Identification of senescent cell surface targetable protein DPP4

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Susenct cell accumulation in aging tissues is linked to age-associated diseases and declining function, prompting efforts to eliminate them. Mass spectrometry analysis revealed that DPP4 (dipeptidyl peptidase 4) was selectively expressed on the surface of senescent, but not proliferating, human diploid fibroblasts. Importantly, the differential presence of DPP4 allowed flow cytometry-mediated isolation of senescent cells using anti-DPP4 antibodies. Moreover, antibody-dependent cell-mediated cytotoxicity (ADCC) assays revealed that the cell surface DPP4 preferentially sensitized senescent cells, but not dividing, fibroblasts to cytotoxicity by natural killer cells. In sum, the selective expression of DPP4 on the surface of senescent cells enables their preferential elimination.

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Cell senescence is a state of terminal growth arrest triggered by stress signals such as critically short telomeres, oxidative damage, oncogene activation, and hypoxia (Kuilman et al. 2010). Compared with proliferating cells, senescent cells exhibit an enlarged morphology, distinct metabolic and gene expression patterns, and increased activity of a neutral β-galactosidase (Crowe et al. 2014). They also display a senescence-associated secretory phenotype (SASP) characterized by the production and secretion of proinflammatory factors, angiogenic factors, and matrix metalloproteases that alter tissue function by promoting angiogenesis, attracting immune cells, and remodeling the extracellular matrix (Coppé et al. 2010).

Cell senescence has a range of complex effects on physiology and disease processes. Among its beneficial effects, senescent cells promote tissue morphogenesis and wound healing and suppress fibrosis and tumorigenesis in young organisms (Prieur and Peper 2008, Muñoz-Espín et al. 2013, Storer et al. 2013, Demaria et al. 2014, Muñoz-Espín and Serrano 2014). On the other hand, extensive detrimental effects have been documented for senescent cells accumulating in tissues of older organisms, as they trigger or exacerbate age-related diseases such as cancer, cataracts, arthritis, and atherosclerosis (Baker et al. 2008, 2011, Prieur and Peper 2008, Muñoz-Espín and Serrano 2014, van Deursen 2014).

The accumulation of senescent cells in aging tissues, as investigated in a genetic mouse model in which senescent cells were selectively eliminated, was found to promote aging-associated declines and diseases. Indeed, the removal of senescent cells from aging mouse tissues—achieved by triggering apoptotic death of cells that expressed the senescence protein p16—enhanced longevity and promoted healthy life span associated with a reduction in tumorigenesis and extended function of the renal, cardiovascular, muscular, and adipose systems (Baker et al. 2011, 2016).

With increasing evidence that senescent cells adversely influence aging-associated declines and diseases, a major goal in the field is to design interventions that recognize and eradicate senescent cells selectively. Senescent cells differ greatly from proliferating cells in the patterns of expressed proteins, including those on the cell surface that can serve as markers and therapeutic targets. Thus, we set out to identify cell surface proteins uniquely present in senescent cells. This strategy is similar to that used to eliminate cancer cells selectively (Rasmussen and Ditzel 2009). Using mass spectrometry (MS) analysis, we identified DPP4 (dipeptidyl peptidase 4; also known as CD26) as a surface protein that was strikingly more abundant in senescent cells. Importantly, senescent cells were preferentially eliminated by antibody-dependent cell-mediated cytotoxicity (ADCC), as the presence of DPP4 on their surface rendered them suitable targets for destruction by natural killer (NK) cells recognizing an anti-DPP4 antibody.

