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Multipotent Stromal Cells (MSCs) are Activated to Reduce Apoptosis in Part by Upregulation and Secretion of Stanniocalcin-1 (STC-1)

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Abstract

Multipotent stromal cells (MSCs) have been shown to reduce apoptosis in injured cells by secretion of paracrine factors, but these factors were not fully defined. We observed that co-culture of MSCs with previously UV irradiated fibroblasts reduced apoptosis of the irradiated cells, but fresh MSC conditioned media was unable reproduce the effect. Comparative Microarray analysis of MSCs grown in the presence or absence of UV irradiated fibroblasts demonstrated that the MSCs were activated by the apoptotic cells to increase synthesis and secretion of stanniocalcin-1 (STC-1), a peptide hormone that modulates mineral metabolism and has pleiotrophic effects that have not been fully characterized. We showed that STC-1 was required but not sufficient for reduction of apoptosis of UV-irradiated fibroblasts. In contrast, we demonstrated that MSC-derived STC-1 was both required and sufficient for reduction of apoptosis of lung cancer epithelial cells made apoptotic by incubation at low pH in hypoxia. Our data demonstrate that STC-1 mediates the anti-apoptotic effects of MSCs in two distinct models of apoptosis *in vitro*.

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INTRODUCTION

Multipotent adherent cells from the bone marrow, known as mesenchymal stem cells or multipotent stromal cells (MSCs), are easily expanded *ex vivo* and maintain their ability to differentiate into a variety of cell phenotypes [1, 2]. Initial observations suggested that MSCs might repair injured tissues through mechanisms involving differentiation and perhaps fusion [3]. Subsequent observations, however, demonstrated that the cells produced functional improvement in several disease models without much evidence of long term engraftment [4-6]. The results suggested that MSCs repaired tissues by multiple interactions that included secretion of paracrine factors to enhance regeneration of injured cells, to stimulate the proliferation and differentiation of the stem-like progenitor cells found in most tissues [7, 8], to decrease immune reactions [9], and to decrease inflammatory reactions [10-12]. Reports that MSCs decreased apoptosis were of special interest. For example, MSCs that were engineered to over-express AKT decreased apoptosis in a mouse model of myocardial infarction [13] by secreting the secreted frizzled related protein-2, an antagonist of Wnt signaling [14]. Also, conditioned medium from human MSCs was shown to contain paracrine factors that inhibited apoptosis in hypoxic human aortic endothelial cells that were not fully defined [15].

In the present study, we first UV irradiated skin fibroblasts to induce apoptosis and then co-cultured the apoptotic fibroblasts in a transwell system with MSCs. The MSCs reduced apoptosis of the UV-irradiated fibroblasts. The strategy allowed us to examine the influence of the apoptotic cells on unperturbed cultures of MSCs. The results indicated that the MSCs were activated by the apoptotic fibroblasts to upregulate and secrete increased amounts of stanniocalcin-1 (STC-1), a peptide hormone that modulates calcium metabolism and has pleiotrophic effects that include increased resistance of cells to damage from hypoxia and other insults under some circumstances [16-21]. Reduction of apoptosis in the UV-irradiated fibroblasts required STC-1; however, recombinant human STC-1 (rhSTC-1) was unable to reduce apoptosis. In another model, we found that MSCs also decreased apoptosis by increased secretion of STC-1 in a co-culture system with lung cancer epithelial cells in which both the MSCs and the epithelial cells were exposed to acidosis and hypoxia. Under these circumstances STC-1 was required and sufficient to reduce apoptosis of the lung epithelial cells.

MATERIALS AND METHODS

Cell Culture and Reagents

Frozen vials of passage 1 human bone marrow MSCs (about 1×10^6 cells) were obtained from Tulane University (www.som.tulane.edu/gene_therapy/distribute.shtml). The cells consistently differentiated into bone, fat, and cartilage in culture, were negative for hematopoietic markers (CD34, CD36, CD117 and CD45), and positive for CD29 (95%), CD44 (>93%), CD49c (99%), CD49f (>70%), CD59 (>99%), CD90 (>99%), CD105 (>99%) and CD166 (>99%). The cells were thawed and plated in a 15 cm diameter dish in complete culture medium (α -MEM (GIBCO/BRL, Carlsbad, CA), 17% fetal bovine serum (FBS, lot-selected for rapid growth of MSCs; Atlanta Biologicals, Atlanta, GA)/100 units/mL penicillin/100 mg/mL of streptomycin/2 mM L-glutamine (GIBCO/BRL)), and

incubated for 24 hr to recover viable cells [22]. The medium was removed, the cultures washed with PBS, and adherent MSCs were recovered by incubation with 0.25% trypsin and 1 mM EDTA (GIBCO/BRL) for 5 min at 37 °C. Donors used: Donor 1: 5064L; Donor 2: 240L; Donor 3: 242L.

