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Research Paper

Cancer-associated Fibroblasts Promote Irradiated Cancer Cell Recovery Through Autophagy

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ABSTRACT

Tumor relapse after radiotherapy is a significant challenge to oncologists, even after recent the advances in technologies. Here, we showed that cancer-associated fibroblasts (CAFs), a major component of cancer stromal cells, promoted irradiated cancer cell recovery and tumor relapse after radiotherapy. We provided evidence that CAFs-produced IGF1/2, CXCL12 and β-hydroxybutyrate were capable of inducing autophagy in cancer cells post-radiation and promoting cancer cell recovery from radiation-induced damage *in vitro* and *in vivo* in mice. These CAF-derived molecules increased the level of reactive oxygen species (ROS) post-radiation, which enhanced PP2A activity, repressing mTOR activation and increasing autophagy in cancer cells. Consistently, the IGF2 neutralizing antibody and the autophagy inhibitor 3-MA reduce the CAF-promoted tumor relapse in mice after radiotherapy. Taken together, our findings demonstrated that CAFs promoted irradiated cancer cell recovery and tumor regrowth post-radiation, suggesting that targeting the autophagy pathway in tumor cells may be a promising therapeutic strategy for radiotherapy sensitization.

1. Introduction

The initiation and progression of tumors are controlled not only by tumor cells but also by their surrounding stromal cells (Lengauer et al., 1998; Ronnov-Jessen et al., 1996; Tlsty, 2001; Carmeliet and Jain, 2000; Sandler et al., 2004). Cancer-associated fibroblasts (CAFs), are major components of cancer stromal cells that account for about 40%–50% of the total cell population in cancers (Xing et al., 2015). CAFs are primarily derived from activated quiescent fibroblasts surrounding the cancer cells, and have been shown to directly promote tumor initiation (Bhowmick et al., 2004; Olumi et al., 1999), progression (Dimanche-Boitrel et al., 1994; Orimo et al., 2005), and metastasis (Grum-Schwensen et al., 2005; Olaso et al., 1997). CAFs produce extracellular matrix-degrading enzymes, secrete growth factors and cytokines, and export a large amount of metabolites, which collectively promote tumor development and progression (Kolch et al., 1995; Saharinen et al., 2011; Boire et al., 2005; Lochter et al., 1997; Chaudhury et al., 2010; Ding et al., 2010) (Bonuccelli et al., 2010; Capparelli et al., 2012; Fiaschi et al., 2012).

Tumor relapse after radiotherapy is a significant challenge to oncologists. Tumor recurrence was generally due to radiation resistance, which was determined by both the intrinsic characteristics and the extrinsic microenvironment of cancer cells (Frank et al., 2010). The unique stem cell properties, including dormancy state (Bao et al., 2013) and enhanced DNA damage repair capacity (Bao et al., 2006), increased the resistance of cancer cells to radiation (Visvader and Lindeman, 2008). The particular niche in tumor tissues also enhanced tumor resistance to radiation (Yang and Wechsler-Reya, 2007). The *in vitro* study has demonstrated that pretreatment with CAF-conditioned medium promoted HeLa cell survival post-radiation (Chu et al., 2014). Further studies demonstrated that preexisting CAFs promoted cancer cell resistance to radiation through the paracrine pathway of insulin-like growth factor (IGF)1/2 (Chen et al., 2014). The IGF1 receptor signaling, in turn, induced tumor stem-like cell formation and increased radiation resistance of immortalized Igf2 null mouse embryonic fibroblasts and glioma stem cells (Burns and Hassan, 2001; Osuka et al., 2013). All these

Abbreviations: CAFs, cancer-associated fibroblasts; IGF1/2, insulin-like growth factor 1/2; CXCL12, C-X-C motif chemokine ligand 12; ROS, reactive oxygen species; PP2A, serine/threonine protein phosphatase 2A; mTOR, mechanistic target of rapamycin; 3-MA, 3-methyladenine.

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observations suggested that preexisting CAFs enhanced radiation resistance of tumor cells before radiation therapy. However, it is not clear whether CAFs play roles in irradiated cancer cell recovery.

