Abstract. Since the discovery of the senescence-associated secretory phenotype, the role of senescent hepatic stellate cells (HSCs) in hepatocellular carcinoma (HCC) development has gained increasing attention. Similar to cytokines, extracellular vesicles (EVs) are essential for intercellular communication. However, the function of EVs derived from senescent HSCs in HCC progression has not been extensively studied. The aims of the present study were to characterize the EVs derived from senescent HSCs and determine their role in the tumor microenvironment.

Cellular senescence was induced in human hepatic stellate cells (HHSteCs) with various concentrations of etoposide. Induction was confirmed using EdU staining and 53BP1 and p21 immunostaining. EVs were isolated by ultracentrifugation and analyzed by nanoparticle tracking analysis. Multiplex immunoassays were used to compare the levels of growth factors secreted from hepatoma cell lines and macrophage cells pretreated with EVs derived from senescent HHSteCs (senescent EVs) with those pretreated with EVs derived from normal cultured HHSteCs (normal EVs).

Treatment with 25 µM etoposide for 3 days was the most effective at inducing senescence in HHSteCs. This finding was confirmed by induction of irreversible cell-cycle arrest, upregulation of 53BP1 and p21 expression, and increased SA-β-gal staining. Senescent HHSteCs released increased quantities of EV particles compared with normally cultured HHSteCs. Multiplex analysis revealed that there was no difference between hepatoma cell lines treated with normal EVs and those treated with senescent EVs in growth factor secretion. In contrast, the secretion of epidermal growth factor (EGF) was increased by macrophage cells treated with senescent EVs compared with those treated with normal EVs. Furthermore, senescent EVs did not affect the viability of hepatoma cells but increased the viability of hepatoma cells co-cultured with macrophage cells. In conclusion, the release of EVs from senescent HSCs was higher compared with normal HSCs. Furthermore, senescent EVs promoted HCC development by upregulating EGF secretion from macrophages.

Introduction

Hepatocellular carcinoma (HCC) is the sixth most common type of cancer and the third leading cause of cancer-associated death in the world (1). HCC development is based on cirrhosis, and the number of patients is expected to increase in the future (1-3). Advances in research regarding the underlying biology and pathophysiology of HCC is required to develop effective means of diagnosis and improved treatments for HCC. The surrounding tumor microenvironment has a notable influence on the development of HCC and is a target of novel cancer therapies (4,5). However, elucidating the underlying mechanisms by which the tumor microenvironment promotes HCC may assist in the development of improved therapeutics (6,7). The HCC tumor microenvironment consists primarily of the surrounding blood vessels, non-parenchymal cells, including fibroblasts, myofibroblasts, macrophages, lymphocytes and sinusoidal endothelial cells, and extracellular proteins such as cytokines and chemokines secreted by these cells (8,9). Hepatic stellate cells (HSCs) are the most abundant type of non-parenchymal cell present in the tumor microenvironment of HCC. They participate in modeling the tumor environment through promoting the transdifferentiation of myofibroblast-like cells, which in turn induces liver fibrosis (10-12). Emerging evidence has shown that tumor-associated macrophages (TAMs) around the tumor lesion facilitate tumor growth (13). TAMs serve important roles in tumor development and have attracted considerable attention as components of the tumor microenvironment (14-16).
Most HCCs develop as a consequence of progression of liver fibrosis (17,18). HSC activation promotes liver fibrosis through the extracellular production of proteins, such as transforming growth factor-β, tumor necrosis factor-α and interleukin-6 (19,20); therefore, numerous studies have focused on HSC activation (21,22). Based on this, Yoshimoto et al (23) proposed that senescent HSCs contribute to the development of HCC through the expression of proinflammatory cytokines associated with the senescence-associated secretory phenotype (SASP). Cellular senescence is thought to be a defense mechanism against tumor progression, but under certain circumstances may eventually promote tumor development. However, to the best of our knowledge, the means by which senescent HSCs contribute to the HCC tumor microenvironment has not been studied.