For the co-culture experiments with irradiated fibroblasts, normal human diploid dermal skin fibroblasts (HS-68; American Tissue Type Culture Collection (ATCC), Rockville, MD) were thawed and plated at 10,000 cells/cm² on a 4.6 cm² transwell inserts (pore size 0.4 µm, #3450; Corning, New York, NY) in 2 mL growth medium (low glucose DMEM (GIBCO/BRL), 10% FBS and 100 units/mL penicillin). Cells were incubated for 24 hrs, and then irradiated with 50J/m² UV light (Stratalinker model 1800; Stratagene, Santa Clara, CA). This amount of UV light was determined to be optimal for achieving apoptosis in 15 to 30 % of the cells in 48 hrs. Irradiated fibroblasts were incubated in co-culture by placing the filters over MSCs that were previously plated at a density of 1000 cells/cm² on regular 6-well dishes (#3516, Corning, New York, NY) and incubated for 5 days, with a media change on the third day. Co-cultures and controls were incubated in 3 mL CCM for 48 hrs. The fibroblasts were then harvested with trypsin/EDTA.

For the co-culture experiments with human A549 lung epithelial cells (ATCC), the A549 cells were plated at 10000 cells/cm² on transwell inserts and incubated in growth medium for 24 hrs. Inserts were then placed on top of MSCs that were previously plated at 1000 cells/cm² and were pre-incubated for 5 days in 3 mL CCM. The CCM was either unmodified or pre-adjusted to a predetermined pH with lactic acid (Sigma-Aldrich, St Louis, MO). For hypoxia experiments, A549 and MSC co-cultures were incubated under 1% O₂, 5% CO₂ and 94% N₂ for 24 hr (Model 3130 incubator; Thermo Electron Corporation, Holsbrook, NY). After a 24 hr incubation period, cells were harvested. pH 5.8 was found to be optimal to induce apoptosis under hypoxia, whereas pH 6.3 was optimal under normoxia.

Mouse Embryonic Fibroblasts from wild-type and STC-1 overexpressing mice were a gift from Dr. Gabriel DiMattia (University of Western Ontario). IMCD3 cells were a gift from Dr. Samir El-Dahr (Tulane University Health Sciences Center).

Donkey anti-STC-1 antibodies from two sources (R & D Systems, Minneapolis, MN and Santa Cruz Biotechnology, Santa Cruz, CA) or an isotype control of donkey anti-IgG (Beckman Coulter, Brea, CA) was added to the media at the indicated concentrations. FLAG tagged recombinant human (rhSTC-1) synthesized in human cells was purchased from BioVendor Laboratory Medicine, Inc. (Czech Republic).

Viability Assays

Viability was assayed with annexin V-fluorescein isothiocyanate and propidium iodide (PI) (Annexin V-FITC Apoptosis Detection Kit; Sigma Aldrich, St Louis, MO) and analyzed with a closed stream flow cytometer (Model FC500; Beckman Coulter, Fullerton, CA). Photomicrographs were prepared by phase contrast microscopy (Eclipse TE200; Nikon, Tokyo, Japan). Cell cycle analysis was performed using the DNA-Prep Reagent System (Beckman Coulter) and analyzed by flow cytometry as described previously [23]. TUNEL staining was performed using the Roche *In Situ* Cell Death Detection Kit (Roche,

Indianapolis, IN) as per manufacturer instructions. Mitochondria were stained using the MitoTracker Red CM-H₂XRos mitochondrial dye as per manufacturer instructions (Invitrogen).

Microarrays

To obtain adequate amounts of RNA for the microarrays, the co-culture experiments were repeated with fibroblasts that were plated at 10,000 cells/cm² on a 9.6 cm² transwell permeable 0.4 µm pore filter (Corning) for 24 hr, and then UV irradiated. The transwell filter was co-cultured with MSCs by placing the filter over MSCs that were previously plated at 100 cells/cm² in a 15cm² dish and incubated for 5 days. The transwell filter was supported over the MSCs with 3×3 mm pieces of sterile silicone that were 1 mm thick (Press-to-seal, Invitrogen, Carlsbad, CA). The samples were incubated in 30 mL CCM for 48 hr. The MSCs were lysed and RNA was isolated (RNeasy RNA extraction kit, Qiagen Inc, Valencia, CA). RNA concentration was assayed by absorbance at 260 nm. Samples were processed by the Microarray Core Facility of the Tulane Center for Gene Therapy [24]. In brief, microarrays were performed using a GeneChip (HGU1332.0; Affymetrix, Santa Clara, CA) for 55,000 human probes for transcripts from over 30,000 human genes. Chips were scanned with Microarray Suite 5.0 (MAS5.0; Affymetrix) and the images were transferred to the dChip1.3+ program [25]. A heat map was generated by clustering genes up-regulated or down-regulated over 2-fold, at 90% confidence.

