatant. As shown in Figure 2A–C, supernatant increased caspase-3 and caspase-8 activation in a dose-dependent manner. In the low-dose supernatant group, 8.2 ± 4.6% and 11.3 ± 3.4% of cells expressed caspase-3 and caspase-8, respectively. In the high-dose supernatant group, the number of caspase-3-positive and caspase-8-positive cells increased to >21% and >26%, respectively.

TUNEL assay

To evaluate the mechanism by which cell death is induced by amniotic epithelial cell supernatant, we further checked for the induction of apoptosis in amniotic epithelial cell supernatant-treated cells by TUNEL assay. The cancer cells treated with high dose of amniotic epithelial cell supernatant clearly showed the presence of more numbers of TUNEL cells than in the low-dose group, indicating that the cancer cells treated with human amniotic epithelial cell supernatant were undergoing apoptosis (Figure 2D–F).

Multi-media

Cancer cells were cultured alone and in the presence of AM-derived medium or amniotic epithelial cell supernatant...
supernatant. The cells were evaluated for a 24-h period by imaging of the cell culture plates every 10 min. When compared with the cancer cells alone (see supplementary Video 1), a decrease in cancer cell motility was evident in the group treated with AM-derived medium or amniotic epithelial cell supernatant, as shown in supplementary Video 2. The recorded media revealed that the AM-derived medium induced cell degradation among cancer cells. Arrows in the media mark some spots of evident cell degradation induced both during cell growth and after cell mitosis (see supplementary Video 2).

Aorta ring assay

In all scraped AM culture samples (without epithelial cells), endothelial-like cells penetrated into the surrounding AM from the aortic ring 24 h after incubation, whereas the non-scraped (with epithelial cells) AM remained unchanged. In the scraped AM cultures (in both epithelial up and mesenchymal up positions), spindle fibroblast cells appeared all over the aortic ring 3 days after incubation. However, no fibroblast cell appeared in non-scraped AM cultures. Capillary formation was detectable 7 days after incubation in scraped mesenchymal up and scraped epithelial up cultures, whereas in non-scraped cultures of the AM, there were no detectable signs of capillary formation even after 7 days of incubation (Figure 3).

Discussion

In our study, we demonstrated a decrease in cancer cell viability and proliferation after treatment with the condition medium of either the AM or the amniotic epithelial cells. As the amount of the amniotic epithelial cell or the AM supernatant that was added to each well of cancer cell plate increased from 200 μL to 800 μL (in increments of 200 μL), the viability was decreased in both types of cancer cells. The condition media both from the AM and from the harvested amniotic epithelial cells had a significant decreasing effect on cancer cell viability. According to these data, both AM and amniotic epithelial cells can have anti-cancer effects. Several studies have been performed more recently on the anti-cancer effect of AM with emphasis on the role of human AM derived-mesenchymal stromal cells on cancer. Jiao et al. (15) confirmed that human AM derived-mesenchymal stromal cells can inhibit the growth of glioma cells, and Magatti et al. (16) have insisted on the anti-cancer property of human AM derived-mesenchymal stromal cells, adding the doubt that an unknown secreted material from the AM can induce cancer cell cycle arrest.

Our study showed a significant increase in caspase-8 and caspase-3 expression in cancer cells treated with amniotic epithelial cells. These results along with the multi-media data of cell degradation and reduction in cancer cell motility after treatment with amniotic epithelial cell supernatant enforce the
possibility of anti-cancer effect of the amniotic epithelial cells and raise the possibility that the amniotic epithelial cells are the key element in secretion of the pro-apoptotic substances from the AM.