Extracellular vesicles (EVs) and cytokines, participate in extracellular communication in the tumor microenvironment (24). EVs are classified as exosomes (40-100 nm), microvesicles (100-1,000 nm) or apoptotic bodies (1-5 μm) (25-27). The contents of EVs vary depending on the conditions of the cells and therefore exert differing biological effects (25-29). Senescent HSCs promote HCC development via pro-inflammatory cytokines induced by the SASP (23). However, whether EVs derived from senescent HSCs inhibit or promote HCC development remains unknown. To attain a more comprehensive understanding of the HCC tumor microenvironment, it is necessary to assess the impact that EVs derived from senescent HSCs have on HCC. The aim of the present study was to elucidate the effects of EVs derived from senescent HSCs on the HCC tumor microenvironment. The characteristics of EVs derived from senescent HSCs and their influence on growth factor secretion from hepatoma cells and macrophages were assessed.

Materials and methods

Cell culture and reagents. Human hepatic stellate cells (HHSteCs) were obtained from SteCM; SciCell Research Laboratories and maintained in stellate cell medium (SciCell Research Laboratories) supplemented with 2% FBS, 1% penicillin/streptomycin solution (SciCell Research Laboratories) and 1% stellate cell growth supplement (SciCell Research Laboratories). The human HCC cell lines Hep3B and HuH7 (American Type Culture Collection) were maintained in DMEM (Wako Pure Chemical Industries Ltd.) supplemented with 10% FBS and 1% PenStrep (Thermo Fisher Scientific, Inc.). The human monocytic leukemia cell line THP-1 (American Type Culture Collection) was cultured in RPMI-1640 medium (Wako Pure Chemical Industries Ltd.) supplemented with 10% FBS and 1% PenStrep (Thermo Fisher Scientific, Inc.). All cells were maintained in a humidified incubator with 5% CO₂ at 37°C. THP-1 cells were induced to differentiate by treating them with 10 mg/ml phorbol-12-myristate-13-acetate (Sigma-Aldrich; Merck KGaA) for 3 days. Etoposide (ETP) was purchased from Sigma-Aldrich (Merck KGaA). Etoposide hydrochloride was purchased from Sigma-Aldrich (Sigma-Aldrich; Merck KGaA) for 3 days. PKH67 expression was observed under a Keyence scope (Keyence Corporation) at x100 magnification. SA-β-gal staining was performed using a Senescence β-Galactosidase Staining kit (Cell Signaling Technology, Inc.) according to the manufacturer's protocol. All assays were performed at least in duplicate.

Immunofluorescence assays. EdU staining and SA-β-gal staining. Cellular senescence was induced by ETP treatment and confirmed by observing p21 and 53BP1 expression in HHSteCs using immunofluorescence assays. A total of 5x10⁴ HHSteCs were mounted on four-chamber slides (Lab-Tek II; Thermo Fisher Scientific, Inc.) and treated with various concentrations of ETP for 3 days. Subsequently, cells were fixed with 4% paraformaldehyde for 30 min at room temperature, permeabilized with ice-cold 70% ethanol and blocked in 1% BSA for 1 h at room temperature. Primary antisera, 1:200 rabbit anti-p21 (cat. no. 29475; Cell Signaling Technology, Inc.) or 1:200 rabbit anti-53BP1 (cat. no. IHC-00001; Bethyl Laboratories, Inc.) were added and the cells were incubated for 1 h at 20-25°C. After washing the cells with PBS, secondary antisera (AlexaFluor 488-conjugated donkey anti-rabbit IgG; 1:1,000; cat. no. A11008; Molecular Probes; Thermo Fisher Scientific, Inc.) was added to the cells and incubated for 1 h at room temperature. The slides were washed, and coverslips were mounted with DAPI Fluoromount-G (SouthernBiotech). The uptake of EdU was observed in the HHSteCs treated with ETP for 3 days, and for cells left to recover, for another 3 days in normal medium following treatment. EdU staining of the HHSteCs was performed using a Click-iT EdU AlexaFluor 594 imaging kit (cat. no. C10339; Thermo Fisher Scientific, Inc.) for 4 h according to the manufacturer's protocol. Images were acquired using a Keyence All-in-One fluorescence microscope (Keyence Corporation) at x100 magnification. SA-β-gal staining was performed using a Senescence β-Galactosidase Staining kit (Cell Signaling Technology, Inc.) according to the manufacturer's protocol. All assays were performed at least in duplicate.