Western Blot Assays

Cells were lysed (RIPA Lysis Buffer; Santa Cruz Biotechnology, Santa Cruz, CA) and suspended in sample buffer (NuPAGE® LDS sample buffer; Invitrogen) containing 5% ̢-mercaptoethanol (Sigma Aldrich), heated for 3 min at 95 °C, and loaded at 20 µg protein/lane onto polyacrylamide gels (NuPAGE® 4-12% Bis-Tris Gels; Invitrogen). Electrophoresis was for 1.5 hr at 180V in running buffer (NuPAGE® MOPS SDS Running Buffer; Invitrogen). PVDF membrane (GeneHunter Corporation, Nashville, TN) was incubated in methanol for 1 min and proteins transferred to the membrane by electrophoresis at 30V for 1.5 hr in transfer buffer (NuPAGE® Transfer Buffer; Invitrogen). The membrane was blocked for 2 hr at room temperature with PBS containing 0.5% Tween 20 (PBST) and 5% skimmed milk (Santa Cruz Biotechnology). The membrane was incubated overnight at 40°C with primary goat antibody to STC-1 (1: 1,000; R&D systems) in 1% skimmed milk. After washing with PBST, the membrane was incubated for 2 hr at room temperature with secondary HRP conjugated donkey anti-goat antibody (1:5,000; Millipore, Temecula, CA) in 1% skim milk in PBST. The membrane was visualized by chemiluminescence (Visualizer™ Spray & Glow™ ECL Western Blotting Detection System; Upstate, Lake Placid, NY). To isolate the 70 kDa band of STC-1, the corresponding region of the gel was excised using the molecular weight standard as a guide. The excised gel was placed in dialysis tubing and was electrophoresed for 1 hr to elute the protein in 5 ml 0.1 × running buffer (NuPAGE® MOPS SDS running buffer; Invitrogen). The sample was then lyophilized (MODULYOD; Thermo Electron Corporation), and resuspended in 100 µL lysis buffer (RIPA lysis buffer; Santa Cruz Biotechnology). Ten µL of sample, containing 1X loading buffer (NuPAGE® LDS sample buffer; Invitrogen) and 5 % 2-mercaptoethanol was boiled for 3 min and electrophoresed 0.1X running buffer (NuPAGE® MOPS SDS running

buffer; Invitrogen). For assays of secreted STC-1, conditioned medium was passed through a 50 kDa filter (Amicon Ultra Centrifugal Filter; Millipore, Temecula, CA), and the filtrate concentrated on a 10 kDa filter. The concentrated sample was then assayed by electrophoresis and Western blotting. Equal loading of protein was confirmed by staining the PVDF membrane with India ink (Pelikan, Hannover, Germany).

Quantitative RT-PCR

Cells were lysed, RNA was isolated (RNeasy RNA extraction kit ; Qiagen, Valencia, CA), and RNA concentration was assayed by absorbance at 260 nm. Reverse transcription was carried out (Superscript III; Invitrogen) and quantitative real-time PCR was performed (ABI Prism 7700 Sequence Detection System using a SYBR green kit; Applied Biosystems, Foster City, CA) using the following primer pairs: STC-1 forward: CAG CTG CCC AAT CAC TTC TC; STC-1 Reverse: TCT CCA TCA GGC TGT CTC TGA; GAPDH Forward: TCA ACG GAT TTG GTC GTA TTG GG; GAPDH Reverse: TGA TTT TGG AGG GAT CTC GC

RNA Interference and Transfection

Three different siRNAs for STC-1 (SilencerR Pre-designed siRNA; Ambion, Austin, TX, Catalogue number; AM16708A) were used with a negative control (SilencerR FAMTM-Labeled Negative Control #1 siRNA; Ambion, Catalogue number; AM4620). siRNA transfection was carried out using a commercial kit (siPORT Neo FX; Ambion). Briefly, 5 µL SiPORT Neo FX in 100 µL OPTI-MEM (Invitrogen) was mixed with either STC-1 100 nM siRNA or 300 nM negative control in 100 µL OPTI-MEM. The mixture was then incubated for 20 min RT and was added to freshly trypsinized MSCs in suspension (5000 cells/800 µL CCM). Final siRNA concentration was 30 nM. The cells were incubated for 16 h at 37°C in a 5% CO₂ incubator and were used for experiments within 48 hr. Following incubation, transfection efficiency was evaluated using flow cytometry. STC-1 knock-down was confirmed by western blotting and RT-PCR. The following three siRNA probes were used simultaneously to knock down STC-1: ID12722; sense; GGGAAAAGCAUUCGUCAAAtt, antisense; UUUGACGAAUGCUUUUCCCTg, ID12905; sense; GGUCUAACUGUGGAAUAUAtt, antisense; UAUAUUCCACAGUUAGACct, ID138790; sense; CGACUAACCUAUCUAUGAAAtt, antisense; UUCAUAGAUAGGUUAGUCGtt.

Immunofluorescence Microscopy

Cells grown on coverslips were fixed for 10 min either with ice cold methanol acetone (1:1) for labeling with antibodies to STC-1 or with 4% paraformaldehyde (Electron Microscopy Science, Hatfield, PA) for labeling with antibodies to both STC-1 and vinculin. The sample was washed three times for 5 min with PBS, blocked for 45 min at room temperature in 5% donkey serum in PBS and incubated for 1 hr at room temperature in donkey anti-STC-1 antibody (1:1,000; R&D systems). For experiments marked 'data not shown' anti-STC-1 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The sample was washed three times for 5 min with PBS and incubated for 1 hr with Alexa-594 conjugated donkey anti-goat IgG (1:1,000; Invitrogen). After three 5 min washes in PBS, cover slips were mounted on slides. For vinculin/STC-1 double labeling, primary antibody solution also

contained mouse anti-vinculin antibody (1:500; Abcam, Cambridge, MA), and an additional secondary (Alexa-488 donkey anti-mouse). Rabbit anti-PML antibody (Millipore/Chemicon, Billerica, MA) was used at a final concentration of 1:1000. Anti-Rabbit Alexa-488 conjugated antibody (Invitrogen) was used at a final concentration of 1:1000. Photomicrographs were obtained with an epifluorescence microscope (model BX51; Olympus, Tokyo, Japan) with a digital CCD camera ORCA-AG (Hamamatsu Photonics, Hamamatsu, Japan). A non-specific goat IgG (Santa Cruz Biotechnology) was used as a negative control. For absorption assays, antibodies were incubated with 50 ng/mL rhSTC-1 45 min prior to incubation with sample. Surface plot rendering of STC-1 staining was performed using ImageJ (<http://rsb.info.nih.gov/ij>).

