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Bystander senescence in human peritoneal mesothelium and fibroblasts is related to thrombospondin-1-dependent activation of transforming growth factor-β1

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ABSTRACT

Senescence bystander effect refers to a phenomenon in which senescent cells elicit the development of senescence phenotype in their nearby young counterparts. In this paper we examined the mechanism of senescence bystander effect triggered by senescent human peritoneal mesothelial cells (HPMCs) in proliferating HPMCs and peritoneal fibroblasts (HPFBs). The results showed that conditioned medium (CM) derived from senescent HPMCs elicited a senescence response (growth inhibition coupled with increased expression of senescence-associated β-galactosidase and accumulation of histone γ-H2A.X) in either early-passage HPMCs or HPFBs. Samples of CM from senescent HPMCs contained increased amounts of numerous soluble mediators of which only transforming growth factor-β1 (TGF-β1) was able to induce senescence phenotype in the both types of peritoneal cells, likely through an induction of reactive oxygen species (ROS) and p38 mitogen-activated protein kinase (MAPK). At the same time, senescent HPMCs released increased amounts of thrombospondin-1 (TSP-1), a major activator of TGF-β1. Significantly, TSP-1 itself was unable to induce senescence phenotype in HPMCs or in HPFBs. The experiments employing anti-TSP-1 antibodies and specific TSP-1 blocking peptide revealed that neutralization of TSP-1 in CM prevented TGF-β1-dependent development of senescence phenotype. Collectively, our findings indicate that senescent HPMCs exhibit senescence-promoting activity toward neighboring young cells (HPMCs and HPFBs), and this effect is, at least partly, related to TSP-1-dependent activation and further ROS- and p38 MAPK-related activity of TGF-β1.

1. Introduction

It has recently been demonstrated that senescent fibroblasts are capable of inducing a senescence phenotype in their nearby proliferating counterparts (Nelson et al., 2012). This phenomenon, being a reminiscence of ionizing radiation-induced bystander effect (Prise and O'Sullivan, 2009), has been attributed to a physical cell-cell contact through junctional connections. In addition, deleterious activity of reactive oxygen species (ROS) in senescence-receiver cells has been identified as a cause of senescence-specific DNA injury, albeit signaling underlying ROS generation in those cells remains unknown. Interestingly, any role of soluble factors released by senescent cells (so-called senescence-associated secretory phenotype; SASP) has been found negligible (Nelson et al., 2012).

In this paper we addressed an issue of SASP engagement as a plausible causative factor (acting next to but not instead of a direct cell–cell interplay) in the senescence bystander reaction in cultured cells. This is based on the seminal observation that cancer cell-derived soluble chemokine (CXCL1/GRO-1) promotes senescence of fibroblasts (Yang et al., 2006). Also our own research revealed that conditioned medium (CM) from co-cultures established from young and senescent human peritoneal mesothelial cells (HPMCs) is able to inhibit growth and stimulate expression of senescence biomarkers in a strict proportion to the size of senescent cells fraction (Książek et al., 2008).

These findings prompted us to design an experimental model in which two co-existing *in vivo* cell types, namely peritoneal mesothelial cells and peritoneal fibroblasts (HPFBs), were

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subjected to conditioned medium (CM) generated by, respectively, young and senescent HPMCs. Using this tool, we confirmed both phenomenologically and mechanistically that soluble agents released at higher amounts by senescent cells to environment elicit molecular signs of senescence in proliferating cultures.

2. Materials and methods

2.1. Chemicals

Unless otherwise stated, all chemicals were purchased from Sigma–Aldrich Corp. (St. Louis, MO, USA). The tissue culture plastics were from Nunc (Roskilde, Denmark). Recombinant exogenous forms of soluble agents were obtained from R&D Systems (Abingdon, UK). Neutralizing monoclonal antibody against TGF-β1 (AF-101-NA) was from (R&D Systems) while the antibody against human TSP-1 (C6.7) was from Lab Vision Corporation (Fremont, CA). TSP-1 blocking peptide, GGWSHW, and a negative control peptide, GGYSHW, were manufactured by QIAGEN (Tokyo, Japan). SB202190 was obtained from Calbiochem/Merck (Warsaw, Poland). The concentrations of certain reagents as well as the incubation times are detailed in legends to figures.

2.2. Cell cultures

Human peritoneal mesothelial cells (HPMCs) and human peritoneal fibroblasts (HPFBs) were isolated from the pieces of omentum by enzymatic digestion, as described in detail by Pronk et al. (1993) and Jorres et al. (1996), respectively. The cells were indentified according to their immunocytochemical staining for HBME-1 antigen (HPMCs) (Zhang et al., 1999) and for an antigen defined by antibody TE-7 (HPFBs) (Witowski et al., 2009). Specimens of omentum were obtained from patients undergoing elective abdominal surgery. The study was approved by the institutional ethics committee and all patients gave their informed consent. HPMCs were propagated in M199 medium supplemented with 10% v/v fetal bovine serum (FBS) while HPFBs were maintained in Hams' F12 with 10% FBS. Replicative senescence of HPMCs was induced by serial passaging at 3-day intervals using a fixed seeding density of 3×10^4 cells/cm^2 (Książek et al., 2006). Cultures were considered senescent when cells failed to increase in number for 4 weeks, showed enlarged morphology and extensive positive staining for SA-β-Gal (Książek et al., 2008).