Results and Discussion

Identification of DPP4 as a novel senescent cell surface marker

To find novel surface markers selectively present on the plasma membrane of senescent cells, we used a well-characterized model of cellular senescence: proliferating WI-38 human diploid fibroblasts (HDFs) at population doubling level (PDL) 23 compared with those that had become senescent (PDL59) following extended culture. Senescent HDFs displayed a characteristic flattened and enlarged morphology and exhibited elevated senescence-associated β-galactosidase (SA-β-gal) activity, a distinctive trait of cellular senescence (Fig. 1A). Analysis of the incorporation of 3H-thymidine or BrdU further indicated that senescent cells had significantly lower proliferating activity (Fig. 1A). We fractionated membrane-associated proteins and cell surface-associated proteins as described (Materials and Methods) and surveyed them by MS.
Candidate on this list was DPP4 (CD26). DPP4 peptides specific to senescent cells are listed in Figure 1C. The leading PSM (peptide spectrum match) count in proliferating relative to senescent cells followed by RT-qPCR analysis revealed that DPP4 mRNA was markedly higher in senescent cells than in proliferating cells. Western blot analysis revealed that DPP4 protein was more highly expressed on the cell surface in senescent (IR-treated) compared with proliferating (untreated) HUVECs and HAECs (Supplemental Fig. S2A,B). Moreover, oncogene (Dox), also led to a rise in DPP4 mRNA abundance in WI-38 cells (Supplemental Fig. S2C). Together, these data indicate that DPP4 levels were highly and broadly elevated in senescent cells relative to proliferating cells at least in part by transcriptional induction of DPP4 mRNA levels and that DPP4 localized on the surface of the plasma membrane.

**Contribution to senescence by DPP4**

Given the rise in DPP4 levels in senescent cells, we asked whether DPP4 itself might help promote cellular senescence. To test this possibility, we overexpressed DPP4 in proliferating WI-38 cells by infection with a lentivirus expressing the oncogene HRAS<sup>G12V</sup> via viral transduction also revealed a robust rise in DPP4 levels (Supplemental Fig. S2D). Together, these data indicate that DPP4 levels were more generally increased in senescent cells, we triggered senescence by exposing proliferating WI-38 and IMR-90 HDFs to ionizing radiation (IR); 10 d later, SA-β-gal activity was selectively elevated in IR-treated cells. Western blot and RT-qPCR analyses indicated that DPP4 and DPP4 mRNA (Supplemental Fig. S1B,C) were up-regulated in IR-induced senescent HDFs, although more modestly than in HDFs undergoing replicative senescence (Fig. 1F,G). We further tested whether DPP4 levels were elevated in other models of senescence by exposing human umbilical vein endothelial cells (HUVeCs) and human aortic endothelial cells (HAECs) to 4 Gy of IR to elicit senescence; 14 d later, RT-qPCR analysis revealed significantly elevated DPP4 mRNA in senescent (IR-treated) compared with proliferating (untreated) HUVeCs and HAECs (Supplemental Fig. S2A,B). Moreover, oncogene-induced senescence (OIS) in mouse embryonic fibroblasts (MEFs) expressing the oncogene HRAS<sup>G12V</sup> via viral transduction also revealed a robust rise in DPP4 levels (Supplemental Fig. S2D). Together, these data indicate that DPP4 levels were highly and broadly elevated in senescent cells relative to proliferating cells at least in part by transcriptional induction of DPP4 mRNA levels and that DPP4 localized on the surface of the plasma membrane.
that expressed only the Myc tag. After selection of infected cells in puromycin for 20 d, protein lysates were prepared, and Western blot analysis was used to assess senescence markers. As shown, markers p16 and p21 were elevated while SIRT1 levels declined in cells overexpressing DPP4 [Fig. 2A], supporting the notion that DPP4 promoted cell senescence. In addition, overexpression of DPP4 in WI-38 cells elevated SA-β-gal activity [Fig. 2B] and decreased 3H-thymidine incorporation [Fig. 2C].