Transient Transfection of A549 cells

mRNA corresponding to STC-1 was obtained from American Tissue Type Culture Collection. About 1×10^5 A549 cells were plated on 22 mm $\times$ 22 mm glass coverslips in a 6-well dish and transfected using Trans-it LT-1 transfection reagent (Mirus Bio, Madison, WI). Briefly, 1 μ g STC-1 DNA was mixed with 1.5 μ g carrier DNA (pBluescript SK+), and incubated with 7.5 μ L Trans-it LT-1 reagent diluted in 250 μ L Optimem, and transferred into well containing 2 mL growth medium.

Statistical Analyses

Unless otherwise indicated all experiments were performed in triplicate, a minimum of 3 times. Analysis of Variance was performed for experiments of more than two groups, otherwise a two-tailed, unpaired Student *t* tests was performed. Statistical analysis was processed using Smiths Statistical Package (<http://www.economics.ponomoma.edu/StatSite/framepg.html>).

RESULTS

MSCs Reduce Apoptosis of UV Irradiated Fibroblasts

To investigate whether soluble factors derived from MSCs could reduce apoptosis, we first irradiated fibroblasts grown on a transwell filter with a sufficient dose of UV light (50J/m²) to induce apoptosis in 15 to 30 % of the cells in 48 hrs. We then placed the irradiated fibroblasts in co-culture with MSCs incubated in a standard 6-well dish, such that the two cell populations were 1 mm apart and separated by the 0.4 μ m pores of the transwell membrane. Forty-eight hrs following UV irradiation, a significant number of the fibroblasts had undergone apoptosis as assayed by annexin-V and PI labeling (Fig. 1A, B) [26-28]. The use of annexin-V/PI staining as a quantitative measure of apoptosis was validated by confirming other hallmarks of apoptosis such as DNA degradation, TUNEL staining, nuclei defragmentation and uptake of mitochondrial dye (Fig S1). When the irradiated fibroblasts were co-cultured with MSCs in the transwell, the level of apoptosis of the fibroblasts was reduced by about half. Similar results were obtained with MSCs from 4 additional donors (not shown), and A549 lung cancer epithelial cells (Fig S2A). In contrast, conditioned media from naïve MSCs (MSC CdM) did not reduce apoptosis of irradiated fibroblasts (Fig. 1A, B). As expected, controls of non-irradiated fibroblasts had no effect in the transwell system with irradiated fibroblasts (Fig. 1C). Phase contrast imaging of the cultures corroborated the

observations. Cultures of irradiated fibroblasts incubated alone became sparse as cells detached from the filter and the cells lost their typical spindle-shaped morphology (Fig 1D, left panel), whereas those co-cultured in transwells with MSCs remained dense and the cells retained a spindle-shaped morphology (Fig 1D, middle panel). Again, co-culture in the transwells with non-irradiated fibroblasts had no effect on irradiated fibroblast morphology (Fig. 1D, right panel). The results indicated that the MSCs had to be activated by soluble factors produced by the irradiated fibroblasts to produce one or more soluble factors that reduced apoptosis in the fibroblasts.

Co-culture with Irradiated Fibroblasts Changed the Transcriptome of MSCs

As a preliminary screen for soluble anti-apoptotic factors produced by the MSCs, we used microarrays to identify changes in the MSC transcriptome following incubation with UV irradiated fibroblasts. Figure 1D displays a heat map analysis of genes shared between two MSC donors after being filtered for genes that were up-regulated or down-regulated by greater than 2-fold when co-cultured with irradiated fibroblasts. MSCs from one donor up-regulated 70 genes, whereas MSCs from a second donor up-regulated 171 (Fig. 1F). The variation in data with the MSCs from the two donors probably reflects different degrees of apoptosis induced by the same exposure to irradiation and differences in the rates of proliferation of the MSCs under the co-culture conditions, an example of the rapid changes in the transcriptome of MSCs as they are expanded in culture [23]. Of special interest were the 11 upregulated genes common to both donors (Fig 1F and Table 1). Of these 11 shared genes, only STC-1 encoded a secreted protein.

STC-1 Expression and Secretion is Upregulated by MSCs in Co-cultures with UV Irradiated Fibroblasts

We next explored the possibility that secretion of STC-1 could account for the anti-apoptotic effects of MSCs in the transwell system. Western blot assays with a polyclonal antibody for STC-1 demonstrated that cell lysates from naïve fibroblasts and MSCs contained a cross-reacting band of 35 kDa, the expected size of the protein [29] (Fig. 2 A and B). The lysates also contained a 70 kDa form of the protein that converted to 35 kDa after more extensive reduction (Fig. 2 A). The specificity of the antibody for Western blots was demonstrated by pre-absorbing the antibody with rhSTC-1 (Fig. S3A) and by confirming reactivity with both 35 kDa and 70 kDa forms of rhSTC-1 (Fig. S3B). Also, Western blots with the antibody demonstrated decreased levels of the protein after the MSCs were transduced with an siRNA for STC-1 (Fig. S4). After irradiation, the STC-1 levels in lysates of fibroblasts decreased (Fig 2A; lanes 2, 3). In contrast, there was an increase in the STC-1 content of MSCs after co-cultured with naïve fibroblasts (Fig 2A; lane 6), and a greater increase after co-cultured with UV irradiated fibroblasts, corroborating our microarray data (Fig 2A; lane 7). (For clarity, a lower exposure of the 35 kDa band is shown in the lower panel.) Western blot assays of conditioned medium indicated that MSCs co-cultured with UV-irradiated fibroblasts showed a marked increase in STC-1 secretion relative to MSCs cultured with non-irradiated fibroblasts or irradiated fibroblasts cultured alone (Fig. 2B). Addition of the antibody against STC-1 decreased the anti-apoptotic effects of MSCs in co-cultures with irradiated fibroblasts (Fig. 2C). An isotype control of IgG had no effect (Fig 2 C). Similar results were obtained with a second commercial source of antibody to STC-1 (not shown).