Extraction and quantification of EVs derived from HHSteCs. To collect EVs, 2.5x10⁴ HHSteCs either untreated or pretreated with ETP were seeded in a 100-mm dish and grown in medium containing exo-free FBS (System Biosciences) for 7-10 days. The medium was collected and centrifuged at 300 x g for 10 min and at 16,500 x g for 20 min at 4°C to remove cells and debris, respectively. After filtration with a 220-nm filter, the supernatant was ultra-centrifuged at 150,000 x g for 120 min at 4°C. The EV pellet was washed and resuspended in PBS and ultra-centrifuged at 150,000 x g for 120 min at 4°C. EVs derived from normal cultured HHSteCs and from senescent HHSteCs were termed ‘normal EVs’ and ‘senescent EVs’, respectively. To measure the particle size and number of normal and senescent EVs, 7.5x10⁴ HHSteCs were seeded in a 60-mm dish and grown for 3 days. The EVs were extracted in the same manner as described above and quantified using nanoparticle tracking analysis (NanoSight; Malvern Panalytical).

Analysis of EV incorporation into hepatoma cells and macrophages. To examine the incorporation of EVs into hepatoma cells and macrophages, Hep3B and THP-1 cells were treated with EVs labeled with PKH67. EVs were labeled using a PKH67 Green Fluorescent Cell Linker Mini kit (cat. no. MIN167-1KT; Sigma-Aldrich; Merck KGaA) for general cell membrane labeling according to the manufacturer's protocol. Labeled EVs were pelleted by ultracentrifugation twice at 150,000 x g for 120 min at 4°C to remove excess dye. Subsequently, 5x10⁴ Hep3B and differentiated THP-1 cells each were seeded on four-chamber slides and treated with 2x10⁴ labeled EVs daily for 3 days. PKH67 expression was observed under a Keyence
All-in-One fluorescence microscope at x100 magnification. In addition to the unlabeled EVs, PBS without EVs based on the same procedure previously described (to confirm the absence of residual PKH67) was prepared as a negative control. These experiments were performed in at least duplicate.

**Quantification of secreted growth factors.** Comprehensive quantification of growth factors secreted by the cells treated with EVs was performed using multiplex immunoassays. A total of 5x10³ Hep3B, Huh7 and differentiated THP-1 cells each were seeded in a 60-mm culture dish and treated with 2x10⁶ EV particles daily for 3 days and the supernatant was subsequently collected. Growth factors were measured using multiplex immunoassays with a Growth Factor 11-Plex Human ProcartaPlex panel (Invitrogen; Thermo Fisher Scientific, Inc.). Epidermal growth factor (EGF) was quantified in the supernatant with a human EGF ELISA kit (cat. no. DEG00; R&D Systems, Inc.) according to manufacturer's protocol.

**RNA extraction and reverse transcription-quantitative (RT-q)PCR.** EGF mRNA expression in differentiated THP-1 cells was analyzed using RT-qPCR. The total RNA was extracted from cells using SuperScript III First-strand Synthesis system (cat. no. 18080051 Thermo Fisher Scientific, Inc.), and cDNA synthesis was performed for 15 min at 42°C using 1 µg total RNA as a template. RT primer and QuantiTect reverse transcriptase (Superscript III; Thermo Fisher Scientific, Inc.). EGF primers were purchased from Takara Bio, Inc. (cat. no. HA159157) but the sequences of the primers were not disclosed. For qPCR, per a reaction, 1 U LightCycler SYBR Green I Master mix (Roche Diagnostics) was used and the cycling conditions were as follows: Pre-incubation for 5 min at 95°C; followed by 30 cycles of 10 sec at 95°C, 10 sec at 60°C and 10 sec at 72°C. GAPDH was used as the endogenous control. The sequences of the GAPDH primers were: Forward, 5’-AGGCCACATCGCTCAGACAC-3’ and reverse, 5’-GCC CAATAGCACCAAATCC-3’. Gene expression was calculated using the 2^ΔΔCq method (30). PCR was performed in triplicate.