We further tested whether DPP4 contributed to eliciting senescence by silencing DPP4 in presenescent WI-38 cells [approximately PDL39–PDL45] and assessing senescence markers. As shown, the robust silencing of DPP4 by 72 h after transfection of siRNA [Supplemental Material] led to marked reductions in the levels of p53, p21, and p16 and increased SIRT1 levels, as determined by Western blot analysis [Fig. 2D]. Likewise, RT-qPCR analysis revealed that the levels of senescence markers p21 mRNA, IL1A mRNA, and IL1B mRNA were significantly reduced in the DPP4 siRNA group [Fig. 2E]. Additionally, DPP4 silencing increased the number of cells in the population [Fig. 2F] and promoted incorporation of 3H-thymidine and BrdU [Fig. 2G,H], further indicating that DPP4 contributed to the growth suppression characteristic of senescence. Interestingly, DPP4 silencing reduced the production of reactive oxygen species (ROS) in presenescence cells [Fig. 2I]. Taken together, our results indicate that DPP4 enhances fibroblast senescence.

Specific selection of senescent cells using cell surface DPP4

Given that DPP4 was identified as a cell surface protein in senescent cells [Fig. 1B,C], we used confocal microscopy to further investigate this localization. Proliferating and senescent WI-38 HDFs were fixed but not permeabilized in order to detect only proteins present on the outside of the plasma membrane. As shown in Figure 3A, DPP4 signal (red) was virtually undetectable in proliferating cells but was highly abundant in senescent cultures and was found throughout the cell surface. In light of these results, we tested whether DPP4 might be a suitable selection marker for senescent cells. We used two mouse antibodies, anti-DPP4-PE and (control) mIgG-PE, to label proliferating and senescent WI-38 HDFs without fixation or permeabilization to analyze cell surface markers. Labeled samples were analyzed by flow cytometry to assess the presence of DPP4 on the cell surface. MFI (mean fluorescence intensity) analysis revealed that DPP4-labeled cells were enriched eightfold in senescent compared with proliferating WI-38 HDFs [Fig. 3B]. Moreover, the number of DPP4-positive cells identified by flow cytometry analysis using DPP4-PE was only 6.05% in proliferating cells but rose to 67.9% in senescent cells [Fig. 3C]. In sum, DPP4 levels are markedly elevated on the surface of senescent fibroblasts and serve as a suitable marker for the specific isolation of senescent cells.

With advancing age, senescent cells accumulate in a variety of tissues, including blood. We thus asked whether DPP4-positive cells might be detected in peripheral blood mononuclear cells (PBMCs) from three healthy young individuals [27–36 yr of age] and three healthy old individuals [78–88 yr of age] [Materials and Methods]. As shown in Figure 3D, the percentage of DPP4-positive cells [as determined by flow cytometry using the DPP4-PE antibody] was higher in “old” PBMCs than in “young” PBMCs [15.5% vs. 10%]. Since most DPP4 signals came from monocytes and lymphocytes, we calculated the percentages of DPP4-positive monocytes and lymphocytes and found that these percentages remained significantly higher in the old donor group than the young donor group [33% vs. 21%]; see Supplemental Figure S3 for contour plots of the individual donors. To determine whether DPP4-positive cells were senescent, PBMCs from four donors [ages 27, 27, 60, and 63 yr] were labeled with DPP4-PE and sorted using anti-PE microbeads by MACS [magnetic-activated cell sorting] analysis. After isolation of RNA from the affinity-purified cells, RT-qPCR analysis was used to assess the expression of senescence markers. As shown [Fig. 3E], DPP4-positive cells in each of the four donors expressed higher levels of DPP4 and DPP4 mRNA by Western blot and RT-qPCR analyses, respectively (to verify...
Selective elimination of DPP4-positive senescent cells by ADCC assay