Surprisingly, rhSTC-1 alone was unable to reduce apoptosis of the UV-irradiated fibroblasts (Fig. 2D). These results were confirmed by treating UV irradiated A549 cells with rhSTC-1 indicating that the effect was not cell type specific (Fig S2B). Therefore the results suggested that in co-cultures with irradiated fibroblasts, STC-1 was a necessary but not sufficient factor to explain the anti-apoptotic effects of the MSCs.

MSCs Reduce Apoptosis in Co-cultures with a Lung Epithelial Cell Line (A549) Incubated at Low pH

To extend and confirm the above observations, we established a model of injury in which apoptosis was induced in lung epithelial cancer cells (A549). Preliminary experiments demonstrated that incubation of the A549 cells in 1 % oxygen did not induce apoptosis. Similar results were previously observed with cultured cardiomyocytes [30, 31]; therefore we induced apoptosis in the A549 cells by incubation in hypoxia at low pH, conditions that were used to induce apoptosis in the cardiomyocytes and that often accompany reduced oxygen conditions *in vivo* as a result of increased lactate accumulation [32]. Cultures of A549 cells became apoptotic when incubated for 24 hrs in 1 % oxygen at pH 5.8 or 5.5 (Fig. 3A). MSCs cultured alone did not undergo apoptosis under the same conditions (Fig. 3B; right panel), but both A549 cells and MSCs underwent apoptosis when the pH was decreased further to 5.0 under hypoxic conditions. Co-culture of A549 cells in transwells with MSCs reduced the apoptosis observed with hypoxia in medium adjusted to pH 5.8 or 5.5 but not if adjusted to pH 5.0 (Fig. 3A). Similar results were obtained with MSCs from two additional donors (not shown). Addition of antibodies to STC-1 reversed the anti-apoptotic effects of the MSCs on the A549 cells under hypoxia and pH 5.8 (Fig. 3B, left panel). To confirm that the MSCs secreted an anti-apoptotic factor or factors, co-cultures of MSCs and A549 cells were incubated for 24 hr under hypoxia at pH 5.8. The conditioned medium (co-culture CdM) was transferred to fresh cultures of A549 cells that then incubated for 24 hrs under hypoxia at pH 5.8. The co-culture CdM inhibited apoptosis in the A549 cells. The anti-apoptotic effects of the co-culture CdM were partially blocked by antibodies to STC-1 but not by a control of IgG (Fig. 3C). Apoptosis of A549 cells incubated under hypoxia at pH 5.8 was also inhibited by rhSTC-1 and the effects were reversed by anti-STC-1 but not by control IgG (Fig. 3D). In further experiments, MSCs were transduced with siRNA for STC-1. The siRNA decreased synthesis of the protein by MSCs (Fig. S4). The MSCs expressing the siRNA were less effective than control MSCs in decreasing apoptosis of A549 cells in the transwell experiment, but the reversal was only partial (Fig. 3 E).

STC-1 Expression and Secretion in Co-cultures of MSCs and A549 Cells

Western blot assays of cell lysates indicated that the levels of STC-1 in MSCs were increased by incubating the cells in 1 % oxygen at pH 7.4 or pH 5.8 (Fig. 4A). In contrast, STC-1 was not detected in A549 cells after incubation at pH 5.8 either under normoxic or hypoxic conditions, even when the blot was overexposed (Fig. 4 B). As expected, increased levels of secreted STC-1 were present in conditioned medium from co-cultures of MSCs and A549 cells incubated under hypoxia at pH 5.8 (Fig. 4C), conditions under which the MSCs reduced the apoptosis of A549 cells. Secreted STC-1 levels were not affected by culturing MSCs with A549 cells under normal conditions (not shown).

MSCs Restore Intracellular STC-1 in Injured Fibroblasts and A549 Cells after Rescue of Apoptosis

To determine the intracellular distribution of STC-1 in the cultures, the cells were examined by immunocytochemistry. STC-1 immuno-reactivity was not detected in non-permeabilized cells (Fig. S5A), indicating that STC-1 was not present in the extracellular matrix or the plasma side of the cell membrane. After the paraformaldehyde fixed cells were permeabilized, or cells were fixed with methanol/acetone, STC-1 immuno-reactivity was observed throughout the cytoplasm. STC-1 also appeared to be enriched in a pattern reminiscent of focal adhesions in all three cell types (Fig 5A). The enrichment of STC-1 in focal adhesions was confirmed by co-labeling the cells with antibodies to STC-1 and the actin binding protein, vinculin (Fig. 5B). Pre-absorption of the antibody with rhSTC-1 abolished immuno-reactivity (Fig S5B). We observed similar distribution of STC-1 in mouse embryonic fibroblasts obtained from wild type or STC-1 overexpressing mice (STC-1-MEF) in that the cells also showed a focal adhesion-like peripheral staining pattern (Fig. 5C). Perinuclear accumulation of the STC-1 was also seen in the STC-1-MEFs, an observation consistent with synthesis of the protein in the rough endoplasmic reticulum (Fig. 5C; inset). Mouse inner medullary collecting duct cells, which are known to express very low amounts of STC-1 [20, 33], were negative for cytoplasmic and focal adhesion staining (Fig. 5C; right panel).