**Cell viability assays.** To evaluate the impact of EVs on proliferation of hepatoma cells, the viability of Hep3B cells treated with EVs was determined using an MTS assay. A total of 2.5x10³ Hep3B cells were seeded in a 96-well plate and treated with either 1x10⁶ or 3x10⁶ EVs daily for 3 days either with or without differentiated THP-1 cells. Subsequently, 20 µl CellTiter96® AQueous One Solution Reagent (Promega Corporation) was added to each well. Following incubation for 2 h at 37°C, the reaction was measured using an automated plate reader (Bio-Rad Laboratories, Inc.) at 490 nm. To evaluate the concentration of erlotinib that could be used whilst maintaining the viability of hepatoma cells, MTS assays were used. A total of 2.5x10³ Hep3B cells were seeded in a 96-well plate and treated with various concentrations of erlotinib for 3 days. MTS assays were performed in at least duplicate.

**Statistical analysis.** Data are presented as the mean ± standard deviation of the mean. Multiple comparisons were performed using an ANOVA with a post-hoc Tukey’s test. P<0.05 was considered to indicate a statistically significant difference. For multiplex immunoassays which were used as a comprehensive quantification of growth factors, >2-fold difference in secretion was used as the threshold of significance.

**Results**

**Induction of senescence in HHSHeCs with ETP treatment.** HHSHeCs were treated with 2.5, 5.0, 25, or 50 µM ETP for 3 days. Alterations in morphology were observed in the treated HHSHeCs compared with the untreated cells. HHSHeCs treated with 50 µM ETP died within 3 days and the number of cells was notably decreased (Fig. S1). For the HHSHeCs treated with 2.5, 5.0 and 25 µM ETP, the expression of p21 (a cell cycle arrest marker), 53BP1 (a DNA damage marker) foci and the uptake of EdU were examined. p21 expression and 53BP1 foci were significantly increased at all three ETP concentrations (Fig. 1A and B), whereas EdU uptake decreased (Fig. 1C) compared with the control. To confirm induction of irreversible cell cycle arrest associated with senescence, HHSHeCs treated with each of the ETP concentrations were grown in fresh ETP-free recovery medium for 3 days. EdU uptake was still reduced in HHSHeCs treated with 25 µM ETP (Fig. 1D). In contrast, EdU uptake slightly recovered in HHSHeCs treated with 2.5 and 5.0 µM ETP. Therefore, 2.5 and 5.0 µM ETP were inadequate for the induction of senescence in HHSHeCs. Finally, induction of senescence was confirmed in HHSHeCs treated with 25 µM ETP using SA-β-gal staining (Fig. 1E). The proliferation of HHSHeCs treated with 25 µM ETP for 3 days was reduced in the recovery medium (Fig. 1F). Thus, 25 µM ETP was used for all subsequent experiments for the induction of senescence in HHSHeCs.

**Particle size and number of senescent HHSHeC-derived EVs.** After extracting the EVs by ultracentrifugation, the particle size and number of senescent EVs were compared to those of normal EVs using nanoparticle tracking analysis. The size of particles were largely ~120 nm, although some particles were ~200 nm in size. Therefore, it was considered that the extracted EVs were primarily composed of exosomes with a small number of other small vesicles (25,26). The median particle size was 126 nm for normal EVs and 120 nm for senescent EVs (Fig. 2A) and this difference was not significant. To determine the number of EV particles released per HHSHeC, the area under the curve (AUC) was calculated using a cell proliferation curve. The cumulative number of EVs in the culture medium was associated with the cumulative number of HHSHeCs, although the quantities differed notably between the normal and senescent HHSHeC cultures. The AUC of the normal cultured HHSHeCs was 1.23x10¹⁰ cells day⁻¹, whereas that of the senescent HHSHeCs was 7.06x10⁹ cells day⁻¹ (data not shown). Therefore, it was estimated that there were 2.5x10³ particles produced cell⁻¹ day⁻¹ for normal EVs and 4.2x10³ particles cell⁻¹ day⁻¹ for senescent EVs. Senescent HHSHeC released ~1.7-fold more EVs per cell than normal cultured HHSHeCs, although the significance of this result could not be analyzed statistically.

