Identification and characterization of secretory proteins during ionizing radiation-induced premature senescence

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1. Introduction

Cellular senescence was first described by Hayflick and Moorhead in 1961 who observed that cultures of normal human fibroblasts had a limited replicative potential and eventually became irreversibly arrest [1]. The majority of senescent cells assume a characteristic flattened and enlarged morphological change, senescence associated β–galactosidase positivity. Recently a large number of molecular phenotypes such as changes in gene expression, protein processing and chromatin organization have been also described [2, 3]. In contrast to apoptosis, senescence does not destroy the cells but leaves them metabolically and synthetically active and therefore able to affect their microenvironment. In particular, senescent fibroblasts and some cancer cells were found to secrete proteins with known or putative tumor-promoting functions such as growth factors or proteolytic enzymes [4]. However, the knowledge about secreted proteins from senescent tumor cells and their functions to surrounding cells is still lacking. In this study, changes of senescence-associated secretory protein expression profile were observed in MCF7 human breast cancer cells exposed to gamma-ray radiation using two dimensional electrophoresis. Also, we identified up-regulated secretory proteins during ionizing radiation-induced cellular senescence. Here, we show that senescent human breast cancer MCF7 cells promote the proliferation, invasion and migration of neighboring cells.

2. Methods and Results

2.1 The Effects of Senescence-Associated Secretory Proteins

Human breast carcinoma MCF7 cells were grown in conditioned media from senescent or presenescent MCF7 cells for 24 h. Cells in the media and attached on the plates were collected separately, and the numbers of viable and dead cells in each fraction were determined using the trypan blue dye exclusion assay. The percentage of cell viability was expressed as the ratio of total viable cells to the sum of total viable and cells counted. Matrigel invasion and Transwell migration assays were performed using modified Boyden Chambers with 8-microcon pore filter inserts for 12-well plates (BD Bioscience). Filters were coated with

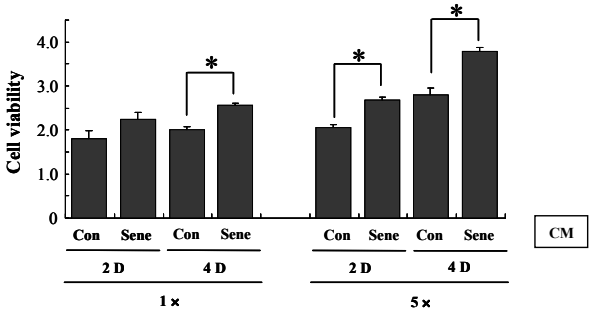

Fig. 1. Senescence-associated secretomes increase cell growth rates of neighboring tumor cells.

basement membrane extract (Matrigel, BD Bioscience). Cells were added to the upper chamber in serum-free medium, while the lower chamber was filled with senescent or presenescent conditioned medium. After 24 h, cells on the underside of the filter were fixed with Diff Quick. Cells on the underside of the filter were counted using light microscopy.

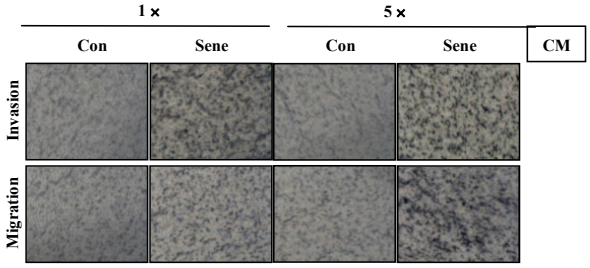

Fig. 2. Senescence-associated proteins stimulate invasion and migration of neighboring tumor cells.

2.2 Identification of Senescence-Associated Secretory Proteins Using by Two Dimensional Electrophoresis

To compare secretory proteins from senescent and presenescent MCF7 cells, proteins in conditioned media were concentrated to 250 ㎍ in a buffer containing 8 M Urea, 2% CHAPS, 2% IPG buffer, and 0.002% of bromophenol blue. Samples were loaded using the passive rehydration method for a minimum of 16 h. Rehydrated strips were focused at a temperature of 20 °C using the IPGphor IEF apparatus. The strips were brought to room temperature and equilibrated for 15 min each to reduce and alkylate the proteins. The equilibration buffer contained 6 M Urea, 2 M Thiourea, 50 mM Tris-Cl pH 8.8, 30% glycerol, 2% SDS, and

0.002% bromophenol blue with 2% DTT for the first step, and 2.5% iodoacetamide replaced DTT for the second step. Strips were then placed on top of a 12.5% polyacrylamide gel and sealed with 0.5% agarose dissolved in electrophoresis buffer. The DALT six apparatus was used for 2DE a constant temperature of 17 °C. For coomasie blue staining, place the gel in stain solution (0.2% Brilliant Blue R250 in 20% methanol, 0.5% acetic acid) for 60 minutes. And then, pour off the stain solution into a hazardous waste container and rinse the gel briefly with distilled water. For distaining, place the gel in 100 ml 30% methanol. Replace with fresh destain solution every half hour. After staining we were used the top of tips for spotting, and the spot were identified from MALDI-TOF MS.