To determine the fate of STC-1 secreted by MSCs, MSCs were co-cultured with IMCD3 cells that do not express STC-1. In the co-cultures, the MSCs were identified by presence of PML, a nuclear protein with a distinct punctate distribution [34]. The mouse IMCD3 cells were identified by the presence of pericentromeric heterochromatin and negative PML staining. In co-cultures, the PML-negative IMCD3 cells acquired increased cytoplasmic immunoreactivity and clear focal adhesion enrichment of STC-1 (Fig. 5D, left panel). The same observation was made when IMCD3 cells were co-cultured with MSCs in transwell culture or treated with rhSTC-1 (Fig. 5D, right panel). To corroborate these findings, A549 cells were transiently transfected with a construct expressing STC-1. The transfected cells showed more pronounced punctate foci (Fig. S5C).

Irradiation of fibroblasts altered the distribution of STC-1. The protein was found near the nucleus, in vesicles (not shown), or disappeared altogether (Fig. 6A, middle row), despite no change in the location of vinculin (Fig. 6A, middle row). After UV irradiated fibroblasts were co-cultured with MSCs, STC-1 was again co-localized with vinculin at focal adhesions of the fibroblasts (Fig. 6A, bottom row, arrows point to focal adhesions). Surface plot diagrams of STC-1 staining intensity are provided to confirm our observations. Similarly, incubation of A549 cells under hypoxia at low pH resulted in no detectable cytoplasmic STC-1; however, co-culture with MSCs restored the intracellular distribution.

DISCUSSION

Co-culture of MSCs with previously irradiated fibroblasts enabled us to assess both the anti-apoptotic effects of MSCs and the effects of apoptotic cells on MSCs under the normal conditions for culture of MSCs. In effect the system simulated conditions *in vivo* in which MSCs tend to home to injured cells and tissues, including those injured by irradiation [35].

The results demonstrated that exposure of MSCs to the irradiated fibroblasts changed their patterns of expressed genes. Although substantial donor variation was observed, transcripts for 11 common genes were upregulated 2-fold or greater in both donor cell populations. Since the signals exchanged by the MSCs and fibroblasts were transmitted through a 0.4 μ m pore transwell filter, the microarray data from the MSCs were queried for up-regulation of transcripts for secreted proteins. The most abundant transcripts for a secreted protein that was up-regulated by 2-fold or more were transcripts from the gene encoding STC-1. The use of microarrays to identify secreted molecules had limitations, and thus, the data did not reflect all of the important changes in the MSC secretome. For example, the microarray data would not have reflected changes in low abundance transcripts and can under-estimate the changes in some transcripts in MSCs [23]. Furthermore, the microarrays could not identify changes that occurred at the post-transcriptional level. However they provided a useful indication of one candidate to examine further, STC-1.

Exposure of MSCs to irradiated fibroblasts increased both synthesis and secretion of STC-1. Interestingly, the upregulation of STC-1 by MSCs was not as apparent in cell lysates as conditioned medium, indicating that the rate of the secretion of the protein may be increased as well. Antibodies targeted against STC-1 decreased the anti-apoptotic effect of the MSCs. Treatment of irradiated fibroblasts with excess rhSTC-1 was unable to reduce apoptosis when used at the same concentrations that gave positive results for A549 cells grown in ischemic conditions. Furthermore, rhSTC-1 had no effect on irradiated A549s indicating that the effect was not cell type specific. Thus, STC-1 was required but not sufficient to reduce apoptosis, and may be enhancing or antagonizing the effects of other secreted factors from the MSC CdM. For example, pretreatment of endothelial cells with STC-1 impaired HGF induced phosphorylation of focal adhesion kinase [36]. Also, pretreatment of a macrophage-like cell decreased intracellular calcium accumulation in response to two cytokines, monocyte chemotactic protein and stromal cell derived factor-1 [37].

Previous studies showed that STC-1 was upregulated in cancer cells during hypoxia [16, 17, 38]. Our results also demonstrated that MSCs decreased apoptosis in co-cultures with a line of pulmonary epithelial cells in which apoptosis was produced by hypoxia under acidic conditions. We then asked whether upregulation of STC-1 by MSCs was responsible for the effect. Interestingly, when MSCs were co-cultured with A549 cells under hypoxia and acidosis, expression of STC-1 was upregulated in MSCs, but abolished in A549 cells. Therefore, the upregulation of STC-1 in MSCs was independent of cross-talk with the A549 cells. Antibody blocking and siRNA knockdown of STC-1 within MSCs, partially impaired the ability of the MSCs to reduce apoptosis. The partial reversal may be the result of inefficient knock down or blocking of STC-1, or indicate the involvement of other MSC derived factors in the reduction of apoptosis. In this system, STC-1 was both necessary and sufficient to reduce apoptosis of the ischemic lung cells. The results were consistent with previous reports that STC-1 was up-regulated by hypoxia in cancer cells.