In this study, we found that CAFs promoted irradiated cancer cell recovery and promoted tumor relapse after radiation therapy, which was further confirmed by the enhancement of IGF2 neutralizaing antibody on radiotherapy results. Moreover, our study demonstrated that CAFs promoted cancer cell recovery through inducing cancer cell autophagy post-radiation and the autophagy inhibitor 3-methyladenine (3-MA) enhanced the efficacy of radiotherapy, suggesting that CAFs are critical factors for tumor recurrence after radiotherapy. Therefore, targeting the autophagy pathway may be a promising therapeutic strategy for radiotherapy sensitization, and we hypothesize that autophagy inhibitors will improve radiotherapy efficacy.

2. Materials & Methods

2.1. Cell Culture and Reagents

Lung cancer A549 and melanoma A375 cells (ATCC, Manassas, VA) were cultured in DMEM with 10% FBS. Glucose-deprived DMEM was purchased from Gibco (Grand Island, NY). Human recombinant TGF-β1, IGF1, IGF2, CSCL12, EGF, was purchased from Peprotech (Suzhou, China). SYBR Green PCR master mix and the TaqMan microRNA reverse transcription kit were purchased from ABI (Foster City, CA). The source for antibodies used for immunoblotting (IB) were as follows: Akt, phospho-AKT (T308), phospho-GSK-3β, S6K, phospho-S6K, mTOR, phospho-mTOR, ERK, phospho-ERK, β-catenin (Cell Signaling Technology, MA, USA), GSK-3β (Epitomics, CA, USA), PP2A (ABclonal, ProteinTech), and β-actin (Santa Cruz Biotechnology, CA, USA). The neutralization antibodies against IGF1, IGF2 and CXCL12 were purchased from the R & D. 3-MA was purchased from the Selleck.

2.2. Isolation and Identification of Cancer-associated Fibroblast

Human normal primary fibroblasts and cancer-associated fibroblasts were isolated from foreskin or from lung cancer tissues, respectively. After posthectomy, the foreskins were immediately transported to the laboratory on ice. The foreskins were minced and then digested with 0.1% type I collagenase and trypsin. After digestion, the tissue was filtered with a 400-mesh sieve, and the filtrate was centrifuged at 1000 ×*g* for 10 min. Cells obtained from the pellet were cultured with DMEM containing 10% FBS for 2 h; the attached cells, verified by F-actin staining (Fig. 1), were fibroblasts. After 3 passages, the cells were frozen in liquid nitrogen for further experiments.

2.3. Cellular ROS (Reactive Oxygen Species) Evaluation

The 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma) was used as a cellular ROS indicator. The DCFH-DA was transformed into 2,7-dichlorodihydrofluorescein (DCFH) by the esterases, which could be further oxidized to a highly fluorescent compound 2,7-dichlorofluorescein (DCF) by ROS. Cells were co-cultured with the 10 μM of DCFH-DA for 20 min, and analyzed by flow cytometry. The intensity of DCF represented intracellular ROS levels.