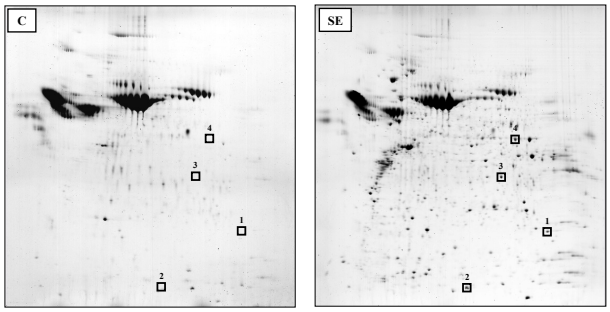

Fig. 3. Senescence -associated secretomes compared with presenescent secretomes by two dimensional electrophoresis.

Table. 1. List of identified senescence-associated secreted proteins from MCF 7 cells by MALDI-TOF MS

Spot no.	Acc. number	pI	MW (KDa)	Cov (%)	Matched Peptides	Protein	IR-induced Expression
1	CH471126.1	9.23	61929	25	11	MAP/microtubule affinity-regulating kinase 4 (MARK4)	Up
2	NM002506.2	7.01	21044	64	10	Eukaryotic Raf kinase inhibitor protein (RKIP)	Up
3	AB019691.1	4.95	451560	9	25	Centrosome- and Golgi-localized PKN-associated protein (CG-NAP, AKAP9)	Up
4	AK222517.1	7.01	47111	50	19	Enolase 1 varient	Up

2.3 The Effects of Senescence-Associated Secreted protein, RKIP

The cells were harvested and then washed with ice-cold PBS twice before the addition of RIPA buffer. Protein concentration was quantified. Equal amounts of protein were loaded into each well and separated by 10% or 15% of SDS-PAGE gel followed by transfer onto nitrocellulose membranes (Watman). Membranes were blocked with 5% nonfat dry milk in PBS containing 5% Tween 20 (PBST) for 30 min at room temperature. The blots were then incubated overnight at 4°C with appropriate primary antibodies. After washing with TBST, membranes were incubated with HRP-

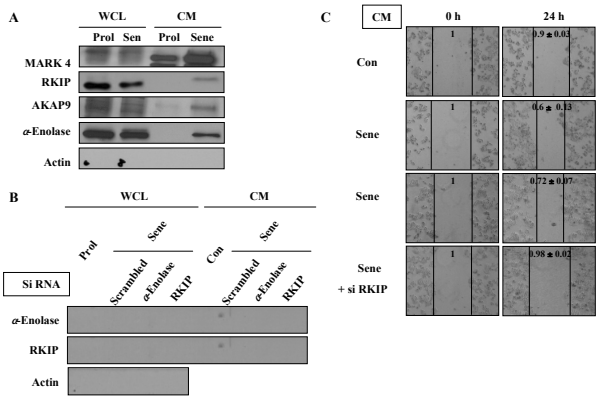

Fig. 4. The expression levels of senescence β-associated proteins confirmed by immunoblot analysis (A) and secreted RKIP have a critical role in migration of neighboring tumor cells (B, C).

conjugated secondary antibodies for 2 h at 4°C. Immunoblots were developed using the enhanced ECL (Amersham Pharmacia Biotech). For wound healing assay, at 24 h after seeding the cells, a linear wound was generated on the confluent monolayers by scraping with a pipette chip. And then discarded media, washed the plated twice using by PBS and add the CM. After 24 hours, cells were stained with Diff Quick and photographed at the same point.

3. Conclusions

In this study, secreted proteins from IR-induced senescent MCF7 cells were analyzed by two dimensional electrophoresis and MALDI-TOF MS. Our data evidenced that senescent cancer cells secreted a lot of secretomes which affect to tumor microenvironments and they stimulated not only neighboring cell growth but also invasion and migration rates. Our results suggest that the senescence associated secretory proteins, RKIP, could be the principal targets that have potentially the effects on the growth of neighboring cell growth.

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