Our initial goal was to devise a method to eliminate senescent cells selectively using a differentially expressed surface protein. Thus, after establishing that DPP4 is located on the surface of the plasma membrane of senescent cells [Figs. 1, 3], we set out to test whether we could eliminate senescent cells selectively using an antibody directed at DPP4 and the ADCC assay. Originally developed for cancer therapy, the ADCC assay uses antibodies to recognize a specific antigen on the cell surface and guide NK cells to selectively destroy the antibody-labeled cells [Weiner 2015]. We carried out ADCC analysis using increasing concentrations of anti-DPP4 antibody [up to 5 µg/mL] to bind the DPP4 surface marker and thereby label proliferating and senescent WI-38 fibroblasts. We then isolated NK cells from human PBMCs and added them to the WI-38 cultures, allowing the NK cells to destroy the cells labeled by anti-DPP4 antibody. Treatment with NK cells from eight different donors in the presence of rabbit or humanized anti-DPP4 antibodies (Fig. 4A) indicated that, in every case, senescent cells exhibited stronger reductions in cell viability, as low as ~40% [as seen with “donor 8” of NK cells], compared with proliferating cells. As shown in Supplemental Figure S4, ADCC assay using a control rabbit IgG [rIgG] revealed that this antibody did not affect cell viability in proliferating or senescent cells.

Figure 3. DPP4 is more highly expressed in senescent cells and localizes on the cell surface. (A) Proliferating (P) or senescent (S) WI-38 HDFs were fixed with methanol, and the endogenous DPP4 protein was detected by confocal microscopy. (B, C) WI-38 [proliferating and senescent] HDFs were harvested and incubated with either anti-DPP4-PE or control mIgG-PE antibodies. (B) DPP4-positive cells were analyzed by flow cytometry, and the MFI of DPP4-PE in proliferating and senescent cells was quantified. The graph represents the means ± SEM. from three independent experiments. (*) P-value < 0.05. (C) Histograms from the flow analysis representing control isotype [left] or DPP4 [right] in proliferating and senescent cells; the percentages of DPP4-PE-positive cells are indicated. The experiment shown is representative of three independent experiments. (D) Human peripheral blood mononuclear cells (PBMCs) obtained from young [27- to 36-yr-old] and old [78- to 88-yr-old] donors were analyzed by flow cytometry using antibodies that recognized DPP4-PE [a] or mouse IgG [b]. The graph shows the percentage of DPP4-PE-positive cells. [Left] PBMC populations. [Right] Monocyte and lymphocyte populations. Each data point represents a young [n = 3 total] or old [n = 3 total] subject; horizontal lines indicate the mean values. (E) Human PBMCs obtained from four donors (27, 27, 60, or 63 yr old) were sorted into DPP4-positive cells and DPP4-negative cells by MACS using a DPP4-PE antibody and anti-PE microbeads. The steady-state levels of DPP4, p16, and ACTB mRNAs were quantified by RT-qPCR analysis after normalization to 18S rRNA levels. Each mRNA in DPP4-negative cells was set as 1.

Figure 4. Eliminating DPP4-positive senescent cells by ADCC. (A) Proliferating and senescent WI-38 cells [5 × 10⁴] were incubated without or with 0.05, 0.5, and 5 µg/mL anti-DPP4 antibody and either 2.5 × 10⁵ NK cells [for rabbit [r]anti-DPP4] or 1.25 × 10⁶ or 2.5 × 10⁶ NK cells [for humanized [h]anti-DPP4 antibody] per well for 4 h. After removing NK cells, WI-38 cells were incubated for another 18 h, and cell viability was measured by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Cell numbers were compared with those that received no anti-DPP4 antibody. Donors 1–4 were incubated with [r]anti-DPP4, and donors 5–8 were incubated with [h]anti-DPP4. (B) Proposed model. [Left] In proliferating cells, DPP4 is expressed at low levels or is undetectable. [Right] In senescent cells, DPP4 mRNA levels increase transcriptionally, leading to increased production of DPP4, which localizes as a dimer on the cell surface, exposed to the extracellular space. The abundance and localization of DPP4 in senescent cells enable the selective elimination of senescent cells by ADCC.
Targeting DPP4 to eliminate senescent cells