2.3. HPMC-derived conditioned medium

Conditioned media (CM) were collected from young (2nd passage) and senescent HPMCs. Briefly, 3×10^5 of young and senescent cells was seeded into 25 cm^2 flasks, allowed to attach for 4 h, and incubated in serum-free medium for 72 h. The samples of CM collected were centrifuged, filtered through a 0.2 μm pore size filter to remove any cellular debris, and then stored in aliquots at −80 °C until required (Mikula-Pietrasik et al., 2012).

2.4. Proliferation measurements

Low-density cultures of HPMCs and HPFBs were subjected to samples of CM from early-passage and senescent HPMCs for 48 h. As a measure of cell proliferative capacity, the percentage of dividing (DNA-synthesizing) cells in the S phase of the cell cycle was employed. In addition, the fraction of cells in the G_1 phase was estimated, as cell accumulation in this particular phase is associated with replicative senescence. In brief, cells were harvested with trypsin-EDTA solution and fixed in ice-cold 70% ethanol overnight at -20 °C. After careful washing with PBS, cells were re-suspended in the Extraction Buffer (0.2 M disodium hydrogen phosphate (Poch SA, Gliwice, Poland) and 0.1 M citric acid) for 5 min followed by 30 min incubation in PI Staining Solution (50 μg/ml of propidium iodide, 50 μg/ml of RNAse A, 3.8 mM sodium citrate (Poch SA, Gliwice, Poland) in PBS). In order to determine the fraction of cells in the S and G_1 phases, one million cells was analyzed using a FACSCalibur™ flow cytometer with ModFit LT™ software (Verity Software House, Topsham, ME, USA).

2.5. Immunocytochemistry for proliferating cell nuclear antigen (PCNA)

For PCNA immunostaining cells were cultured in Lab-Tek™ Chamber Slides (Nunc, Roskilde, Denmark) and fixed with 70% ethanol. PCNA antigen was detected with the use of specific monoclonal antibody (clone PC10; Dako, Glostrup, Denmark), diluted 1:500 in 0.05 Tris-HCl pH 7.6, 2% bovine serum albumin (BSA) for 20 min at a room temperature. After washing with the phosphate buffered saline (PBS), the cells were treated with 0.3% H_2O_2 to quench endogenous peroxidase activity. Bound antibodies were detected by immunoperoxidase staining using the EnVision$^+$ System (Dako) as per manufacturer's instructions.

2.6. Senescence-associated β-galactosidase (SA-β-Gal) expression and activity

The presence of SA-β-Gal was detected according to Dimri et al. (1995). Briefly, low-density cultures of young HPMCs and HPFBs were grown on Lab-Tek™ Chamber Slides and exposed to CM samples from early-passage and senescent HPMCs for 48 h. Afterwards they were fixed with 3% formaldehyde, washed and exposed for 6 h at 37 °C to a solution containing 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM $MgCl_2$ and 40 mM citric acid, pH 6.0. In addition, SA-β-Gal activity was quantified using fluorescence method in which the rate of conversion of 4-methylumbelliferyl-β-D-galactopiranose to 4-methylumbelliferone was measured at pH 6.0, essentially as described by Gary and Kindell (2005). In some experiments, the measurements of SA-β-Gal activity were preceded by cell incubation in serum free medium with p38 MAPK inhibitor, SB 202190 (at 10 μM for 6 h), and/or with spin-trap ROS scavenger, alpha-phenyl-*N*-*tert*-butyl-nitrone (PBN at 800 μM for 6 h).

2.7. Immunofluorescence for histone γ-H2A.X

The presence of senescence-specific DNA injury (double-strand breaks) was examined according to visualization of phosphorylated variant of histone H2A.X (γ-H2A.X). To this end, low-density cultures of young HPMCs and HPFBs were cultured in Lab-Tek™ Chamber Slides and subjected to CM from early-passage and senescent HPMCs for 48 h. Then the cells were fixed with 3% formaldehyde and incubated with monoclonal antibody against γ-H2A.X (Ser 139) (Upstate Biotechnology, Lake Placid, NY, USA), diluted 1:2000 for 1 h at room temperature. Next, cells were washed and treated with Alexa Fluor 594 goat anti-mouse IgG (Molecular Probes, Eugene, OR, USA), diluted 1:4000. After that cells were stained with 1 μg/ml DAPI. The specimens were mounted and inspected in the Zeiss Axio Obserwer D1 microscope.

2.8. Senescence-associated secretory phenotype (SASP) examination

SASP accompanying senescence of HPMCs has been analyzed at mRNA (quantitative real-time PCR) and protein levels. For real-time PCR, DNA was isolated from cells using mini columns (GenElute™ Mammalian Genomic DNA Miniprep Kit, Sigma–Aldrich, St Louis,