Previous studies observed the presence of STC-1 in many different cellular compartments frequently as a receptor/ligand complex. In sections of mouse kidney, STC-1 was present throughout the cytoplasm with apparent enrichment within mitochondria [39]. In cardiomyocytes over-expressing a STC-1-FLAG fusion protein, STC-1 was seen in

mitochondria [17]. STC-1 was also found in the nucleus in cardiomyocytes [40] and in mouse lactiferous duct cells during pregnancy [41]. Here we observed pan-cytoplasmic staining of STC-1 with enriched immuno-reactivity at focal adhesion plaques. Focal adhesion distribution was observed in all three human cell types as well as two primary MEF cultures. The presence of STC-1 in focal adhesion plaques was supported by co-localization with the actin binding protein, vinculin. Following injury of both cell types in each condition, STC-1 was depleted from focal adhesions but localization was restored after rescue by MSCs. We provided evidence that secreted STC-1 localized to focal adhesions of STC-1-null IMCD3 target cells, indicating that STC-1 leaves the cell prior to localizing to focal adhesions. Therefore, MSC-derived STC-1 may localize to focal adhesions in injured cells and promote viability by a mechanism that has yet to be determined.

The importance of STC-1 in mammalian systems is not well understood; however, a growing body of evidence indicates that it may be a critical stress response protein. STC-1 upregulation has been observed in multiple models of injury, including the ischemic brain [42], obstructed kidney [37], and the hypoxia preconditioned heart and brain [17]. Furthermore, the STC-1 gene is readily activated by multiple cytokines [36, 37, 43]. Thus, the upregulation of STC-1 in MSCs by irradiated fibroblasts is likely due to the presence of similar cytokines released by the injured cells. These factors have yet to be determined. Previous observations on pro- or anti-apoptotic effects of STC-1, however, were inconsistent. For example, increased expression after hypoxiapreconditioning of heart and brain suggested STC-1 was anti-apoptotic [16]. In contrast, STC-1 was pro-apoptotic in chondrocytes during bone development [44], and transgenic mice over-expressing STC-1 had defects in bone growth [29, 45]. We have demonstrated that MSC derived STC-1 can have anti-apoptotic effects. Thus, the cytoprotective effects of MSCs may be explained in part by the upregulation of STC-1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

MSCs Reduce Apoptosis of UV Irradiated Fibroblasts. (A) Fibroblasts were incubated on a transwell filter, UV irradiated and then transferred for co-culture with MSCs in a 6-well dish. Forty-eight hrs later, viability and apoptosis were assayed after labeling for annexin-V staining and PI incorporation by flow cytometry. (B) Quantification of annexin-V/PI positive cells. * = $p < 0.05$. Error bars = SD. (C) Irradiated fibroblasts were incubated alone or in transwell co-cultures with naïve fibroblasts. (D) Representative phase-contrast images of UV irradiated fibroblasts incubated alone and in co-culture with MSCs or naïve

fibroblasts. Scale bar = 100 μ m. Magnification = 40X. **(E)** Microarray heat map analysis of shared genes from two MSC donors up-regulated or down-regulated greater than 2.0-fold when incubated either alone (lanes 1,4), in co-cultures with naïve fibroblasts (lanes 2,5), or in co-cultures with irradiated fibroblasts (lanes 3,6) n=1. **(F)** Venn diagram of genes upregulated 2.0-fold in each MSC donor cell line when co-cultured with UV irradiated fibroblasts versus naïve fibroblasts. Genes upregulated in MSCs from donor 1 (green); donor 2 (red); both donors (yellow) n=1. Abbreviations: Fib, naïve fibroblasts; UV-Fib, irradiated fibroblasts; MSC, MSC in transwell co-culture; MSC CdM = conditioned media from naïve MSCs. Data from experiments performed with MSC donor 1, unless otherwise stated.

Figure 2.

Upregulation and Secretion of STC-1 by MSCs Is Required, but not Sufficient for Reduction of Apoptosis of UV Irradiated Fibroblasts. **(A)** Western blot analyses of cell lysates. Left panel: Fibroblasts incubated alone or with irradiated fibroblasts. Middle panel: MSCs incubated alone, with naïve fibroblasts or with irradiated fibroblasts. A low exposure of 35 kDa band is provided for clarity. Right panel: 70 kDa band was excised, again denatured and reduced before re-electrophoresis. Actin was the loading control. Non-reduced controls taken at the same exposure are shown adjacent to each blot to highlight antibody specificity. **(B)** Western blot analysis of secreted STC-1 in conditioned media. Albumin on the PVDF membrane was stained with India ink as a loading control. **(C)** Apoptosis of UV irradiated fibroblasts cultured alone, or co-cultured with MSCs with or without antibodies to STC-1. * = $p < 0.05$. Error bars = SD. **(D)** Fibroblasts were treated with 50 ng/mL or 100 ng/mL for 48 hours following irradiation. Apoptosis was measured using flow cytometry. All experiments shown were performed with MSC donor 1.