In sum, we identified DPP4 as a protein robustly up-regulated on the plasma membrane of senescent cells. DPP4 levels increased via transcriptional induction of DPP4 mRNA in senescent cells. Whether this increase might be mediated via transcription factors HNF and/or STAT1α in fibroblasts, as shown previously in B lymphocytes and intestinal epithelial cells (Bauvois et al. 2000; Erickson et al. 2000; D’Angelo et al. 2010), remains to be tested. We further found that senescent cells can be selectively targeted by anti-DPP4 antibodies and eliminated. Accordingly, we propose a model (Fig. 4B) in which the highly abundant DPP4 on the exposed surface of a senescent cell allows it to be recognized and eliminated by anti-DPP4 antibody-directed NK-elicited cell death. These results underscore the usefulness of targeting for selective destruction proteins differentially present on the plasma membrane of senescent cells.

DPP4 is best known as a protease that inactivates two hormones named incretins: glucose-dependent insulinotropic peptide (GIP) and glucagon-like peptide-1 (GLP-1). Incretins trigger a rapid release of insulin from pancreatic β cells after a meal; since they suppress a sudden rise in blood glucose, they are particularly beneficial in diabetes (Wu et al. 2016). Although the significance of having elevated DPP4 in senescent cells is unclear, DPP4 might influence some of the changes in glucose homeostasis that occur with aging. For example, a rise in DPP4 levels from accumulating senescent cells could contribute to the impairment of glucose metabolism that leads to hyperglycemia in the elderly. In this regard, it will be interesting to test whether DPP4 inhibitors (e.g., sitagliptin) (Scott 2017), which enhance the so-called “incretin effect” and have been used to treat diabetes, recapitulate the enhanced proliferation and suppressed senescence seen after silencing DPP4 (Fig. 2).

DPP4 and senolytic therapy

Similarly, as incretin-independent actions of DPP4 are emerging, it will be important to study whether the health benefits of DPP4 inhibitors depend partly on their impact on senescent cells irrespective of DPP4-mediated inactivation of incretins. For example, DPP4 present on the plasma membrane of lymphocytes (which we found to be elevated with age) (Fig. 3D) associates with CAV1, a membrane protein that also rises with senescence, leading to the activation of IRAK-1 and NF-κB (Ohnuma et al. 2005, 2008). Likewise, DPP4 present on the membrane of antigen-presenting cells and inflammatory cells in adipose tissue promotes inflammation independent of its effects on insulin production (Zhong et al. 2013). These actions of DPP4 as well as its interaction with extracellular matrix components such as collagen and fibronectin (Löster et al. 1995; Zhong et al. 2013) might implicate DPP4 as a factor related to SASP traits, which are modulated by NF-κB and profoundly affect extracellular matrix remodeling.

SASP has been linked to the disease-enhancing impact of senescent cells that accumulate in aging tissues, since SASP factors (cytokines, growth factors, and MMPs) perturb tissue metabolism locally and systemically. Accordingly, senescent cell elimination via genetic manipulation improved age-related pathologies (Baker et al. 2008, 2011, 2016). To achieve the same goal, several drugs called “senolytics” have been identified that selectively destroy senescent cells. Senolytics ABT737 (Yosef et al. 2016) and ABT263 (Zhu et al. 2016) were BH3 mimetic inhibitors of anti-apoptotic proteins [Bcl-XL, Bcl-2, and Bcl-w] originally developed for cancer therapy, although inhibiting Bcl-XL was found to have severe side effects. Other senolytics, such as dasatinib and quercetin (Roos et al. 2016; Zhu et al. 2016), trigger apoptosis in subsets of senescent cells, while piperlongumine appears to work best in combination with other senolytics (Wang et al. 2016). In this context, the identification of DPP4 as a targetable senescence marker can complement interventions aimed at eliminating senescent cells.