The AM is believed to have anti-angiogenesis properties (7,8,17). There are reports that anti-angiogenic factors such as thrombospondin-1, interleukin (IL)-1 receptor antagonist, collagen XVIII, IL-10 and tissue inhibitor of metalloprotease (TIMP-1, TIMP-2, TIMP-3, and TIMP-4) have been detected mainly in AM-derived epithelial cells (17). The mesenchymal cells of the AM secrete factors such as intravascular adhesion molecule, IL-8, IL-6, growth-related oncogene, monocyte chemoattractant protein-1 and migration inhibitory factor, which have angiogenesis promoting activity (18). Consistent with these reports, the results from our aorta ring assay showed a difference in anti-angiogenic effect of different sides of the AM. Our results showed that the scraped AM that has no epithelial cells can induce angiogenesis on both of its sides, whereas there were no signs of capillary formation even on the mesenchymal side of the non-scraped AM. This suggests that the whole AM can have both anti-angiogenesis and angiogenesis effects, which is consistent with previous claims that the AM may have a role in angiogenesis (19). As described, we observed no signs of angiogenesis in the presence of the amniotic epithelial cells on the AM. It is likely that the existence of amniotic epithelial cells completely prevented angiogenesis on the membrane, an effect that itself is desirable in cancer therapy. These findings raise the doubt that the epithelial cells of the AM may be the main compartment of the AM responsible for anti-angiogenesis effect of this membrane because most of the factors that give the AM its anti-angiogenic property are derived from the amniotic epithelial cells (8). Based on these results, both amniotic epithelial cells and AM can have anti-cancer properties, but the possible angiogenesis effect of the whole AM makes its use contraindicated in cancer therapy because angiogenesis plays the main role in cancer metastasis.

One issue still to be resolved is which mechanism is responsible for anti-cancer properties of the AM. Magatti et al. (16) reported more recently that anti-proliferative effect of the amniotic mesenchymal cells is associated with induction of cell cycle arrest in G0/G1 phase by unknown soluble factors. These investigators suggested that the amniotic cells can down-regulate expression of cancer cells genes associated with cell cycle progression such as cyclins (cyclin D2, E1 and H) and cyclin-dependent kinases (cdk2, cdk4 and cdk6). Based on our results, another mechanism for anti-cancer characteristics of the AM is induction of apoptosis in cancer cells. These results are consistent with a previous study, which demonstrated that glioma cells highly expressed apoptotic markers Bax, caspase-8 and caspase-3 and decreased expression of Bcl-2 when treated by the amniotic mesenchymal cells (15). We also showed more recently in an animal model that epithelial cells of the AM prevent angiogenesis (19).

Regarding these findings, we thought that induction of apoptosis, inhibition of angiogenesis and cell cycle arrest in cancer cells mediated by the AM are through inhibition of heat shock protein 90 (HSP90). HSP90 is an adenosine triphosphate (ATP)-dependent molecular chaperone that can protect cancer cells through inhibition of cell cycle arrest and apoptosis and enhancement of angiogenesis (20). We have started to investigate the mechanism of HSP90 modulation by the AM. Our first results show that condition medium of the AM can reduce the levels of some HSP90 client proteins in cancer cells, as investigated by Western blot analysis. The levels of Akt and Cdk4 declined in a dose-dependent manner in response to treatment with condition medium of the AM. After overnight treatment, 400 μL of condition medium down-regulated Akt and Cdk4 by 40–50%, and 800 μL of condition medium decreased these client proteins by 70–75%. In addition, treatment with condition medium of the AM decreased the amount of ATP-bound HSP90 (as investigated by ATP-Sepharose [Sigma] binding assay), which suggests that the AM affects the ATP-binding capacity of HSP90. These preliminary data support the hypothesis that unknown substances from the AM inhibit HSP90, which leads to inducing of apoptosis, inhibition of angiogenesis and cell cycle arrest in tumor cells. However, further research is required to identify the mechanism by which the AM affects cancer cells.

Other than having anti-angiogenesis and pro-apoptotic properties, amniotic epithelial cells have many characteristics such as anti-inflammatory, anti-fibrosis, anti-bacterial and immunoregulatory properties (7) that can make them candidates for transplantation in cell therapy. The immunoregulatory effect of the AM is mainly due to the amniotic epithelial cells because it has been proven that these cells express no HLA-A, HLA-B, HLA-C or HLA-DR antigens (21,22). Also, Li et al. (23) reported the inhibitory effect of amniotic epithelial cells on both innate and adaptive immune system cells. Compared with the whole AM, amniotic epithelial cells are a good alternative for cancer therapy because they have both anti-angiogenesis and anti-cancer effects along with many other properties (e.g., immunoregulatory) that can make them easily implantable in the human body.

In conclusion, the anti-angiogenesis and anti-cancer properties of amniotic epithelial cells through
induction of apoptosis along with their immunoregulatory effect make amniotic epithelial cells potential candidates for new therapies to control cancer cell growth and metastasis.

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References


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