were used at a working dilution of 1: 2,000 * = $p < 0.05$. Error bars = SD. **(C)** Effects of conditioned medium from A549 cells and co-cultures. Conditioned media was prepared by incubating A549 cells alone (A549 CdM) or in co-culture with MSCs (Co-culture CdM) for 24 hr at pH 5.8 under hypoxia and then transferred to A549 cells incubated under the same conditions for 24 hr with or without addition of antibodies to STC-1 or non-immune IgG (1: 2,000). * = $p < 0.05$. Error bars = SD. **(D)** Effect of rhSTC-1 on A549 viability after exposure to hypoxia and low pH for 24 hours. rhSTC-1 was used at a final concentration of 50 ng/mL. anti-STC-1 was used at a final dilution of 1:1,000. * = $p < 0.05$. Error bars = SD. **(E)** Knockdown of STC-1 in A549 cells by siRNA. A549s were co-cultured in transwell with MSCs (MSC transwell), MSCs transfected with a control siRNA (MSC Control siRNA) or siRNA targeting STC-1 (MSC STC-1 siRNA). Apoptosis was measured using flow cytometry. * = $p < 0.05$. Error bars = SD. All experiments shown were performed with MSC donor 2.

Figure 4.

Up-regulation and Secretion of STC-1 in MSCs Is Required for Reduction of Apoptosis of A549 Cells Incubated under Hypoxia at Low pH. **(A)** Western blot for STC-1 in MSC cell lysates when incubated under hypoxia at the indicated pH. Actin was used as a loading control. **(B)** Western blots for STC-1 in A549 cell lysates when incubated under normoxia and hypoxia at the indicated pH. Note: Panels in A and B are from the same Western taken at the same exposure. Legend: Norm = pH 7.4 under all conditions. Low = pH 6.3 when cells cultured in normoxic conditions or pH 5.8 in under hypoxia. **(C)** Analysis of conditioned media for secreted STC-1 from of A549 cells incubated alone (lanes 4-6) or in co-culture with MSCs (lanes 1-3) at the indicated pH in hypoxic conditions. Loading control was albumin. All experiments shown were performed with MSC donor 2.

Figure 5.

STC-1 Located at Focal Adhesions. **(A)** Cells were fixed with methanol acetone and labeled with antibodies to STC-1. Magnification = 600X. **(B)** Cells fixed with paraformaldehyde and co-labeled with antibodies to STC-1 and vinculin, an F-actin binding protein located in focal adhesions. Magnification = 600X for top panels; 200X for bottom panels; 400X for inset. **(C)** Mouse cells were fixed with methanol/acetone and labeled for STC-1. Magnification = 200X. MEF= Mouse Embryonic Fibroblast; STC-1-MEF=STC-1 overexpressing MEF; mIMCD3=Mouse Inner Medullary Collecting Duct Cell (negative

control). Magnification=200X. Inset=400X **(D)** Left panel: IMCD3 cells co-cultured directly with MSCs and stained with antibodies to STC-1 and human specific PML. Right panel: IMCD3 cells were treated with rhSTC-1 or co-cultured with MSCs in transwell culture and labeled for STC-1. White arrows indicate prominent focal adhesion staining. Magnification=200X. Inset=400X. For all, levels were adjusted linearly for clarity.

Figure 6.

STC-1 Location Was Disrupted in Injured Cells but Preserved When Co-cultured with MSCs. **(A)** Fibroblasts were fixed with 4% paraformaldehyde, and co-labeled with antibodies to STC-1 (green) and vinculin (red). Irradiation displaced the STC-1 but not the vinculin from focal adhesions. STC-1 was present in focal adhesions in co-cultures. Magnification = 600X. **(B)** A549 cells were incubated in hypoxia at physiologic or acidic pH, in the absence or presence of MSCs. Cells were stained with antibodies to STC-1. Inset of middle panel is shown with enhanced signal, to display the faint outlines of the cell. Inset of right panel is shown with enhanced signal, to display the faint outlines of the cell.

of right panel shows distinct focal adhesion staining (arrow). Magnification = 200X upper panels; 400X insets. For all, levels were adjusted linearly for clarity.

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Table 1

Gene transcripts that are upregulated in MSCs when co-cultured with irradiated fibroblasts. Threshold for upregulation = 2-fold.

Gene Name	Secreted	Accession	Fold Increase	
			Donor 1	Donor 2
cornichon homolog 3 (Drosophila)	N	AF070524	4.17	4.62
endothelial cell-specific molecule 1	N	NM_007036	2.2	3.99
ets variant gene 1	N	BE881590	2.52	4.02
hypothetical protein MGC11324	N	BC006236	3.31	3.89
IGF-II mRNA-binding protein 3	N	AU160004	5.03	3.62
	N	NM_006547	2.28	3.54
integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	N	NM_002203	3.57	2.28
	N	N95414	3.38	2.51
matrix metalloproteinase 1 (interstitial collagenase)	N	NM_002421	5.55	4.68
phosphatidylinositol 3,4,5-trisphosphate-dependent RAC exchanger 1	N	BF308645	2.89	2.41
		AL445192	2.04	2.55
protein tyrosine phosphatase, non-receptor type 22 (lymphoid)	N	NM_015967	3.28	3.52
Ras-induced senescence 1	N	BF062629	2.91	2.83
stanniocalcin-1	Y	AI300520	3.49	4.6
		U46768	2.83	2.94
		NM_003155	3.04	5.55
		AW003173	2.78	6.96