In closing, DPP4 is overexpressed in cancers such as malignant pleural mesothelioma (MPM) and renal cell and colorectal carcinoma. A recent study found that DPP4 was more highly expressed on the plasma membrane of MPM than normal mesothelioma cells (Angevin et al. 2017). Importantly, in preclinical trials, MPM tumor growth was delayed by treatment with anti-DPP4 antibody, suggesting that it was inhibitory in both cultured cells and patients. Antibody modifications such as conjugation with a toxin might enhance the cytotoxic activity of the anti-DPP4 antibody on DPP4-bearing cells. In summary, our findings have identified the cell membrane-associated protein DPP4 senescence marker as a promising target of therapeutic intervention in conditions in which it is desirable to eliminate senescent cells.

Materials and methods

Cells, cell culture, IR, and SA-β-gal assay

The source and culture of HDFs WI-38 and IMR-90, MEFs, HUVECs, and HAECs are indicated in the Supplemental Material. Proliferating WI-38 HDFs were used at PDLs ranging between PDL18 and PDL23, and senescent cells were used after additional culture (PDL47–PDL55). SA-β-gal analysis and siRNA transfections using Lipofectamine 2000 (Invitrogen) are described (Supplemental Material). Human PBMCs were isolated from participants of the Baltimore Longitudinal Study of Aging (BLSA) (Ferrucci 2008) aged 27–36 yr old (young) or 76–88 yr old (old). Proliferating WI-38 cells (approximately PDL25) and IMR-90 cells (approximately PDL25) were rendered senescent by exposure to 10 Gy of IR; cells were harvested 10 d later. Proliferating WI-38 cells were transduced with lentiviruses that expressed either DPP4-Myc or Myc (GeneCopoeia, Inc.) and selected using 1 µg/mL puromycin for 20 d before harvest.

RNA isolation, reverse transcription, and quantitative real-time PCR

Total RNA was extracted using TriPure isolation reagent (Roche), and cDNA was synthesized using random hexamers and reverse transcriptase (Invitrogen) as described (Supplemental Material). Reactions for qPCR amplification contained SYBR Green master mix (Kapa Biosystems) and were performed using an Applied Biosystems 7300 instrument. The gene-specific primers used are listed in the Supplemental Material.

Protein analysis

The preparation and analysis of cell membrane and cell surface proteins by Western blotting and MS as well as the detection of DPP4 using immunofluorescence are explained in the Supplemental Material.

Flow cytometry of WI-38 cells and PBMCs and MACS

Proliferating and senescent WI-38 cells were counted using a TC20 cell counter (Bio-Rad), washed using FACS buffer (0.5% BSA in PBS), and seeded into 96-well plates. Dead cells were stained with Zombie Aqua fixable viability kit (BioLegend); after washing, human TruStain FcX (BioLegend) was added to block the Fc receptor, and cells were labeled with DPP4-PE
ADCC assay

Peripheral blood from participants of the BLSA (National Institute on Aging) and healthy volunteers was collected under Human Subject Protocol 2003054 and Tissue Procurement Protocol 2003-076. White blood cells were isolated by ACK lysis, and PBMCs were isolated using Ficoll-Ficoll-Paque Plus, GE Healthcare gradients following the manufacturer’s instructions. NC cells were isolated from PBMCs using a human NC cell isolation kit (Miltenyi Biotec, Inc.). Proliferating and senescent WI-38 cells growing on 24-well plates [5 × 10^5 cells per well] were incubated with different concentrations of control IgG or with rabbit or humanized monoclonal antibodies recognizing 0.05, 0.5, and 5 µg/mL DPP4 for 15 min at 37°C. The isolated NC cells [2.5 × 10^5 cells per well for rabbit antibody and 1.25 × 10^5 or 2.5 × 10^6 for humanized antibody] were added to a ratio of 3:1 [proliferating] or 25:1 or 50:1 [senescent; NC cells/fibroblasts [proliferating or senescent]] and incubated for 4 h at 37°C. NC cells were removed by washing, fibroblasts were returned to the incubator, and, 18 h later, cell viability was analyzed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Sigma).

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