**Incorporation of EVs derived from HHSHeCs into hepatoma cell lines.** To confirm whether EVs secreted by HHSHeCs were incorporated into both hepatoma cells and macrophages, EVs labeled with PKH67 were added to Hep3B cells and THP-1
cells daily for 3 days. PKH67 expression was observed in both cells (Fig. 2B), which suggests that EVs derived from HHSteCs were incorporated into cells.

Growth factor secretion from hepatoma cell lines and differentiated THP-1 cells treated with EVs derived from senescent HHSteCs. To assess the effect of senescent EVs on growth factor secretion from hepatoma cells, Hep3B and Huh7 cells were treated with either normal or senescent EVs daily for 3 days and the secretion of growth factors into the supernatant was measured using a panel of multiplex immunoassays. The difference in secretion of growth factors between normal and senescent EV treatments was <2-fold (Fig. S2). Several studies have shown that TAMs participate in hepatoma cell proliferation via changes in cytokine expression levels (31,32). Thus, the effect of senescent EVs on growth factor secretion from THP-1 cells was assessed. Differentiated THP-1 cells were treated daily for 3 days with either normal or senescent EVs and growth factor secretion was measured. THP-1 cells treated with senescent EVs secreted significantly more EGF compared with those treated with normal EVs and the change was >2-fold (Fig. 3A).

EGF expression in THP-1 cells treated with EVs was further assessed by RT-qPCR and EGF protein-specific ELISA. EGF secretion from THP-1 cells treated with EVs was significantly higher compared with both the THP-1 cells treated with normal EVs and control (Fig. 3B). To further confirm the results obtained by multiplex immunoassays, EGF secretion was measured using ELISA. EGF secretion from THP-1 cells treated with senescent EVs was also increased compared with THP-1 cells treated with normal EVs and the control (Fig. 3C).
The effect of EVs derived from senescent HHSteCs on the proliferation of Hep3B cells. Effect of EVs derived from senescent HHSteCs on the proliferation of Hep3B cells. The effect of EVs derived from senescent HHSteCs on hepatoma cell viability was assessed. As shown in Fig. 4A, neither treatment with normal nor senescent EVs affected the proliferation of Hep3B cells. However, both EV treatments significantly increased the viability of Hep3B cells when co-cultured with differentiated THP-1 cells. Notably, this effect was significantly greater with senescent EVs compared with normal EVs (Fig. 4B). To validate the effect of EGF secretion from THP-1 cells on hepatoma cell lines, the cell viability of hepatoma cells in the presence or absence of erlotinib, an EGFR tyrosine kinase inhibitor, was determined. The concentration of erlotinib was set to 2.5 µM to maintain Hep3B cell viability at >80% (Fig. 5A). As shown in Fig. 5B, senescent EVs were more effective at enhancing Hep3B cell viability compared with EVs in the absence of erlotinib; however, this enhancing effect was inhibited in the presence of erlotinib. This suggests that the effect of senescent EVs on the proliferation of hepatoma cells co-cultured with THP-1 cells was dependent on EGF secreted from THP-1 cells.
more conducive tumor microenvironment for proliferation of hepatoma cells.