2.4. Lentiviruses-mediated Stable Cell Lines

Short-hairpin sequences targeting IGF1 (NM_001111283.2), IGF2 (NM_000612.5), and CXCL12 (NM_000609.6), ATG5 (NM_001111283.2) was synthesized (Sangon Biotech) and separately inserted into the pGIPZ lentiviral vector (Open Biosystems, Lafayette, CO). These lentiviral plasmids were co-transfected into 293T cells with psPAX2 and pMD2G to generate the lentiviruses using Lipofectamine 2000 (Invitrogen, CA). Viruses were collected from the supernatant of transfected 293T cells 3 days after transfection. The specific shRNA sequences were as following: IGF1-SH1: sense: 5′-CCGGCCCGTCCCTATCGACAAACAACTCGAGTTGTTTGTCGATAGGGACGGGTTTTTG-3′; antisense: 5′-AATTCAAAAACCCGTCCCTATCGACAAACAACTCGAGCCCGTCCCTATCGACAAACAA-3′. IGF1-SH2: sense: 5′-CCGGGCTTCTCACCTTCTTGGCCTTCTCGAGAAGGCCAAGAAGGTGAGAAGCTTTTTG-3′; antisense: 5′-AATTCAAAAAGCTTCTCACCTTCTTGGCCTTCTCGAGAAGGCCAAGAAGGTGAGA-AGC-3′. IGF1-SH3: sense: 5′-CCGGCCAATATGACACCTGGAAGCACTCGAGTGCTTCCAGGTGTCATATTGGTTTTTG-3′; antisense: 5′-AATTCAAAAACCAATATGACACCTGGAAGCACTCGAGTGCTTCCAGGTGTCA-TATTGG-3′. IGF1-SH4: sense: 5′-CCGGCTCGTGCTGCATTGCTGCTTACTCGAGTAAGCAGCAATGCAGCACGAGTTTTTG-3′; antisense: 5′-AATTCAAAAACTCGTGCTGCATTGCTGCTTACTCGAGTAAGCAGCAAT-GCAGCACGAG-3′. IGF2-SH1: sense: 5′-CCGGGCATCGTTGAGGAGTGCTGTTCTCGAGAACAGCACTCCTCAACGATGCTTTTTG-3′; antisense: 5′-AATTCAAAAAGCATCGTTGAGGAGTGCTGTTCTCGAGAACAGCACTCCTCAACGATGC-3′. IGF2-SH2: sense: 5′-CCGGGAGTGCAGGAAACAAGAACTACTCGAGTAGTTCTTGTTTCCTGCACTCTTTTTG-3′; anti-sense: 5′-AATTCAAAAAGAGTGCAGGAAACAAGAACTACTCGAGTAGTTCTTGTTTCCTGCACTCC-3′. IGF2-SH3: sense: 5′-CCGGCCTCCCAAATTGCTGGGATTACTCGAGTAATCCCAGCAATTTGGGAGGTTTTTG-3′; antisense: 5′-AATTCAAAAACCTCCCAAATTGCTGGGATTACTCGAGTAATCCCAGCAATTTGGGAGGC-3′. CXCL12-SH1: sense: 5′-CCGGCAAACTGTGCCCTTCAGATTGCTCGAGCAATCTGAAGGGCACAGTTTG-TTTTTG-3′; antisense: 5′-AATTCAAAAACAAACTGTGCCCTTCAGATTGCTCGAGCAATCTGAAGGGCACAGTTTG-3′. CXCL12-SH2: sense: 5′-CCGGCCGTCAGCCTGAGCTACAGATCTCGAGATCTGTAGCTCAGGCT-GACGGTTTTTG -3′; antisense: 5′-AATTCAAAAACCGTCAGCCTGAGCTACAGATCTCGAGCCGTCAGCCTGAGCTACAGAT -3′. ATG5-SH1: sense: 5′-CCGGGCACCCATCTTTCCTTAACGAAACTCGAGTTTCGTTAAGGAAAGATGGGTTTTTTTG -3′; antisense: 5′-AATTCAAAAACACCCATCTTTCCTTAACGAAACTCGAGTTTCGTTAAGGAAAGATGGGTT-3′. ATG5-SH4: sense: 5′-CCGGGCATGAAAGAAGCTGATGCTTTACTCGAGTAAAGCATCAGCTTCTTTCATATTTTTG-3′; antisense: 5′-AATTCAAAAACATGAAAGAAGCTGATGCTTTACTCGAGTAAAGCATCAGCTTCT-TTCATA-3′.

2.5. Immunoprecipitation and Western Blotting

The cells were placed on ice and washed with ice-cold PBS. Total protein extract was prepared with the appropriate amount of RIPA lysis buffer (25 mM tris-HCl at pH 7.5, 2 mM EDTA, 25 mM NaF and 1% Triton X-100) containing 1 × protease inhibitor mixture (Roche Inc., CH, Switzerland) and 1 × PMSF.

The proteins were resolved on 7–15% SDS-polyacrylamide gels and transferred by electroblotting to nitrocellulose membranes (Bio-Rad Inc., CA, USA). The membranes were blocked with 5% nonfat dry milk in TBST (the mixture of tris-buffered saline and Tween 20) for 1 h. Proteins of interest were detected with specific antibodies, blots were scanned using an Odyssey infrared imaging system (LI-COR), and proteins were quantitatively analyzed using the Odyssey software.

2.6. Cytokine Array

The CAFs or fibroblasts were cultured in serum-free medium when they grew to 90% confluence. Growing for another four days, the media were collected and concentrated, and followed by dialysis. After labeling with biotin, samples were incubated with the antibody array chip (AAH-B LG-1, Raybiotech) for 2 h. Then, chips were reacted with streptavidin-conjugated fluorescene dye, and detected by the Axon Genepix scanner The data were normalized to the total protein.

2.7. Patients and Eligibility

This study was approved by the Ethical Review Board of the Medical Faculty of the Shanghai Jiao-Tong University School of Medicine.

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