Senescent HSCs affect their surrounding cells, inducing alterations to the hepatic microenvironment. Krizhanovsky et al. (33) examined the role of senescent HSCs in the hepatic microenvironment in detail. They showed that hepatic fibrosis develops in the absence of HSC senescence induction and that senescent HSCs promote the activity of NK cells to eliminate the activated HSCs which cause progression of hepatic fibrosis. However, they also hypothesized that senescent HSCs accumulate in the presence of sustainable and excessive liver damage, caused by viruses or hepatic steatosis, beyond the means of a physiological immune response. In addition, Yoshimoto et al. (23) showed that senescent HSCs promote progression of liver cancer. At present, therapy targeting senescent cells has also been studied. Ogrodnik et al. (34) reported that a combination of the selective senolytic agents dasatinib and quercetin reduced hepatic steatosis (34). Senescent cells are now regarded as attractive targets for novel therapeutic strategies. However, it is necessary to further elucidate their contributions to the tumor microenvironment.

To date, there have been numerous reports on the effects of EVs secreted by hepatocytes on stellate cells (35-37); however, to the best of our knowledge, there are no report on the effects of EVs secreted by stellate cells on hepatocytes or surrounding cells. In addition, it has been reported that the efficiency of EV uptake varies depending on the type of recipient cells (38). Accordingly, EV uptake by hepatoma cells and macrophage cells was initially determined and confirmed, and EVs affected the secretion of cytokines. Li et al. (39) reported that EVs containing oncomiRs secreted from hepatoma cells are incorporated into HSCs, and that EVs secreted from HSCs promote HCC progression as a positive feedback mechanism. Furthermore, Wan et al. (40) showed that exosomes secreted from activated HSCs are taken up by Kupffer cells, as well as the HSCs themselves, which increases the expression of GLUT1 and PKM2. Therefore, EVs secreted by HSCs are actively taken up by surrounding cells including hepatocytes and macrophage cells with functional effects on the hepatic microenvironment.

Several studies have shown that the impact of EVs on surrounding cells varies by EV content and is regulated by cell conditions (41,42). Regarding the effect of EVs derived from senescent cells on cancer cells, Takasugi et al. (43) showed that EVs derived from senescent cells are absorbed by several breast cancer cell lines via EphA2, which resulted in SASP factor-like cancer progression. Similarly, in the present study, it was shown that EVs derived from senescent HSCs increased secretion of EGF from THP-1 cells, which in-turn promoted the proliferation of the hepatoma cells co-cultured with these cells. EVs derived from senescent HSCs may thus indirectly contribute to the formation of an environment conducive to the development of HCC.

There are some limitations to the present study. The primary limitation is that these results are based entirely on in vitro assays. Additionally, the number of EVs used for treatment was based on an approximate prediction method. The ratio of stellate cells in vivo is equivalent to ~10% of the number of parenchymal cells (21,44). Thus, the same ratio was used in the present study. As described in the results section, a single HHSteC secreted 2,500-4,000 particles per day. Therefore, the number of EVs used for treatment was set to ~400x the number of hepatoma cells. However, it is unclear the number of EVs secreted from HSCs in the liver microenvironment in vivo. It is also possible that various changes to the liver state may result in changes of the number of EVs secreted from HSCs. In addition, as the experiments in the present study were only performed in vitro, the conditions are considerably different from in vivo where immune cells are also involved. As reported by Krizhanovsky et al. (33), senescent HSCs can modulate the immune system in vivo. Therefore, immunomodulatory signals from EVs derived from senescent HSCs.
should be explored in the future. However, the findings of the present study highlight the role of EVs derived from senescent HSCs in promoting tumor growth, similar to that observed with the SASP. Therefore, in our future studies, the effect of EVs derived from senescent HSCs in vivo will be assessed. In conclusion, senescent HSCs released increased quantities of EVs compared with normal HSCs. Similar to, SASP factors, EVs from senescent HSCs promote HCC development by upregulating EGF production in macrophages.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

All authors have contributed to this work and read and approved the manuscript. YM and SM designed the study, performed the experiments, wrote the article and interpreted the data. YK, SN, RS, MH and HS performed the experiments. TH, HM, NT and KN interpreted and analyzed the data. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patients consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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